

ESTABLISHING A REPRODUCIBLE METHOD FOR THE CULTURE OF  
PRIMARY EQUINE CORNEAL CELLS AND EVALUATION OF ANTIFUNGAL  
DRUG EFFECTS ON EQUINE CORNEAL KERATOCYTES

by

RACHEL MATHES

(Under the Direction of David Hurley)

ABSTRACT

For culture of primary equine corneal epithelial cells, keratocytes and endothelial cells, corneas from horses recently euthanized for reasons unrelated to the study were collected and enzymatically separated into individual layers for cell capture. The cells were plated and grown in culture with complete media. The cells were passaged once confluent and continued for at least three passages. Each cell type was characterized and described with morphologic descriptions and immunocytochemical staining. All three equine corneal cell types were successfully grown in culture. Equine keratocytes and endothelial cells were able to be cryopreserved and recovered. Effects of different antifungal drugs and delivery vehicles on equine corneal keratocyte morphology and proliferation were evaluated. This work provides a construct for reproducible cell culture of all three equine corneal cells and is designed to describe cell collection, morphology, cytoskeletal expression and characteristics in culture.

INDEX WORDS: Cornea, Equine ophthalmology, Cell culture, Antifungal drugs

ESTABLISHING A REPRODUCIBLE METHOD FOR THE CULTURE OF  
PRIMARY EQUINE CORNEAL CELLS AND EVALUATION OF ANTIFUNGAL  
DRUG EFFECTS ON EQUINE CORNEAL KERATOCYTES

by

RACHEL MATHES

B.A., Franklin College, 2001

D.V.M., Purdue University, 2005

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial  
Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2009

© 2009

Rachel Mathes

All Rights Reserved

ESTABLISHING A REPRODUCIBLE METHOD FOR THE CULTURE OF  
PRIMARY EQUINE CORNEAL CELLS AND EVALUATION OF ANTIFUNGAL  
DRUG EFFECTS ON EQUINE CORNEAL KERATOCYTES

by

RACHEL MATHES

Major Professor: David Hurley

Committee: Ursula Dietrich  
James Moore

Electronic Version Approved:

Margie Lee  
Dean of the Graduate School  
The University of Georgia  
August 2009

## **ACKNOWLEDGEMENTS**

Thank you to Dr. Ursula Dietrich for believing in me, conceiving this project idea, providing support and being her generous, patient self. She makes me want to strive for excellence, learn something new every day and live life to the fullest.

Thank you to Dr. Adrian Reber for sticking with me during the project, showing me how to be a true researcher, giving me the tools to succeed and providing his time, patience and kind words. His humor and empathy allowed me to continue forging ahead, asking questions and refusing to let the project be less than complete.

Thank you to Drs. David Hurley and James Moore for allowing me to work in their laboratory, supporting me in this endeavor and accepting an ophthalmology resident into the world of cell culture.

Special thanks to SoYoung Kwon, Londa Berghaus, Dr. Kelsey Hart, Kim Galland, Natalie Norton, Dr. Amelia Woolums, Dr. Michel Vandenplas, Dr. Wanchun Sun and Dr. Monica Figueiredo for all their sympathy and support.

Thank you to my parents, Lincoln and Nancy Mathes, for their unwavering support and unquestioning love. Without their prayers and dedication, I never would have been able to pursue my dreams. I love you both.

Finally, thank you to all the horses that participated in the study. I am certain they gave tremendously to this field and will never be taken for granted.

## TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS .....	iv
CHAPTER	
1 INTRODUCTION .....	01
2 LITERATURE REVIEW .....	04
SECTION I: EQUINE CORNEAL ANATOMY, DAMAGE AND HEALING .....	04
SECTION II: EQUINE CORNEAL PATHOGENS .....	12
SECTION III: TREATMENT OF EQUINE CORNEAL DISEASE .....	15
SECTION IV: CORNEAL CELL CULTURE .....	20
REFERENCES .....	26
3 ESTABLISHING A REPRODUCIBLE METHOD FOR THE CULTURE OF PRIMARY EQUINE CORNEAL CELLS .....	41
ABSTRACT.....	42
INTRODUCTION .....	43
MATERIALS AND METHODS .....	45
RESULTS .....	51
DISCUSSION .....	54

	REFERENCES .....	59
	FIGURES .....	66
4	EFFECTS OF ANTIFUNGAL DRUGS AND DELIVERY VEHICLES ON THE MORPHOLOGY AND PROLIFERATION OF EQUINE CORNEAL KERATOCYTES <i>IN VITRO</i> .....	69
	ABSTRACT .....	70
	INTRODUCTION .....	71
	MATERIALS AND METHODS .....	73
	RESULTS .....	78
	DISCUSSION .....	81
	REFERENCES .....	85
	FIGURES .....	90
5	CONCLUSION .....	95
	REFERENCES .....	99

**CHAPTER 1**  
**INTRODUCTION**  
***PURPOSE OF STUDY***

**Background and Purpose**

The purpose of this study was to develop a reproducible method for the culture of primary equine corneal epithelial cells, keratocytes and endothelial cells to expand *in vitro* equine corneal cell research. While cell culture methods have been described for many other species, methods for the collection and culture of these three types of equine corneal cells have not been previously established. This study characterized each of the three equine corneal cell types based on morphologic description and immunocytochemical staining of cytoskeletal elements, also previously unreported. In addition, methods for cryopreservation of each of the three corneal cell types were evaluated. This preservation method allowed the use of equine corneal cells long after collection.

In order to demonstrate that the identified, cultured equine corneal cells could be utilized for research purposes, the effects of several antifungal drugs and common delivery vehicles for these drugs on equine corneal keratocyte morphology and proliferation in culture were evaluated. Through evaluation of factors directly related to cell health, a quantifiable assessment of substance effect on equine keratocytes *in vitro*

was performed. These results suggest that *in vitro* cell culture may provide useful information for research in equine corneal disease and therapy.

This work provides a foundation for expansion of equine corneal research through demonstration of functional, reproducible cell culture methods that provide the basis for practical application of *in vitro* research to clinically relevant problems in the treatment of ocular diseases in horses.

## **Hypotheses**

The hypotheses that were tested in these studies were:

1. Each of the three equine corneal cell types can be recovered in a viable state after mechanical and enzymatic disassociation, placed in simple culture medium and grown through several passages based on the methods established in other species.
2. A simple method for freezing and recovery of viable corneal cells can be established for each of the three types cultured that will allow for long-term utilization of the cells in a series of experiments, and
3. Cultures of equine corneal cells can be utilized to address questions that are important to equine eye disease.

## **Objectives**

The following objectives will be addressed in this study:

1. Establish methods for equine corneal epithelial cell, keratocyte and endothelial cell collection and culture conditions.

2. Passage all three types of corneal cells for two to seven passages.
3. Demonstrate morphological criteria and biochemical cell markers that allow definitive characterization of *in vitro* equine corneal epithelial cells, keratocytes and endothelial cells.
4. Determine whether all three equine corneal cells from the passages two to six can be frozen in liquid nitrogen and recovered in viable and functional condition.
5. Utilize equine corneal keratocytes for drug application studies *in vitro* and quantifiably assess cytopathological effects of different drugs based on changes in cellular morphology and proliferation.

## **CHAPTER 2**

### **LITERATURE REVIEW**

#### ***SECTION I: EQUINE CORNEAL ANATOMY, DAMAGE AND HEALING***

##### **Introduction**

Corneal disease is one of the most common ocular conditions affecting horses, is often serious and can be vision threatening. It is estimated that 20-40% of horses presented to veterinary ophthalmologists are evaluated for some form of corneal disease.<sup>1,2</sup> Factors likely contributing to development of corneal disease include lateral placement of their large eyes, equine behavior patterns, the environment in which they are housed and certain types of work for which they are used. Despite the large number of equine cases seen, relatively little is known about the development, pathogenesis, progression and treatment of corneal disease in horses. Equine corneal disease encompasses a wide variety of conditions including superficial corneal abrasions, deep infected ulcers, perforations, abscesses, evaporative syndromes and neoplasia. Fungal, bacterial, parasitic and viral pathogens may enter the cornea as a result of opportunistic invasion or more aggressive receptor-mediated mechanisms.<sup>3,4</sup> Novel therapies are greatly needed for equine corneal disease, in particular, as it remains a frustratingly complex disease to treat. Rapid corneal deterioration, poor treatment efficacy, inefficient medication delivery and marked organism pathogenic potential all contribute to making

equine corneal disease an aggressive and often sight-threatening problem. Only through expansion of current knowledge and development of new techniques to study the equine cornea will we be able to better understand, prevent and treat equine corneal disease.

### **Corneal Anatomy, Physiology and Protective Qualities**

The cornea is a unique avascular structure that remains in contact with the external environment, serving to transmit light into the eye while protecting the delicate intraocular structures. The cornea itself is partially protected by the precorneal tear film composed of three layers, the lipid layer, the aqueous layer and the mucin layer. This tear film serves to wash away debris and potential invaders while keeping the surface of the cornea moist and lubricated.<sup>5</sup> The tear film is produced by some of the surrounding adnexa or extraocular tissues. The upper, lower and third eyelids, bony orbit, extraocular muscles, glands and hair also help to protect the globe and cornea from external trauma, UV light, dust, other environmental substances and desiccation.<sup>6</sup>

The cornea is composed of three layers, the superficial stratified squamous non-keratinized epithelial layer, the middle stromal layer and the deep monolayer of modified corneal posterior epithelium or so-called corneal endothelium. The latter layer sits on a well-formed basement membrane termed Descemet's membrane. The epithelium arises from embryologic ectoderm while the stroma and endothelium are derived from neural crest cells. The cornea also has an extensive and very sensitive nerve fiber meshwork with highest densities in the epithelial layer and superficial one-third of the stromal layer.<sup>7,8</sup> The horse has an especially sensitive cornea, likely as an adaptation for its laterally placed, exposed and very large eyes.<sup>6</sup> The normal cornea is notably free of

immune cells, pigment, vessels, goblet cells or other cell types that could interfere with light transmission.<sup>6</sup>

Approximately 3-5 cell layers thick, the epithelium maintains some characteristics of normal “skin epithelium” likely due to its ectodermal origin. However, the epithelium is highly modified and differentiated. Because the epithelium rests on a poorly-formed basement membrane, not readily observed on routine light microscopy, there is a close association with the underlying stroma. It is also in direct contact with the precorneal tear film. The epithelial cells have very close cell-to-cell adhesions through tight junctions that allow selective permeability of the cornea.<sup>9</sup> These associations help the epithelial cells respond to changes in both the stroma and external environment as well as facilitate cellular signaling between areas of the cornea superficially. The epithelium is also constantly replenished and repaired. Stem cells move into the basal layer from the limbus, the most peripheral corneal area at the junction of the cornea and conjunctiva, as part of the normal turn-over of corneal cells. The cells in the basal layer of the cornea then differentiate into squamous cells as they move superficially and are eventually sloughed, similar to skin epithelial turnover.<sup>6,10</sup> This epithelial layer is relatively impenetrable to pathogens in its normal uncompromised state, primarily as a result of the precorneal tear film characteristics, resident normal ocular flora, inherent epithelial cell expression of immune components<sup>11</sup>, tight junctions between cells, constant supply of renewable cells and close association to the middle stromal layer.<sup>12</sup>

The stromal or middle layer of the cornea, comprises approximately 90% of the cornea and is composed of keratocytes, or the stromal cells, and a highly organized extracellular matrix. This layer arises from neural crest cells during development. The

keratocytes possess some morphologic characteristics similar to fibroblasts and are capable of differentiating into fibroblasts when the cornea is damaged. The keratocytes produce the extracellular matrix which is composed of proteoglycans and collagen fibrils, layered parallel to each other.<sup>6,13</sup> The stroma exists in a dehydrated state to allow for maximal light transmission as excess fluid causes dispersion of light waves. Any change in the arrangement of collagen fibrils, as occurs with scarring, or imbibement of fluid into the stroma will result in stromal opacities. The stromal cells produce the extracellular matrix in which they reside, have the capability to differentiate into other cell types, signal with surrounding tissues, and may facilitate migration of other cells and/or vessels into the stroma during disease states. The stromal layer affords the most protection to the eye as it is firm and relatively resistant to blunt trauma, thus maintaining the integrity of the ocular structure.

The endothelial layer, also arising from neural crest cells, is a very specialized cellular monolayer that is in direct contact with the aqueous humor in the anterior chamber of the eye. These cells are primarily responsible for maintaining the stroma in a relatively dehydrated state. They serve to actively pump water from the stroma to the anterior chamber through ATPase-mediated pumps. These cells have very little to no regenerative capabilities, depending on the species.<sup>14,15</sup> Horses, in particular, are thought to have no regenerative capabilities of the endothelium. These cells are capable of marked migration, sliding and morphologic change, presumably as an adaptation for their lack of regenerative powers.<sup>16,17</sup> Although these cells are relatively fragile and do not regenerate, they are afforded a protected state, by nature, due to their location on the internal aspect of the eye. Nutrition is provided by the aqueous humor that is in direct

contact with these cells, and waste products are immediately flushed away, an essential component for such metabolically active cells.

### **Corneal Damage and Healing**

Even with all of the protective qualities and specialized features of the cornea, it remains very susceptible to damage due to its location, lack of vasculature and close association with potential pathogens. Because the cornea is so specialized and relatively small, seemingly minor damage can have devastating consequences. All three cell layers of the cornea respond differently to injury. These responses are very extensive, complex and incompletely understood. While a full explanation of all corneal responses to injury is beyond the scope of this thesis, an understanding of basic responses to injury and healing ability in these three layers is warranted.

Of the three layers, the corneal epithelium has the largest capacity to heal and respond to injury very quickly due to its renewable source of cells. Superficial abrasions that do not penetrate the basement membrane result in rapid healing due to active migration of the basal cells across the denuded surface. These small disturbances will generally heal with very little or no scarring. The intricate cellular responses responsible for this response are not fully elucidated, but healing requires many elements. Presumably, abnormal healing patterns and migration of these basal cells account for the development of some non-healing ulcers in many species, although the mechanisms underlying most non-healing ulcers have yet to be identified.<sup>18-21</sup> It is known, however, that epithelial cells respond to injuries, in part, by producing growth factors, including epidermal growth factor<sup>22,23</sup>, that mediate epithelial migration during healing. Epithelial

cell migration also relies on protein synthesis by these cells and may be facilitated by serine proteases.<sup>24</sup> The re-epithelialization of the cornea after an injury is also facilitated by application of amniotic fluid, suggesting that factors in amniotic fluid are likely capable of signaling to the corneal epithelial cells.<sup>25</sup> Proper re-epithelialization also requires interactions among the basal cell layer, the stroma and basement membrane in order for the basal cells to form proper adhesions.<sup>26,27</sup> Pinin, a desmosome-associated molecule, has a role in converting quiescent, attached basal cells into migrating, non-adherent epithelial cells that are capable of covering defects.<sup>28</sup> Although larger defects that extend into the stroma are also covered by mitotically active epithelial cells, the substrate to which they attach is different than the basement membrane.<sup>6</sup> These epithelial cells have a remarkable ability to respond to factors produced in other locations, produce factors and proteins themselves, alter their shape, become mobile, differentiate terminally, reform a multi-cellular layer after injury and develop attachments to components of the stroma or basement membrane.

Although keratocytes do not have the high mitotic capability of epithelial cells, they have other unique responses to injury that can be important for corneal clarity. Stromal injuries may result in scarring, fluid accumulation or ocular rupture. Defects that extend to the stromal layer cause rapid formation of corneal edema, as any break in the epithelium allows fluid from the tears to passively enter the hyperosmolar stroma. In response to deeper stromal defects, neutrophils migrate from the surrounding adnexa into the stroma and keratocytes differentiate into fibroblasts for more rapid production of extracellular matrix to facilitate healing. Depending on the extent of the injury, vessels may begin to enter the stroma to allow for even more effective delivery of healing factors

and cells to the site. During stromal repair, however, the extracellular matrix of collagen and other components is not as organized as it was before the injury. For this reason, stromal defects almost always result in a stromal scar, or area of corneal opacity that persists long after the cornea has returned to its normal state.<sup>6</sup>

The stroma is uniquely susceptible to a specific type of degeneration called liquefaction, which is facilitated by extracellular products acting on the stromal collagen and keratocytes. Repair is reliant on organized and regulated formation of the extracellular matrix. Normal turnover of this matrix, which entails some remodeling or degradation of old or damaged fibers, is facilitated by proteinases, predominantly the matrix metalloproteinases. These enzymes are secreted in the tear film and aqueous humor<sup>1,29</sup> by epithelial cells, stromal cells and inflammatory cells. Several other growth factors and cytokines are also produced by these cells; however, proteinases are increasingly important due to their noted ability to degrade collagen fibers and other extracellular matrix components. The activities of these proteinases are modulated by proteinase inhibitors to prevent unregulated degradation of the stroma. In disease states, the normal interactions between the proteinases and their inhibitors are interrupted and imbalances are created. While a great deal of research has been performed to help expand our understanding of this process, many of the mechanisms involved remain to be identified. Regardless of the processes involved, the end result of the loss of control of proteinase activity is a rapid, often uncontrolled, liquefaction of the corneal stroma with marked destruction of the extracellular matrix, a process termed keratomalacia. Once this cascade is started, inflammatory cells migrate to the area and produce additional proteinases, thus exacerbating the problem.<sup>6</sup>

Endothelial cells have a limited repertoire of available responses to injury as they lack the ability to regenerate. They make up for this deficiency by being able to alter their cellular morphology while maintaining normal cellular function. Damage to the endothelium initiates a sliding and expansion response of adjacent cells to cover the defect. After the denuded area has been repaired, cell-to-cell contact is re-established and normal pumping of fluid from the stroma resumes.<sup>6,30</sup> The cell-to-cell adhesions between adjacent endothelial cells are especially important for this cell layer as non-adherence would result in movement of fluid through the gaps and formation of corneal edema. Therefore, the most common result of irreversible endothelial damage is corneal edema. In humans, 400-700 endothelial cells/mm<sup>2</sup> are needed to retain corneal clarity.<sup>30</sup> While it is unknown what density is critical in horses, normal endothelial density in the equine eye is approximately  $3,155 \pm 765$  cells/mm<sup>2</sup>, depending on the horse's age;<sup>15</sup> other domestic species have similar endothelial densities.<sup>31</sup> Damage to the endothelium does not usually accompany stromal and epithelial damage unless there is progression to the point of ocular perforation. Most endothelial changes are the result of intraocular changes that occur as a result of a particular disease state. Treatment modalities, however, for stromal and epithelial disease become very important in regards to the endothelium as these layers are permeable to many drugs. Knowledge of endothelial drug toxicity will help prevent inadvertent loss of this layer due to drug exposure during treatment.

