

**Checklist:**

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This grant application includes all of the following required components:

- Completed and signed cover page (page 1)
- Completed abstract and project duration (page 3)
- Completed budget that does not exceed \$5000 in total costs (pages 4 and 5)
- Complete description of the resources available for the proposed research (page 6)
- Complete research plan (beginning page 7; sections a through e)
- Complete literature cited (section f)
- Complete description of investigators and key personnel
- Check if letters of cooperation are included
- Curriculum Vitae (use the 2-page NIH format)
- Check if appendices are included
- Check if IACUC or Hospital Executive Committee approval is included
- Check if an Informed Consent form is included
- All page restrictions are met
- PDF size restriction (no larger than 5MB) is met
- Submitted an electronic PDF to [chanderler.111@osu.edu](mailto:chanderler.111@osu.edu)

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**Scientific Abstract:**

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**PURPOSE:** To determine if the equine cornea will make a suitable *ex vivo* model by adequately replicating the extent of equine corneal fibrosis observed *in vivo*. Our study aims are: (1) to assess the equine cornea's extracellular matrix at 3 and 7 days after using two different culture techniques (either (a) an air/liquid interface or (b) immersion system) to determine the best *ex vivo* equine corneal model; (2) determine if the equine cornea in an *ex vivo* model will develop corneal fibrosis after an alkali burn.

**MATERIALS AND METHODS:** Eight equine corneas and 2-3 mm of perilimbal sclera will be harvested from horses undergoing euthanasia for reasons unrelated to this study and free of ocular disease. To fulfill aim 1, one sclero-corneal ring (SCR) from each horse will be placed in the air/liquid interface organ culture system (ALC), with the contralateral SCR being placed in an immersion condition (IC) organ culture system for 3 or 7 days. All SCRs will be evaluated using photography, histology, and molecular assay. To fulfill aim 2, 8 additional equine SCRs will be harvested. SCRs will be divided into two groups, one receiving a central corneal alkali burn (n=4 eyes) and a control group (n=4 eyes). The SCRs will be cultured using the culture system which best preserves the cornea's cellular matrix as determined in aim 1. The SCRs will be cultured for either 3 or 7 days and evaluated similarly to aim 1.

**EXPECTED OUTCOME:** Preliminary data shows that these models maintain the cornea's cellular matrix and produces fibrotic changes within the corneal stroma, supporting our hypothesis.

**SIGNIFICANCE:** We are confident that the equine cornea will be suitable to be utilized in an *ex vivo* corneal fibrosis culture system. Data generated from this study will be used to further evaluate anti-fibrotic therapies in horses.

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**Project Duration:**

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6 months (*ex vivo* work); 12 months total

All experiments detailed in this proposal (both *ex vivo* and bench-top processing/analysis of results) will be accomplished within 12 months. The *ex vivo* experimentation will be completed in 6 months and the additional bench top processing/analysis of tissue samples will be completed in 12 months from funding start date. It is expected that Dr. Marlo will be directly responsible for the completion of all aspects of experimentation and data analysis described herein. Furthermore, Dr. Marlo **is guaranteed 3 months of protected off-clinic time and freed from emergency duty annually throughout his 3 year ABVO-approved residency program** to ensure that a minimum of two and potentially three hypothesis-driven manuscripts will be published and contributed to the scientific literature. These publications will partially fulfill requirements of his graduate program at the University of Missouri. **The project time line is considered highly doable for a resident to complete in his structured program.**

**Estimated Budget:****Animals, Per Diem, and/or Owner Compensation:**

.....\$0

**Equipment:**

NaOH..... \$30  
 Fume hood, microscopes, digital photography\* ..... \$0  
 PCR\* ..... \$0  
 Optical coherence tomography\* ..... \$0

**Expendable Supplies:***Cell Culture Supplies:*

Tissue culture dishes..... \$500  
 Pipettes ..... \$650  
 Cell culture media..... \$1500  
 Routine culture reagents ..... \$2500  
 (Sodium Bicarb, MEM essential amino acid, L-glutamine, Non-essential amino acids,  
 Penicillin/Streptomycin, Sodium Pyruvate, MEM vitamins, FBS, Trypsin)

*Primers for RT-PCR:*

$\alpha$ -SMA,  $\beta$ -actin ..... \$1000

*Assay Kits*

TUNEL assay ..... \$50

*Immunohistochemistry for  $\alpha$ -SMA, collagen type III, fibronectin, thrombospondin, and integrin*

Antibodies ..... \$1000

**Other:***Laboratory Tests:*

Histopathology and specialized stains ..... \$300

\*all necessary equipment is established and in good working order in Dr. Mohan's laboratory, College of Veterinary Medicine.

