The Effects of Hyaluronic Acid on Lens Epithelial Cell Migration *In Vitro*

Master's Thesis

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Abstract

Purpose. To determine if canine lens epithelial cells (LEC) contain the hyaluronic acid (HA) receptors CD44 and RHAMM, and if HA can impact posterior capsular opacification (PCO) formation *in vitro*.

Methods. Normal (n=10) and cataractous (n=10) canine LEC were evaluated using immunohistochemistry and RT-PCR for expression of CD44 and RHAMM. *In vitro* models of PCO formation were used to determine if various HA concentrations altered LEC migration. A one-millimeter scratch was created in cultures of confluent canine LEC. Cells were treated with 0, 0.02, 0.012, or 1.0 mg/mL (n=6 per group) of HA dissolved in culture media. Migration of LEC into the scratch was quantified. Mock cataract surgery was performed on canine cadaver eyes. Capsules were re-distended using one of the following (n=6 per group): PBS only, hydroxypropyl methylcellulose viscoelastic, 1.2% HA (12 mg/ml HA) viscoelastic, or 2.0% (20 mg/ml HA) HA viscoelastic. LEC migration within the capsule was evaluated.

Results. Normal and cataractous LEC were positive for CD44 and RHAMM protein and mRNA. CD44 showed a statistically significant increase ($p<0.01$) in mRNA expression in LECs treated with 1.0 mg/ml of HA as compared to the control and 0.02 mg/ml HA

Cultured LECs that were treated with 1.0 mg/mL of HA compared to control showed a significantly smaller area devoid of cells ($p=0.001$) in the scratch test model compared with the control. The number of LECs increased when capsules were treated with 2% HA (20mg/ml HA) viscoelastic as compared to control ($p < 0.0109$).

Conclusions. Canine LEC possess receptors to respond to HA signaling. Treatment with HA significantly increased the rate of LEC migration in a dose dependent manner. CD44 was upregulated in canine LEC after being treated with 1.0mg/ml of HA. RHAMM was slightly upregulated in canine LEC after being treated with 1.0mg/ml of HA. Viscoelastics containing HA promoted LEC migration and proliferation *in vitro*. The introduction of exogenous HA in the form of viscoelastics during cataract surgery may promote LEC migration and contribute to PCO formation *in vivo*.

I would like to dedicate this thesis to my three best friends, my mother, my father, and my loving wife, Jayme. My mother has taught me that through patience and dedication, all things are possible. My father has taught me that through hard work and diligence, all things are possible. My Jayme has taught me that through love and perseverance, all things are possible.

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CHAPTER 1

INTRODUCTION

The normal crystalline lens is isolated by a basement membrane, the lens capsule. The lens capsule is formed by a single layer of cuboidal lens epithelial cells (LEC) that lines the interior portion of the anterior lens capsule. The lens is composed of fibers that are arranged as a central nucleus and a peripheral cortex. Cataracts can be defined as an opacity to the crystalline lens[1-2]. Cataracts have numerous etiologies and these may vary by age and species. Nutritional cataracts can develop secondary to many causes; some examples of this include feeding puppies a diet that consists of a milk replacer deficient in arginine[3], or raising marsupials on cow's milk[4]. Cow's milk has a high content of lactose and marsupials lack galactokinase and transferase, which are necessary to metabolize galactose. This then leads to cataract formation. Various species have been shown to develop cataracts secondary to irradiation[5-6]. Trauma to the lens, both a sharp trauma as well as blunt trauma, can also stimulate cataractogenesis in a variety of species[2], various pharmaceuticals given to various species have shown be cataractogenic[7] and congenital or inherited cataracts have been well-documented in a variety of species[8]. Metabolic causes of cataractogenesis include hyperglycemia secondary to diabetes

mellitus in dogs and humans[9-10], hypocalcemia in dogs, as well as hypercupremia in humans[2]. Toxins have also been found to initiate cataracts[2], as have cataracts resulting from senility[2], cataracts resulting from electric shock[2], retinal atrophy[2], secondary to uveitis[2] and secondary to enzymatic abnormalities[11].

Cataract surgery is currently the only accepted effective treatment for cataracts and related vision loss and is the current standard of care in both animals and humans. Extracapsular cataract extraction utilizing phacoemulsification with intraocular lens (IOL) implantation currently holds a success rate of greater than 90% with long term follow-up[12] and is the most commonly performed ophthalmic surgical procedure worldwide[13]. During cataract surgery, part of the anterior lens capsule is removed by a capsulectomy. The lens fibers are removed via phacoemulsification, and the residual LECs may be removed by irrigation/aspiration, also known as polishing, prior to IOL implantation. During cataract surgery, viscoelastic materials are used intraoperatively to maintain the anterior chamber as well as to inflate the lens capsule prior to IOL implantation. Viscoelastics protect the corneal endothelial cells, maintain the anterior chamber, provide space, manipulate tissues and facilitate the ability to insert an IOL into the capsular bag. Quality of life following cataract surgery is markedly improved due to restored vision since the lens opacity has been removed, as reported by humans who have had cataract surgery performed. There is also the perception, by owners, of a markedly improved quality of life in canine companions that have had cataract surgery performed.

Complications of cataract surgery in humans include capsule phimosis, corneal endothelial decompensation, residual lens material, cystoid macular edema, retinal detachment, glaucoma, incisional dehiscence, endophthalmitis, astigmatism, IOL decentration, and the most common postoperative complication, posterior capsule opacification (PCO)[14-15]. Following cataract surgery, residual LECs may migrate across the inner surface of the lens capsule, across the IOL optic, or they may migrate around the capsulotomy edge onto the outer surface of the capsule[16]. Aberrant movement of LECs onto the posterior capsule may create folds and wrinkles of the posterior capsule. Proliferation of cells on the posterior capsule creates PCO and decreases the patient's visual acuity[17]. Elschnig's pearls are one type of PCO that typically occurs near the equator of the lens capsule or on the posterior lens capsule and are LECs that have become a clear pearl shape[16]. Elschnig's pearls are likely formed by the germinal cells of the lens equator and histologically are very similar to subcapsular cataracts[16]. Another type of PCO that is commonly seen is the fibrosis-type[16], occurring when cells at the leading edge of the capsulotomy site undergo fibrous metaplasia[16].

Nishi and Nishi have shown that blood aqueous barrier breakdown occurs at the onset of cataract surgery; however, with phacoemulsification and IOL placement, residual post-operative LECs can lead to an additional inflammatory response[18]. After the anterior capsule is surgically disrupted, cell signaling takes place, and remaining LECs on the anterior and equatorial capsule undergo epithelial-mesenchymal transition (EMT)[19].

After EMT occurs, LECs migrate and proliferate and cause PCO[19], which in turn, causes vision impairment. Lens epithelial cells possess specialized phenotypic markers such as cytokeratin 5[20], PAX-6[21], FoxE3[21], and alpha-crystalline[21] and after cells undergo EMT, they will shed these epithelial characteristics and become more mesenchymal-like cells[22]. Lens epithelial cells that transform into mesenchymal-like cells have a flattened appearance and possess other phenotypic markers such as α –smooth muscle actin (α-SMA), which allows them to behave in a more invasive manner[23]. Lens epithelial cells also have the ability to synthesize prostaglandin E_2[24] from arachidonic acid and this synthesis is mediated by cyclooxygenase produced by EMT[25]. Cyclooxygenase-2 (COX-2) overexpression has recently been shown to increase proliferation and decrease apoptosis rates of certain cancer cells[26]. Recently, Chandler *et al.* found that normal canine LECs show minimal COX-2 immunoreactivity and cataractous LECs show diffuse cytoplasmic immunoreactivity to COX-2[27]. Chandler's study showed that COX-2 levels were increased in LECs undergoing EMT and that exogenous COX-2 inhibitors decreased LEC proliferation and increased LEC apoptosis *ex vivo*[27]. Cyclooxygenase-2 is a precursor to inflammation, therefore it is possible that inflamed eyes may have more PCO. Unfortunately, the use of diclofenac has not shown the ability to reduce PCO two years after cataract surgery in humans[28]. The degree of inflammation both pre and post-operatively in canine patients did not significantly influence PCO[29]. The process of PCO development is not completely understood for any species. The pathway to PCO

formation is complex and potentially offers numerous sites for possible inhibition chemically, pharmacologically, or mechanically. No single inhibition site will ultimately prevent PCO, however, a multifactorial approach to inhibition may prove to minimize of even prevent PCO.

Both types of PCO, the fibrosis-type and the Elschnig pearl-type, contribute to decreased vision by way of decreasing visual acuity, decreasing contrast sensitivity, increasing glare and decentering the IOL[30-31]. Posterior capsule opacification occurs in up to 50% of adult humans up to five years postoperatively[16,32-33], up to 70.8% of children under the age of one year up to 12 years post-operatively[34], and 100% of dogs within one year post-operatively[29]. In addition, Mootha et al revealed that 23% of human patients experienced some form of PCO when examined six weeks postoperatively[32].

Hyaluronic acid (HA) is a glycosaminoglycan composed of glucuronic acid and N-acetylglucosamine[35]. Hyaluronic acid is commonly found in connective tissues of the body such as the skin, as well as being found in synovial fluid, the vitreous, the umbilical cord, and the aqueous humor[36-37]. Hyaluronic acid has the ability to influence both normal and abnormal cells through signaling pathways resulting in cell proliferation and differentiation[37].

The biological functions of HA are essentially mediated by cell surface HA receptors, including CD44 and the receptor for HA mediated motility (RHAMM). The CD44 ectodomain will bind with HA, and once HA is bound to CD44, CD44 can then

interact with other signaling molecules. Occasionally, interaction between CD44 and HA can influence a particular cell's behavior, most commonly through induction of motility and proliferation[38]. Using immunohistochemistry, Nishi *et al.*[39] and Saika *et al.*[40] determined that normal human LECs expressed reactivity to CD44 and human patients after cataract surgery expressed immunoreactivity to CD44; however, CD44 expression is unknown in the canine lens.