## ***SECTION II: EQUINE CORNEAL PATHOGENS***

### **Corneal Pathogens**

While the cornea may be affected by neoplasia, immune-mediated diseases and other diseases, most corneal pathology is caused by pathogenic organisms, namely fungi and bacteria.<sup>6,32,33</sup> The location of the cornea makes it a prime target for attack by external invaders. The list of potential pathogens to the cornea is exhaustive and depends on the geographic location of the patient. In addition, potential pathogens change for different species, sometimes for reasons that are unclear. Resident ocular flora present on the surface of the conjunctiva and cornea help to prevent pathogenic invasion by competing for resources and maintaining a competent immune system in the host. While the resident flora of most domestic animal species is bacteria, horses have both bacterial and fungal ocular flora. The resident fungal organisms are presumed to contribute to the high number of fungal corneal infections that occur in horses.<sup>34</sup> Because pathogen invasion of the cornea is usually opportunistic, the likelihood of a corneal infection occurring is markedly increased if there is a break in the normal defense system of the host.<sup>6</sup> Other pathogenic organisms in the environment that are not resident flora may enter the tissues after the ocular immunity has been compromised. Invasion of the corneal tissues, even after minor disruptions in the tissue, may have devastating effects.<sup>4,6</sup>

In horses, the significance of corneal viral pathogens is unknown, although it is suspected that equine herpesvirus type 2 may play a role in keratoconjunctivitis. However, the reports of equine herpesvirus as a confirmed pathogen are rare, and often are based on response to treatment rather than concrete evidence related to corneal

invasion and pathogenicity.<sup>3,35</sup> Reports of adenovirus affecting the cornea exist, but this is not considered a true corneal pathogen.<sup>36</sup> To date, no other viruses have been implicated in equine corneal disease.<sup>6</sup>

There is a report of *Thelazia lacrymalis* being recovered from the surface of equine corneas.<sup>37</sup> However, this is the only report for a parasite being associated with the cornea in horses and *Thelazia lacrymalis* has never been reported to penetrate the equine cornea and cause infection.<sup>37,38</sup> In a study comparing the binding of *Acanthamoeba castellani* to corneas from various animal species, there was no evidence that *Acanthamoeba castellani* was able to bind to equine corneal cells nor was there any evidence of cytopathic effects in the equine corneal epithelium.<sup>39</sup> Thus, it appears that parasites do not cause corneal disease in horses.

The major pathogens in equine corneal diseases are bacteria and fungi. These invaders have abundant specializations for specific and opportunistic invasion. In addition, they often act synergistically and many corneal lesions have multiple organisms present. Equine corneal disease is especially serious as the pathogens involved are often highly aggressive and exceedingly diverse.

The scope of the bacterial species that are able to invade the cornea and the methods they use for invasion are astounding. In addition, treatment of any corneal disease with antibiotics may kill resident flora, setting up an appropriate environment for other bacterial and fungal invaders.<sup>34</sup> When compared to other types of organisms, bacterial pathogens also have a unique ability to become resistant to antimicrobial therapy in a relatively short amount of time. The most common bacterial isolates cultured in horses with ulcerative keratitis are *Pseudomonas aeruginosa*, *Streptococcus*

*equi* subspecies *zooepidemicus* and *Staphylococcus* species, regardless of location in the United States.<sup>6,40-42</sup> Other bacteria that may cause infection in the cornea are *Leptospira*, *Listeria*, *Clostridium*, *Peptostreptococcus*, *Actinomyces*, *Fusobacterium*, *Bacteriodes*, *Enterococcus*, *Escherichia*, *Pasteurella* and *Acinetobacter*.<sup>43-45</sup> To further demonstrate the diversity of bacterial pathogens in equine corneal diseases, the results of a three-year study performed in Florida documented the isolation of 38 different bacterial species from 266 positive corneal bacterial cultures.<sup>46</sup> The mainstay of treatment for bacterial corneal disease remains antibiotic therapy.

Fungal corneal disease is relatively common in horses. This is in direct contrast to other domestic species, in which fungal keratitis is rare. Although the mechanisms responsible for the development of fungal corneal diseases in horses remain to be identified, it has been hypothesized that the increased prevalence in horses may be due to the large surface area of the cornea, resident fungal species, exposure to environmental substances, including hay and dust, and possible immune deficiencies in horses when compared to other species.<sup>6</sup> Fungal organisms are extremely aggressive, difficult to treat successfully, rapidly spread to deep corneal tissues, difficult to detect with routine testing and cause loss of vision in as many as 50% of horses that are infected.<sup>6</sup>

The most common species of fungi that invade the cornea in horses are *Aspergillus* sp. and *Fusarium* sp.<sup>6,46-49</sup> Both of these are filamentous organisms with septate hyphae that are surrounded by a cell wall and a plasma membrane containing ergosterol. Other types of fungi that may invade the equine cornea include *Penicillium*, *Cladosporidium*, *Mucor*, *Phycomyces* and many others. Yeasts that are capable of invading the cornea include *Candida*, *Cryptococcus*, *Histoplasma* and others.<sup>48,50</sup> Fungal

organisms have the ability to adhere to corneal cells and structures, release proteases that degrade corneal stroma and move into deeper layers of the cornea. Fungal elements also stimulate a severe inflammatory response by attracting large numbers of immune cells that ultimately can release additional proteases that result in rapid keratomalacia. With their ability to mutate and their propensity to colonize equine corneas, fungi can be extremely successful pathogens in this location. Even after a fungus dies in the cornea, damage will continue as a result of the ongoing inflammatory response.<sup>6</sup>

### ***SECTION III: TREATMENT OF EQUINE CORNEAL DISEASE***

#### **Introduction**

Successful treatment of horses with corneal disease depends on a variety of factors, including time of presentation, duration of disease, number of pathogens present in the cornea, client compliance and patient behavior. Treatments available may be classified as surgical, medical or a combination of the two. Treatment selection depends on collaboration between the clinician and client to determine the best scenario for that particular patient. Both medical and surgical options create unique challenges for the clinician when dealing with horses due to patient size, housing, behavior and restraint. Advances made in the field of equine surgery, especially in equine corneal surgery, have expanded our options for therapy in the last twenty years.

## **Surgical Treatment of Equine Corneal Disease**

Surgical management of equine corneal diseases typically involves either conjunctival grafting over the diseased area or replacement of the abnormal cornea with healthy corneal tissue obtained from a donor or other biomaterial. Corneal transplantation is challenging due to transplant rejection, spread of the infection to the transplant and, most importantly, opacity of the transplant after surgery. Despite the fact that there are no methods available that result in a transparent cornea post-surgically, corneal transplantation remains an effective treatment modality for many equine patients. In addition to corneal tissue, amniotic membranes, porcine small intestinal submucosa, oral mucous membrane, vaginal mucous membrane and peritoneum have been tested for their ability to replace diseased cornea.<sup>51-54</sup> Finding novel transplant materials or improving upon current transplant methods remains a priority for most equine corneal transplant research. Based on recent promising evidence in human medicine, it may be possible to use corneal cell culture techniques to provide the material needed for corneal transplantation in this field.<sup>103, 108, 109</sup> Possibility exists for some of these techniques to be used in future equine transplantation research as well.

The most common method employed for corneal transplantation is surgical replacement of diseased cornea with donor fresh or frozen corneal buttons. Because the tissue is from another individual, there is always some degree of rejection of the graft and an inflammatory response by the host.<sup>6</sup> The donor corneal tissue provides tectonic support while being biologically similar to the host's tissue. Because all healthy host tissue should be preserved, corneal transplants are used to replace only the damaged area of the cornea. Depending on the amount of cornea involved, surgeons choose to use

either full thickness or partial thickness grafts. Rejection of the graft by the host may occur and is manifested by vascular tissue invading the graft, corneal edema around the graft and fibrous tissue development in the area after the acute inflammatory response. Development of corneal edema is facilitated in this area by the loss of the donor's endothelial layer of cells. Because this cell layer is fragile, functional endothelial cells are infrequently present in fresh corneal grafts and never in frozen corneal grafts.<sup>6,52,55</sup>

Conjunctival grafting involves surgical placement of the host's conjunctival vascular tissue over the corneal defect.<sup>6</sup> This procedure is effective for treating small or superficial defects, however, would not be appropriate, when used alone, for deep or large defects as conjunctival tissue lacks structural support. Furthermore, conjunctival grafts heal opaque, which will impair vision in the area under the graft. In some cases, conjunctival grafts are used in combination with other procedures, such as corneal transplantation, to facilitate healing. While the healing potential of a conjunctival graft is unmatched by other techniques, its use is limited because of the corneal opacity post-operatively and lack of support to the cornea when used alone to repair corneal defects.<sup>6,51</sup>

Corneal transplantation using amniotic membranes is an alternative treatment for horses with corneal disease. Amniotic membranes not only provide structural support to the area, but also have anti-inflammatory, anti-angiogenic and anti-fibrotic properties.<sup>53,56</sup> These beneficial effects of amniotic membranes are especially desirable features in the cornea as less vascular ingrowth and scarring will result in a better visual outcome. Amniotic membranes also have a similar composition to that of the cornea.<sup>6,57</sup> Additionally, amniotic membranes may be processed to remove the cellular components,

leaving the extracellular meshwork that can then serve as a scaffold for other cells.

While recovery and storage of amniotic membranes can be challenging, there is evidence that these membranes may provide excellent support, healing and visual outcome when used in corneal transplantation procedures.

### **Medical Treatment of Equine Corneal Disease**

Medical management of horses with corneal disease is imperative for any degree of success and is always a component of the treatment regimen, even after ocular surgery. Administration of medications topically can be difficult in horses, especially when several types of medications are required multiple times each day. Because keratomalacia is so prevalent in horses with corneal disease, specific treatments are directed not only at the pathogen, but also at inhibiting local proteinase activity. Medications currently available for treatment of infectious keratopathies include antibacterials, antifungals, proteinase inhibitors and novel therapies.<sup>6</sup> Other medications, such as steroids and non-steroidal anti-inflammatory drugs, are used topically in the treatment of specific types of corneal diseases in horses. In addition, other oral medications are sometimes used to aid treatment and can be detected in therapeutic concentrations in the tears and aqueous humor.<sup>58</sup> While corneal exposure to these drugs is significant, this thesis focuses on topical ophthalmic antifungal medications used for equine fungal keratitis.

Because horses appear to be predisposed to fungal keratitis, especially in certain geographic locations, antifungals comprise a significant portion of medications used for treatment of equine corneal diseases. Generally, these drugs are insoluble in aqueous

solutions, a characteristic that makes them somewhat difficult to use topically. In addition, MIC's and fungal resistance properties are not fully established for antifungal drugs, especially when compared to antibacterial medications.<sup>59</sup>

The three primary categories of antifungal agents are the azoles, polyenes and allylamine/thiocarbamates,<sup>60</sup> with only the azoles and polyenes currently being used topically. Azoles, or N-substituted imidazoles, function to deplete ergosterol and promote accumulation of non-functional sterols that cause the fungal cell wall to become unstable. These changes in the fungal cell wall result in death of the organism, in most cases, or adversely affect the organism by increasing permeability and resulting in leakage of cytoplasmic fluids.<sup>60</sup> Miconazole, ketoconazole, and itraconazole are examples of azoles used topically for ophthalmic disease. Unlike the azoles, polyenes purportedly produce aqueous pores in the fungal cell membrane, causing increased permeability, leakage of intracellular contents and death of the organism. Commonly used topical polyenes include nystatin and natamycin. Although allylamines are used commonly to kill dermatophytes, this class of compounds has not been used for ophthalmic use. Recent evidence indicates that terbinafine, an allylamine, is able to penetrate the rabbit cornea in sufficient concentrations to inhibit the growth of fungi.<sup>61</sup> However, concentrations of the drug decreased relatively rapidly when applied topically. The pharmacokinetic properties of these drugs make them more clinically useful for the control of dermatophytes.<sup>60,62</sup>

Presently, little is known about the direct effects of antifungals on corneal cells, even those antifungal agents used in human medicine, and there are no reports in the literature regarding the direct effects of antifungal medications on corneal cells in culture.

Furthermore, little is known about the effects of antifungal medications on the host,<sup>47</sup> despite the completion of *in vivo* studies in humans and horses in which antifungal drugs have been applied to either healthy or diseased cornea.<sup>64-70</sup> These studies have not looked at the specific cellular changes, if any, related to antifungal application. It has been suggested that targeted administration of these drugs in liposomal preparations may facilitate the delivery these drugs to fungal organisms, thereby minimizing exposure of the host cells to the drug. Liposomal application of these drugs to the cornea holds promise, but research is ongoing and no products are available currently.<sup>63</sup> More work is needed to investigate the effects of antifungal drugs on the cornea and corneal cells.

#### ***SECTION IV: CORNEAL CELL CULTURE***

##### **Human Corneal Cell Culture**

For the past 40 years, advancements in human corneal disease therapy have been based on the results of *in vitro* and *in vivo* research studies, mostly involving rabbit and human models. Although *in vitro* conditions may not replicate the *in vivo* situation, it is generally accepted that results obtained in *in vitro* studies often translate to the *in vivo* situation. This is exemplified by the myriad of uses for cell culture already employed in human medicine. The advantages of *in vitro* research techniques over *in vivo* studies include more tightly regulated control of variables, better reproducibility of results, lower costs, decreased personnel requirements and large sample sizes. While a review of every study involving corneal cell culture in human medicine is beyond the scope of this thesis, it is important to appreciate the four major categories in which corneal cell culture has

been instrumental: corneal cell behavior and biology, cellular responses to pathogens, responses to medications, and corneal transplantation.

Corneal cell behavior and biology has been studied *in vitro* using organ and cell culture. In 1975, cell culture techniques were used to investigate corneal epithelial and endothelial cell biology as it pertained to migration properties of epithelial cells and the effect of contact inhibition on wound healing *in vivo*.<sup>17</sup> Similarly, the physiologic properties regulating fluid transport, osmotic water flow, electrical potential differences and impedance, biochemical parameters and morphologic appearance of endothelium were discovered using an organ culture model with rabbit tissues.<sup>71</sup> The discovery that endothelial cell migration occurs as the result of actin and tubulin synthesis caused by increased transcriptional activity in these cells was made using a treated organ culture model.<sup>16</sup> In addition, knowledge of the signals that regulate extracellular matrix production by keratocytes *in vivo* has been greatly enhanced by examining cultured keratocyte behavior.<sup>72</sup> Research studies using cell culture techniques have also demonstrated that collagenase production in the cornea is affected by the total relative concentrations of stromal and epithelial cells in the cornea. This finding is applicable to the understanding of corneal lesions *in vivo* as knowledge of the processes that direct collagenase production will help researchers develop better treatment modalities for the diseased cornea.<sup>27</sup> The ability to study cells in a controlled, regulated environment provides a unique opportunity to investigate cellular interactions, behavior and biology. Furthermore, recent advancements in human cell culture techniques have allowed researchers to create *in vitro* environments that promote cellular development in a manner that mimics the situation *in vivo*.<sup>73-75</sup> This knowledge can be integrated with information

about cell and organ behavior *in vivo* to expand our understanding of corneal biology and to guide more effective treatment modalities.

Most corneal pathogens arise from inhabitants of the resident flora that invade the damaged cornea. Therefore, a better understanding of the interactions between corneal cells and bacterial and fungal pathogens may provide insight into the pathophysiology of corneal disease, thus helping to improve treatment options. For example, corneal epithelial cells up-regulate the genes responsible for the synthesis of inflammatory cytokines when exposed to *Pseudomonas aeruginosa* bacteria, a response that has been linked to the expression of a specific Toll-like receptor (TLR5).<sup>76</sup> Similarly, exposure to lipopolysaccharide, a component of the cell walls of gram negative bacteria, initiates the synthesis of several pro-inflammatory cytokines in corneal cells.<sup>77</sup> Unmitigated inflammatory cytokine production can cause destruction of corneal tissues, leading to vision loss and even ocular rupture. Thus, treatments directed at modulating specific cellular responses may help prevent or reduce tissue damage in the diseased state, provided the initial pathogen is targeted during treatment as well.<sup>76-79</sup>

In addition to up-regulating genes and cytokines during the diseased state, corneal epithelial cells normally produce antimicrobial peptides.<sup>80</sup> These peptides along with specific membrane-associated mucins help enhance ocular surface immunity by providing barriers to prevent pathogen entry. Deficiencies in these mucins allow *Staphylococcus aureus* organisms to bind to corneal epithelial cells, whereas binding is prevented when these glycoproteins are present. Mucins produced by corneal epithelial cells also help protect, lubricate and hydrate the surface epithelium, important components of normal ocular surface immunity.<sup>81</sup> Through the use of cell culture, it was

discovered that specific expression of protective substances on the ocular surface epithelium is a vital element in prevention of corneal pathogen entry. Many important discoveries regarding specific corneal cell behavior related to prevention of pathogen entry as well as targeted responses to this entry have been made *in vitro*.

The responses of human corneal epithelial cells to fungal toxins and organisms have been well studied using cell culture models. Using this approach, it has been determined that exposure of corneal epithelial cells to *Aspergillus fumigatus* conidia elicits expression of TLR2, TLR4, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 8 (IL-8).<sup>82</sup> Furthermore, there is evidence that the inflammatory response to certain pathogens, like mycotoxins, is dependent on TLR signaling in the cornea.<sup>82</sup> Production of specific mycotoxins may trigger certain corneal cell responses, suggesting that corneal cells react to particular pathogen properties.<sup>83</sup> By integrating what is known about *in vitro* cellular responses to fungal elements with *in vivo* pathophysiology of fungal keratitis, it may be possible to identify the specific mechanisms responsible for lesion development in this disease.<sup>84-86</sup>

Specific cellular responses to ophthalmic medications have been examined *in vitro* using corneal cell research. These substances include antibiotics<sup>87,88</sup>, multipurpose solutions<sup>89</sup>, growth factors<sup>23</sup>, irrigation solutions<sup>90</sup>, surfactants<sup>91</sup>, amniotic fluid<sup>25</sup>, plasma<sup>92</sup>, serum<sup>92</sup> and estrogen.<sup>93</sup> Although information obtained from studies with cultured cells can be very useful, it is also important to ensure that the findings obtained using cell culture are similar to those observed *in vivo*.<sup>94</sup> By using cell culture as an initial tool for testing ophthalmic drugs, information can be obtained about specific cellular toxicity, thus helping to determine if the drug would be a candidate for *in vivo*

study. Determination of drug concentrations within the cornea is also important for examining cellular toxicity as most cytotoxic drug effects are concentration dependent. In 2003, an ocular pharmacokinetic model for measuring drug concentrations in a stratified corneal epithelial layer was created using cell culture that was shown to closely mimic *in vivo* drug application studies.<sup>74</sup> Before that, in 2001, it was shown that a cell culture model could be used to accurately predict *in vivo* ocular drug absorption through intact corneal epithelium.<sup>95</sup> It has been demonstrated that *in vitro* corneal epithelial cell culture may be used to gather information directly applicable to the clinical setting.<sup>96</sup>

Finally, cell culture application to corneal transplantation work in human medicine hold great and exciting promise. Currently, materials used in corneal transplants cause post-operative scarring that may interfere with vision and prolong healing. A number of clinical studies have examined the possibility of using cells grown in culture to restore and repair damaged corneal tissues in affected patients. The results of these studies suggest that the morphological and functional properties of cultured corneal epithelial and endothelial cells are similar to *in vivo* cells and that much promise exist for their use in human patients.<sup>97,98</sup> A considerable amount of work has been performed to optimize carriers for the transplantation of these *in vitro* epithelial and endothelial cell sheets.<sup>75,99-101</sup> While the majority of these studies have been performed in rabbit models of corneal injury, a few studies have investigated transplantation of cultured corneal cells in human patients. For example, in one study, cultured epithelial cells, grown on different carriers, were transplanted onto the corneal surface of nineteen human patients with a variety of corneal diseases. While the results of that study were inconsistent across patients, some had marked improvements in their condition.<sup>102</sup> In a

similar study, corneal epithelial cells cultured on amniotic membrane were transplanted onto the corneal surface of six human patients, and all patients had improved vision and corneal clarity.<sup>103</sup> The results of these preliminary studies indicate that cell culture may become an integral part of corneal transplantation procedures in the future. This is especially interesting for equine ophthalmology as available corneal transplants currently heal with marked scarring and opacities.