**Cost Justification:**

Data obtained in this experiments will provide the foundation for future research regarding the use of anti-fibrotic treatment modalities to inhibit equine corneal fibrosis *in vivo*. The experimental results obtained from this research project are critical to future planned external grant applications to funding agencies including the Morris Animal Foundation and USDA. The data generated from this study represent the next logical step in our laboratory's on-going investigations and results will be presented at national and international ophthalmology meetings, including the American College of Veterinary Ophthalmologists annual meeting. Support from the VAF is requested to help cover costs associated with the purchase of laboratory supplies. **All remaining components of budget will be financed by co-investigators and collaborators, Dr. Rajiv Mohan and Dr. Elizabeth Giuliano.**

<b>Total Direct Project Costs</b>	<b>\$7530</b>
<b>The ACVO-VAF does not support any institutional F&amp;A</b>	<b>\$0</b>
<b>Total Costs Requested from ACVO-VAF Resident Research Fund</b>	<b><u>\$5,000</u></b>

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**Resources:**

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**Facilities:**

**Laboratory:**

The Veterinary Ophthalmology Service of the University of Missouri, Veterinary Medical Teaching Hospital (VMTH), offers excellent referral services to practicing veterinarians in Missouri and surrounding states. Small and large animal patients are provided comprehensive diagnostic, medical and surgical services in veterinary ophthalmology. Dr. Rajiv Mohan recently has been appointed as the Ruth M. Kraeuchi Endowed Professor of Ophthalmology at the College of Veterinary Medicine, University of Missouri. He continues to serve in a joint appointment at the School of Medicine, University of Missouri. He has a well-established working relationship with Dr. Giuliano (co-investigator and the PI's primary resident mentor), and directs extremely productive laboratories located in the University of Missouri's College of Veterinary Medicine and the Harry S. Truman Veterans Hospital in Columbia, Missouri.

**Clinical:**

The Veterinary Ophthalmology Service of the University of Missouri Veterinary Medical Teaching Hospital (VMTH), offers state of the art referral services to practicing veterinarians in Missouri and surrounding states. Complete veterinary ophthalmology diagnostic, medical and surgical services are provided for companion animal, food animal and equine species.

**Animals:**

All animals that are used in this experiment will be undergoing humane euthanasia due to reasons unrelated to this study at the University of Missouri Veterinary Teaching Hospital. All tissue used in this experiment will be collected immediately post-mortem and will be handed in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and the University of Missouri's institutional care and use of animals protocol.

**Major Equipment:**

Dr. Mohan's laboratories and the MU-VMTH contain all of the necessary equipment required to complete this project, including stereomicroscopy, PCR, slit-lamp biomicroscopy, and digital photography.

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**Research Plan:**

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**A. Hypothesis and Specific Aims:**

**To our knowledge, the equine cornea has never been evaluated in an *ex vivo* model system.** Utilizing organ culture protocols established in other mammalian (pigs, rabbits, and human) corneal *ex vivo* models (1-3), we seek to develop an *ex vivo* model of equine corneal fibrosis. This equine corneal *ex vivo* model will be used to evaluate anti-fibrotic modalities proven successful at reducing corneal fibrosis *in vitro* (4-8). It is our hypothesis that this model will sufficiently replicate equine corneal fibrosis similar to what is observed in spontaneously occurring disease *in vivo*, yielding the first *ex vivo* model of equine corneal fibrosis.

## Specific Study Aims:

1) To evaluate the equine cornea at 3 and 7 days utilizing two different organ culture techniques (either (a) an air/liquid or (b) an immersion system). Data generated from this aim will be utilized to fulfill aim 2.

2) To evaluate if the optimal equine corneal *ex vivo* model (as determined in aim 1) can reproduce corneal fibrosis to a similar degree as that observed *in vivo* through application of a central corneal alkali burn with 1 N NaOH. Data from this study would establish an equine *ex vivo* corneal fibrosis model to be used in future studies aimed at examining various anti-fibrotic therapeutic strategies.

**B. Background and Significance:**

Equine corneal fibrosis can result following various insults (both surgical and traumatic) and as a sequel to infectious keratitis (8). The cornea comprises two-thirds of the total refractive power of the eye, therefore maintaining corneal clarity is of the utmost importance in preserving vision (9). In horses in particular, as with most prey animals, their eyes are laterally placed, allowing for nearly a 360° degree visual field (10). Any degree of corneal fibrosis resulting in vision loss can have detrimental consequences for both the horse and handler. Corneal fibrosis can become so significant that the horse may need to be retired from their function (e.g. eventing, barrel racing, jumping), and potentially need to be euthanized if deemed to be a danger to themselves or the owner.