RHAMM is a protein that can be found in most tissues both intracellularly and extracellularly[41]. RHAMM binds HA to the cell and HA can then promote reorganization of the cytoskeleton to help arrange actin filaments and promote the adhesion of cell to the cell matrix. Hyaluronic acid can initiate cell locomotion through binding with RHAMM, which then induces a protein tyrosine kinase pathway[42]. To date, expression of RHAMM has not been examined in the human or canine lens. The contribution of the two types of HA receptors (CD44 and RHAMM) and the intracellular signaling pathways involved in HA-mediated effects on LECs remain unknown. It is well established in other tissue types that HA will bind with cell receptors CD44 and RHAMM and increase cellular migration and proliferation[38,43-44]. Hyaluronic acid interactions with CD44 and RHAMM have previously been shown to induce EMT in other cell types such as meningiomas[44].

In patients with breast and ovarian cancer, the level of HA within the tumor can be a means of predicting malignancy[43]. Nykopp *et al.* evaluated ovarian tissue biopsies and found that there was a significant higher levels of HA in patients with malignant

ovarian tumors as compared to benign tumors and normal ovaries[45]. In addition, a study performed by Corte *et al.* showed that patients with invasive ductal carcinoma exhibited increased immunoreactivity against HA compared to patients with carcinoma *in situ*[46]. These two studies show that there are increasing levels of HA associated with aggressive ovarian and breast cancer and HA content within these tumors could be a predictor for survivability. It is possible that the HA within these tumors may bind to cell surface receptors CD44 as well as RHAMM leading to enhanced cellular proliferation as well as migration, thereby making the tumor more aggressive within the host.

In intraocular surgery, viscoelastic materials are used to maintain the anterior chamber and lens capsule and HA is a common component of many viscoelastics. In 1958, HA was known to exist in the vitreous as well as the joint fluid. It was proposed at that time that an inert intraocular compound could be created utilizing HA to aid in surgery[47]. Hyaluronic acid was obtained and further purified from both human umbilical cords and rooster coombs where it is found in the high concentrations[47] and can be synthesized by eukaryotic organisms as well as simple bacteria[48]. After purifying and solidifying HA with high molecular weight, a viscoelastic was created[47]. In the early 1980's there were many publications based on the benefits of using Healon®, the first commercially available viscoelastic[49-50]. These beneficial properties included protection of intraocular tissues, maintenance of space for tissue manipulation during IOL implantation, and coating of the corneal endothelial cells[47]. There are various forms of

commercial viscoelastics available to the surgeon and these are generally separated by their rheologic properties into cohesive or dispersive viscoelastics[47]. Cohesive viscoelastics have a high viscosity and molecular weight whereas dispersive viscoelastics have a low viscosity and molecular weight. Viscoelastics are typically created by utilizing various concentrations of HA, hydroxypropylmethylcellulose, or chondroitin sulfate[51]. Viscoelastics that are cohesive are typically used to inflate the anterior chamber and lens capsule as well as to dilate the pupil[51]. These viscoelastics are typically easier to remove from the eye at the conclusion of surgery. Viscoelastics that are dispersive are typically used to coat the endothelium and are generally more difficult to remove from the eye at the conclusion of surgery[51].

The introduction of exogenous HA in the form of viscoelastics during cataract surgery may promote LEC migration and proliferation, and subsequently, PCO formation. This study was performed to evaluate if canine LECs are capable of responding to HA signaling through CD44 and RHAMM as well as to evaluate if LEC migration and proliferation increased using viscoelastics with higher HA content.

CHAPTER 2

MATERIALS & METHODS

Samples

Normal eyes were obtained by enucleation from dogs in good general health that were humanely euthanized at a local animal shelter for population control purposes. All dogs used in this study were estimated to be between one and eight years of age, based on dentition and thickness of the anterior lens capsule. Globes were collected within one hour of death and placed in dilute betadine solution until dissection. Normal lenses and lens capsules were obtained by dissection and placed in 10% neutral-buffered formalin.

Cataractous lens capsules were obtained from dogs undergoing elective cataract surgery at The Ohio State University College of Veterinary Medicine. These capsules were harvested from immature, mature, and hypermature cataracts. The most common etiologies for the cataracts were diabetes mellitus and cataracts presumed to be inherited. These capsules were then used for immunohistochemistry, western blots, and qualitative real-time polymerase chain reaction (qRT-PCR).

Positive Controls for CD44 and RHAMM

A total of five paraffin-embedded neoplasms from dogs were utilized in this study for CD44 immunohistochemical positive controls. One canine squamous cell carcinoma, one canine complex mammary adenoma, one canine mammary carcinoma, one canine mixed mammary tumor, and one canine cutaneous hemangiosarcoma. To confirm RHAMM immunoreactivity in canine tissue, four paraffin-embedded neoplasms (sertoli cell tumor, and three squamous cell carcinomas) and three normal testicles were utilized. Control samples were obtained from the Department of Veterinary Biosciences, at The Ohio State University and were diagnosed by various board-certified veterinary pathologists.

Immunohistochemistry of CD44 and RHAMM

The standard avidin-biotin-peroxidase complex (ABC) technique was used with diaminobenzidine (DAKO, Carpinteria, CA) as the chromagen and Mayers hematoxylin (Signet Laboratories, Dedham, MA) as the counterstain. Paraffin-embedded samples were sectioned (5um) and placed on Probe-On Plus slides (Fisher Scientific, Pittsburgh, PA). Ten cataractous lens capsules and ten normal lens capsules were evaluated for the presence of CD44. Sixteen cataractous lens capsules and ten normal lens capsules were evaluated for the presence of RHAMM. Slides were decerated in xylenes, rehydrated through a graded series of alcohols, and rinsed in phosphate-buffered saline (PBS). Slides were incubated with peroxidase block (DAKO) for ten minutes at room temperature,

washed in PBS, and then incubated with a protein block (DAKO) for twenty minutes at room temperature. Sections were incubated for one hour at 37° C in a humidified chamber with one of the following primary antibodies diluted to the following ratios in antibody diluent (DAKO): CD44 (Santa Cruz Biotechnology, INC., Santa Cruz, CA) 1:400 or RHAMM (Santa Cruz Biotechnology, INC.) 1:50. Sections were then rinsed in PBS and incubated with anti-rat secondary antibody (Biogenex, San Ramon, CA) solution for thirty minutes at room temperature. Slides were washed a final time in 1X PBS before incubation with the chromagen and counterstain. Slides were washed again with tap water until clear and then dehydrated. Slides were gradually passed through 70%, 85%, 100% ethanol until a final two-minute incubation in xylenes. Slides were then coversliped with Permount mounting solution. Following staining, the slides were evaluated and assessed for staining intensity and location.

Antibody specificity was previously confirmed with cellular lysate from canine corneal epithelium and canine Madin-Darby Canine Kidney cells using western blot analysis. Western blots were then used to evaluate the expression of CD44 and RHAMM in normal and cataractous LEC (Figs. 2a, 3a).

qRT-PCR of Normal and Cataractous Lens for CD44 and RHAMM

RNA was extracted from normal and cataractous anterior lens capsule samples according to the suggested protocol using Absolutely RNA Microprep Kit (Stratagene, La

Jolla, CA). The ImPromII Reverse Transcriptase kit (Promega, Madison, WI) was used to synthesize the first strand of cDNA. Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using the Mx3000p Multiplex Quantitation System (Stratagene) as follows: 95° C for fifteen minutes, then forty cycles at 94° C for thirty seconds, 60° C for thirty seconds, and 70° C for thirty seconds, using the QuantiTect SYBR Green PCR kit (Stratagene). Primers to amplify sequences of CD44 and RHAMM were designed based on previously published sequence data. Primers to amplify the sequence for glyceraldehydes-3-phosphate dehydrogenase (GAPDH; housekeeping control) were based on previously published sequence data. Primers employed were:

CD44 Forward: 5'-TATTGCTTCAATGCTTCAGCTCCA-3'

CD44 Reverse: 5'-AGGTTGTGTTTGCTCCACCTTCTTGAC-3'

RHAMM Forward: 5′-TCTGCACTTTTCTCAGCCCTGGT-3′

RHAMM Reverse: 5′-TGC TGCTCACAGTCAAGCCACT-3′

GAPDH forward: 5′-GCCGTGGAATTTGCCGT-3'

GAPDH reverse: 5′-GCCATAAATGACCCCTTCAT-3'

All samples were run in duplicate three separate times. The threshold cycle value was calculated for each sample by the instrument software. The relative amount of CD44, RHAMM, and GAPDH mRNA was calculated using the LinRegPCR software (v. 11.x;

JM Ruijter, S van der Velden, A Ilgun, Amsterdam, the Netherlands). The results were expressed as the ratio of the target gene (CD44 or RHAMM) to the HPRT housekeeping gene. An unpaired t-test was used to analyze the difference in mRNA between normal and cataractous lenses.

qRT-PCR of Normal Lens Epithelial Cells Treated with Varying Concentrations of Hyaluronic Acid

Cultured anterior lens epithelial cells were treated with vehicle only, 0.02 mg/ml of HA, or 1.0 mg/ml of HA and allowed to incubate. RNA was extracted from normal anterior lens capsule samples according to the suggested protocol using Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA). Quantitative RT-PCR was performed as described above. The results were expressed as the ratio of the target gene (CD44 or RHAMM) to the GAPDH housekeeping gene. The differences in mRNA expression after wound healing were evaluated using a one-way ANOVA and Tukey's adjustment for multiple comparisons.