### **Corneal Cell Culture in Veterinary Medicine**

Corneal cell culture in veterinary medicine is in its infancy, as only recently have corneal cells from domestic species been successfully cultured. For example, feline epithelial cells have recently been cultured and experimentally-infected with feline herpesvirus-1 (FHV-1), a common pathogen in feline corneal disease. The infected cells responded to FHV-1 with marked morphological changes.<sup>104</sup> The ability of interferon- $\alpha$ <sup>105</sup> and cidofovir<sup>106</sup> to prevent FHV-1 infection in the corneal cells was also examined. The results of these studies provided new information regarding the effects of FHV-1 on corneal cells and may help guide the use of specific medications in clinically affected animals. Feline corneal cell culture has also been recently used to further expand endothelial cell transplantation research.<sup>107-109</sup> The cytoskeletal arrangement of feline corneal keratocytes and effects of growth factors on feline endothelial cell morphology in culture has also been studied.<sup>110</sup>

In a series of studies performed with canine corneal epithelial cells in culture, the cytotoxic effects of antibiotics<sup>111</sup>, anti-inflammatory drugs and preservatives<sup>112</sup> on cellular morphology and migration characteristics were evaluated. Most recently, all

three types of canine epithelial cells, keratocytes and endothelial cells were successfully grown in cell culture. These cells were characterized by their morphologic and immunocytochemical staining properties<sup>113</sup> and evaluated for their responses to dexamethasone exposure.<sup>113</sup> There is promise that veterinary corneal cell culture may be expanded and used for further corneal research advancement.

### **Equine Corneal Cell Culture**

There is a single report in the literature regarding the growth of equine keratocytes and epithelial cells in culture. Although the effects of epidermal growth factor, platelet derived growth factor-BB and transforming growth factor- $\beta$ 1 on the proliferation of equine keratocytes and epithelial cells were evaluated in that study<sup>114</sup>, no attempt was made to characterize those cells using either immunocytochemistry or other methods. In that study, incubation with either epidermal growth factor or platelet derived growth factor-BB for 24 hours significantly increased proliferation of both epithelial cells and keratocytes, whereas incubation with transforming growth factor- $\beta$ 1 had the opposite effect.<sup>114</sup> Based on these initial findings, it appears that studying equine corneal cells *in vitro* may become a valuable research tool.

### **REFERENCES**

1. Strubbe DT, Brooks DE, Schultz GS, et al. Evaluation of tear film proteinases in horses with ulcerative keratitis. *Vet Ophthalmol* 2000;3:111-119.

2. Ollivier FJ, Brooks DE, Kallberg ME, et al. Evaluation of various compounds to inhibit activity of matrix metalloproteinases in the tear film of horses with ulcerative keratitis. *Am J Vet Res* 2003;64:1081-1087.
3. Kershaw O, von Oppen T, Glitz F, et al. Detection of equine herpesvirus type 2 (EHV-2) in horses with keratoconjunctivitis. *Virus Res* 2001;80:93-99.
4. Fleiszig SM, Zaidi TS, Pier GB. *Pseudomonas aeruginosa* invasion of and multiplication within corneal epithelial cells in vitro. *Infect Immun* 1995;63:4072-4077.
5. Crispin SM. Tear-deficient and evaporative dry eye syndromes of the horse. *Vet Ophthalmol* 2000;3:87-92.
6. Gelatt KN. *Veterinary ophthalmology*. 4th ed. Ames, Iowa: Blackwell Pub., 2007.
7. Kaps S, Richter M, Spiess BM. Corneal esthesiometry in the healthy horse. *Vet Ophthalmol* 2003;6:151-155.
8. Kafarnik C, Fritsche J, Reese S. Corneal innervation in mesocephalic and brachycephalic dogs and cats: assessment using in vivo confocal microscopy. *Vet Ophthalmol* 2008;11:363-367.
9. Nakamura T, Yamada M, Teshima M, et al. Electrophysiological characterization of tight junctional pathway of rabbit cornea treated with ophthalmic ingredients. *Biol Pharm Bull* 2007;30:2360-2364.
10. Vossmerbaeumer U, Kuehl S, Bieback K, et al. Cultivation and differentiation characteristics of human limbal progenitor cells. *Tissue Cell* 2008;40:83-88.

11. Jin X, Qin Q, Tu L, et al. Toll-like receptors (TLRs) expression and function in response to inactivate hyphae of *Fusarium solani* in immortalized human corneal epithelial cells. *Mol Vis* 2007;13:1953-1961.
12. Doughty MJ. Normal features of superficial nondesquamating cells of the rabbit corneal epithelium assessed by scanning electron microscopy. *Vet Ophthalmol* 2008;11:81-90.
13. Biros DJ, Brooks DE, Brown MP, et al. Regional and zonal variations in the sulfation patterns of chondroitin sulfate in normal equine corneal stroma. *Am J Vet Res* 2002;63:143-147.
14. Labbe A, Liang H, Martin C, et al. Comparative anatomy of laboratory animal corneas with a new-generation high-resolution in vivo confocal microscope. *Curr Eye Res* 2006;31:501-509.
15. Andrew SE, Ramsey DT, Hauptman JG, et al. Density of corneal endothelial cells and corneal thickness in eyes of euthanatized horses. *Am J Vet Res* 2001;62:479-482.
16. Gordon SR, Buxar RM. Inhibition of cytoskeletal reorganization stimulates actin and tubulin syntheses during injury-induced cell migration in the corneal endothelium. *J Cell Biochem* 1997;67:409-421.
17. Yanoff M. Biology in vitro of corneal epithelium and endothelium. *Doc Ophthalmol* 1976;41:157-204.
18. Cooley PL, Wyman M. Indolent-like corneal ulcers in 3 horses. *J Am Vet Med Assoc* 1986;188:295-297.

19. Bentley E, Murphy CJ. Thermal cautery of the cornea for treatment of spontaneous chronic corneal epithelial defects in dogs and horses. *J Am Vet Med Assoc* 2004;224:250-253, 224.
20. Michau TM, Schwabenton B, Davidson MG, et al. Superficial, nonhealing corneal ulcers in horses: 23 cases (1989-2003). *Vet Ophthalmol* 2003;6:291-297.
21. Brunott A, Boeve MH, Velden MA. Grid keratotomy as a treatment for superficial nonhealing corneal ulcers in 10 horses. *Vet Ophthalmol* 2007;10:162-167.
22. Zieske JD, Takahashi H, Hutcheon AE, et al. Activation of epidermal growth factor receptor during corneal epithelial migration. *Invest Ophthalmol Vis Sci* 2000;41:1346-1355.
23. Zhang Y, Akhtar RA. Effect of epidermal growth factor on phosphatidylinositol 3-kinase activity in rabbit corneal epithelial cells. *Exp Eye Res* 1996;63:265-275.
24. Zieske JD, Bukusoglu G. Effect of protease inhibitors on corneal epithelial migration. *Invest Ophthalmol Vis Sci* 1991;32:2073-2078.
25. Castro-Combs J, Noguera G, Cano M, et al. Corneal wound healing is modulated by topical application of amniotic fluid in an ex vivo organ culture model. *Exp Eye Res* 2008;87:56-63.
26. Jumblatt MM, Neufeld AH. A tissue culture assay of corneal epithelial wound closure. *Invest Ophthalmol Vis Sci* 1986;27:8-13.
27. Johnson-Muller B, Gross J. Regulation of corneal collagenase production: epithelial-stromal cell interactions. *Proc Natl Acad Sci U S A* 1978;75:4417-4421.

28. Shi Y, Tabesh M, Sugrue SP. Role of cell adhesion-associated protein, pinin (DRS/memA), in corneal epithelial migration. *Invest Ophthalmol Vis Sci* 2000;41:1337-1345.
29. Wang L, Pan Q, Xue Q, et al. Evaluation of matrix metalloproteinase concentrations in precorneal tear film from dogs with *Pseudomonas aeruginosa*-associated keratitis. *Am J Vet Res* 2008;69:1341-1345.
30. Krachmer JH, Mannis MJ, Holland EJ. *Cornea*. 2nd ed. Philadelphia: Elsevier Mosby, 2005.
31. Kafarnik C, Fritsche J, Reese S. In vivo confocal microscopy in the normal corneas of cats, dogs and birds. *Vet Ophthalmol* 2007;10:222-230.
32. Gilger BC, Michau TM, Salmon JH. Immune-mediated keratitis in horses: 19 cases (1998-2004). *Vet Ophthalmol* 2005;8:233-239.
33. Plummer CE, Smith S, Andrew SE, et al. Combined keratectomy, strontium-90 irradiation and permanent bulbar conjunctival grafts for corneolimbic squamous cell carcinomas in horses (1990-2002): 38 horses. *Vet Ophthalmol* 2007;10:37-42.
34. Gemensky-Metzler AJ, Wilkie DA, Kowalski JJ, et al. Changes in bacterial and fungal ocular flora of clinically normal horses following experimental application of topical antimicrobial or antimicrobial-corticosteroid ophthalmic preparations. *Am J Vet Res* 2005;66:800-811.
35. Borchers K, Wolfinger U, Ludwig H, et al. Virological and molecular biological investigations into equine herpes virus type 2 (EHV-2) experimental infections. *Virus Res* 1998;55:101-106.

36. Gleeson LJ, Studdert MJ, Sullivan ND. Pathogenicity and immunologic studies of equine adenovirus in specific-pathogen-free foals. *Am J Vet Res* 1978;39:1636-1642.
37. Lyons ET, Drudge JH. Occurrence of the eyeworm, *Thelazia lacrymalis*, in horses in Kentucky. *J Parasitol* 1975;61:1122-1124.
38. Lyons ET, Tolliver SC, Drudge JH, et al. Eyeworms (*Thelazia lacrymalis*) in one- to four-year-old Thoroughbreds at necropsy in Kentucky (1984 to 1985). *Am J Vet Res* 1986;47:315-316.
39. Niederkorn JY, Ubelaker JE, McCulley JP, et al. Susceptibility of corneas from various animal species to in vitro binding and invasion by *Acanthamoeba castellanii* [corrected]. *Invest Ophthalmol Vis Sci* 1992;33:104-112.
40. Sauer P, Andrew SE, Lassaline M, et al. Changes in antibiotic resistance in equine bacterial ulcerative keratitis (1991-2000): 65 horses. *Vet Ophthalmol* 2003;6:309-313.
41. Keller RL, Hendrix DV. Bacterial isolates and antimicrobial susceptibilities in equine bacterial ulcerative keratitis (1993--2004). *Equine Vet J* 2005;37:207-211.
42. Brooks DE, Andrew SE, Biros DJ, et al. Ulcerative keratitis caused by beta-hemolytic *Streptococcus equi* in 11 horses. *Vet Ophthalmol* 2000;3:121-125.
43. Ledbetter EC, Scarlett JM. Isolation of obligate anaerobic bacteria from ulcerative keratitis in domestic animals. *Vet Ophthalmol* 2008;11:114-122.
44. Wada S, Yoshinari M, Katayama Y, et al. Nonulcerative keratouveitis as a manifestation of Leptospiral infection in a horse. *Vet Ophthalmol* 2003;6:191-195.

45. Sanchez S, Studer M, Currin P, et al. Listeria keratitis in a horse. *Vet Ophthalmol* 2001;4:217-219.
46. Scotty NC, Brooks DE, Schuman Rose CD. In vitro efficacy of an ophthalmic drug combination against corneal pathogens of horses. *Am J Vet Res* 2008;69:101-107.
47. Weinstein WL, Moore PA, Sanchez S, et al. In vitro efficacy of a buffered chelating solution as an antimicrobial potentiator for antifungal drugs against fungal pathogens obtained from horses with mycotic keratitis. *Am J Vet Res* 2006;67:562-568.
48. Ledbetter EC, Patten VH, Scarlett JM, et al. In vitro susceptibility patterns of fungi associated with keratomycosis in horses of the northeastern United States: 68 cases (1987-2006). *J Am Vet Med Assoc* 2007;231:1086-1091.
49. Brooks DE, Andrew SE, Dillavou CL, et al. Antimicrobial susceptibility patterns of fungi isolated from horses with ulcerative keratomycosis. *Am J Vet Res* 1998;59:138-142.
50. Richter M, Hauser B, Kaps S, et al. Keratitis due to *Histoplasma* spp. in a horse. *Vet Ophthalmol* 2003;6:99-103.
51. Alexander GR, Chester Z. Use of free conjunctival grafts in horses: ten cases. *Aust Vet J* 2004;82:206-210.
52. Brooks DE, Plummer CE, Kallberg ME, et al. Corneal transplantation for inflammatory keratopathies in the horse: visual outcome in 206 cases (1993-2007). *Vet Ophthalmol* 2008;11:123-133.

53. Lassaline ME, Brooks DE, Ollivier FJ, et al. Equine amniotic membrane transplantation for corneal ulceration and keratomalacia in three horses. *Vet Ophthalmol* 2005;8:311-317.
54. Bussieres M, Krohne SG, Stiles J, et al. The use of porcine small intestinal submucosa for the repair of full-thickness corneal defects in dogs, cats and horses. *Vet Ophthalmol* 2004;7:352-359.
55. Brooks DE, Andrew SE, Denis H, et al. Rose bengal positive epithelial microerosions as a manifestation of equine keratomycosis. *Vet Ophthalmol* 2000;3:83-86.
56. Ollivier FJ, Kallberg ME, Plummer CE, et al. Amniotic membrane transplantation for corneal surface reconstruction after excision of corneolimbic squamous cell carcinomas in nine horses. *Vet Ophthalmol* 2006;9:404-413.
57. Tsuzuki K, Yamashita K, Izumisawa Y, et al. Microstructure and glycosaminoglycan ratio of canine cornea after reconstructive transplantation with glycerin-preserved porcine amniotic membranes. *Vet Ophthalmol* 2008;11:222-227.
58. Baker A, Plummer CE, Szabo NJ, et al. Doxycycline levels in precorneal tear film of horses following oral administration. *Vet Ophthalmol* 2008;11:381-385.
59. Thomas PA. Current perspectives on ophthalmic mycoses. *Clin Microbiol Rev* 2003;16:730-797.
60. Ghannoum MA, Rice LB. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin Microbiol Rev* 1999;12:501-517.
61. Sun XG, Wang ZX, Wang ZQ, et al. Pharmacokinetics of terbinafine in the rabbit ocular tissues after topical administration. *Ophthalmic Res* 2007;39:81-83.

62. Jensen JC. Clinical pharmacokinetics of terbinafine (Lamisil). *Clin Exp Dermatol* 1989;14:110-113.
63. Kaur IP, Rana C, Singh H. Development of effective ocular preparations of antifungal agents. *J Ocul Pharmacol Ther* 2008;24:481-493.
64. Shi W, Li S, Liu M, et al. Antifungal chemotherapy for fungal keratitis guided by in vivo confocal microscopy. *Graefes Arch Clin Exp Ophthalmol* 2008;246:581-586.
65. Kalavathy CM, Parmar P, Kaliyamurthy J, et al. Comparison of topical itraconazole 1% with topical natamycin 5% for the treatment of filamentous fungal keratitis. *Cornea* 2005;24:449-452.
66. Ball MA, Rebhun WC, Gaarder JE, et al. Evaluation of itraconazole-dimethyl sulfoxide ointment for treatment of keratomycosis in nine horses. *J Am Vet Med Assoc* 1997;211:199-203.
67. Oji EO. Development of quantitative methods of measuring antifungal drug effects in the rabbit cornea. *Br J Ophthalmol* 1981;65:89-96.
68. O'Day DM, Robinson R, Head WS. Efficacy of antifungal agents in the cornea. I. A comparative study. *Invest Ophthalmol Vis Sci* 1983;24:1098-1102.
69. O'Day DM, Ray WA, Head WS, et al. Influence of the corneal epithelium on the efficacy of topical antifungal agents. *Invest Ophthalmol Vis Sci* 1984;25:855-859.
70. Clode AB, Davis JL, Salmon J, et al. Evaluation of concentration of voriconazole in aqueous humor after topical and oral administration in horses. *Am J Vet Res* 2006;67:296-301.

71. Fischbarg J. Active and passive properties of the rabbit corneal endothelium. *Exp Eye Res* 1973;15:615-638.
72. Funderburgh ML, Mann MM, Funderburgh JL. Keratocyte phenotype is enhanced in the absence of attachment to the substratum. *Mol Vis* 2008;14:308-317.
73. Toropainen E, Ranta VP, Vellonen KS, et al. Paracellular and passive transcellular permeability in immortalized human corneal epithelial cell culture model. *Eur J Pharm Sci* 2003;20:99-106.
74. Ranta VP, Laavola M, Toropainen E, et al. Ocular pharmacokinetic modeling using corneal absorption and desorption rates from in vitro permeation experiments with cultured corneal epithelial cells. *Pharm Res* 2003;20:1409-1416.
75. Chen KH, Azar D, Joyce NC. Transplantation of adult human corneal endothelium ex vivo: a morphologic study. *Cornea* 2001;20:731-737.
76. Zhang J, Xu K, Ambati B, et al. Toll-like receptor 5-mediated corneal epithelial inflammatory responses to *Pseudomonas aeruginosa* flagellin. *Invest Ophthalmol Vis Sci* 2003;44:4247-4254. Song PI, Abraham TA, Park Y, et al.
77. The expression of functional LPS receptor proteins CD14 and toll-like receptor 4 in human corneal cells. *Invest Ophthalmol Vis Sci* 2001;42:2867-2877.
78. Zhang J, Wu XY, Yu FS. Inflammatory responses of corneal epithelial cells to *Pseudomonas aeruginosa* infection. *Curr Eye Res* 2005;30:527-534.
79. Ni M, Tam C, Verma A, et al. Expression of surfactant protein D in human corneal epithelial cells is upregulated by *Pseudomonas aeruginosa*. *FEMS Immunol Med Microbiol* 2008;54:177-184.

80. Huang LC, Jean D, Proske RJ, et al. Ocular surface expression and in vitro activity of antimicrobial peptides. *Curr Eye Res* 2007;32:595-609.
81. Blalock TD, Spurr-Michaud SJ, Tisdale AS, et al. Functions of MUC16 in corneal epithelial cells. *Invest Ophthalmol Vis Sci* 2007;48:4509-4518.
82. Zhao J, Wu XY. Triggering of toll-like receptors 2 and 4 by *Aspergillus fumigatus* conidia in immortalized human corneal epithelial cells to induce inflammatory cytokines. *Chin Med J (Engl)* 2008;121:450-454.
83. Naiker S, Odhav B. Mycotic keratitis: profile of *Fusarium* species and their mycotoxins. *Mycoses* 2004;47:50-56.
84. Das S, Samant M, Garg P, et al. Role of confocal microscopy in deep fungal keratitis. *Cornea* 2009;28:11-13.
85. Miller WL, Giannoni AG, Perrigin J. A case of fungal keratitis: a clinical and in vivo confocal microscopy assessment. *Cont Lens Anterior Eye* 2008;31:201-206.
86. Gopinathan U, Ramakrishna T, Willcox M, et al. Enzymatic, clinical and histologic evaluation of corneal tissues in experimental fungal keratitis in rabbits. *Exp Eye Res* 2001;72:433-442.
87. Sosa AB, Epstein SP, Asbell PA. Evaluation of toxicity of commercial ophthalmic fluoroquinolone antibiotics as assessed on immortalized corneal and conjunctival epithelial cells. *Cornea* 2008;27:930-934.
88. Kim SY, Lim JA, Choi JS, et al. Comparison of antibiotic effect and corneal epithelial toxicity of levofloxacin and moxifloxacin in vitro. *Cornea* 2007;26:720-725.