Corneal fibrosis is due to a complex cascade of cytokines, notably TGF $\beta$ , which results in myofibroblast proliferation and increased extracellular matrix (ECM) deposition (4). Anti-fibrotic agents and gene therapies have been evaluated using equine corneal fibroblasts *in vitro* (4-8). However, these therapies have never been tested in an *in vivo* equine patient. Our group has been actively involved in the investigation of equine corneal fibrosis treatment modalities by establishing and using *in vitro* models. *In vitro* models utilize monocultures of representative corneal cell populations and cannot fully evaluate potential pathologic and toxic changes that can occur in the multi-layered native cornea following treatment. Presently, significant limitations are associated with direct application of our *in vitro* findings to an *in vivo* equine model. Due to the increasing financial, regulatory, and terminal (due to mandatory corneal collection) requirements necessary to conduct *in vivo* animal research, it is both prudent and responsible from an animal welfare perspective to conduct pilot studies using an animal model. As a result, greater numbers of corneal experiments are being performed using *ex vivo* animal models (2, 3, 11). *Ex vivo* models allow for the evaluation of pathologic changes while maintaining normal anatomical and

cellular divisions of the desired organ allowing for histological rather than cytological evaluation. *Ex vivo* models also avoid many of the constraints associated with *in vivo* work. **Currently, no *ex vivo* model of the equine cornea exists.**

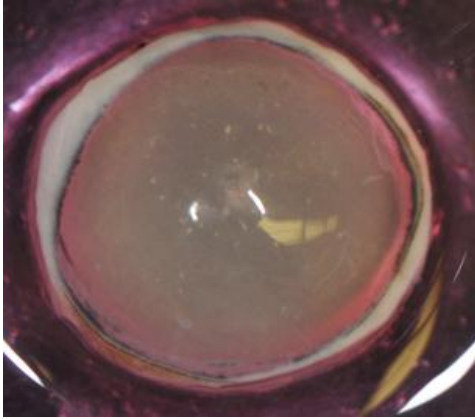
*Ex vivo* models are currently utilized to evaluate a variety of different ocular diseases (e.g. keratoconjunctivitis sicca, corneal fibrosis, and corneal ulceration) in humans, pigs, and rabbits models (1-3, 11). We seek to develop an *ex vivo* model which can adequately replicate equine corneal fibrosis to a similar level as we observe in a clinical setting. This model would then complement existing *in vitro* study data generated from our laboratory and represents the next logical step prior to *in vivo* clinical trials. We hypothesize that **this model will adequately replicate *in vivo* equine corneal fibrosis, therefore yielding the first *ex vivo* model of equine corneal fibrosis.** Our research has significant implications to improve vision in horses, a multi-billion dollar industry annually.

### **C. Preliminary Data:**

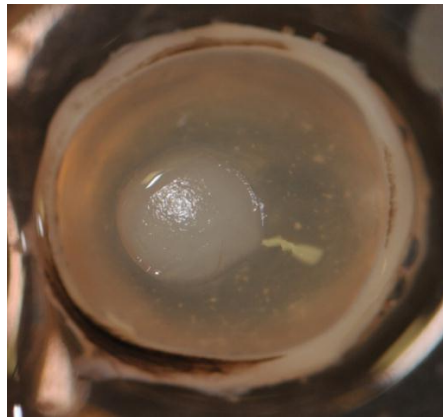
Our comparative corneal research group at the University of Missouri, College of Veterinary Medicine has extensive research experience investigating corneal fibrosis using various models in a variety of animal species (dogs, cats, horses and humans). Recent pilot studies investigating this planned *ex vivo* model have yielded promising results showing that we are able to adequately maintain the cornea's cellular matrix for up to 7 days in both control and wounded corneas (e.g. after a central corneal alkali burn with 1 N NaOH).