Primary canine lens epithelial cell cultures

Anterior lens capsules with adherent LEC were incubated in trypsin (0.25% trypsin and 1X EDTA, Gibco, Carlsbad, CA) 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% CO_2. LEC were grown until 90% confluence prior to re-plating.

Scratch model to induce epithelial-mesenchymal transition

Lens epithelial cells in each well were allowed to grow to 90% confluence in unsupplemented DMEM in a twelve-well laminin coated culture dish. A vertical one-mm scratch was then made on the cellular surface. Cells were then treated with unsupplemented DMEM, 0.012 mg/mL of HA, 0.02 mg/mL of HA, and 1 mg/mL of HA (n=6) and allowed to recover for sixteen hours. The HA (Sigma) was prepared in unsupplemented DMEM. Digital images were taken immediately after the initial scratch was made and sixteen hours later. ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA) was then used to quantify the area of the scratch before and after the scratch was performed. A one-way Analysis of Variance with a Bonferroni Multiple Comparison post-hoc test was performed to evaluate differences in the area of the scratch devoid of cells between the four treatment groups both at time zero and at time sixteen hours.

Mock Cataract Surgery

A stab incision was made into the anterior chamber avoiding the lens (n=24). The cornea was completely removed with Steven's tenotomy scissors and the iris was removed using Vannas scissors. An anterior capsulorhexis was performed using Vannas scissors exposing the lens nucleus and cortex. The lens nucleus and cortex were removed using a lens loop. Residual LECs 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 or posterior lens capsule. For the control group (n=6), the lens capsule was irrigated a second time using PBS and the PBS was allowed to remain in the capsule for five minutes. The remaining eyes were then divided into three treatment groups (n=6 per group) and treated with 2% hyaluronic acid viscoelastic (contains 20 mg/mL HA; Acri.Vet, Hennigsdorf, Germany), 1.2% hyaluronic viscoelastic (contains 12 mg/mL HA; Acri.Vet), or hydroxypropylmethylcellulose (HPMC) viscoelastic (contains 0 mg/mL HA; Acri.Vet,). To simulate the exposure during cataract surgery, PBS (control) as well as the various viscoelastic agents were left in the lens capsule for five minutes. The capsular bag was then irrigated and aspirated with PBS to ensure removal of the viscoelastic material. Capsules were then removed from their zonular and vitreal attachments with Vannas scissors and placed in cell culture dishes with five milliliters of unsupplemented DMEM. The plates were then placed in a 37° C / 5% carbon dioxide incubator. Phase contrast photomicrographs were taken of the capsules immediately

following mock cataract surgery, and every twenty-four hours after the treatment until 72 hours after treatment to monitor LEC migration onto the posterior capsule. The lens capsule was then placed in formalin and submitted for histological processing and staining with hematoxylin and eosin. Lens epithelial cells were then manually counted from the anterior and posterior capsule from four different five-micron sections (Fig. 1). A Kruskal-Wallis one-way analysis of variance was performed with a Dunn's Multiple Comparison post-hoc test to evaluate the LEC counts of each treatment group.

CHAPTER 3

RESULTS

Immunohistochemistry of CD44 and RHAMM

All normal canine LEC exhibited minimal to no detectable expression of CD44 (Fig. 2b). All ten cataractous lens capsules that were evaluated were positive for CD44 expression (Fig. 2c). Ten normal lens capsules were evaluated for RHAMM expression and of these, eight were faintly positive for RHAMM expression (Fig. 3b) and two were negative. Ten cataractous lens capsules were evaluated for RHAMM expression and all were positive; staining intensity varied from faint to moderate (Fig. 3c). All LECs that were positive for CD44 and RHAMM staining exhibited cytoplasmic staining, as observed in our positive controls.

qRT-PCR of Normal and Cataractous Lens for CD44 and RHAMM

Both cataractous and normal LEC were evaluated for CD44 or RHAMM mRNA expression. Normal canine LEC had significantly lower expression of CD44 mRNA compared to cataractous LEC ($p<0.01$) (Fig. 4). In contrast, while normal LEC had reduced expression of RHAMM mRNA compared to cataractous LEC, the difference was not considered significant (Fig. 4).

qRT-PCR of Normal Lens Epithelial Cells Treated with Varying Concentrations of Hyaluronic Acid for CD44 and RHAMM

Normal LECs were evaluated for CD44 and RHAMM mRNA expression after being treated with vehicle only, 0.02 mg/ml of HA or 1.0 mg/ml of HA. CD44 mRNA expression significantly increased ($p<0.01$) in LECs treated with 1.0 mg/ml of HA as compared to control or 0.02 mg/ml of HA (Fig. 5).

Scratch model to evaluate lens epithelial cell migration and proliferation

Lens epithelial cell cultures were evaluated immediately after creation of a scratch (referred to as time zero) (Figs. 6a, 6c, 6e, 6g) and sixteen hours after creating the scratch (Figs. 6b, 6d, 6f, 6h). All time zero cultures compared with time sixteen hours within the same treatment group showed a statistically significant difference between the area devoid of cells, indicating cellular migration and proliferation occurred as expected in all treatment groups. There was a significant decrease ($p<0.001$) in the area devoid of cells at time sixteen hours in cells treated with 1.0 mg/ml of HA compared to the area devoid of cells at time sixteen hours in cells treated with DMEM only (Fig. 7).

Mock Cataract Surgery

Capsules were evaluated by phase contrast microscopy to determine the extent of migration and proliferation of LECs from the anterior capsule to the posterior lens capsule. At time zero, LECs were found only on the anterior lens capsule of all treatment groups (Figs. 8a, 8c, 8e, 8f). After 72 hours, cells from the control group (PBS only) were consistently found only on the anterior lens capsule (Fig. 8b). Migration of cells in the HPMC group was limited, similar to the control group, as LECs consistently remained only on the anterior lens capsule after 72 hours (Fig. 8d). Cells from the 1.2% HA treated group showed minimal migration onto the posterior lens capsule; however, the LECs did not cross the midline, or into the visual axis, on the posterior lens capsule (Fig. 8f). Cells from the 2.0% HA treated group showed extensive migration onto the posterior lens capsule and frequently migrated beyond the posterior capsule midpoint into the region of the visual axis, achieving 90% confluence on the posterior capsule by 72 hours (Fig. 8h). Capsules treated with 2% (20 mg/ml HA) HA viscoelastic showed significantly more LECs compared to capsules treated with PBS ($p=0.0109$) (Fig. 9).

CHAPTER 4

DISCUSSION

Posterior capsule opacification is seen in up to 50% of adult humans up to five years postoperatively[16,32-33], up to 70.8% of children under the age of one year old postoperatively[34] and up to 100% of dogs within one year post-operatively[29]. Surgical techniques and medical modalities need to be further assessed to ascertain a way to decrease or prevent PCO, which can be threatening to vision. Posterior capsule opacification, the most common post-operative cataract complication is primarily caused by residual LECs[16]. Davidson *et al.* reported that LECs remained in the capsule post-operatively using techniques such as ECCE, phacoemulsification, and phacoemulsification with vacuuming of LECs from the capsule. Immediately post-operatively, the initial cell concentration on the lens capsules were 31.6 +/- 19.3%, 16.1 +/- 8.9%, and 7.7 +/- 5.7% for groups ECCE, phacoemulsification only, and phacoemulsification with vacuuming, respectively[52]. Days until LECs reached confluence was 15.6 +/- 2.7, 17.0 +/- 3.4, and 22.33 +/- 3.7 for groups ECCE, phacoemulsification only, and phacoemulsification with vacuuming, respectively. These rates are different from our study, where capsules treated with 2.0% HA reached confluence within 72

hours. The capsules treated with DMEM, HPMC, and 1.2% HA did not reach confluence within that 72-hour period.

Disrupting the anterior LECs by creating a capsulorhexis has been shown to induce LEC metaplasia as well as promote LEC migration and proliferation[19]. The LECs are then thought to undergo EMT and become either the fibrosis-type of PCO, or the pearl type of PCO[16]. A study by Nishi *et al.*, showed remaining LECs after ECCE promote inflammation[18] and there are studies that have shown that increased levels of uveitis post-operatively contribute to PCO[53]; however, other reports suggest that topical corticosteroid or non-steroidal therapy is ineffective in inhibiting PCO[28,54]. Potentially, corticosteroids and NSAIDs are ineffective at prohibiting PCO because HA has already bound to CD44 on the LECs and LECs have already begun to migrate and proliferate.