89. Imayasu M, Shiraishi A, Ohashi Y, et al. Effects of multipurpose solutions on corneal epithelial tight junctions. *Eye Contact Lens* 2008;34:50-55.
90. Yagoubi MI, Armitage WJ, Diamond J, et al. Effects of irrigation solutions on corneal endothelial function. *Br J Ophthalmol* 1994;78:302-306.
91. Xu KP, Li XF, Yu FS. Corneal organ culture model for assessing epithelial responses to surfactants. *Toxicol Sci* 2000;58:306-314.
92. Hartwig D, Herminghaus P, Wedel T, et al. Topical treatment of ocular surface defects: comparison of the epitheliotropic capacity of fresh frozen plasma and serum on corneal epithelial cells in an in vitro cell culture model. *Transfus Med* 2005;15:107-113.
93. Suzuki T, Sullivan DA. Comparative effects of estrogen on matrix metalloproteinases and cytokines in immortalized and primary human corneal epithelial cell cultures. *Cornea* 2006;25:454-459.
94. Pfister RR, Burstein N. The effects of ophthalmic drugs, vehicles, and preservatives on corneal epithelium: a scanning electron microscope study. *Invest Ophthalmol* 1976;15:246-259.
95. Toropainen E, Ranta VP, Talvitie A, et al. Culture model of human corneal epithelium for prediction of ocular drug absorption. *Invest Ophthalmol Vis Sci* 2001;42:2942-2948.
96. Castro-Munozledo F. Corneal epithelial cell cultures as a tool for research, drug screening and testing. *Exp Eye Res* 2008;86:459-469.

97. Aboalchamat B, Engelmann K, Bohnke M, et al. Morphological and functional analysis of immortalized human corneal endothelial cells after transplantation. *Exp Eye Res* 1999;69:547-553.
98. Higa K, Shimmura S, Kato N, et al. Proliferation and differentiation of transplantable rabbit epithelial sheets engineered with or without an amniotic membrane carrier. *Invest Ophthalmol Vis Sci* 2007;48:597-604.
99. Gotze T, Valtink M, Nitschke M, et al. Cultivation of an immortalized human corneal endothelial cell population and two distinct clonal subpopulations on thermo-responsive carriers. *Graefes Arch Clin Exp Ophthalmol* 2008;246:1575-1583.
100. Kim HJ, Ryu YH, Ahn JI, et al. Characterization of immortalized human corneal endothelial cell line using HPV 16 E6/E7 on lyophilized human amniotic membrane. *Korean J Ophthalmol* 2006;20:47-54.
101. Ishino Y, Sano Y, Nakamura T, et al. Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation. *Invest Ophthalmol Vis Sci* 2004;45:800-806.
102. Schwab IR. Cultured corneal epithelia for ocular surface disease. *Trans Am Ophthalmol Soc* 1999;97:891-986.
103. Tsai RJ, Li L, Chen J. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells(1). *Am J Ophthalmol* 2000;130:543.
104. Sandmeyer LS, Keller CB, Bienzle D. Culture of feline corneal epithelial cells and infection with feline herpesvirus-1 as an investigative tool. *Am J Vet Res* 2005;66:205-209.

105. Sandmeyer LS, Keller CB, Bienzle D. Effects of interferon-alpha on cytopathic changes and titers for feline herpesvirus-1 in primary cultures of feline corneal epithelial cells. *Am J Vet Res* 2005;66:210-216.
106. Sandmeyer LS, Keller CB, Bienzle D. Effects of cidofovir on cell death and replication of feline herpesvirus-1 in cultured feline corneal epithelial cells. *Am J Vet Res* 2005;66:217-222.
107. Wencan W, Mao Y, Wentao Y, et al. Using basement membrane of human amniotic membrane as a cell carrier for cultivated cat corneal endothelial cell transplantation. *Curr Eye Res* 2007;32:199-215.
108. Proulx S, Audet C, d'Arc Uwamaliya J, et al. Tissue Engineering of Feline Corneal Endothelium Using a Devitalized Human Cornea as Carrier. *Tissue Eng Part A* 2009.
109. Proulx S, Bensaoula T, Nada O, et al. Transplantation of a tissue-engineered corneal endothelium reconstructed on a devitalized carrier in the feline model. *Invest Ophthalmol Vis Sci* 2009.
110. Jester JV, Barry PA, Lind GJ, et al. Corneal keratocytes: in situ and in vitro organization of cytoskeletal contractile proteins. *Invest Ophthalmol Vis Sci* 1994;35:730-743.
111. Hendrix DV, Ward DA, Barnhill MA. Effects of antibiotics on morphologic characteristics and migration of canine corneal epithelial cells in tissue culture. *Am J Vet Res* 2001;62:1664-1669.

112. Hendrix DV, Ward DA, Barnhill MA. Effects of anti-inflammatory drugs and preservatives on morphologic characteristics and migration of canine corneal epithelial cells in tissue culture. *Vet Ophthalmol* 2002;5:127-135.
113. Werner A, Braun M, Kietzmann M. Isolation and cultivation of canine corneal cells for in vitro studies on the anti-inflammatory effects of dexamethasone. *Vet Ophthalmol* 2008;11:67-74.
114. Haber M, Cao Z, Panjwani N, et al. Effects of growth factors (EGF, PDGF-BB and TGF-beta 1) on cultured equine epithelial cells and keratocytes: implications for wound healing. *Vet Ophthalmol* 2003;6:211-217.

**CHAPTER 3**  
**ESTABLISHING A REPRODUCIBLE METHOD FOR THE CULTURE OF**  
**PRIMARY EQUINE CORNEAL CELLS**

---

<sup>1</sup> R. L. Mathes, U. M. Dietrich, T. M. Krunkosky, D. J. Hurley, A. J. Reber.  
Submitted for publication in *Veterinary Ophthalmology*

## ABSTRACT

**Objective:** To establish a reproducible method for the culture of primary equine corneal epithelial cells, keratocytes and endothelial cells and to describe each cell's morphologic characteristics, immunocytochemical staining properties and conditions required for cryopreservation.

**Procedures:** Corneas from eight horses recently euthanized for reasons unrelated to this study were collected aseptically and enzymatically separated into three individual layers for cell isolation. The cells were plated, grown in culture and continued for several passages. Each cell type was characterized by morphology and immunocytochemical staining.

**Results:** All three equine corneal cell types were successfully grown in culture. Cultured corneal endothelial cells were large, hexagonal cells with a moderate growth rate.

Keratocytes were small, spindle cells that grew rapidly. Epithelial cells had heterogeneous morphology and grew slowly. The endothelial cells and keratocytes stained positive for vimentin and were morphologically distinguishable from one another. The epithelial cells stained positive for cytokeratin. Keratocytes and endothelial cells were able to be cryopreserved and recovered. The cryopreserved cells maintained their morphological and immunocytochemical features after cryopreservation and recovery.

**Discussion:** This work establishes reproducible methods for isolation and culture of equine corneal keratocytes and endothelial cells. Cell morphology and cytoskeletal element expression for equine corneal epithelial cells, keratocytes and endothelial cells is also described. This has not previously been reported for equine corneal cells. This report also demonstrates the ability to preserve equine keratocytes and endothelial cells

for extended periods of time and utilize them long after primary cell collection, a feature that has not been reported for veterinary corneal cell culture.

## INTRODUCTION

Corneal disease is one of the most common ocular conditions affecting horses and is frequently vision threatening and difficult to treat.<sup>1-4</sup> Horses are thought to be predisposed to corneal disease for a variety of reasons. Lateral placement of large eyes on the head, equine behavior, the environment in which they are housed, types of work for which they are used and presence of normal fungal and bacterial flora on and around the cornea are all thought to be contributing factors. Rapid corneal deterioration, poor treatment efficacy, difficulty in medication application and marked organism pathogenic potential contribute to the seriousness of equine corneal disease after development.<sup>1,5-7</sup> Despite the large number of equine cases seen in veterinary medicine, relatively little is known about the pathogenesis and treatment of corneal disease.

Advancement in human corneal disease has long been based on *in vitro* and *in vivo* work. Human and rabbit *in vitro* cell and organ culture has been used extensively to investigate not only corneal cell biology<sup>8-11</sup>, signaling and characteristics<sup>12</sup>, but also efficacy of corneal treatment regimes and effects of substances applied to the cornea.<sup>13-15</sup> Human corneal cells have been immortalized through cell culture work to allow a consistent, predictable cell type to be used.<sup>16-19</sup> In addition to this, recent work has made the possibility of using cultured cells in corneal transplantation a reality in human medicine.<sup>20-27</sup> Corneal cell culture is an accepted, valuable component of corneal research in human medicine for many reasons. Expanded knowledge related to corneal

cell pathology, better therapies for corneal disease and superior transplant implementation have resulted from corneal cell culture research.

Obtaining corneal cells in culture from veterinary species has recently been explored. In 2001, canine corneal epithelial cells were cultured and exposed to antibiotics and benzalkonium chloride.<sup>28</sup> The cellular morphology and migration properties were evaluated after agent application. In 2002, canine corneal epithelial cells were also exposed to anti-inflammatory drugs and preservatives with similar properties evaluated after substance application.<sup>29</sup> Selection of different agents in a clinical setting may be influenced by cytotoxicity studies *in vitro*. In 2008, all three types of canine corneal cells were successfully grown in primary cell culture.<sup>30</sup> These cells were exposed to dexamethasone to establish a measured cellular inflammatory response. Similarly, feline corneal epithelial cells have been primarily cultured and exposed to feline herpesvirus-1.<sup>31</sup> The cellular responses as well as potential protective qualities of interferon<sup>32</sup> and cidofovir<sup>33</sup> during viral infection were examined. These models will not only help expand knowledge of canine and feline corneal disease, but will likely lead to novel treatment modalities.

Research related to equine corneal disease has been heavily devoted to *in vivo* work.<sup>3,6,34-41</sup> Despite this work, equine corneal disease remains a frustratingly complex disease to treat. Inherent limitations exist for *in vivo* research and include large variability between subjects, small sample numbers, management of animals, definition and management of individual subject discomfort and facility limitations for animal housing. The *in vitro* work done thus far is preliminary and has inconsistent application to the clinical setting.<sup>6,42,43</sup> Although equine corneal epithelial cells and keratocytes have

been cultured<sup>44</sup>, these cells have never been characterized or described. In fact, there is no current method for establishing all three equine corneal cells in culture nor is there any information on characterizing them through immunocytochemical staining. A basic methodology for obtaining primary equine corneal epithelial, keratocyte and endothelial cells in culture, as well as characterizing, maintaining and cryopreserving them could be the basis for significant contributions to our current knowledge base of equine corneal disease. The possibilities for using these cells would be to discover additional information about their properties, behavior, signaling, response to applied substances, growth and response to pathogens. In addition, potential exists for the use of these cells in equine corneal transplantation work. The purpose of this project was to establish a reproducible method for culturing primary equine corneal epithelial cells, keratocytes and endothelial cells, as well as determine the conditions required for their preservation.

## **MATERIALS AND METHODS**

### *Isolation of equine corneal cells*

Corneas (n=16) of eight horses recently euthanized (<0.5hr) for reasons not related to the study were excised using a sterile scalpel, forceps and scissors after aseptic preparation of the site with 0.2% betadine solution in isotonic, sterile phosphate buffered solution. The corneas were used only if the eyes and periocular tissues were free of disease. A suture was aseptically placed in the peripheral cornea before excision to aid with handling of the cornea and orientation of the corneal layers. The corneas were placed in a 0.2% betadine solution for 10-15 minutes for further decontamination and transport to the laboratory. After transport, corneas were subjected to systematic enzyme

digestion in a laminar flow hood for aseptic collection of each cell type. Corneas were placed separately endothelial side down to contact 0.5% trypsin solution<sup>a</sup> for 8 minutes at 37°C and 5% CO<sub>2</sub>. This step was performed in a sterile petri dish to allow the corneas to lie flat in the trypsin solution and to prevent the epithelial side from contacting the trypsin. A sterile cell scraper<sup>b</sup> was used to gently scrape off the endothelium from Descemet's membrane, leaving Descemet's with the stromal layer, as determined by gross examination. The endothelial cells were suspended in DMEM-F12<sup>b</sup> supplemented with 5% fetal bovine serum<sup>b</sup>, 200 IU/mL penicillin<sup>a</sup>, 200 µg/mL streptomycin<sup>a</sup>, 0.5 µg/mL amphotericin B<sup>c</sup>, 20 ng/mL epidermal growth factor<sup>d</sup>, 5.6 µg/mL insulin<sup>d</sup> and 4 mM L-glutamine<sup>b</sup>, and centrifuged at 800 x g for 7 minutes to remove trypsin. The supernant was removed and the cell pellet was suspended in complete media and plated into 3-4 wells in commercially available 6-well rat tail collagen coated plates<sup>b</sup>. These collagen coated plates were used for initial cell growth of all three types of equine corneal cells. The remaining stroma and epithelium were incubated in Dispase II<sup>e</sup> for 1 hour at 37°C and 5% CO<sub>2</sub>. The epithelium was then scraped off with a sterile cell scraper, suspended in complete media and plated into 3-4 wells in 6-well collagen coated plates. The media used was the same as described for the endothelium and each cornea was processed and plated separately. Most of Descemet's membrane was mechanically removed from the remaining stroma using a sterile cell scraper and was noted to be overall removed by gross examination. The stroma was then cut into approximately 3-4 mm pieces, 2-3 explants per well were placed into 4-6 wells in a 6-well collagen coated plate and complete media was added to each well.

### *Culture of equine corneal cells*

Culture media was changed every 1-3 days and the cells were washed with sterile phosphate buffered solution<sup>b</sup> (PBS) before adding fresh media. Endothelial cells were passaged every 7-10 days once they grew to confluency. For this, 0.5% trypsin solution was added to the 6-well collagen coated wells and incubated (~ 8 minutes) at 37°C and 5% CO<sub>2</sub> until the cells were detached and separated. Media was added to the cells to inhibit the enzymatic reaction and they were centrifuged for 7 minutes at 800 x g in sterile 50mL centrifuge tubes<sup>b</sup>. The supernatant was removed and cell pellet suspended in media and plated in either a 25 cm<sup>2</sup> or 75 cm<sup>2</sup> tissue culture flask<sup>b</sup>. The stromal explants were removed after 3 days and the keratocytes were allowed to grow to confluency. . After this, they were passaged as described above. The epithelial cells were passaged by adding 0.25% trypsin for 5 minutes at 37°C and 5%CO<sub>2</sub>. Media was added to stop the enzymatic reaction and the cells were divided and plated into collagen coated 6-well plates for continued growth.

### *Morphology and immunocytochemistry of equine corneal cells*

For identification, the endothelial cells and keratocytes were plated at a concentration of  $1 \times 10^4$  cells per well into 4-well tissue culture slide chambers<sup>b</sup> and allowed to grow for 24-48 hours until they were attached and confluent. The epithelial cells were plated at a concentration of  $1 \times 10^4$  cells per well into 4-well rat tail collagen coated slide chambers<sup>b</sup> and allowed to grow for 24-48 hours until they were attached. The cells were morphologically described and subsequently photographed using an Olympus IX70 inverted microscope containing an Olympus DP70 digital camera and

associated software. This was done for three different passages at different times for all cell types. Once photographed, the cells were fixed with 100% acetone and the slides allowed to dry overnight at -20°C. The endothelial and stromal cells were verified using both direct and indirect immunohistochemistry using Clone V9 monoclonal anti-vimentin antibodies (Catalog number: V6389)<sup>d</sup> at a dilution of 1:200 and Clone V9 CY3 conjugate directly conjugated monoclonal anti-vimentin antibodies (Catalog number: C-9080)<sup>d</sup> at a dilution of 1:200. Epithelial cells were identified using multiple cytokeratin antibodies; including Clone 34BE12 high molecular weight pre-diluted monoclonal anti-cytokeratin antibody (Catalog number: A00071.0025)<sup>f</sup> and Clone ST1 monoclonal wide molecular weight range anti-cytokeratin antibody (Catalog number: A20075)<sup>f</sup>.

Immunocytochemistry was performed on three separate passages at different times for each of the three cell types. All three cell types were incubated with all types of anti-cytokeratin and the anti-vimentin antibodies, allowing the stromal and endothelial cells to serve as negative controls for the epithelial cells and the epithelial cells to be a negative control for the endothelial and stromal cells. Since stromal and endothelial cells had different morphologic characteristics, cellular morphology was used to differentiate between the two cell types. Samples were also incubated with secondary antibodies alone to confirm the absence of nonspecific binding of secondary antibodies to each cell type.

All three cell types underwent indirect immunofluorescence staining. Once fixed, the cells were washed three times with PBS containing 0.05% polyoxyethylenesorbitan monolaurate Tween-20<sup>d</sup> (PBST), incubated with 10% goat serum<sup>d</sup> in PBST for 1 hour at room temperature and incubated with primary antibody for 1 hour at room temperature.

After this, they were washed with PBST, incubated with either Alexa Fluor 488 goat anti-mouse IgG (H+L) (Catalog number: A11001)<sup>c</sup> or Alexa Fluor 546 goat anti-mouse IgG (H+L) (Catalog number A11030)<sup>c</sup>. After removal of the secondary antibody, cells were washed three times with PBST and subsequently mounted with slow fade Gold antifade reagent containing DAPI<sup>c</sup>. The cells were then photographed and images were generated using either an Olympus IX70 inverted microscope containing an Olympus DP70 digital camera and associated software or a Zeiss LSM 510 META confocal microscope.

#### *Cryopreservation of equine corneal cells*

After growth in flasks, the primary cultured endothelial cells and keratocytes from passages 2-5 were detached from the tissue culture 75cm<sup>2</sup> flasks as previously described with 0.5% trypsin. Once detached, complete media was added in an equal volume to that of the trypsin solution to inhibit the enzymatic reaction. This suspension was centrifuged for 7 minutes at 800 x g. The supernant was removed and cell pellets suspended in DMEM-F12 with 10% dimethyl sulfoxide<sup>b</sup> and 10% fetal bovine serum at a concentration of  $\sim 1.5 \times 10^6$  cells/mL. The cells were divided into labeled cryovials<sup>b</sup> in 1mL aliquots. These were then placed in a -80°C freezer for 24-48hr before being transferred into liquid nitrogen for long term storage.

After a minimum of 7 days and maximum of 4 months, each cell type was recovered by briefly placing the cryovial containing the cells in a warm water bath at 37°C until thawed (about 2-3 minutes). The cell suspension ( $1.5 \times 10^6$  cells) was then pipetted into a 75 cm<sup>2</sup> tissue culture flask with complete media. The cells were allowed to adhere and grow. The media was changed after 48hrs and every 1-3 days thereafter.

To examine cell viability, cells were evaluated using trypan blue exclusion after 24hr of growth following recovery.