**Figures:**

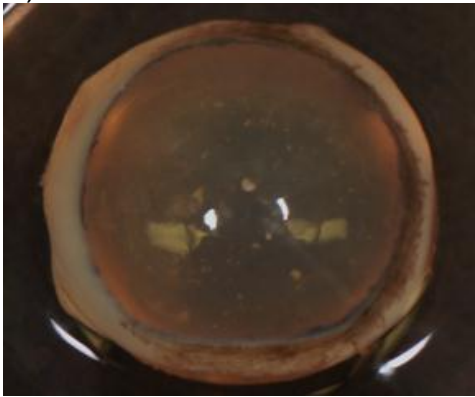
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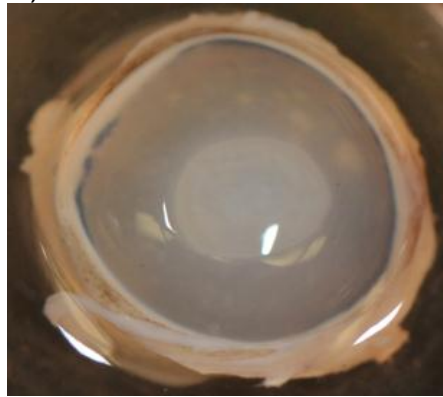
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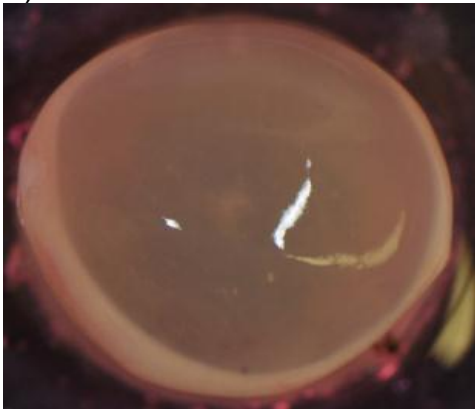
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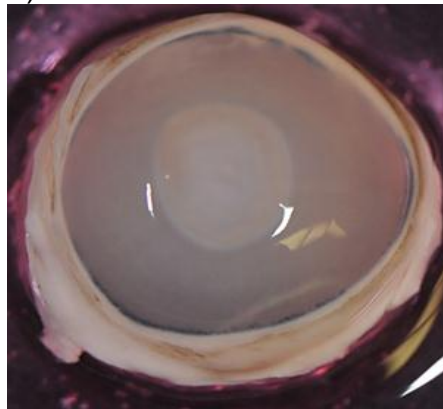
D)



E)

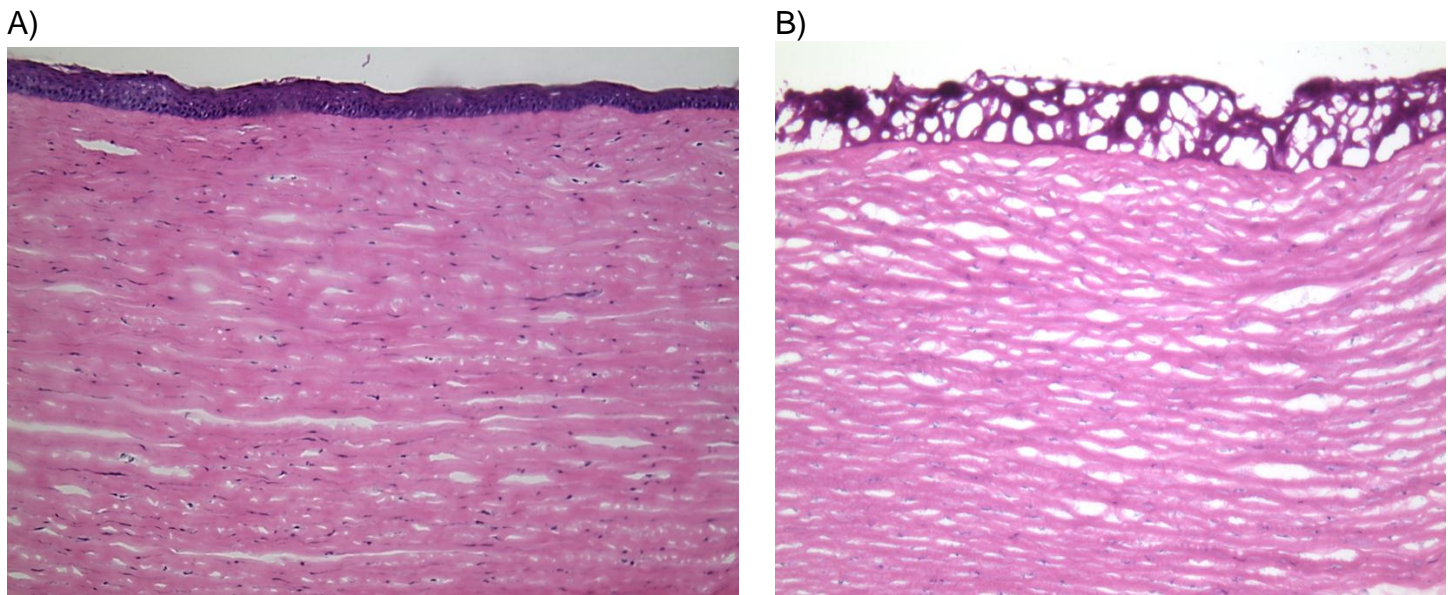


F)



**Figure 1:** Representative gross photography images of corneas through various stages of *ex vivo* organ culture. A) A normal dog cornea immediately after placement into the *ex vivo* ALC organ culture system. B) A cornea immediately after a central corneal alkali burn with 1 N NaOH. C) A normal cornea at 3 days. D) A cornea which received an alkali burn at 3 days in ALC organ culture conditions. E) Normal cornea after 7 days. F) A cornea which received an alkali burn 7 days previously.