Proteins of the TGF-β family are cytokines that can be found in the lens[55] as well as in the aqueous humor[56]. The three isoforms of TGF-β found in the lens and lens capsule are TGF-β1, TGF-β2, and TGF-β3[55,57]. In cataract surgery, disruption of the anterior lens capsule simulates LECs. Saika *et al.* reported that following experimental injury to the lens capsule of the mouse, TGF-β2 levels increased as compared to uninjured lens capsules[58]. TGF-β2 can induce LECs to transform into myofibroblast-like cells, which helps to create extracellular matrix (ECM) and regulate cell proliferation[59]. The creation of ECM is a prominent step in the wound healing process[60,61] and the most

prominent form of ECM found histologically in PCO contains collagen types I, III, and IV[62]. Following trauma, such as a creation of a capsulorhexis or cataract surgery, the cuboidal LECs undergo EMT and interact with the ECM. The ECM then allows the fibroblastic cells to migrate and proliferate as well and also creates folds and wrinkles on the posterior capsule. The folds, wrinkles, and accumulation of cells on the posterior capsule all contribute to PCO[16]. Growth factors such as insulin-like growth factor, epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor (FGF) have all been shown to induce cell proliferation in various tissue types. Increased expression of FGF has also been found in LECs after injury *in vitro* and *in vivo*[63,64], thus encouraging cells to continue to proliferate rather than undergo apoptosis[64]. Lens epithelial cells have also been shown to produce hepatocyte growth factor (HGF)[65]. *In vitro* studies have shown that when cultured LECs are treated with exogenous HGF, they continued to proliferate, thereby contributing to PCO[65]. *In vitro* studies in humans, rabbits, and cows have shown that following EMT, there is positive immunoreactivity to α-SMA in LECs[66-67]. Alpha-SMA expression is characteristic of myofibroblasts which have the ability to contract, causing wrinkles or folds in the lens capsule. These wrinkles and folds increase glare, decrease visual acuity, and cause IOL decentration[17,67]. Previous reports by Colitz *et al.*and Chandler *et al.* identified cytokeratin, vimentin, α-SMA, TGF-β, fibronectin, lumican, Slug, Snail, and tenascin from canine LECs thereby proving that PCO and EMT in our canine patients is similar to PCO and subcapsular cataracts in

humans[27,68]. These studies indicate that by identifying positive immunoreactivity to proliferating cell nuclear antigen and α-SMA, these cells are actively proliferating and migrating[27]. Telomeres are genetic structures that protect the ends of chromosomes from degradation and telomerase is an enzyme that protects DNA from becoming unable to replicate[69]. Cataractous LECs have been shown to have a significant increase in telomerase activity when compared to normal LECs which may enable cataractous LECs to replicate without termination[70]. Recently, it was found that cataractous LECs exhibit an increased expression of phosphorylated Akt (pAkt)[71]. Phosphorylated Akt has been shown to interact with telomerase when cells undergo EMT[71]. Use of a pAkt inhibitor has been shown to decrease LECs *in vitro* in canine lens capsules in a dose dependent manner and this could potentially decrease PCO *in vivo*[71]. The pathway that leads to the formation of PCO is very complex and is associated with many molecular signaling pathways and thus offers many potential targets to inhibit or suppress PCO. No single pathway is responsible to PCO. Therefore, multiple inhibition sites will likely need to be explored to reduce PCO.

Since the first modern IOL was implanted by Ridley in 1949, IOLs have been the subject of intense research to help improve vision and decrease PCO. Many IOL designs have been studied to evaluate capabilities of reducing PCO. The IOL implanted by Ridley in 1949 did not completely fill or stretch the capsular bag, allowing LECs to migrate between the IOL and the capsule, resulting in PCO[16]. In the early 1980s, Hoffer designed

an IOL that had a distinct edge to the IOL, thus creating a "wall" where residual LECs could not penetrate underneath the IOL[16]. Other companies have manufactured IOLs with a posteriorly concave surface allowing the IOL to be vaulted against the posterior capsule. This would allow the posterior surface of the IOL to stay in contact with the posterior lens capsule, preventing LEC migration under that particular IOL[16]. Nagata *et al*. showed that the squared edge to the optic prevented PCO to a greater degree than a rounded degree[72] and Ursell *et al.* found that acrylic IOLs significantly reduced PCO pared to PMMA IOLs and silicone IOLs[73]. It is now known that the incidence of PCO is multifactorial, not solely dependent on the IOL implanted into the lens capsule[16]. Recently, Stone *et al.* found that implanting a capsular tension ring (CTR) with an IOL subjectively reduced PCO formation *in vitro.* Placement of a CTR stretched the capsule and allowed the posterior capsule to have greater contact with the posterior surface of the IOL[74]. Combining a CTR with a square edged optic IOL supports the barrier effect on LEC migration and proliferation and does not allow the cells to migrate under the IOL optic[74]. Kim *et al.* placed a CTR in human capsules *in vivo* and compared PCO of capsules implanted with a CTR against controls[75]. Capsules that had a CTR placed had significantly less PCO when evaluated up to 5 years post-operatively[75]. This supports the theory that PCO can be reduced mechanically with a CTR when combined with an acrylic lens, and reduced pharmacologically by utilizing lower concentrations of HA viscoelastic and removing this viscoelastic at the conclusion of surgery.

The effect of different IOL biomaterials on the presence and severity of PCO has also been studied. In a study by Gift *et al.*, 120 canine eyes received either a polymethyl-methacrylate (PMMA) IOL, a hydrophilic acrylic IOL, or a hydrophobic acrylic IOL and PCO presence and severity was evaluated[76]. Eyes that received a PMMA IOL showed significantly more PCO than the hydrophilic acrylic IOL but PCO was not clinically significantly different between the hydrophilic acrylic IOL and the hydrophobic acrylic IOL. An additional study of 180 eyes were evaluated in people implanted with either a heparin-surface PMMA lens, a hydrophobic acrylic lens, or a silicone lens[77]. Rates of capsulotomy performed by Nd:YAG laser were significantly increased in eyes receiving a heparin-surface PMMA IOL as compared to the silicone and acrylic implants at five years post-operation[77]. Intraocular lens design and biomaterial has been studied extensively in the prevention of PCO. Although hydrophilic acrylic IOLs, hydrophobic acrylic IOLs, and squared edged optics of the IOLs has shown to effectively reduce PCO, none of these measures have been able to eliminate PCO.

Many pharmacologic trials have been evaluated to assess efficacy of reducing PCO by removing LECs. These trials typically assess whether the pharmacologic agent inhibits LEC proliferation or induces apoptosis. Studies of agents that have evaluated inhibition of proliferation of LECs included utilizing cyclooxygenase-2[27], retinoic acid[78], mitomycin-c[79], cyclosporine[80], and daunomycin[81]. Studies of pharmacologic agents used to lyse LECs include distilled-deionized water[82], methotrexate[83], and 5-fluorouracil (5-

FU)[84]. Pot *et al.* recently showed that IOLs coated with selenocystamine and implanted into canine cadaver lens capsules had significantly less PCO than canine cadaver lens capsules that had either no IOL implanted or a control IOL implanted[85]. In addition, EDTA and trypsin have been studied to evaluate their ability to break the bonds between LECs and the capsule during hydrodissection in surgery[86]. This study showed that 15 mM and 30 mM of EDTA injected into human cadaver eyes had the ability to de-epithelialize the capsule but 2% trypsin caused proteolytic damage to the iris and also damage to the lens zonules. Although these pharmaceuticals may effectively lyse LEC or prevent proliferation, they have the ability to have toxic effects on the corneal endothelium, ciliary body, retina, and other intraocular structures. Recently, a sealed-capsule irrigation device has been developed that allows the surgeon to administer treatment to the lens capsule and remaining LECs without exposing the remaining intraocular structures, such as the corneal endothelium[87]. In a recent study by Kim *et al.*, the sealed-capsule irrigation device was used to deliver varying dosages of mitomycin-C or distilled water into rabbit lens capsules to lyse LECs[88]. This *in vitro* study showed that there was no damage that occurred to the corneal endothelium. In a recent study by Histed *et al.* a sealed-capsule irrigation device was trialed in six canine patients *in vivo*. In this study, one out of the six capsules had leakage of 5-FU into the anterior chamber thereby damaging the endothelium. Capsules treated with 5-FU showed subjectively less PCO than controls[89].

Many studies and techniques have been performed to either minimize or eliminate PCO. The current gold standard to treat PCO, in human medicine, is to utilize a Nd:YAG laser to create a posterior capsulotomy to improve visual acuity[90]. Utilizing an Nd:YAG laser to create a posterior capsulotomy is associated with potential complications such as malignant glaucoma[91], short-term intraocular pressure increase[92-93], glaucoma[92-93], retinal detachment[92-93], IOL decentration or damage[94-95], cystoid macular edema[92-93,95], and iris hemorrhage[95]. A study performed on normal canine cadaver eyes showed that higher laser energy levels are required to disrupt the lens capsule in dogs as compared to humans due to the thickness of the posterior lens capsule[96]. In addition, when these higher energy levels were used, there were many pits created in the IOL[96]. As a result, Nd:YAG capsulotomy does not appear to be clinically useful for canine PCO. Another technique utilized in cataract surgery to minimize PCO formation is to perform surgery as atraumatically as possible. In addition, by keeping intraoperative and postoperative inflammation to a minimum and by decreasing blood aqueous breakdown, the surgeon can potentially decrease PCO[97]. Hollick *et al.* showed that large capsulorhexes where the edge of the capsulorhexis was not in contact with the edge of the IOL developed more PCO within 1 year post-operatively compared to smaller capsulorhexes where the edge of the capsulorhexis was in contact with the IOL[98]. Other techniques used to attempt to decrease PCO include a primary posterior continuous curvilinear capsulorhexis with or

without an anterior vitrectomy[99], and as previously discussed, aggressive capsule polishing of the anterior and posterior capsule[52].

Hyaluronic acid is classified as a glycosaminoglycan, composed of glucuronic acid and N-acetylglucosamine[35]. Hyaluronic acid can be found in numerous tissues in the body, with the umbilical cord, the joint fluid, and the vitreous having the highest concentrations[36-37]. The aqueous humor also contains concentrations of HA[36]. However, during some cellular events such as wound repair, inflammation, or tissue invasion through cancerous processes, HA can interact with cells and influence their behavior by binding to cell surface receptors[37-38,43,100-101]. Toll-like receptors are utilized by the body's immune system to initiate a defense as well as to recognize microbes. Toll–like receptors can form a cascade of events utilizing proteins such as MyD88, IL-1 receptor-associated kinase-4 (IRAK 4), IRAK-1, TNF-associated factor 6, and TGF-β-activated kinase which then activates NF-κβ[102]. Lung epithelial cells have been prevented from undergoing apoptosis after being treated with HA and NF-κβ has been shown to have a role in this mechanism[102]. Potentially, this same mechanism plays a vital role in LECs after cataract surgery where the HA from the viscoelastic acts to protect the remaining LECs from apoptosis, thus enhancing the potential for proliferation; however, Toll-like receptors have yet to be found in LECs.