#### *Flow Cytometry*

Keratocytes and endothelial cells were examined for expression of surface MHC class I (Clone PT85A)<sup>g</sup> and MHC class II (Clone TH14B)<sup>g</sup> using monoclonal antibody staining and flow cytometry. Epithelial cells were not examined due to insufficient cell numbers and the inability to obtain monocultures. The keratocytes and endothelial cells were determined to be greater than 95% viable by trypan blue exclusion prior to staining. Cells were plated into round bottom 96-well plates at a concentration of  $5 \times 10^5$  cells/well and stained for 20 minutes at 4 °C. The primary antibodies were diluted 1:50 in FACS buffer (0.2% bovine serum albumin<sup>d</sup> and 0.15 sodium azide<sup>d</sup> in PBS) and cells were stained with 20μL of each diluted antibody. Cells were then washed three times with cold, sterile FACS buffer and labeled with FITC-conjugated goat anti-mouse IgG<sup>d</sup> for 20 minutes at 4°C; cells were stained using 20μL of diluted (1:100) secondary antibody per well. Cells were then washed three times with cold, sterile FACS buffer and suspended in 500μL of cold FACS buffer. Samples were immediately analyzed using an Accuri C6 flow cytometer<sup>h</sup>. Cellular debris and aggregates were gated out using forward vs. side scatter; at least 10,000 cells were analyzed per sample.

## RESULTS

### *Isolation, culture and morphology of equine corneal cells*

Each corneal cell type had distinct morphologic characteristics allowing differentiation from one another. The isolation methods employed provided pure cultures of endothelial cell and keratocyte populations without difficulty.

Endothelial cells and keratocytes were initially passaged after 7-10 days after primary recovery. After this, they were passaged every 2-6 days depending on confluency. Equine keratocytes reached confluence in 7 days. The endothelial cells reached confluence in 10 days. The keratocytes were noted to grow relatively rapidly. The keratocytes and endothelial cells could be carried out for at least nine passages without marked changes to cell morphology. Epithelial cells were passaged 14-21 days after primary recovery and passaged every 7-10 days thereafter. Epithelial cells reached approximately 80% confluence in the wells within 14-21 days, but did not cover every extent of the wells due to clustering. These cells were carried for three passages; however, they did not form confluent monolayers. The epithelial cells were observed to grow better in cell groups or clusters. Our epithelial cell cultures were also frequently contaminated with keratocytes. After two to three passages, epithelial cells were often overgrown by keratocytes due to their rapid growth rate. Passaging the epithelial cell clusters appeared to optimize space for keratocyte growth.

The keratocytes were approximately half the size of the endothelial cells. The endothelial cells were polygonal to hexagonal cells and grew into confluent monolayers with cobblestone-like appearance. Before confluency, the endothelial cells sent out cytoplasmic processes and exhibited markedly flattened morphology (Figure 3.1A). After

the cells divided and became confluent, the endothelial processes regressed and the cells assumed their characteristic polygonal to hexagonal shape in a monolayer (Figure 3.1B). The keratocytes were small spindloid cells with homogenous morphologic characteristics (Figure 3.1C). These cells had a high proliferation rate and exhibited a typical spindle shape whether subconfluent or confluent. The epithelial cells were polygonal to hexagonal cells with heterologous morphologic features (Figure 3.1D). These cells readily formed clusters with more densely packed areas in a monolayer. The epithelial cells were also observed to decrease proliferation in culture if the cell clusters were broken apart or if the cells were seeding into wells at a low concentration.

#### *Immunocytochemistry of equine corneal cells*

Immunocytochemical staining was performed on all three cell types. The endothelial cells and keratocytes stained positive for vimentin (Figures 3.2A and 3.2B, respectively) and negative for cytokeratin (data not shown). The epithelial cells showed positive staining with high molecular weight anti-cytokeratin primary antibody (data not shown) and wide molecular weight range anti-cytokeratin primary antibody (Figure 3.2C) and negative for vimentin (data not shown). Staining was performed on three different passages between 2-7 at different times for each cell type. Each cell type was photographed after staining.

#### *Cryopreservation of equine corneal cells*

The keratocytes and endothelial cells were recovered after preservation using the described methods for continued growth after cryopreservation. To date, >60 vials of keratocytes and >40 vials of endothelial cells have been cryopreserved. Each cell type

exhibited their specific morphologic characteristics within 24 hours after recovery from freezing. Both cell types grew into confluent monolayers in 1-5 days and were able to be passaged. At least one passage from each cell type was assessed after cryopreservation and recovery for morphologic description and immunocytochemistry. These cells could not be distinguished morphologically or immunocytochemically from the original primary cultured cells. All vials recovered ( $n \geq 20$  for each cell type) have resulted in good cell growth. Twenty-four hours after recovery from liquid nitrogen, all cell cultures showed  $>95\%$  viability as determined by trypan blue exclusion. Cryopreservation was not attempted with the epithelial cells due to keratocyte contamination that was noted after multiple passages.

#### *Flow Cytometry*

Previous studies in other species have demonstrated that corneal cells express MHC class I, but do not express MHC class II under normal physiological conditions.<sup>45-48</sup> To determine whether our purified corneal primary cells demonstrated a similar pattern of expression, cells were stained with monoclonal antibodies specific for equine MHC class I and MHC class II. Staining with goat anti-mouse IgG was used to determine non-specific binding; fluorescence greater than this staining was determined to be positive. Both keratocytes and endothelial cells expressed MHC I (Figure 3.3A and 3.3B, respectively) and did not express MHC II (Figure 3.3C and 3.3D, respectively). Ninety percent of keratocytes were determined to be positive for MHC class I, while 70% of endothelial cells stained positive (Figure 3.3E).

## DISCUSSION

A reproducible method for obtaining and identifying equine corneal epithelial cells, keratocytes and endothelial cells was established using described techniques. This cell isolation method is a modification from previously established canine and feline corneal cell culture.<sup>28-31</sup> The keratocytes and endothelial cells grew very well in the described conditions. These two cell types were grown in tissue culture wells and flasks after initial passaging due to the availability and lower cost of these containers when compared to the collagen coated wells. The explant method for keratocyte growth was a reliable method for obtaining large numbers of equine keratocytes in culture. Equine corneal cells may become contaminated with fungal organisms, a feature not described in other literature. This is likely due to equine normal fungal flora present on the surface of their conjunctiva and cornea. Because we observed fungal contamination in some of the corneal cell cultures, we removed the stromal explants after three days, instead of leaving them in longer, as has been previously reported for canine keratocyte growth.<sup>30</sup> Although, prevention of contamination through earlier removal of the stromal explants was likely conjecture, we obtained good growth of the keratocytes using this method. The methods presented in this study are effective at preventing fungal contamination in the cultures.

As previously reported, epithelial cells were the most difficult cells to obtain in culture and had a noticeably slow growth rate. Epithelial cells grew and adhered in collagen-coated wells with the described media. We observed that cell capture and adherence was markedly enhanced with collagen treated wells, although epithelial cells did grow and adhere in tissue culture treated wells. To optimize equine corneal epithelial cell growth in culture, limbal epithelial cells were included in the corneal collection. This

has been described to help increase the proliferation rate of canine corneal epithelial cells in culture as the limbal epithelial cells are less differentiated and presumably more proliferative than the central epithelial cells.<sup>30</sup> Despite this, keratocyte growth after multiple epithelial passages was noted in our epithelial cell cultures. Fibroblast cell growth has been commonly reported for primary cell culture in many other epithelial cell cultures<sup>49,50</sup> and multiple methods for fibroblast removal have been suggested.<sup>51-53</sup> Attempts to purify our epithelial populations by selective enzymatic detachment of the keratocytes with different concentrations of ethylenediaminetetraacetic acid (EDTA) (0.04mg/mL, 0.1mg/mL, 0.4mg/mL and 1.0mg/mL)<sup>50</sup> were unsuccessful. We observed keratocyte contamination after 2-3 passages. This was likely due to two factors. The high growth rate of keratocytes likely allows them to overtake the epithelial cells. Multiple passaging also breaks up the epithelial cell clusters, providing more space for the keratocytes to grow. The previous study with equine corneal epithelial cells used cells from passages 1-3 and may be the reason why keratocyte contamination was not reported.<sup>44</sup> In addition, this study used definitive corneal cell identification criteria, allowing us to distinguish between epithelial cells and keratocytes. By using identification, we were able to recognize the contamination.

It has been previously reported that corneal endothelial cells maintain polygonal to hexagonal cell morphology when the serum concentration is 4%.<sup>10</sup> For this reason, we chose to grow the cells in media with 5% FBS. While the other cells seemed unaffected by an increase in serum concentration, the endothelial cells tended to develop a more elongated morphology when the FBS concentration approached 10%, as previously reported.<sup>10</sup> Because all the cell types grew well in 5% FBS, we recommend this

concentration for cell growth of all three cell types. Equine corneal cells also do not require the use of cholera toxin in the media. This additive has been reported to optimize the effects of epidermal growth factor and insulin<sup>31</sup>, however we observed growth of all three types of equine corneal cells without this. .

This study is the first to characterize equine corneal cells based on cytoskeletal expression. Endothelial cells and keratocytes express vimentin, an intermediate protein component of cytoplasmic microfilaments expressed in mesenchymal cells. Unlike the endothelial cells and keratocytes, corneal epithelial cells arise from ectoderm.

Cytokeratin is another intermediate protein found in epithelial cells; however, there are over twenty different recognized types of cytokeratin. Cytokeratin 3 and 12 are unique to corneal epithelia.<sup>54-56</sup> Previously, it was shown that fixed bovine cornea expressed high levels of cytokeratin 3 (MW 64) and cytokeratin 12 (MW 56) with moderate to low levels of cytokeratin 4,5 and 6.<sup>57</sup> One of the only anti-cytokeratin antibodies commercially available with published equine cross-reactivity (Clone 34BE12) recognizes cytokeratin 1, 5, 10 and 14. Because it was not known if these cytokeratins would be expressed in equine corneal epithelial cells in culture, we chose to also stain these cells with a wide molecular weight range cytokeratin (Clone ST1). This antibody had not been tested on equine cells; however, we demonstrate here that this antibody is capable of staining equine corneal epithelial cells. Furthermore, this antibody did not stain keratocytes and endothelial cells, cells known to be negative for cytokeratin in other species.<sup>30</sup>

Cytokeratin 1 and 10 are only expressed in cornified epithelium.<sup>55</sup> This would indicate that the corneal epithelial cells are expressing cytokeratin 5, 14 or both. Cytokeratin 5 is expressed in bovine corneal epithelium<sup>57</sup> and multiple reports show that human and rabbit

corneal epithelium express cytokeratin 5 and 14.<sup>9,58</sup> It is likely that equine corneal epithelial cells express both as well. The staining properties of these cells would indicate that there are multiple cytokeratins expressed in equine corneal epithelial cells and two known anti-cytokeratin antibodies would be ideal for immunochemical characterization of these cells.

This report also describes cryopreservation of primary corneal cells, an issue not previously addressed in the veterinary literature. Continued passaging of primary cells in culture has been shown to cause altered cell characteristics.<sup>49</sup> It is therefore important to use early-passage cells for *in vitro* research purposes.<sup>49</sup> Utilization of corneal cells after preservation would be especially helpful. This report is the first one in veterinary medicine to characterize and describe corneal cells after cryopreservation. These cells do not differ morphologically or immunocytochemically from the primary cells. This allows for use of early-passage corneal cells long after they have been collected.

In order to determine the expression of major histocompatibility I (MHC-I) and MHC-II cell surface receptors on equine corneal keratocyte and endothelial cells, flow cytometry was performed on these cell types. Unfortunately, the low numbers of epithelial cells obtained in culture precluded their evaluation. Previously, it has been reported that cultured human corneal epithelial cells, keratocytes and endothelial cells express MHC-I, but not MHC-II in the normal, healthy state.<sup>46-48</sup> MHC-I surface antigens are expressed in most cells of the body. MHC-II surface antigens induce cell-mediated immune responses and are upregulated in certain disease processes.<sup>46</sup> We found that normal equine corneal keratocytes and endothelial cells expressed MHC-I, but

not MHC-II (Figure 3.3). This information may be useful for further investigation into inflammation studies with cultured equine corneal cells.

The methods reported in this study are modifications of corneal cell culture methods utilized in other species.<sup>28-31</sup> These methods have been used reproducibly and predictably to obtain three distinct equine corneal cell types and to identify these cells in culture. This work may form a basis for expanding equine corneal *in vitro* research. Future work would include measuring cellular responses to applied stimuli, evaluating cell behavior and biology in culture and possibly investigation into culturing these cells on various carriers to determine if they may be used in transplantation work. This work is intended to provide a construct for continued development of equine corneal cell culture research.

<sup>a</sup> Mediatech Inc., Manassas, VA

<sup>b</sup> Fisher Scientific, Norcross, GA

<sup>c</sup> Invitrogen, Carlsbad, CA

<sup>d</sup> Sigma Chemical Co., St. Louis, MO

<sup>e</sup> Gibco, Burlington, ON, Canada

<sup>f</sup> Scytek Laboratories, Logan, UT

<sup>g</sup> VMRD, Pullman, WA

<sup>h</sup> Accuri, model C6, Ann Arbor, MI

## REFERENCES

1. Brooks DE. Inflammatory stromal keratopathies: medical management of stromal keratomalacia, stromal abscesses, eosinophilic keratitis, and band keratopathy in the horse. *Vet Clin North Am Equine Pract* 2004;20:345-360, vi.
2. Gilger BC, Michau TM, Salmon JH. Immune-mediated keratitis in horses: 19 cases (1998-2004). *Vet Ophthalmol* 2005;8:233-239.
3. Andrew SE, Brooks DE, Smith PJ, et al. Equine ulcerative keratomycosis: visual outcome and ocular survival in 39 cases (1987-1996). *Equine Vet J* 1998;30:109-116.
4. Gaarder JE, Rebhun WC, Ball MA, et al. Clinical appearances, healing patterns, risk factors, and outcomes of horses with fungal keratitis: 53 cases (1978-1996). *J Am Vet Med Assoc* 1998;213:105-112.
5. Ollivier FJ, Gilger BC, Barrie KP, et al. Proteinases of the cornea and precorneal tear film. *Vet Ophthalmol* 2007;10:199-206.
6. Scotty NC, Brooks DE, Schuman Rose CD. In vitro efficacy of an ophthalmic drug combination against corneal pathogens of horses. *Am J Vet Res* 2008;69:101-107.
7. Strubbe DT, Brooks DE, Schultz GS, et al. Evaluation of tear film proteinases in horses with ulcerative keratitis. *Vet Ophthalmol* 2000;3:111-119.
8. Blalock TD, Spurr-Michaud SJ, Tisdale AS, et al. Functions of MUC16 in corneal epithelial cells. *Invest Ophthalmol Vis Sci* 2007;48:4509-4518.
9. Kurpakus MA, Maniaci MT, Esco M. Expression of keratins K12, K4 and K14 during development of ocular surface epithelium. *Curr Eye Res* 1994;13:805-814.

10. Yanoff M. Biology in vitro of corneal epithelium and endothelium. *Doc Ophthalmol* 1976;41:157-204.
11. Funderburgh ML, Mann MM, Funderburgh JL. Keratocyte phenotype is enhanced in the absence of attachment to the substratum. *Mol Vis* 2008;14:308-317.
12. Zhang J, Xu K, Ambati B, et al. Toll-like receptor 5-mediated corneal epithelial inflammatory responses to *Pseudomonas aeruginosa* flagellin. *Invest Ophthalmol Vis Sci* 2003;44:4247-4254.
13. Castro-Munozledo F. Corneal epithelial cell cultures as a tool for research, drug screening and testing. *Exp Eye Res* 2008;86:459-469.
14. Fleiszig SM, Zaidi TS, Pier GB. *Pseudomonas aeruginosa* invasion of and multiplication within corneal epithelial cells in vitro. *Infect Immun* 1995;63:4072-4077.
15. Imayasu M, Shiraishi A, Ohashi Y, et al. Effects of multipurpose solutions on corneal epithelial tight junctions. *Eye Contact Lens* 2008;34:50-55.
16. Suzuki T, Sullivan DA. Comparative effects of estrogen on matrix metalloproteinases and cytokines in immortalized and primary human corneal epithelial cell cultures. *Cornea* 2006;25:454-459.
17. Toropainen E, Ranta VP, Vellonen KS, et al. Paracellular and passive transcellular permeability in immortalized human corneal epithelial cell culture model. *Eur J Pharm Sci* 2003;20:99-106.
18. Aboalchamat B, Engelmann K, Bohnke M, et al. Morphological and functional analysis of immortalized human corneal endothelial cells after transplantation. *Exp Eye Res* 1999;69:547-553.

19. Kim HJ, Ryu YH, Ahn JI, et al. Characterization of immortalized human corneal endothelial cell line using HPV 16 E6/E7 on lyophilized human amniotic membrane. *Korean J Ophthalmol* 2006;20:47-54.
20. Chen KH, Azar D, Joyce NC. Transplantation of adult human corneal endothelium ex vivo: a morphologic study. *Cornea* 2001;20:731-737.
21. Lai JY, Chen KH, Hsiue GH. Tissue-engineered human corneal endothelial cell sheet transplantation in a rabbit model using functional biomaterials. *Transplantation* 2007;84:1222-1232.
22. Pan Z, Zhang W, Wu Y, et al. Transplantation of corneal stem cells cultured on amniotic membrane for corneal burn: experimental and clinical study. *Chin Med J (Engl)* 2002;115:767-769.
23. Tsai RJ, Li L, Chen J. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells(1). *Am J Ophthalmol* 2000;130:543.
24. Higa K, Shimmura S, Kato N, et al. Proliferation and differentiation of transplantable rabbit epithelial sheets engineered with or without an amniotic membrane carrier. *Invest Ophthalmol Vis Sci* 2007;48:597-604.
25. Hitani K, Yokoo S, Honda N, et al. Transplantation of a sheet of human corneal endothelial cell in a rabbit model. *Mol Vis* 2008;14:1-9.
26. Kim JT, Chun YS, Song KY, et al. The effect of in vivo grown corneal epithelium transplantation on persistent epithelial defects with limbal stem cell deficiency. *J Korean Med Sci* 2008;23:502-508.

27. Liu XW, Zhao JL. [Corneal endothelial cell transplantation for cornea endothelium cell destruction in rabbits]. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao* 2007;29:407-412.
28. Hendrix DV, Ward DA, Barnhill MA. Effects of antibiotics on morphologic characteristics and migration of canine corneal epithelial cells in tissue culture. *Am J Vet Res* 2001;62:1664-1669.
29. Hendrix DV, Ward DA, Barnhill MA. Effects of anti-inflammatory drugs and preservatives on morphologic characteristics and migration of canine corneal epithelial cells in tissue culture. *Vet Ophthalmol* 2002;5:127-135.
30. Werner A, Braun M, Kietzmann M. Isolation and cultivation of canine corneal cells for in vitro studies on the anti-inflammatory effects of dexamethasone. *Vet Ophthalmol* 2008;11:67-74.
31. Sandmeyer LS, Keller CB, Bienzle D. Culture of feline corneal epithelial cells and infection with feline herpesvirus-1 as an investigative tool. *Am J Vet Res* 2005;66:205-209.
32. Sandmeyer LS, Keller CB, Bienzle D. Effects of interferon-alpha on cytopathic changes and titers for feline herpesvirus-1 in primary cultures of feline corneal epithelial cells. *Am J Vet Res* 2005;66:210-216.
33. Sandmeyer LS, Keller CB, Bienzle D. Effects of cidofovir on cell death and replication of feline herpesvirus-1 in cultured feline corneal epithelial cells. *Am J Vet Res* 2005;66:217-222.
34. Brooks DE, Andrew SE, Biros DJ, et al. Ulcerative keratitis caused by beta-hemolytic *Streptococcus equi* in 11 horses. *Vet Ophthalmol* 2000;3:121-125.