**Figure 2:** Representative images (100x magnification) of axial corneal histologic samples from corneas cultured for 24 hours in the *ex vivo* ALC organ culture model stained with hematoxylin and eosin (H&E). A) Normal dog cornea B) Cornea which received a central alkaline burn (Note the degree of stromal edema and lamellar disorganization).

#### D. Experimental Plan:

**Specific Aim #1:** To determine if the equine cornea is suitable to be studied in an *ex vivo* model system.

**Rationale:** Currently *ex vivo* models are utilized to study a variety of ophthalmic diseases (e.g. keratoconjunctivitis sicca, corneal ulceration and corneal fibrosis) (10-13). Currently several types of organ culture techniques are utilized to maintain a cornea's cellular matrix *ex vivo*. We seek to evaluate if the equine cornea is suitable to be cultured in an *ex vivo* setting by utilizing two different organ culture techniques that are reported in the literature. It is our hypothesis that the air/liquid interface technique (ALC) will more adequately support the equine corneal cellular matrix.

**Experimental design:** We will aseptically harvest 8 equine corneas from 4 horses undergoing humane euthanasia for reasons unrelated to this study. Horses will be examined prior to euthanasia by slit-lamp biomicroscopy and determined to be free of anterior segment disease. At the time of tissue collection, the entire equine cornea as well as its surrounding (e.g. perilimbal) 2-3 mm of scleral rim will be procured. These sclero-corneal rings (SCRs) will be placed in Modified Eagle Medium (MEM) and immediately transported to the lab on ice for further processing. The SCRs from each horse will be randomly (as determined by a coin flip) placed in either an air/liquid interface organ culture (ALC) system (1) or placed in an immersion condition (IC) system (1) (4 corneas from 4 different horses in each culture system). The ALC procedure will involve placing the SCR on top of a conformer in a single well of a sterile 6 well plate with the corneal epithelial side facing up and filling the well to a level where the endothelium is continually bathed in organ culture medium, yet allowing for the axial corneal epithelium to be exposed to air. The conformer will consist of a 50 ml sterile test tube end cut off at the first indicator line with a sterile saw blade.



The conformer will then be attached to the bottom of single well in a 6 well plate concave side down using sterile acrylic glue (3). This conformer will allow the corneal endothelium to remain in contact with the culture media while also maintaining normal corneal curvature. The ALC samples will have 1 ml of organ culture medium applied directly to the axial corneal epithelium 6 times daily in a drop-wise manner to prevent epithelial desiccation (1). The IC procedure will involve placing the SCR in a sterile well of a 6 well plate without a conformer and continuously submerging the SCR in 10 mls of organ culture medium. The medium for all procedures will be Modified Eagle Medium (MEM) containing a 1:9 ratio of 10% fetal bovine serum, 0.01  $\mu$ l/ml penicillin-streptomycin, and 0.001  $\mu$ l/ml fungizone. The plates will be cultured at 37°C in 5% CO<sub>2</sub> for either 3 (4 corneas/2 in each organ culture system) or 7 (4 corneas/2 in each organ culture system) days. The medium in all samples will be changed every other day. Corneas will be sterilely evaluated daily by keeping the lid plate on at all times unless underneath a cell culture hood, to ensure that all corneas are still in the proper position. All culture plates will be evaluated using gross photography, histology, apoptosis assays (TUNEL), and RT-PCR at the end of their respective cultured time periods. Due to multiple assays requiring sections of axial cornea from both culture groups (ALC and IC), the axial cornea of each sample will be sub-sectioned to an appropriate diameter as required by each evaluation modality. All modalities used for analysis will have at least a 2 mm diameter representative sample of axial cornea for analysis from each cornea.

#### **Evaluation of corneal transparency using gross photography:**

Corneal transparency will be evaluated through daily stereomicroscopy and photo documentation. Prior to, and every day while in culture, corneal transparencies for all corneas in aim 1 (ALC and IC groups) will be obtained. Corneas will aseptically placed within a sterile clear plastic petri dish with the epithelial corneal surface positioned dorsally. To ensure that all corneas are handled in a sterile manner, the culture plates will only be opened while in a cell culture hood and lids of both the culture plates and petri dishes will be kept closed at all times while corneas are outside of the cell culture hood. The petri dish containing the cornea will then be placed directly over a white page of paper containing a single line of 11 point text in Arial font that has been positioned on the stage of the stereomicroscope as previously described (1). Representative images of Arial font as viewed through the equine cornea from both culture conditions in aim 1 will be captured and analyzed.