HA has the ability to bind to hyaladherins, which are proteins such as CD44 and RHAMM[102-103]. CD44 is an 85-kDa protein found on the cell surface and is the most

prominent hyaladherin[102,104]. Saika *et al.* were able to prove that HA and CD44 were present in the ECM of cataractous lens capsules in humans. In addition, it was shown that CD44 was immunolocalized in LECs and Saika's study concluded that CD44 and HA are involved in healing mechanisms, cell migration, and cell proliferation[40]. In our study, utilizing immunohistochemistry and western blots, we were able to demonstrate that normal LECs had minimal to no detectable expression of CD44 protein and that all of the cataractous LECs showed robust positive expression for CD44. In order to quantify CD44 mRNA expression in both cataractous and normal LECs, qRT-PCR was utilized. Although CD44 was found to be present in normal LECs, cataractous LECs showed a significant increase in mRNA when compared to normal LECs. This supports our hypothesis that CD44 is upregulated in LEC that have undergone EMT and may contribute to cell migration and proliferation *in vitro*, potentially enhancing PCO *in vivo*. Furthermore, LECs treated with 1.0 mg/ml of HA showed a significant increase in CD44 mRNA expression as compared to LECs treated with 0.2 mg/ml HA and or the vehicle only. This result provides evidence that CD44 is upregulated in canine LECs when treated with HA.

RHAMM, also classified as a hyaladherin, is found in many tissue types in the body, can be found in multiple areas within the cell, and is primarily associated with cellular motility[41]. Previous to this study, RHAMM expression had not been evaluated in the lens of any species. Hyaluronan-RHAMM interactions induce a series of cellular

cascades that promotes a cell to migrate. In our study, immunohistochemistry and western blot analysis were used to identify RHAMM in normal and cataractous LECs. We have shown that normal LECs were variably positive for expression of RHAMM and all of the cataractous LECs showed positive expression of RHAMM. In addition, by utilizing qRT-PCR, expression of RHAMM mRNA was found in normal LECs and was slightly increased in cataractous LECs. Although not statistically significant, there was a slight increase in RHAMM mRNA expression in LECs after being treated with 1.0 mg/ml of HA as compared to LECs treated with 0.2 mg/ml of HA, or LECs treated with the vehicle only. The expression of RHAMM in LECs and its ability to respond to HA may influence cellular motility *in vitro* and may slightly enhance PCO *in vivo* as compared to CD44.

Through the use of a scratch test model that has been previously described[27], we were able to monitor cellular migration and proliferation *in vitro*. There was a significant decrease in the area of the scratch devoid of cells between the LECs treated with 1.0 mg/mL of HA and the control group. Therefore, *in vitro*, LECs respond to HA by increasing the rate of migration and proliferation, and the response appears to be concentration dependent. Corneal wounds have also been proven to heal faster *in vitro* when treated with HA as compared to PBS and this happens in a concentration dependent manner[105-106]. Although LEC proliferation and migration was monitored through the

scratch test model, use of immunohistochemistry to further identify α-SMA in our LECs, would have further confirmed that LECs were migrating and proliferating.

A previous study by Davidson *et al.* has shown that *ex vivo* canine lens capsular sac explants provide an adequate model to evaluate PCO and LEC proliferation and migration in both canines as well as humans[107]. Using this model, viscoelastic with varying concentrations of HA were added to lens capsules to simulate *in vivo* surgery in our study. As previously discussed, viscoelastics are materials that can contain various concentrations of HA or no HA at all. This study employed HPMC, 1.2% HA, and 2% HA for the mock cataract surgery experiments. Capsules treated with 2.0% HA viscoelastic reached confluence on the posterior lens capsule 72 hours after treatment. Capsules treated with HPMC or PBS did not show any migration of LECs onto the posterior lens capsule at 72 hours and capsules treated with 1.2% HA viscoelastic showed minimal progression onto the posterior lens capsule at the same time point. Previous studies of *ex vivo* canine lens capsules have shown that LECs reached confluence on the posterior capsule in 10-14 days[27,85]. If our control groups were allowed more time, they would have likely continued to proliferate and migrate, ultimately resulting in confluence on the posterior capsule. The results of this study show that viscoelastics containing HA promote LEC migration and proliferation *in vitro* and this effect appears to be concentration dependent. A similar effect may occur *in vivo* and contribute to PCO formation. The surgeon should always keep in mind that by adding exogenous HA,

through use of HA-containing viscoelastics, migration and proliferation of any remaining LEC within the capsule may be potentiated.

This study elucidates another step in understanding the process of LEC migration and proliferation. It reveals future targets for pharmacologic intervention for the prevention of cell migration and proliferation and thus PCO. It is feasible that a pharmaceutical agent could be used to block cell surface receptors CD44 and RHAMM from binding to HA thereby hindering LECs from migrating and proliferating. The results of this study are not meant to discourage the use of viscoelastics that contain HA, but illustrate that concentration of the HA may be one factor for the surgeon to consider when selecting the appropriate viscoelastic. As well, the surgeon should remove the viscoelastic from the anterior chamber and capsular bag when possible.

Potential limitations of this study would include that the experiments were performed *in vitro.* Although laboratory conditions can be carefully controlled, they can never completely mimic what happens *in vivo*. Additionally, although the correct power calculations were utilized in the experimental model, increasing the sample size would inevitably increase the statistical power. In this study, all dogs were determined to be between one and eight years of age, however the size of the dog was not recorded. In a recent study by Bras *et. al.* although the age of the dog was not significant, younger dogs had a tendency to develop more PCO as compared to older dogs. In our study, we used dogs between the ages of one and eight years of age, but we did not follow the incidence

of PCO that correlated to those individual dogs. Future studies *in vivo* should evaluate PCO in different sizes of dogs, different ages of dogs, and type of IOL inserted in comparison to the intraoperative viscoelastic utilized.

CHAPTER 5

CONCLUSION

This study has proven that canine LECs possess the appropriate receptors, CD44 and RHAMM, to respond to HA signaling and that cataractous LECs show significant upregulation of CD44 and a slight upregulation of RHAMM. CD44 mRNA expression significantly increased ($p<0.01$) in LECs treated with 1.0 mg/ml of HA as compared to control or 0.02 mg/ml of HA. As with previous studies of the corneal epithelium[105-106], the canine LECs migrate and proliferate *in vitro* at a greater rate when treated with HA as compared to controls and this response to HA appears to be concentration dependent. Therefore, the lower the concentration of HA used *in vitro* showed a decreased rate of LEC migration and proliferation. Clinically, the surgeon should remove the viscoelastic after surgery when it is appropriate to do so. As well, the surgeon should pick the HA containing viscoelastic that is most appropriate for the surgical procedure being performed.

Future studies should include *in vivo* trials to assess if higher concentrations of HA significantly increase PCO in our clinical patients. Many factors *in vivo* such as age of the animal, pre-operative inflammation, post-operative inflammation, medications

administered pre-operatively, stage of cataract, and the etiology of the cataract may influence LEC migration and proliferation that cannot be mimicked in the laboratory setting. These factors may increase or decrease the degree of PCO post-operatively.

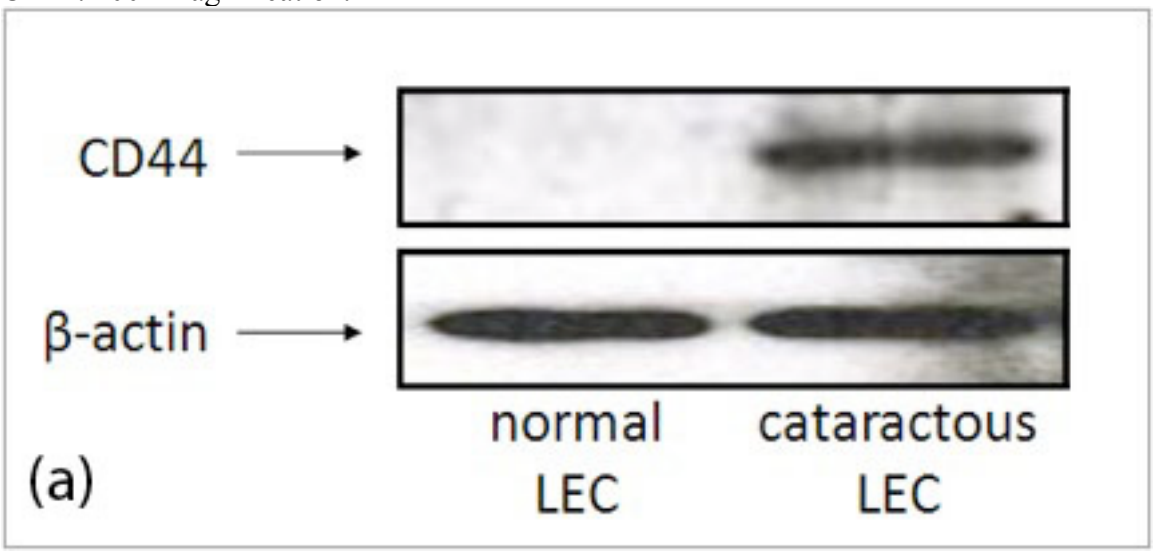
An additional study that should be performed is the application of an anti-CD44 antibody to the lens capsule prior to the administration of HA *in vitro* as well as *in vivo.* Recent studies have shown that the addition of anti-CD44 antibodies prevents adhesion of HA to CD44 and prohibits tumor metastasis[108]. Addition of an anti-CD44 antibody to the lens capsule prior to the addition of HA containing viscoelastic could potentially stop the migration and proliferation of LECs. If proven successful at preventing PCO, future studies should also include the analysis of toxicity of applying anti-CD44 antibodies intraocularly.