35. Brooks DE, Andrew SE, Denis H, et al. Rose bengal positive epithelial microerosions as a manifestation of equine keratomycosis. *Vet Ophthalmol* 2000;3:83-86.
36. Brooks DE, Plummer CE, Kallberg ME, et al. Corneal transplantation for inflammatory keratopathies in the horse: visual outcome in 206 cases (1993-2007). *Vet Ophthalmol* 2008;11:123-133.
37. Hendrix DV, Brooks DE, Smith PJ, et al. Corneal stromal abscesses in the horse: a review of 24 cases. *Equine Vet J* 1995;27:440-447.
38. Lassaline ME, Brooks DE, Ollivier FJ, et al. Equine amniotic membrane transplantation for corneal ulceration and keratomalacia in three horses. *Vet Ophthalmol* 2005;8:311-317.
39. Ollivier FJ, Brooks DE, Van Setten GB, et al. Profiles of matrix metalloproteinase activity in equine tear fluid during corneal healing in 10 horses with ulcerative keratitis. *Vet Ophthalmol* 2004;7:397-405.
40. Ollivier FJ, Kallberg ME, Plummer CE, et al. Amniotic membrane transplantation for corneal surface reconstruction after excision of corneolimbal squamous cell carcinomas in nine horses. *Vet Ophthalmol* 2006;9:404-413.
41. Bistner SI, Riis RC. Clinical aspects of mycotic keratitis in the horse. *Cornell Vet* 1979;69:364-374.
42. Weinstein WL, Moore PA, Sanchez S, et al. In vitro efficacy of a buffered chelating solution as an antimicrobial potentiator for antifungal drugs against fungal pathogens obtained from horses with mycotic keratitis. *Am J Vet Res* 2006;67:562-568.

43. Brooks DE, Andrew SE, Dillavou CL, et al. Antimicrobial susceptibility patterns of fungi isolated from horses with ulcerative keratomycosis. *Am J Vet Res* 1998;59:138-142.
44. Haber M, Cao Z, Panjwani N, et al. Effects of growth factors (EGF, PDGF-BB and TGF-beta 1) on cultured equine epithelial cells and keratocytes: implications for wound healing. *Vet Ophthalmol* 2003;6:211-217.
45. Lee SK, Choi BK, Kang WJ, et al. MCP-1 derived from stromal keratocyte induces corneal infiltration of CD4+ T cells in herpetic stromal keratitis. *Mol Cells* 2008;26:67-73.
46. Donnelly JJ, Li WY, Rockey JH, et al. Induction of class II (Ia) alloantigen expression on corneal endothelium in vivo and in vitro. *Invest Ophthalmol Vis Sci* 1985;26:575-580.
47. Whitsett CF, Stulting RD. The distribution of HLA antigens on human corneal tissue. *Invest Ophthalmol Vis Sci* 1984;25:519-524.
48. Dreizen NG, Whitsett CF, Stulting RD. Modulation of HLA antigen expression on corneal epithelial and stromal cells. *Invest Ophthalmol Vis Sci* 1988;29:933-939.
49. Freshney RI. *Culture of animal cells : a manual of basic technique*. 5th ed. Hoboken, N.J.: Wiley-Liss, 2005.
50. Freshney RI, Freshney MG. *Culture of epithelial cells*. 2nd ed. New York: Wiley-Liss, 2002.

51. Jensen PK, Therkelsen AJ. Cultivation at low temperature as a measure to prevent contamination with fibroblasts in epithelial cultures from human skin. *J Invest Dermatol* 1981;77:210-212.
52. Linge C, Green MR, Brooks RF. A method for removal of fibroblasts from human tissue culture systems. *Exp Cell Res* 1989;185:519-528.
53. Halaban R, Alfano FD. Selective elimination of fibroblasts from cultures of normal human melanocytes. *In Vitro* 1984;20:447-450.
54. Franke WW, Schiller DL, Moll R, et al. Diversity of cytokeratins. Differentiation specific expression of cytokeratin polypeptides in epithelial cells and tissues. *J Mol Biol* 1981;153:933-959.
55. Moll R, Franke WW, Schiller DL, et al. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* 1982;31:11-24.
56. Chen WY, Mui MM, Kao WW, et al. Conjunctival epithelial cells do not transdifferentiate in organotypic cultures: expression of K12 keratin is restricted to corneal epithelium. *Curr Eye Res* 1994;13:765-778.
57. Paladino G, Marino C, La Terra Mule S, et al. Cytokeratin expression in primary epithelial cell culture from bovine conjunctiva. *Tissue Cell* 2004;36:323-332.
58. Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* 1986;103:49-62.

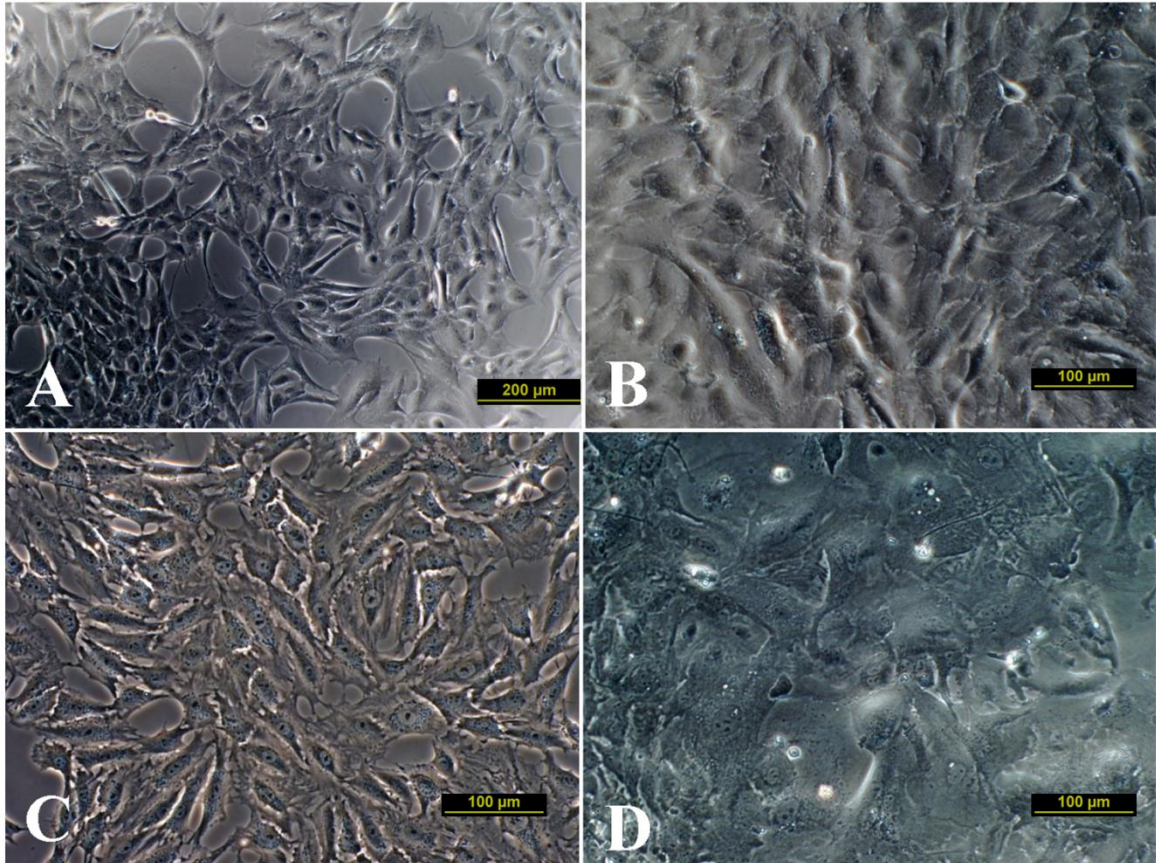


Figure 3.1—Photomicrographs of primary equine corneal cells plated in glass chamber slides for morphologic evaluation. When subconfluent, endothelial cells demonstrated long cytoplasmic processes and markedly flattened morphology. A representative culture photographed at 100X magnification is depicted (A). At confluency, endothelial cells assumed a polygonal shape with cobble-stone like appearance (B). Keratocytes were small, spindloid cells with homogenous morphology exhibiting a rapid growth rate (C). The epithelial cells were large, polygonal cells with heterologous morphology which readily formed clusters (D). Confluent endothelial cells (B), keratocytes (C) and epithelial cells (D) are photographed here at 200X magnification.

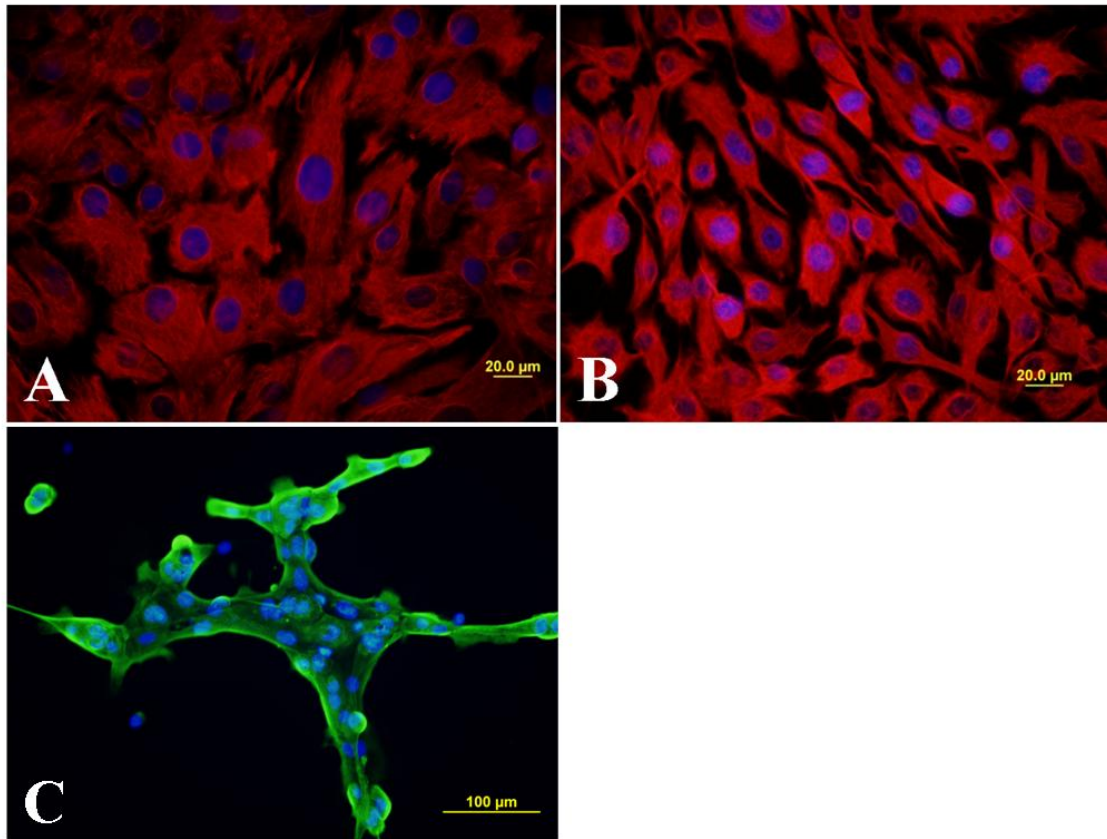


Figure 3.2—Primary equine corneal endothelial cells (A) and keratocytes (B) depicted at 400X magnification stained positive with anti-vimentin antibody CY3. Vimentin positive cells appear red with DAPI-stained nuclei appearing blue. Primary equine corneal epithelial cells stained positive with high molecular weight anti-cytokeratin antibody 34BE12 and wide molecular weight range anti-cytokeratin antibody ST1 (C). These cells clustered in culture, as represented at 200X magnification. A representative culture stained with antibody ST1 is depicted. Cytokeratin positive cells appear green with DAPI-stained nuclei appearing blue.

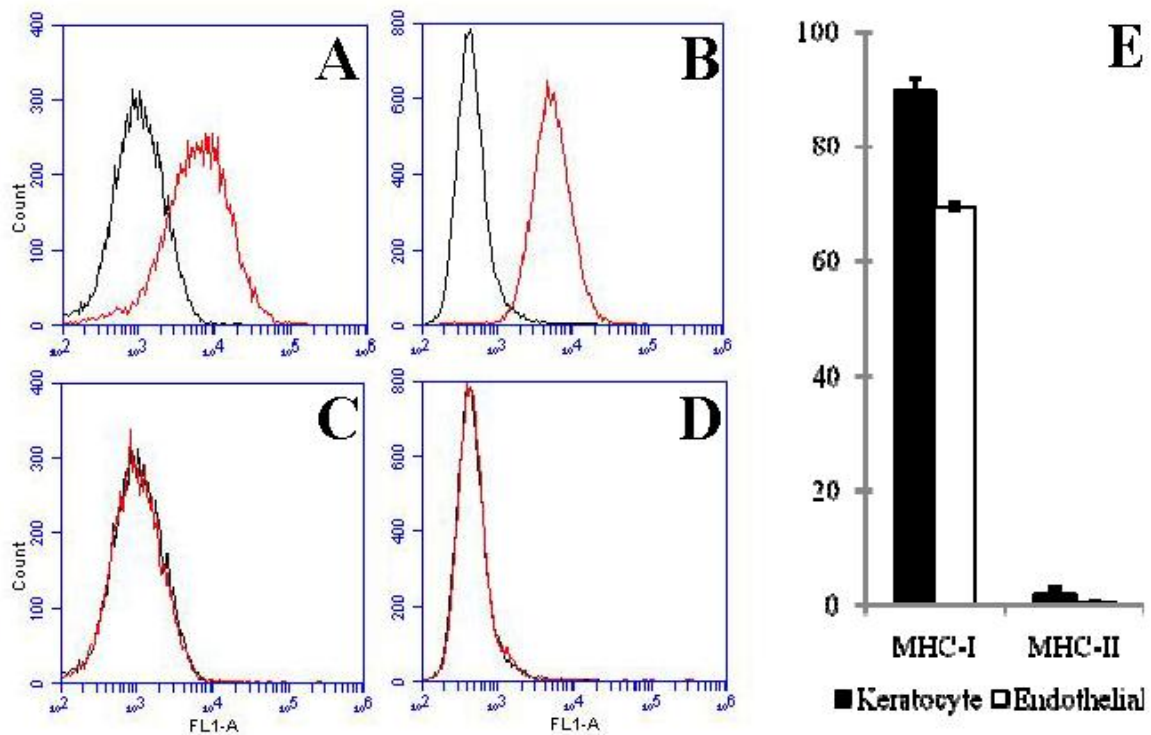


Figure 3.3—Flow cytometric analysis demonstrates surface expression of MHC class I on equine corneal endothelial cells (A) and keratocytes (B), following staining with monoclonal antibody PT85A. Representative flow cytometry histograms are depicted showing cells stained with goat anti-mouse IgG in black and positive monoclonal antibody PT85A staining in red. Neither cell type [endothelial cells (C) or keratocytes (D)] showed surface expression of MHC class II following staining with monoclonal antibody TH14B. Ninety percent of equine keratocytes showed MHC class I expression while 70% of equine endothelial cells showed expression (E). Both cell types did not express MHC class II. Data represent the average of replicate experiments for each cell type. Error bars represent standard error of the mean.

**CHAPTER 4**

**EFFECTS OF ANTIFUNGAL DRUGS AND DELIVERY VEHICLES ON**

**MORPHOLOGY AND PROLIFERATION OF EQUINE CORNEAL**

**KERATOCYTES *IN VITRO***

---

<sup>1</sup> R. L. Mathes, A. J. Reber, D. J. Hurley, U. M. Dietrich.  
Submitted for publication in the *American Journal of Veterinary Research*

## ABSTRACT

**Objective:** To evaluate effects of topical antifungal drugs and delivery vehicles on the morphology and proliferation rate of cultured equine corneal keratocytes.

**Procedures:** Primary cultures of equine keratocytes were exposed to several concentrations of three commonly used topical antifungal drugs, natamycin, itraconazole and miconazole. In addition, effects of these drug delivery vehicles, dimethyl sulfoxide, benzalkonium chloride, carboxymethylcellulose and a combination vehicle composed of polyethylene glycol, methylparaben and propylparaben, were also evaluated.

Morphologic changes and cellular proliferation were assessed 24hr, 48hr and 72hr after application.

**Results:** At the highest concentrations tested, all antifungal drugs caused marked cellular morphologic changes and inhibited proliferation. At lower concentrations, natamycin and miconazole induced rounding, shrinking and detachment of the cells with inhibition of cellular proliferation. Natamycin caused the most severe cellular changes.

Itraconazole, at the lower concentrations, caused minimal morphologic changes and had minimal effect on proliferation. All vehicles tested demonstrated significantly lower effects on cellular morphology and proliferation when compared to the antifungal drugs except for the combination vehicle of polypropylene glycol, methylparaben and propylparaben. This vehicle caused severe morphologic changes and inhibited proliferation, even at low concentrations. It was additionally found that dimethyl sulfoxide had minimal effects on cellular morphology and proliferation, even at higher concentrations.

**Conclusions:** Natamycin and miconazole induced marked cellular changes on equine keratocytes. In contrast, itraconazole had notably milder cellular effects. Dimethyl sulfoxide, although not commonly used as a carrier for topical ophthalmic antifungal drugs, produced minimal cellular changes and warrants further consideration as a potential ophthalmic vehicle.

## INTRODUCTION

Fungal keratitis is a serious, vision-threatening disease that is relatively common in horses, compared to other domestic species.<sup>1-3</sup> In addition to normal bacterial flora, horses have fungal flora present on the surface of their cornea and conjunctiva. Ninety-five percent of horses, in one study, were found to have normal fungal flora, in contrast to only 40% of cats and 22% of dogs with fungi found normally on their conjunctiva.<sup>4</sup> Under normal physiological conditions, the cornea is protected by both mechanical and physical barriers to help prevent pathogen mediated entry. If a break occurs in this normal defense; however, invasion of bacteria and fungi into the cornea can easily occur.<sup>1,5,6</sup> Horses likely develop fungal keratitis readily due to normal fungal flora and close proximity to fungal organisms in their environment. Many features of fungal keratitis are unknown, in part because this disease is relatively unique to the horse and data cannot be extrapolated from other domestic species. In addition, most research investigating fungal keratitis in horses has been based on *in vivo* studies<sup>2,3,6-10</sup> which are inherently limited by patient variables, patient numbers, housing, personnel and reproducibility.