#### **Histologic evaluation:**

All corneas in aim 1 will have the axial cornea subsectioned so that a 2 mm diameter section of cornea will be fixed in 10% formalin for 24 hours and then embedded in paraffin blocks. Fixed corneas will be sectioned routinely and stained with hematoxylin and eosin (H&E). The presence or absence of artifactual change and histology of the normal cornea's cellular matrix in aim 1 will be evaluated.

#### **Apoptosis assay:**

A 2 mm diameter axial corneal section will be utilized to determine the equine corneal apoptotic response in aim 1. This will be determined by using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Corneal sections will be incubated with TUNEL reaction and counterstained with DAPI (1 mg/ml) (3). Digital images will be obtained using a fluorescent microscope equipped with a CCD digital camera after tissue processing is complete.

#### **Real-time PCR detection of $\alpha$ -smooth muscle actin ( $\alpha$ SMA):**

Total RNA will be extracted from a representative axial corneal stromal subsection of all cultured corneas in aim 1 with an RNeasy kit. RNA will then be stored in a -80° freezer until ready to be

analyzed. Real-time PCR will then be performed using Step One Plus real-time PCR system according to manufacturer's instructions. The forward and reverse  $\alpha$ SMA primer sequences are 5' TGG GTG ACG AAG CAC AGA GC 3' and 5' CTT CAG GGG CAA CAC GAA GC 3' respectively. The  $\beta$ -actin gene has historically been used by our laboratory as a loading control for PCR; it will be used as a housekeeping gene for the normalization of data (4-7). The forward and reverse primers of  $\beta$ -actin are 5' CGG CTA CAG CTT CAC CAC CA 3' and 5' CGG GCA GCT CGT AGC TCT TC 3' respectively. Cycle threshold (CT) will be used to detect increases in signal related to the exponential growth of PCR product during the log-linear phase.

**Data Analysis:** A two-way ANOVA and post hoc Tukey's multiple comparisons test. A *P* value of less than 0.05 will be considered significant.

**Expected Results:** We expect that equine corneas placed in the ALC condition will adequately maintain the equine cornea's cellular matrix for at least 7 days.

**Limitations, Potential Pitfalls and Alternative Strategies:** Acquiring an adequate number of equine corneas from freshly euthanized horses could cause a potential problem. To facilitate acquiring at least 8 corneas from 4 horses at one time for euthanasia, other investigators at the University of Missouri performing terminal equine research and multiple equine rescue groups which routinely bring in large groups of unadoptable horses at one time for donation and euthanasia, will be contacted to help supplement the normal equine donation case load at the MU-VMTH. This approach has been highly successful for our equine *in vitro* work to date. Second, contamination of the culture medium could reduce nutrients available to the cornea. Standard operating procedures designed to limit the degree of contamination of the cultures will be implemented throughout all phases of this project. If these procedures are inadequate to control contamination, alternative antimicrobials in the culture medium would be utilized. Finally; a 6 well culture plate's individual wells are not large enough to fulfill the required amount of culture media that is needed to either immerse the large equine cornea or create the air/liquid interface, corneas will then be placed in 100 mm cell culture plates and organ culture media would be applied as previously described.

**Specific Aim #2:** To determine if the equine cornea can develop corneal fibrosis in an *ex vivo* model after an axial corneal alkaline burn with 1 N NaOH.

**Rationale:** The alkaline burn has proven successful in creating corneal scarring (fibrosis) *in vivo*; however, this technique has been used primarily in rabbits and rats (12-14). TGF $\beta$ 1 has been proven *in vitro* to be a potent inducer of equine fibroblast to myofibroblast transformation (15, 16). Utilizing the *ex vivo* organ culture system determined in aim 1, we hypothesize that the equine cornea will be able to develop corneal fibrosis *ex vivo* optimally after an alkali burn and in the presence of TGF $\beta$ 1.