APPENDIX

THE EFFECTS OF HYALURONIC ACID ON LENS EPITHELIAL CELL MIGRATION *IN VITRO*: IMMUNOHISTOCHEMISTRY, WESTERN BLOTS, QRT-PCR, PHASE CONTRAST MICROSCOPY, & STATISTICAL ANALYSIS

Figure 1: Graphical representation – Four different five-micron planes of histologic sectioning of the lens capsule.

Figure 2: Evaluation of CD44. (a) Western Blot expression of CD44 of normal LECs and cataractous LECs. CD44 was not detected in normal LECs. (b) Immunohistochemistry of normal LECs showing no detectable cytoplasmic staining for CD44. 100x magnification. (c) Immunohistochemistry of cataractous LECs showing positive cytoplasmic staining of CD44. 100x magnification.



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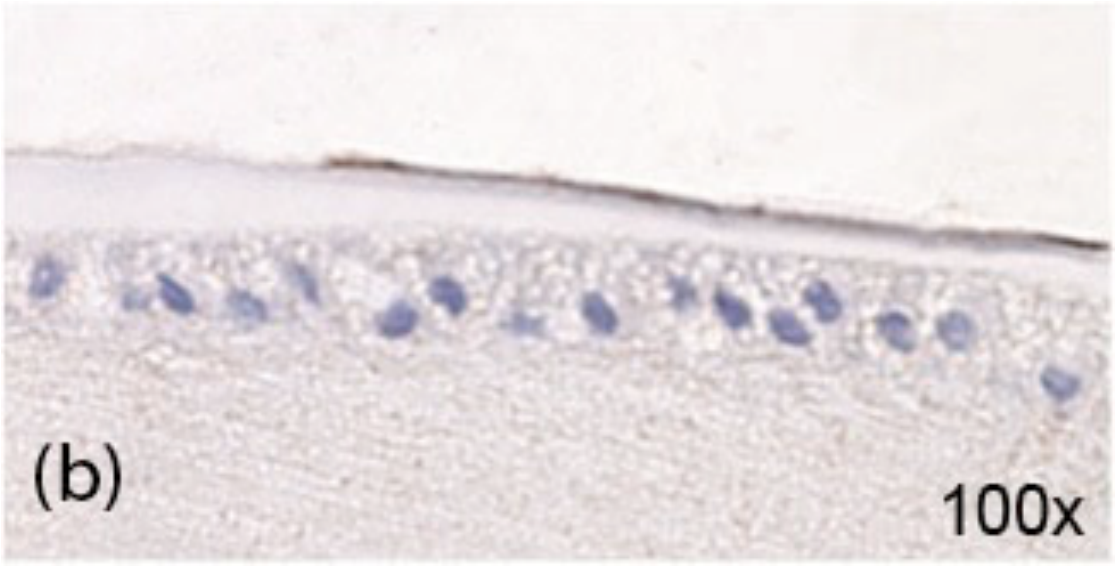

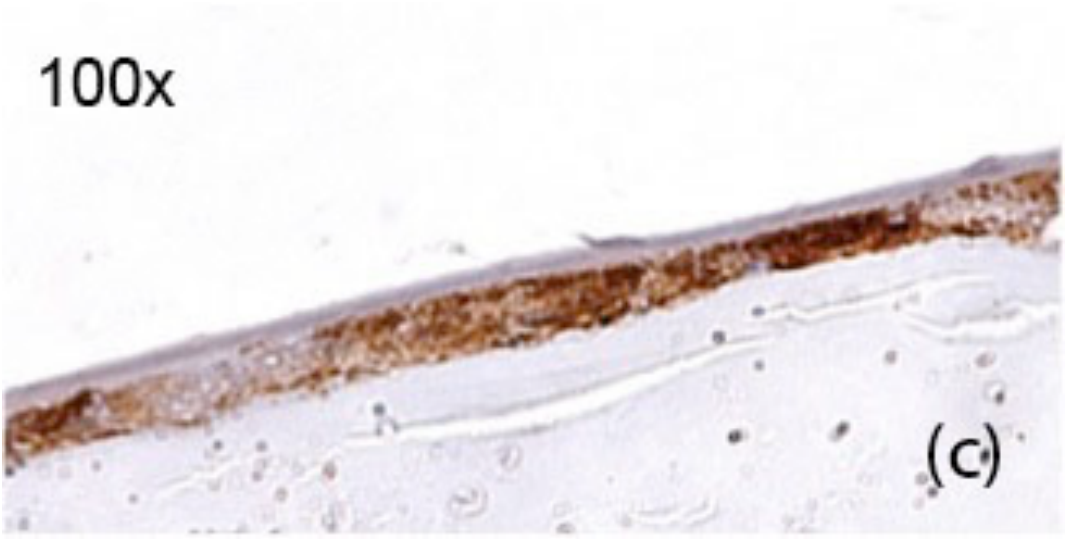

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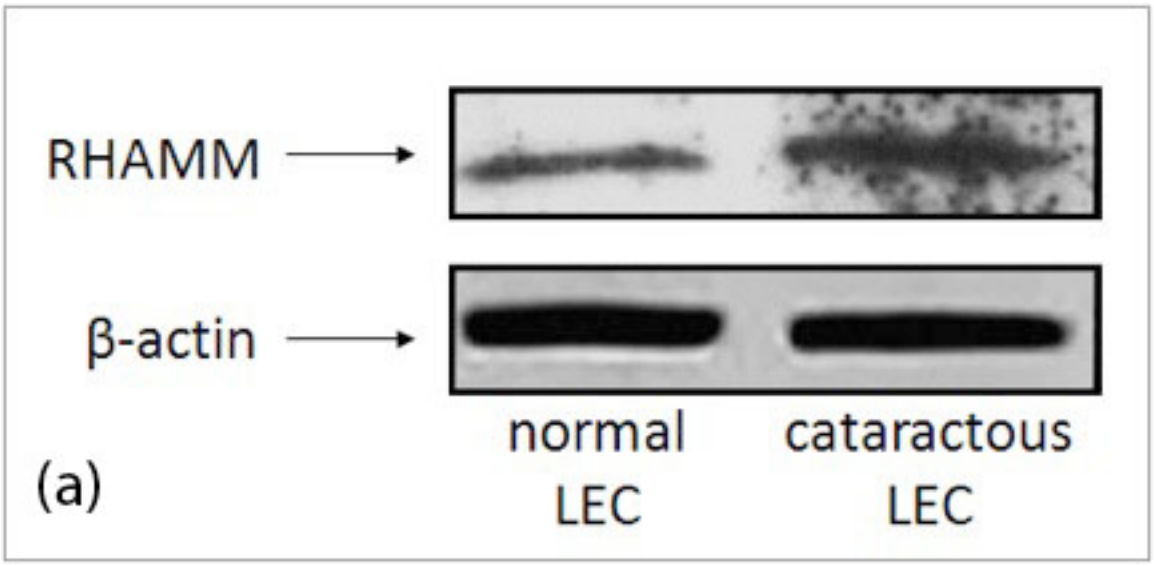
Figure 3. Evaluation of RHAMM. (a) Western Blot expression of RHAMM positive in normal and cataractous LECs. (b) Immunohistochemistry of normal LECs showing faint positive cytoplasmic staining for RHAMM. 100x magnification. (c) Immunohistochemistry of cataractous LECs showing positive cytoplasmic staining for RHAMM. 100x magnification.



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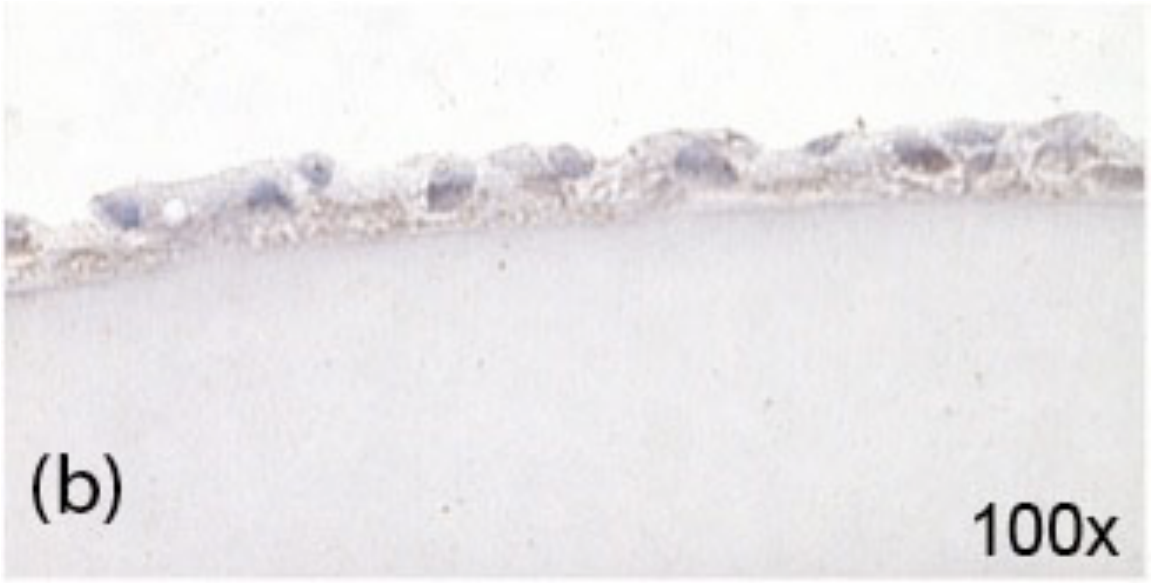