Equine fungal keratitis is difficult to treat, in part, due to the marked pathogenic potential of fungal organisms.<sup>11,12</sup> Fungal organisms may cause proteinase-mediated corneal destruction, or keratomalacia, allowing them to invade into the corneal stroma.<sup>12</sup> These proteinases may be produced by the immune system or fungal organisms themselves.<sup>13-16</sup> In addition to causing stromal destruction, fungal organisms are thought to have a specific affinity for Descemet's membrane, causing them to travel deep into the posterior stroma.<sup>17</sup> Fungal keratitis, as a result, often presents as a deep stromal defect with extensive corneal involvement.<sup>18,19</sup>

Topical ophthalmic antifungal drugs are the primary medications employed to treat fungal keratitis.<sup>20,21</sup> Drugs from the azole and polyene families are currently the only antifungal medications used for topical ophthalmic administration.<sup>5,22-25</sup> Both classes of drugs function to interfere with normal synthesis or function of the fungal cell wall.<sup>5,20,23</sup> Natamycin, a polyene, is the only commercially available topical ophthalmic antifungal medication available in the United States. It has the broadest spectrum of activity against fungal organisms<sup>20,26</sup> and has greater efficacy against all isolates of *Fusarium sp.* when compared to other antifungal drugs.<sup>6,26</sup> Other commonly used topical antifungal medications include miconazole, itraconazole, fluconazole, voriconazole and ketoconazole.<sup>26-28</sup> These products are compounded for use in the eye.<sup>29</sup> Miconazole was effective against all isolates of *Aspergillus sp.* and had good activity against other fungal isolates in a susceptibility study.<sup>26</sup> Miconazole also has good corneal penetration when given topically.<sup>30,31</sup> Itraconazole has a broader spectrum of activity against fungal organisms than ketoconazole and fluconazole and is very effective against *Aspergillus sp.*<sup>21,25,26</sup> Itraconazole has also been shown to be effective for treatment of clinical equine

keratomycosis.<sup>25</sup> Voriconazole, a new azole antifungal, has recently been investigated for use in horses, but its use topically is somewhat limited due to cost and unknown safety.<sup>23,32</sup>

Very little is currently known about potential local corneal toxicity of antifungals when administered topically in horses. Itraconazole (1%) ointment suspended in dimethyl sulfoxide (DMSO) caused no corneal histologic or gross abnormalities in six normal horses.<sup>24</sup> A pharmaceutical study with voriconazole assessed ocular toxicity based on clinical descriptions of epiphora, blepharospasm and conjunctival hyperemia, however, no direct corneal toxicity was evaluated.<sup>32</sup> The purpose of this study was to evaluate the direct effects of commonly used antifungal drugs, natamycin, itraconazole and miconazole, along with their respective drug delivery vehicles on equine corneal keratocyte morphology and cellular proliferation *in vitro*.

## **MATERIALS AND METHODS**

### *Primary Equine Keratocyte Culture*

Equine keratocytes were obtained in primary cell culture according to previously described methods.<sup>33</sup> Briefly, corneas were aseptically collected from horses recently euthanized (< 0.5hr) for reasons unrelated to the study. The corneal layers were enzymatically separated first by incubation for 8min in 0.5 % trypsin<sup>a</sup> at 37° C and 5 % CO<sub>2</sub> to separate the endothelium from the stroma and epithelium. This was followed by incubation of the stroma and epithelium in Dispase II<sup>b</sup> (2.4 U/mL) for 1hr at 37° C and 5 % CO<sub>2</sub>. After removal of the epithelium mechanically with a sterile cell scraper, the stromal layer was sectioned into several 3-4 mm explants and 2-3 explants per well were

placed into 6-well collagen coated plates<sup>c</sup>. After placement of the explants into wells, DMEM-F12<sup>c</sup> supplemented with 5 % fetal bovine serum<sup>c</sup>, 200 IU/mL penicillin, 200 µg/mL streptomycin<sup>d</sup>, 0.5 µg/mL amphotericin B<sup>a</sup>, 20 ng/mL epidermal growth factor<sup>e</sup>, 5.6 µg/mL insulin<sup>e</sup> and 4 mM L-glutamine<sup>c</sup> was added to each well and the plates were incubated at 37° C and 5 % CO<sub>2</sub>. The explants were removed after 3 days and media was changed every 1-3 days in the wells. After the cells became confluent in approximately 7 days, 0.5 % trypsin was added per well and incubated at 37° C and 5 % CO<sub>2</sub> for 8min until the cells were detached and separated. Media was added to the cells to stop the enzymatic reaction and they were centrifuged for 7min at 800 x G. The supernant was removed and the cell pellet was resuspended in media and plated in either a 25 cm<sup>2</sup> or 75 cm<sup>2</sup> tissue culture flask. Keratocytes were passaged in the described manner when confluency was reached in the flasks. Keratocytes were identified morphologically and immunocytochemically with anti-vimentin antibody staining as previously described.<sup>33</sup> Passages 2-8 were used for the study.

#### *Equine Keratocyte Morphologic Evaluation*

For morphologic evaluation, keratocytes were plated into 24-well tissue culture plates<sup>c</sup> at a density of  $2 \times 10^4$  cells per well. After plating, the cells were allowed to adhere and grow for 48hrs. At this time, the selected antifungal drugs in their respective vehicles and vehicles alone were added in media at the following concentrations: benzalkonium chloride<sup>f</sup> (BC) (20 µg/mL, 2 µg/mL, 0.2 µg/mL), carboxymethylcellulose<sup>g</sup> (CM) (600 µg/mL, 60 µg/mL, 6 µg/mL), polyethylene glycol 40 castor oil<sup>g</sup> (PG) (1.15 %, 0.115 %, 0.0115 %)/methylparaben<sup>g</sup> (72.5 µg/mL, 7.25 µg/mL, 0.725

µg/mL)/propylparaben<sup>g</sup> (7.5 µg/mL, 0.75 µg/mL, 0.075 µg/mL), dimethyl sulfoxide<sup>c</sup> (DMSO) (6000 µg/mL, 600 µg/mL, 60 µg/mL), natamycin<sup>g</sup> (5000 µg/mL, 500 µg/mL, 50 µg/mL), itraconazole<sup>g</sup> (1000 µg/mL, 100 µg/mL, 10 µg/mL) and miconazole<sup>g</sup> (1000 µg/mL, 100 µg/mL, 10 µg/mL). All substances had a neutral pH of 7.0-7.5. Morphology of the cells was evaluated at 24hr, 48hr and 72hr. Each application and grading was done four times on separate cell passages (passage 2-8). All trials were done with a control well consisting of media and an equal volume of phosphate buffered solution<sup>c</sup> added at the highest treatment volume. The highest concentration of all antifungal drugs and delivery vehicles represented the physiologic concentration after topical administration, accounting for tear film dilution.<sup>34-39</sup> Concentrations of all drugs and vehicles were diluted ten-fold for each lower concentration.

Morphologic changes were based on the following grading scale: 0 – no evidence of any morphologic changes, no cell detachment from the plate, normal cell adherence and appearance in culture, 1 – normal cell adherence to the plate, < 30 % of cells showing rounding or loss of typical spindloid appearance, 2 – 10-20 % cell detachment from the plate, 30-60 % of cells showing rounding or loss of normal spindloid appearance, 3 – 20-50 % detachment from the plate, > 60 % of cells showing rounding, shrinking or loss of normal spindloid appearance, 4 – 50-90 % detachment of cells from plate, severe rounding of all cells and complete loss of spindloid appearance in all cells, 5 – 100 % detachment from plate, no recognizable keratocytes in culture, loss of cells, severe shrinking and rounding. It was not possible to blind the investigators to the treatments as the addition of antifungal drugs and carriers made easily distinguishable differences in

media appearance between treatment groups. This was due to viscosity differences in the drug vehicles and poor solubility of the antifungal drugs.

#### *Equine Keratocyte Cellular Proliferation*

For cellular proliferation, keratocytes were plated into 96-well tissue culture plates<sup>c</sup> at a concentration of  $5 \times 10^3$  cells per well. The cells were allowed to adhere and grow for 48hr before application of the specific treatments. After this, 100  $\mu$ L of the treatments at the following concentrations were added to each well: benzalkonium chloride (BC) (20  $\mu$ g/mL, 2  $\mu$ g/mL, 0.2  $\mu$ g/mL), carboxymethylcellulose (CM) (600  $\mu$ g/mL, 60  $\mu$ g/mL, 6  $\mu$ g/mL), polyethylene glycol 40 castor oil (PG) (1.15 %, 0.115 %, 0.0115 %, 0.00115 %, 0.000115 %)/methyparaben (72.5  $\mu$ g/mL, 7.25  $\mu$ g/mL, 0.725  $\mu$ g/mL, 0.0725  $\mu$ g/mL, 0.00725  $\mu$ g/mL)/propylparaben (7.5  $\mu$ g/mL, 0.75  $\mu$ g/mL, 0.075  $\mu$ g/mL, 0.0075  $\mu$ g/mL, 0.00075  $\mu$ g/mL), dimethyl sulfoxide (DMSO) (25,000  $\mu$ g/mL, 6000  $\mu$ g/mL, 600  $\mu$ g/mL, 60  $\mu$ g/mL), natamycin (5000  $\mu$ g/mL, 500  $\mu$ g/mL, 50  $\mu$ g/mL, 5  $\mu$ g/mL, 0.5  $\mu$ g/mL), itraconazole (1000  $\mu$ g/mL, 100  $\mu$ g/mL, 10  $\mu$ g/mL) and miconazole (1000  $\mu$ g/mL, 100  $\mu$ g/mL, 10  $\mu$ g/mL, 1  $\mu$ g/mL, 0.1  $\mu$ g/mL). The highest concentration of all antifungal drugs and delivery vehicles represented the physiologic concentration after topical administration, accounting for tear film dilution. Concentrations of all drugs and vehicles were diluted ten-fold for each lower concentration. Antifungal drugs and vehicles that inhibited cellular proliferation at 1,000-fold were diluted further in two more 10-fold dilutions to reach a point at which cellular proliferation was not inhibited. Each proliferation evaluation was done at least three times on different passages (passages 2-5) and evaluated at 24hr, 48hr and 72hr. Early passages were used for

proliferation to help eliminate variability between inherent proliferation properties of early vs. late passage cells.

To evaluate cellular proliferation, 20  $\mu$ L of 1:500 5'-bromodeoxyuridine<sup>h</sup> (BrdU) was added to each well and allowed to incubate for 24hrs for each time point. Cells were processed according to the manufacture's published protocol. Briefly, cells were fixed for 30min. After multiple washings, the cells were then incubated with mouse anti-BrdU monoclonal antibody (1:200) for 1hr. This was followed by another series of washes and incubation with peroxidase goat anti-mouse IgG antibody 1:2000 for 30min. After another series of washes, pre-diluted TMB Peroxidase was added for 30min. After incubation in the dark, stop solution was added and the plates were read at 450 nm using a spectrophotometric microtiter plate reader. For each trial, wells were included in assay processing that contained cells, but did not have BrdU added. This baseline reactivity was subtracted from all values.

### *Statistical Evaluation*

Morphologic evaluation was evaluated using a 1-way ANOVA with a Bonferroni correction to compare among multiple groups. Cellular proliferation was evaluated using a 1-way ANOVA with a Bonferroni correction to compare among groups at the 10-fold, 100-fold and 1,000-fold dilutions. Because natamycin and miconazole dilutions were carried further for cellular proliferation data, these two groups were compared using an unpaired T-test, at the 10,000-fold and 100,000-fold dilutions. Results were considered significant at a P value of  $\leq 0.05$ .

## RESULTS

### *Growth and Morphology Equine Corneal Keratocytes*

Keratocytes were small spindloid cells in culture with a high growth rate. These cells were morphologically and immunocytochemically confirmed to be a monoculture.<sup>33</sup>

### *Effects of Applied Substances to Morphologic Characteristics of Equine Keratocytes*

Cellular morphology was graded at all time points for all concentrations of antifungal drugs and delivery vehicles. Each experiment was repeated four times and the scores were averaged for comparison between groups. Morphologic scores for control wells were consistent with Grade 0 at all time points for all experiments. Morphologic changes were compared between each antifungal drug in its vehicle and the vehicle alone to ensure that any changes were due to the active drug and not due to vehicle effects (Figure 4.1). The scores for the natamycin and itraconazole were significantly higher than their vehicle scores when compared at each concentration tested. The scores for miconazole and its vehicle, PG, were not significantly different, making it impossible to determine if the cellular effects were due to the active drug or due to the vehicle. Miconazole was therefore suspended in a new vehicle, CM and DMSO, to determine whether the morphologic effects were due to the active drug or the carrier (Figure 4.2). The new vehicle showed significantly lower scores when compared to the miconazole, indicating that the morphologic changes were due to miconazole and not to its carrier.

Antifungal drug effects were then compared with each other at all concentrations. Only the scores for miconazole in the new vehicle (CM/DMSO) were used for comparison among antifungals. This was done to ensure that morphologic scoring was

not confounded by drug vehicle effects from the combination carrier, PG. Itraconazole had the least effect on cellular morphology for all time points when compared to natamycin and miconazole. Natamycin caused the most severe morphologic changes to the cells. Results are summarized in figure 4.3. All antifungal drugs showed marked morphologic changes with cell rounding, shrinking and detachment at the highest concentration tested. At all time points, the 100-fold dilution of itraconazole had significantly lower effects on cellular morphology than natamycin and miconazole at the same concentration. At 1000-fold dilution, natamycin showed significantly greater morphologic effects than itraconazole and miconazole for all time points.

#### *Effects of Antifungal Drugs and Delivery Vehicles on Cellular Proliferation*

Cellular proliferation was also evaluated at specified time points for each substance at different concentrations. Effects of antifungal drugs in the respective vehicles were compared with vehicles alone to ensure that effects on proliferation were due to the active drug and not due to their carriers. The results observed when comparing the vehicles and antifungal drugs were similar to the results seen when comparing morphologic changes to the cells after substance application (Figure 4.1). Natamycin and itraconazole vehicles had a significantly lower effect on cellular proliferation than did the drugs in their respective vehicles (data not shown). The miconazole vehicle, PG, did not have significantly different inhibition of proliferation when compared to miconazole in PG. Miconazole was therefore suspended in the alternative carrier, CM/DMSO, as was done for morphology, and proliferation data was collected for this substance. Miconazole effects on proliferation were significantly higher when compared to the alternative carrier

(data not shown). Cellular proliferation data was compared between all antifungal groups using the data from miconazole in this vehicle, CM/ DMSO, as was done for morphologic comparisons to eliminate confounding vehicle effects. Results are expressed as a percentage of control proliferation, after subtracting baseline, and are summarized in figure 4.4. The 10-fold concentration of all antifungal drugs virtually eliminated all cellular proliferation when applied to the keratocytes at all time points. At 24 hours, itraconazole inhibited proliferation less than did natamycin and miconazole at the 100-fold and 1,000-fold dilutions; however, these results were not significant. Natamycin and miconazole dilutions were continued to reach a point at which cellular proliferation was not inhibited to allow comparison between these two antifungals. At 24 hours, natamycin and miconazole inhibited proliferation below that of the control, even at 10,000-fold and 100,000-fold dilutions. At 48 hours, itraconazole showed minimal effects on cellular proliferation while natamycin and miconazole markedly inhibited proliferation at 100-fold dilutions. At 1,000-fold dilutions, itraconazole did not affect proliferation while natamycin caused marked inhibition. At further dilutions, natamycin and miconazole did not affect cellular proliferation at 48 hours. Similar results were seen at 72 hours with itraconazole showing the least effects on cellular proliferation when compared to natamycin and miconazole. At the lowest concentration, a dilution of 100,000-fold, miconazole showed statistically less inhibition to cellular proliferation than did natamycin.

## DISCUSSION

Equine keratocytes have several ideal characteristics *in vitro*, making them amenable to drug application studies. Keratocytes have predictable small, spindloid cell morphology in culture whether subconfluent or confluent and readily form a monolayer.<sup>33,40</sup> These two characteristics allow for definitive grading criteria when evaluating changes after drug application. Keratocytes are also able to be carried out for multiple passages readily without changes to their morphology or immunocytochemical staining properties.<sup>33</sup> In addition, keratocytes have a high growth rate, allowing for cellular proliferation evaluation after drug application as a measurement of cell health. While it would have been ideal to additionally evaluate equine corneal epithelial cells in this study, our purified equine corneal epithelial cells did not lend themselves to this type of evaluation due to their slow growth, clustering and short life-span in culture.<sup>33</sup> Other studies have demonstrated similar problems with purified corneal epithelial cells.<sup>40,41</sup>

Clinically, cases of equine keratomycosis are almost always associated with stromal disease and are ulcerative in most cases.<sup>2,12,20,26</sup> Because of this, topical antifungal drugs are almost always in contact with keratocytes directly when treating fungal keratitis. It has been shown that antifungal drug penetration is markedly increased when the corneal epithelium is removed<sup>42</sup> and epithelial debridement is often performed in cases of equine fungal keratitis to optimize drug delivery.<sup>2</sup> Keratocyte health would presumably be of utmost importance, therefore, in cases of equine keratomycosis.

Drug concentrations for this study were selected based upon clinically relevant concentrations of topically applied drugs. Topical ophthalmic medications are immediately diluted due to the normal tear film properties.<sup>35-39</sup> This dilution is quite

significant and it is estimated that anywhere from 1-10% of a topically applied drug is actually available on the corneal surface immediately after application.<sup>35,36,39</sup> While surfactants and suspending agents may increase the viscosity of the drug suspension, allowing for potentially longer contact time, there is still very little drug remaining in contact with the corneal cells after 4-23 minutes.<sup>36,38</sup> Tear production is further increased from baseline in cases of corneal disease and after application of topical medication due to irritation.<sup>35,36,43</sup> Because of this, the highest applied concentration of antifungal drugs and vehicles reflected the concentration of drug that would be found in contact with corneal cells immediately after application, taking into account tear dilution properties. This concentration is 10% of the topical ophthalmic drug preparation. The first concentration was then subsequently diluted 10-fold for the concentrations following. Because the drugs and vehicles were maintaining constant contact with the cells, the lower concentrations are likely more indicative of the clinical exposure of the keratocytes to the antifungal drug, even with frequent topical administration.

Antifungal drugs and delivery vehicles used in clinical settings were selected for this study to evaluate *in vitro* cellular effects. When antifungal drug effects were compared to drug vehicle effects, we found that one vehicle, PG, a combination of polyethylene glycol 40 castor oil, methylparaben and propylparaben, caused marked morphologic changes, even at the lower concentrations tested (Figure 4.1). Polyethylene glycol (PEG) is a commonly used surfactant, likely desirable as an antifungal vehicle as it helps increase topical drug contact time due to its viscosity. Methylparaben and propylparaben are commonly used as preservatives. The ingredients in this vehicle are similar to those used across many compounding pharmacies.<sup>29</sup> This vehicle was

determined to be representative for a clinical setting as similar techniques are employed to optimize delivery of antifungal drugs due to their low solubility. Benzalkonium chloride, another common preservative, and carboxymethylcellulose, a suspending agent, both had very little to no cellular effect at the lower concentrations tested (Figure 4.1). Various surfactants and suspending agents are used as delivery vehicles to optimize topical antifungal drug contact time. Our results would indicate that certain delivery vehicles may have less cytopathological effects than others, although more work would need to be done to fully determine *in vivo* effects. This study; however, underscores the importance of discriminate selection of delivery vehicles based not only on their surfactant and suspending properties, but also on their potential deleterious cellular effects.

Because many antifungal drugs have low solubility in aqueous solutions, we tested a commercially available solvent, dimethyl sulfoxide (DMSO), on the keratocytes. DMSO increases the penetration of itraconazole to allow for better drug delivery in the cornea<sup>24,25</sup> and likely has this effect for other antifungal drugs. In our study, DMSO had very little effect on keratocytes (Figure 4.1) even at the higher concentrations tested. Because DMSO has been shown to increase drug delivery in the cornea and has very low cytopathological effects on equine keratocytes *in vitro*, it may be an ideal antifungal drug delivery vehicle for topical ophthalmic administration.