**Experimental Design:** Eight SCR's from 4 freshly euthanized horses free of ophthalmic disease and undergoing euthanasia for reasons unrelated to this study, will be collected in a similar manner to aim 1. One cornea from each horse will randomly be assigned (as determined by a coin flip) to a control or experimental group. Each cornea will be placed in the organ culture system yielding the most intact corneal cellular matrix (either ALC or IC conditions) and cultured for either 3 or 7 days pending results in aim 1. Immediately prior to placing the experimental group's SCR's into their respective well of a 6 well plate, the axial equine cornea will be wounded with 1 N NaOH applied to the axial cornea for 60 seconds (8 mm diameter filter paper disc). Following 1 N NaOH

application, the cornea will be gently rinsed three times with 1x Phosphate Buffered Saline (PBS) before being placed in culture. The experimental group's SCRs will then be immediately exposed to TGF $\beta$ 1, at a dose of 10 ng/ml (1) added directly to the media. TGF $\beta$ 1 is used as it is a known inducer of corneal fibrosis by causing transformation of corneal fibroblasts to myofibroblasts (4, 5, 15-17). TGF $\beta$ 1 will be supplemented to the media of the experimental group only, at each media change for the remainder of the experiment. Based on our work to date, we believe that the combination of alkali burn and culture medium supplementation with TGF $\beta$ 1 will optimize our chances of creating a reliable model of equine corneal fibrosis *ex vivo*. Both the experimental and control group SCRs will remain in culture for either 3 or 7 days (pending results in aim 1). Each cornea will have the axial area sub-sectioned into 2 mm sections to allow for complete analysis by all evaluation modalities and analyzed in parallel to tissue sections in aim 1 with the addition of immunohistochemistry. The results of each individual horse's experimental and control SCR will be compared to each other.

#### **Evaluation of corneal transparency using gross photography and stereomicroscopy:**

Corneal transparency will be evaluated through daily stereomicroscopy and photo-documentation. Prior to, and every day while in culture, corneal transparencies for all corneas in aim 2 (experimental and control groups) will be obtained. Corneas will aseptically placed within a sterile clear plastic petri dish with the epithelial corneal surface positioned dorsally. To ensure that all corneas are handled in a sterile manner, the culture plates will only be opened while in a cell culture hood and lids of both the culture plates and petri dishes will be kept closed at all times while corneas are outside of the cell culture hood. The petri dish containing the cornea will then be placed directly over a white page of paper containing a single line of 11 point text in Arial font that has been positioned on the stage of the stereomicroscope as previously described. Representative images of Arial font as viewed through the equine cornea from both control and wounded corneas in aim 2 will be captured and analyzed.

#### **Histologic evaluation:**

A 2 mm diameter section of all corneas in aim 2 will be fixed in 10% formalin for 24 hours and then embedded in paraffin blocks. Fixed corneas will sectioned routinely and stained with hematoxylin and eosin (H&E). The presence of corneal fibrosis (graded as mild, moderate or severe) (1) in experimental corneas compared to controls will be indicative of the model's ability to faithfully reproduce histological features of corneal fibrosis.

#### **Apoptosis assay:**

A representative axial corneal sub-section will be utilized to further characterize the cornea's apoptotic response to wounding in aim 2. This will be determined by using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Corneal sections will be incubated with TUNEL reaction and counterstained with DAPI (1 mg/ml) (3) as previously described. Digital images will be obtained using a fluorescent microscope equipped with a CCD digital camera after tissue processing is complete.

#### **Real-time PCR detection of $\alpha$ -smooth muscle actin ( $\alpha$ SMA):**

Total RNA will be extracted from a representative axial corneal stromal subsection of all cultured corneas in aim 2 with an RNeasy kit. RNA will then be stored in a -80 $^{\circ}$  freezer until ready to be analyzed. Real-time PCR will then be performed using Step One Plus real-time PCR system according to manufacturer's instructions. The forward and reverse  $\alpha$ SMA primer sequences are 5' TGG GTG ACG AAG CAC AGA GC 3' and 5' CTT CAG GGG CAA CAC GAA GC 3' respectively. The  $\beta$ -actin gene has historically been used by our laboratory as a loading control for PCR; it will

be used as a housekeeping gene for the normalization of data (4-7). The forward and reverse primers of  $\beta$ -actin are 5' CGG CTA CAG CTT CAC CAC CA 3' and 5' CGG GCA GCT CGT AGC TCT TC 3' respectively. Cycle threshold (CT) will be used to detect increases in signal related to the exponential growth of PCR product during the log-linear phase.