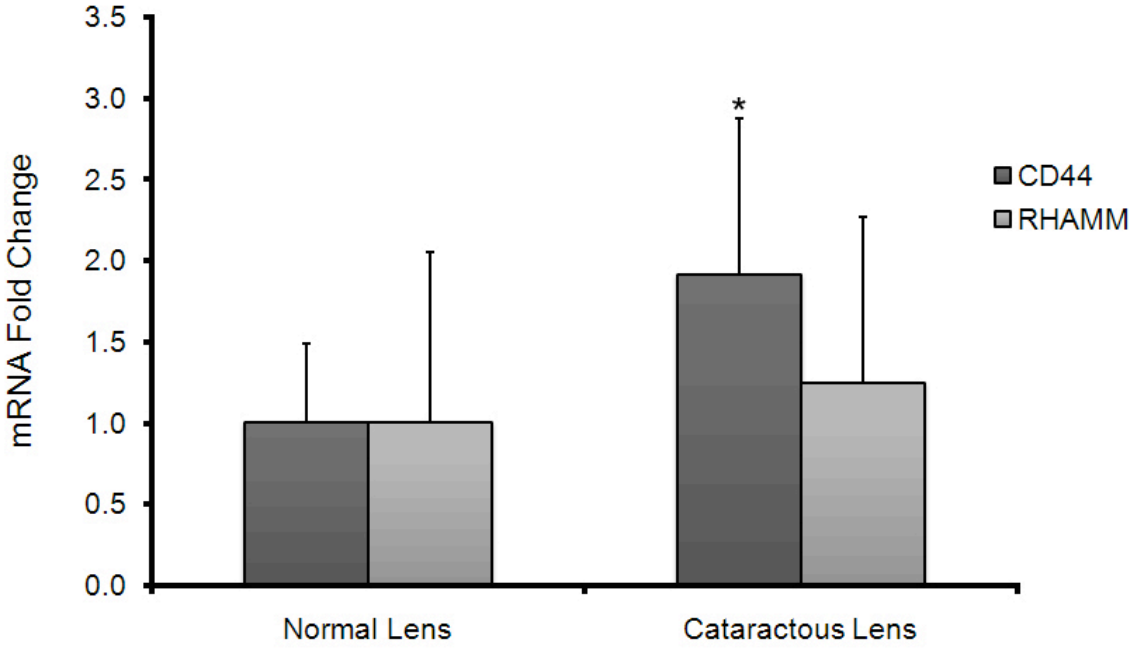


Figure 4. mRNA Expression of CD44 and RHAMM in Cataractous and Normal LECs - CD44 showed a statistically significant increase ($p<0.01$) in mRNA expression in cataractous lens epithelial cells as compared to normal lens epithelial cells.

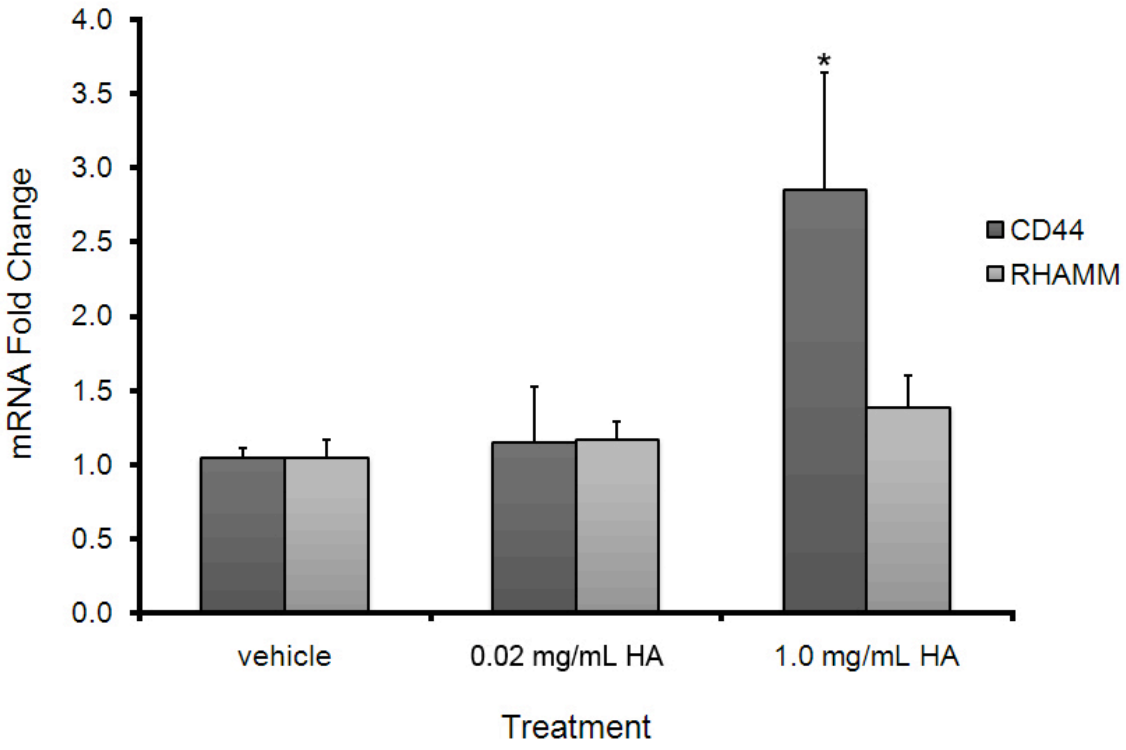
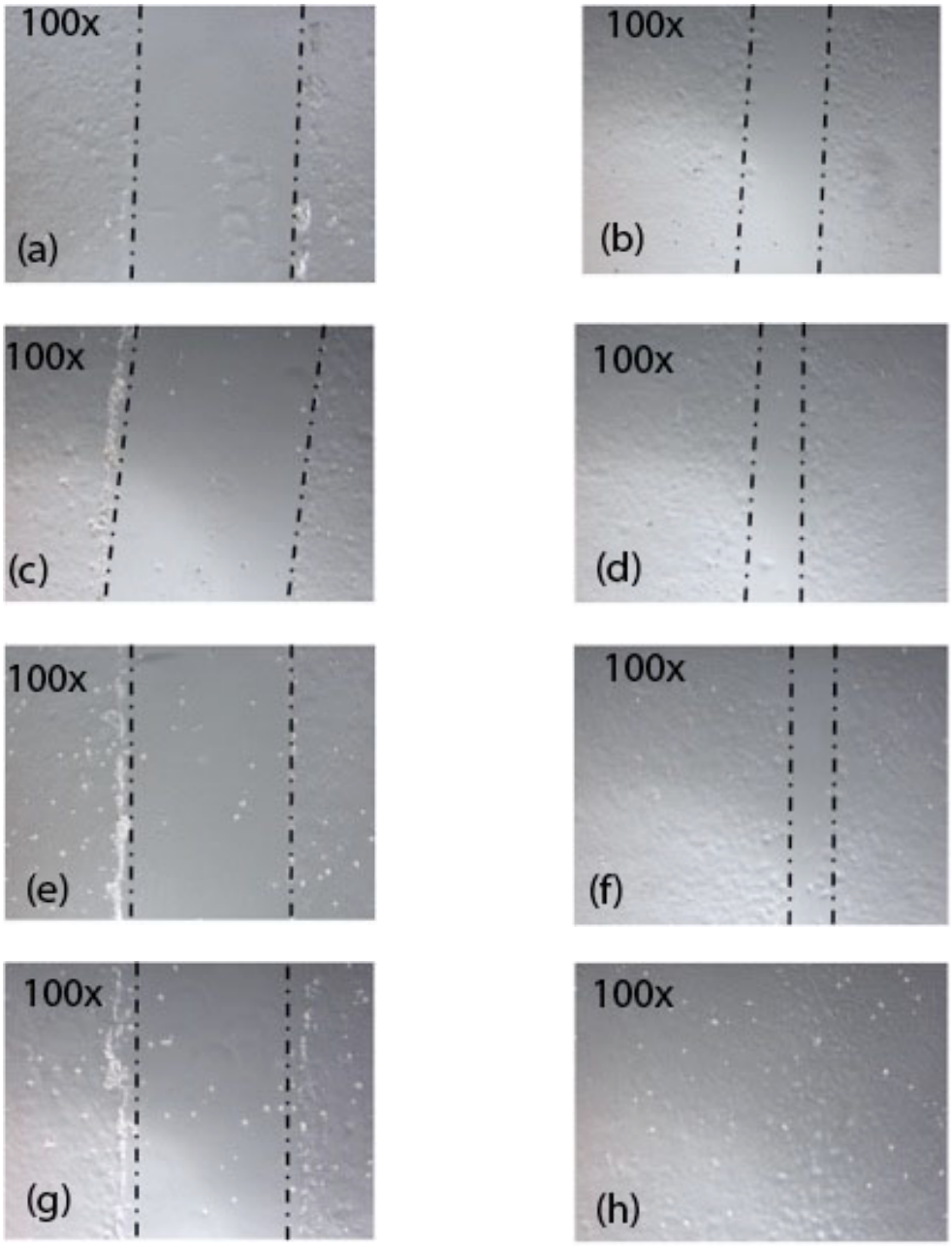


Figure 5. mRNA Expression of CD44 and RHAMM After HA Treatment - Expression of CD44 and RHAMM mRNA in normal LECs that were treated with vehicle only, 0.02 mg/ml of HA and 1.0 mg/ml for 24 hours. CD44 showed a statistically significant increase ($p<0.01$)) in mRNA expression in LECs treated with 1.0 mg/ml of HA as compared to vehicle only and 0.02 mg/ml of HA.