#### **Immunohistochemical evaluation:**

Following either 3 or 7 days of culture as determined in aim 1, all corneas evaluated in aim 2 will have representative axial corneal subsections stained to confirm the presence of biochemical markers closely associated with corneal fibrosis:  $\alpha$ -smooth muscle actin ( $\alpha$ SMA), collagen type III (COL), fibronectin (FN), thrombospondin (TSP), and integrin (INT) (1). Corneal sections will be fixed with 4% paraformaldehyde, permeabilized with 0.025% triton for 15 min, and incubated with 5% bovine serum albumin (BSA) for 30 min. Primary incubation with mouse monoclonal antibodies will be as follows: anti- $\alpha$ SMA, anti-COL, anti-FN, anti-TNC, and anti-TSP for 90 min at room temperature respectively. Following incubation with the primary antibodies, sections will be washed twice with 1x PBS and then incubated with Alexa 488 goat anti-mouse IgG secondary antibody for 1 h, washed three times in 1x PBS, and mounted in Vectashield containing 4'-6'-Diamidino-2-phenylindole (DAPI). Immunostained sections will be viewed and photographed with a fluorescent microscope equipped with a CCD digital camera. Representative images for each biochemical marker in both experimental and control groups will be collected individually and merged through SPOT Microscopy Imaging Software to allow for semi-quantitative analysis of corneal fibrosis development following corneal insult in the experimental group as compared with the contralateral representative untreated controls

**Data Analysis:** A two-way ANOVA and post hoc Tukey's multiple comparisons test will be used. A *P* value of less than 0.05 will be considered significant for all experiments.

**Expected Results:** We expect that the *ex vivo* equine cornea will adequately develop hallmark signs of cornea fibrosis (e.g. stromal disorganization, increased stromal cellularity, myofibroblast transformation).

**Limitations, Potential Pitfalls and Alternative Strategies:** It is unlikely but possible that that previously reported dose of TGF $\beta$ 1 supplemented to the media will be unable to induce corneal fibrosis. If this occurs the dose will be adjusted accordingly. Other possible pitfalls have been discussed in aim 1.

#### **E. Timeline for the Experimental Plan:**

The proposed timeline for the experimental plan is approximately 6 months (~180 days). The project will commence in early February 2015 with anticipated completion in mid-August 2015. Bench-top analysis of collected tissue samples will be completed within 12 months of project start date. Manuscript preparation with anticipated submission of one or more scientific abstracts to the annual ACVO conference will follow immediately upon project completion. The primary investigator (TM) is guaranteed 3 months of protected "off-clinic and off-call" time per each year of his residency program to be used in the pursuit of scientific investigations. **The project time line is considered highly doable for a MU veterinary ophthalmology resident to complete in his structured ABVO-approved training program.**

**F. Literature Cited:**

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**Investigator Information:**

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**A. Role of Investigators and Key Personnel:**

**Todd Marlo, DVM**, Principal Investigator (PI), is a resident in comparative ophthalmology at the University of Missouri, Veterinary Medical Teaching Hospital, where he is receiving training on microsurgical specialty procedures and is responsible for primary care for small and large animal patients. Dr. Marlo's responsibilities as the principal investigator will be to aid in experimental design, carry out **all aspects** of experimental plan, perform data collection and statistical analysis and serve as primary author on grant applications and publications. Dr. Marlo will devote 90% effort to this project.

**Elizabeth Giuliano, DVM, MS, DACVO**, co-investigator and primary resident advisor to Dr. Marlo, is a board-certified veterinary ophthalmologist with eighteen years of veterinary experience. Her primary research interests at present include investigations of novel treatment strategies for equine periocular neoplasia and corneal wound healing strategies. Currently, she is a tenured faculty ophthalmologist and Section Head of the ophthalmology service at the University of Missouri Veterinary Medical Teaching Hospital, where she continues to be responsible for primary patient care and on-going clinical studies, including data collection on naturally occurring ophthalmic disease. She has been the direct resident supervisor for nine ACVO-board certified ophthalmologists, co-advisor for two ECVO diplomates, and chief advisor to several graduate students who have all successfully completed their degrees. She has served on numerous MU College and ACVO committees, is currently vice president of the ACVO. She is highly capable and extremely committed to directing Dr. Marlo's research efforts. Dr. Giuliano serves as chair of Dr. Marlo's graduate committee and will assist with experimental design and data analysis, aid in the preparation of grant applications and publications, and assist the PI to prepare for national and international scientific abstract presentation. Dr. Giuliano will devote 6% effort to this project.

**Rajiv Mohan, Ph.D.**, co-investigator, is a professor who holds joint appointments at the School of Medicine and is the Ruth M. Kraeuchi Endowed Professor of Ophthalmology, College of Veterinary Medicine at the University of Missouri. He has received national and international recognition for his research involving the cornea; particularly corneal wound healing and gene therapy. He has a well-established collaborative working relationship with Dr. Giuliano for over eight years. His laboratory at the University of Missouri, College of Veterinary Medicine will provide the necessary facilities and state of the art equipment to execute the proposed studies. He will assist with experimental design, data analysis, aid in the preparation of grant applications and publications, and help to prepare the resident for national scientific abstract presentation. Dr. Mohan will devote 4% effort to this project.

**B. Letters of Cooperation:**

None needed.

**Appendices:**

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Not Applicable