Figure 6. Phase Contrast Microscopy of cultured LECs after scratch test - Each time 0 hours has a 1mm bore scratch represented by the area devoid of cells between the black dashed lines. (a) Time 0 hours of LECs as a control treated with DMEM only. (b) Time 16 hours of LECs as a control treated with DMEM only. (c) Time 0 hours of LECs treated with 0.012 mg/ml of HA. (d) Time 16 hours of LECs treated with 0.012 mg/ml of HA. (e) Time 0 hours of LECs treated with 0.02 mg/ml of HA. (f) Time 16 hours of LECs treated with 0.02 mg/ml of HA. (g) Time 0 hours of LECs treated with 1.0 mg/ml of HA. (h) Time 16 hours of LECs treated with 1.0 mg/ml of HA. Notice that these cells reached confluence.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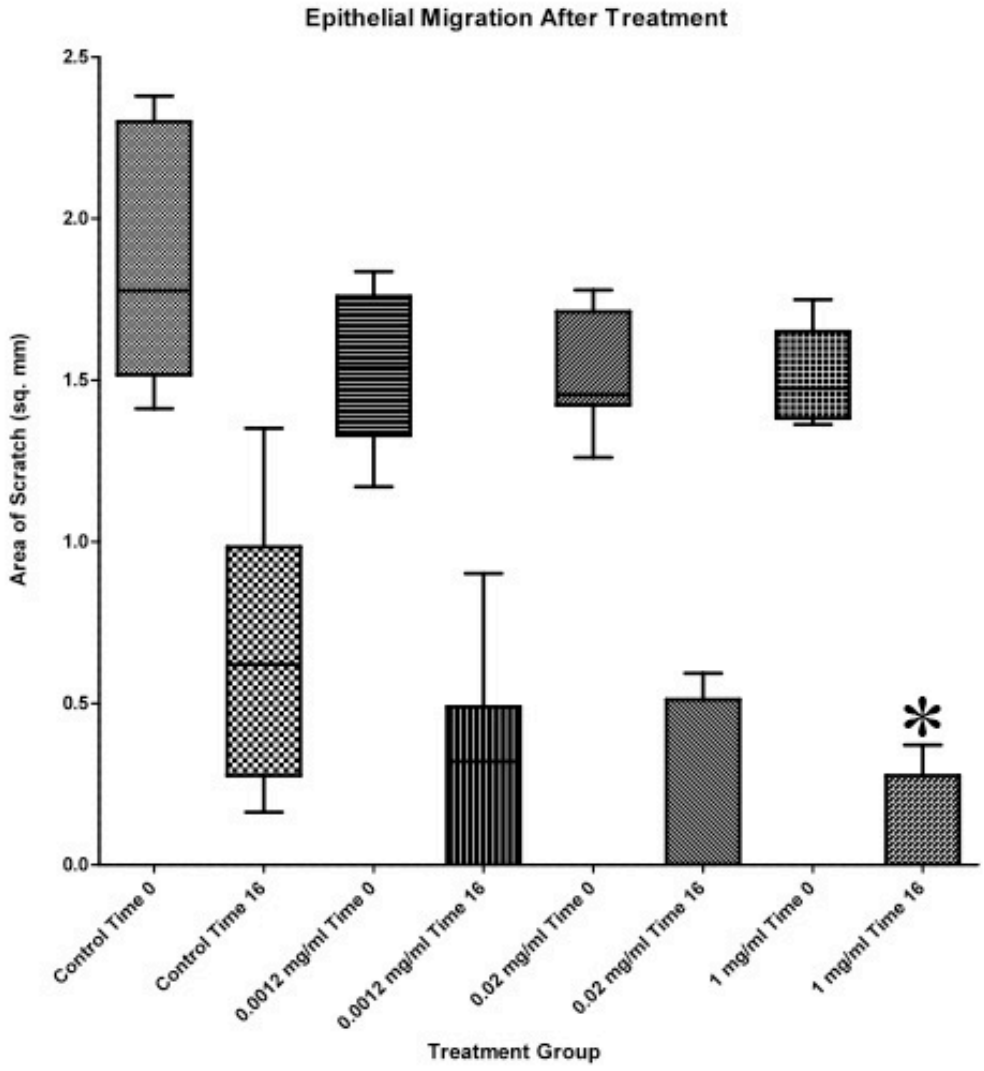
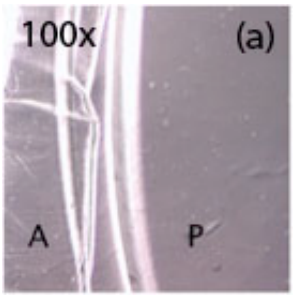


Figure 7. Area of Scratch Pre and Post Treatment - Area of scratch devoid LECs at each time point (hours) and the associated treatment group. Cells treated with 1.0 mg/ml of HA showed a statistically significant smaller area devoid of cells than the control group at 16 hours ($p<0.001$).

Figure 8. Phase Contrast Microscopy of LECs Following Mock Cataract Surgery – A and P represent the anterior and posterior capsule, respectively. The dashed red line indicates progression of LEC migration and proliferation on the posterior capsule. (a) Appearance of capsule treated with PBS 24 hours after surgery. (b) Appearance of capsule treated with PBS 72 hours after surgery. This capsule lacks posterior LEC migration and proliferation. (c) Appearance of capsule treated with HPMC 24 hours after surgery. (d) Appearance of capsule treated with HPMC 72 hours after surgery showing minimal posterior LEC migration and proliferation. (e) Appearance of capsule treated with 1.2% HA viscoelastic 24 hours after surgery. (f) Appearance of capsule treated with 1.2% HA viscoelastic 72 hours after surgery showing LECs migration and proliferation approaching the visual axis. (g) Appearance of capsule treated with 2.0% HA viscoelastic 24 hours after surgery. (h) Appearance of capsule treated with 2.0% viscoelastic 72 hours after surgery showing LEC migration and proliferation that have reached confluence.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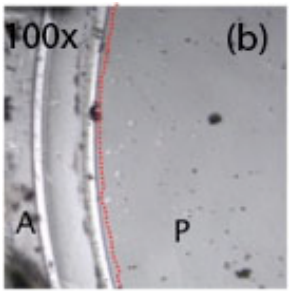
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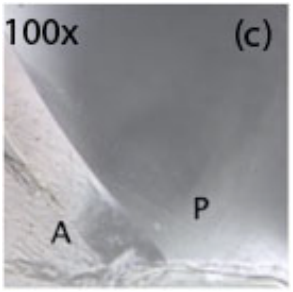
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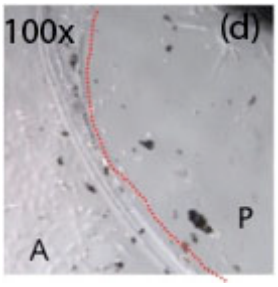
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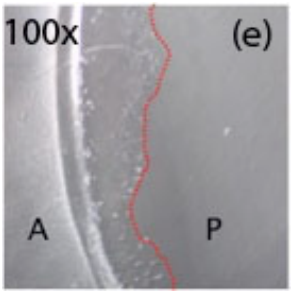
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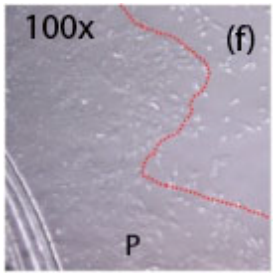
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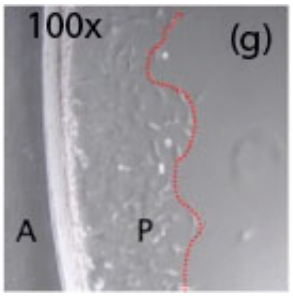
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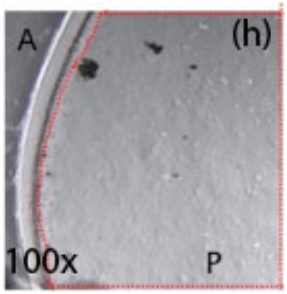
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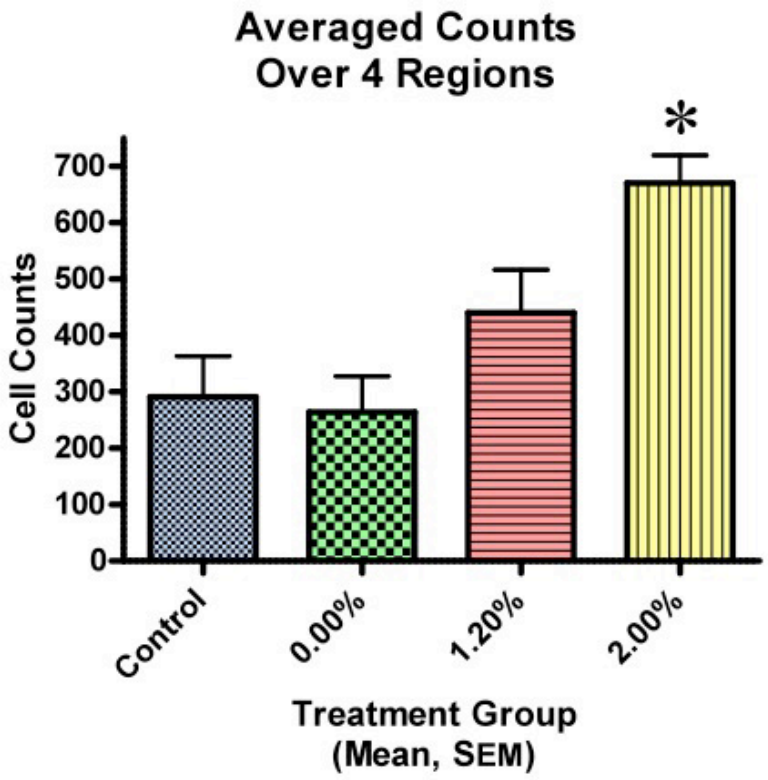

Figure 9: Histopathology LEC Counts After Mock Cataract Surgery - Averaged histopathologic cell counts from four different five-micron sections for each capsule treated with PBS (control), HPMC (0.00% HA), 1.2% HA, and 2.0% HA. The 2.0% HA treated group showed significantly more LECs when compared to the control group and the HPMC group.

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