In our study, itraconazole had markedly lower cytopathological effects in comparison to miconazole and natamycin. At the lower concentrations, miconazole was less cytotoxic than natamycin. For cellular proliferation studies, miconazole and natamycin were diluted further to reach a point at which the drug concentration used did

not have a significant effect. This was done to more effectively compare the two drugs at lower concentrations. While more work is needed to fully evaluate the *in vivo* cellular effects, this study demonstrates measurable cytopathic effects of antifungal drugs on keratocytes at clinically relevant concentrations. Although selection of an antifungal agent is based on many factors, cytopathologic effects should be considered, especially with repeated administration of these agents for extended periods of time. This study emphasizes the marked differences in cytopathologic effects between antifungal drugs when applied to corneal cells and may help guide the clinician in selection of a topical antifungal drug.

The purpose of this study was to evaluate the effects of antifungal drugs and their vehicles on cultured equine keratocytes. *In vitro* drug testing has many benefits including high reproducibility, lower cost, elimination of patient issues, decreased personnel requirements, enhanced control of variables and large sample sizes. Equine corneal keratocytes may be grown in culture and used to evaluate direct drug effects using criteria directly related to cell health in culture, such as cell morphology and proliferation. This study showed that itraconazole has significantly lower cellular effects than the other two antifungal drugs tested. It was also shown that different drug vehicles have markedly different cellular effects. Effects of antifungal drugs and vehicles on corneal cell health should be accounted for when selecting a drug for *in vivo* application as increased drug cytotoxicity may decrease corneal healing.

<sup>a</sup> Invitrogen, Carlsbad, CA

<sup>b</sup> Gibco, Burlington, ON, Canada

<sup>c</sup> Fisher Scientific, Norcross, GA

<sup>d</sup> Mediatech, Manassas, VA

<sup>e</sup> Sigma Chemical, St. Louis, MO

<sup>f</sup> Winthrop-Breon Laboratories, New York, NY

<sup>g</sup> Wedgewood Pharmacy, Swedesboro, NJ

<sup>h</sup> Exalpha Biological, Watertown, MA

## REFERENCES

1. Barton M. Equine Keratomycosis. *Compend Contin Educ Pract Vet* 1992;14:936-944.
2. Gaarder JE, Rebhun WC, Ball MA, et al. Clinical appearances, healing patterns, risk factors, and outcomes of horses with fungal keratitis: 53 cases (1978-1996). *J Am Vet Med Assoc* 1998;213:105-112.
3. Andrew SE, Brooks DE, Smith PJ, et al. Equine ulcerative keratomycosis: visual outcome and ocular survival in 39 cases (1987-1996). *Equine Vet J* 1998;30:109-116.
4. Samuelson DA, Andresen TL, Gwin RM. Conjunctival fungal flora in horses, cattle, dogs, and cats. *J Am Vet Med Assoc* 1984;184:1240-1242.
5. Thomas PA. Current perspectives on ophthalmic mycoses. *Clin Microbiol Rev* 2003;16:730-797.
6. Ledbetter EC, Patten VH, Scarlett JM, et al. In vitro susceptibility patterns of fungi associated with keratomycosis in horses of the northeastern United States: 68 cases (1987-2006). *J Am Vet Med Assoc* 2007;231:1086-1091.

7. Rosa M, Cardozo LM, da Silva Pereira J, et al. Fungal flora of normal eyes of healthy horses from the State of Rio de Janeiro, Brazil. *Vet Ophthalmol* 2003;6:51-55.
8. Brooks DE, Andrew SE, Denis H, et al. Rose bengal positive epithelial microerosions as a manifestation of equine keratomycosis. *Vet Ophthalmol* 2000;3:83-86.
9. Andrew SE, Nguyen A, Jones GL, et al. Seasonal effects on the aerobic bacterial and fungal conjunctival flora of normal thoroughbred brood mares in Florida. *Vet Ophthalmol* 2003;6:45-50.
10. Grahn BW, J; Keller, C; et al. Equine keratomycosis: clinical and laboratory findings in 23 cases. *Prog Vet Comp Ophthalmol* 1993;3:2-7.
11. McLaughlin SAG, B. C.; Whitley, R. D. . Infectious keratitis in horses: evaluation and management. *Compend Contin Educ Pract Vet* 1992;14:372-379.
12. Brooks DE. Inflammatory stromal keratopathies: medical management of stromal keratomalacia, stromal abscesses, eosinophilic keratitis, and band keratopathy in the horse. *Vet Clin North Am Equine Pract* 2004;20:345-360, vi.
13. Strubbe DT, Brooks DE, Schultz GS, et al. Evaluation of tear film proteinases in horses with ulcerative keratitis. *Vet Ophthalmol* 2000;3:111-119.
14. Ollivier FJ, Gilger BC, Barrie KP, et al. Proteinases of the cornea and preocular tear film. *Vet Ophthalmol* 2007;10:199-206.
15. Monod M, Capoccia S, Lechenne B, et al. Secreted proteases from pathogenic fungi. *Int J Med Microbiol* 2002;292:405-419.
16. Tomee JF, Kauffman HF. Putative virulence factors of *Aspergillus fumigatus*. *Clin Exp Allergy* 2000;30:476-484.

17. Gelatt KN. *Veterinary ophthalmology*. 4th ed. Ames, Iowa: Blackwell Pub., 2007.
18. Gilger BC. *Equine ophthalmology*. St. Louis, Mo.: Elsevier Saunders, 2005.
19. Bistner SI, Riis RC. Clinical aspects of mycotic keratitis in the horse. *Cornell Vet* 1979;69:364-374.
20. Scotty NC. Equine Keratomycosis. *Clin Tech Equine Pract* 2005;4:29-36.
21. Ford MM. Antifungals and their use in veterinary ophthalmology. *Vet Clin North Am Small Anim Pract* 2004;34:669-691.
22. Xu KP, Li XF, Yu FS. Corneal organ culture model for assessing epithelial responses to surfactants. *Toxicol Sci* 2000;58:306-314.
23. Colitz CM, Latimer FG, Cheng H, et al. Pharmacokinetics of voriconazole following intravenous and oral administration and body fluid concentrations of voriconazole following repeated oral administration in horses. *Am J Vet Res* 2007;68:1115-1121.
24. Ball MA, Rebhun WC, Trepanier L, et al. Corneal concentrations and preliminary toxicological evaluation of an itraconazole/dimethyl sulphoxide ophthalmic ointment. *J Vet Pharmacol Ther* 1997;20:100-104.
25. Ball MA, Rebhun WC, Gaarder JE, et al. Evaluation of itraconazole-dimethyl sulfoxide ointment for treatment of keratomycosis in nine horses. *J Am Vet Med Assoc* 1997;211:199-203.

26. Brooks DE, Andrew SE, Dillavou CL, et al. Antimicrobial susceptibility patterns of fungi isolated from horses with ulcerative keratomycosis. *Am J Vet Res* 1998;59:138-142.
27. Sansom J, Featherstone H, Barnett KC. Keratomycosis in six horses in the United Kingdom. *Vet Rec* 2005;156:13-17.
28. Weinstein WL, Moore PA, Sanchez S, et al. In vitro efficacy of a buffered chelating solution as an antimicrobial potentiator for antifungal drugs against fungal pathogens obtained from horses with mycotic keratitis. *Am J Vet Res* 2006;67:562-568.
29. Malmberg GJ, Lupo R. Compounding in veterinary ophthalmology. *Vet Clin North Am Small Anim Pract* 2004;34:825-838.
30. Foster CS, Stefanyszyn M. Intraocular penetration of miconazole in rabbits. *Arch Ophthalmol* 1979;97:1703-1706.
31. Foster CS. Miconazole therapy for keratomycosis. *Am J Ophthalmol* 1981;91:622-629.
32. Clode AB, Davis JL, Salmon J, et al. Evaluation of concentration of voriconazole in aqueous humor after topical and oral administration in horses. *Am J Vet Res* 2006;67:296-301.
33. Mathes RM, Dietrich UD, Krunkosky TM, et al. Establishing a Reproducible Method for the Culture of Primary Equine Corneal Cells. Athens, GA: University of Georgia, 2009.
34. Hendrix DV, Stuffle JL, Cox SK. Pharmacokinetics of topically applied ciprofloxacin in equine tears. *Vet Ophthalmol* 2007;10:344-347.

35. Myrna KE, Herring IP. Constant rate infusion for topical ocular delivery in horses: a pilot study. *Vet Ophthalmol* 2006;9:1-5.
36. Baeyens V, Gurny R. Chemical and physical parameters of tears relevant for the design of ocular drug delivery formulations. *Pharm Acta Helv* 1997;72:191-202.
37. Zhang J, Wang L, Gao C, et al. Ocular pharmacokinetics of topically-applied ketoconazole solution containing hydroxypropyl beta-cyclodextrin to rabbits. *J Ocul Pharmacol Ther* 2008;24:501-506.
38. Wilson CG. Topical drug delivery in the eye. *Exp Eye Res* 2004;78:737-743.
39. Scotty NC, Brooks DE, Schuman Rose CD. In vitro efficacy of an ophthalmic drug combination against corneal pathogens of horses. *Am J Vet Res* 2008;69:101-107.
40. Haber M, Cao Z, Panjwani N, et al. Effects of growth factors (EGF, PDGF-BB and TGF-beta 1) on cultured equine epithelial cells and keratocytes: implications for wound healing. *Vet Ophthalmol* 2003;6:211-217.
41. Werner A, Braun M, Kietzmann M. Isolation and cultivation of canine corneal cells for in vitro studies on the anti-inflammatory effects of dexamethasone. *Vet Ophthalmol* 2008;11:67-74.
42. O'Day DM, Ray WA, Head WS, et al. Influence of the corneal epithelium on the efficacy of topical antifungal agents. *Invest Ophthalmol Vis Sci* 1984;25:855-859.
43. Hendrix DV, Ward DA, Barnhill MA. Effects of anti-inflammatory drugs and preservatives on morphologic characteristics and migration of canine corneal epithelial cells in tissue culture. *Vet Ophthalmol* 2002;5:127-135.

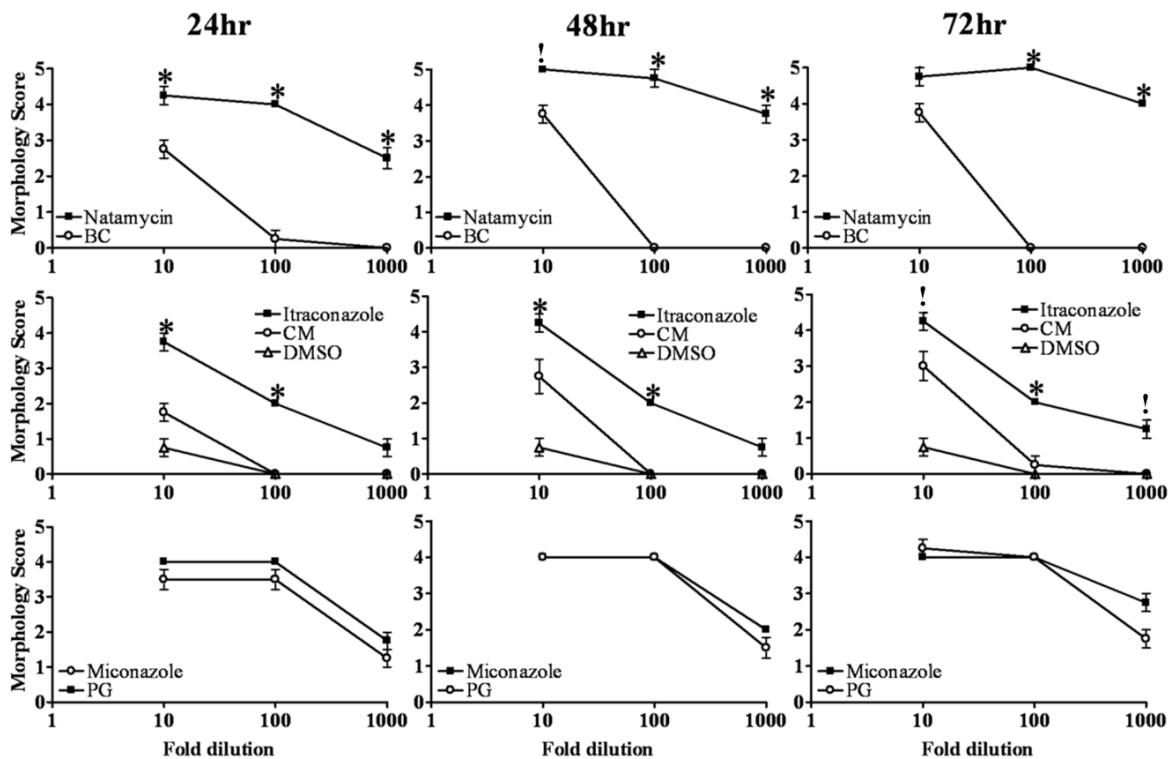


Fig 4.1: Equine keratocytes were exposed to antifungal drugs in their respective vehicles or their vehicles alone at clinically relevant concentrations, adjusting for tear film dilution. Morphologic changes were scored for each concentration at 24hr, 48hr and 72hr. Statistical significance is denoted by the following: \* p<0.001, ! p<0.01.

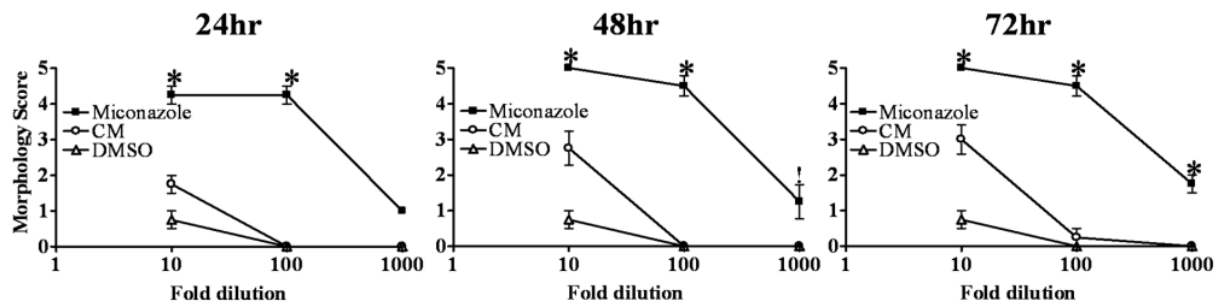


Fig 4.2: Morphologic scoring was compared for miconazole suspended in carboxymethylcellulose (CM) and dimethyl sulfoxide (DMSO) vs. CM and DMSO alone at clinically relevant concentrations at 24hr, 48hr and 72hr. The highest concentration tested (10-fold dilution) is consistent with topical antifungal drug concentration accounting for tear film dilution. Miconazole had significantly higher effects on keratocyte morphology than did the vehicle. Statistical significance is denoted by the following: \*  $p < 0.001$ , !  $p < 0.01$ .

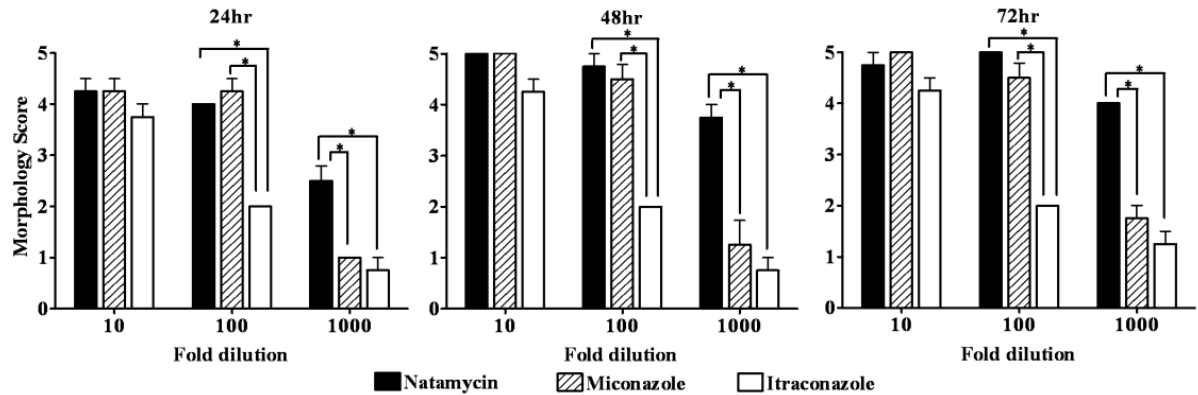


Fig 4.3: Keratocytes were exposed to different antifungal drugs at clinically relevant concentrations for 24hr, 48hr and 72hr, accounting for tear film dilution. Morphologic changes were evaluated and scored at each time point. Antifungal drugs were compared to each other to assess cellular morphologic effects. At the highest concentration, all antifungal drugs had severe effects on cell morphology at all time points. Itraconazole had significantly lower cellular effects on morphology than did natamycin and miconazole at a 100-fold dilution. At a 1,000-fold dilution, natamycin had significantly greater effects on cell morphology than miconazole and itraconazole. Statistical significance is denoted by the following: \*  $p < 0.001$ .

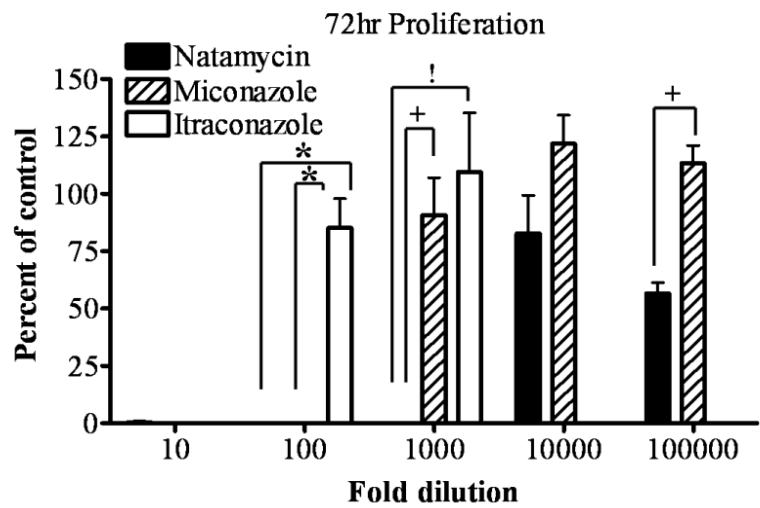
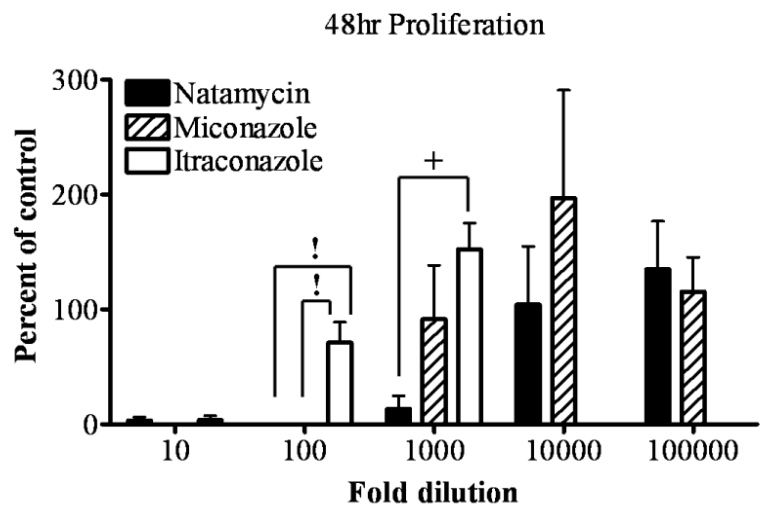
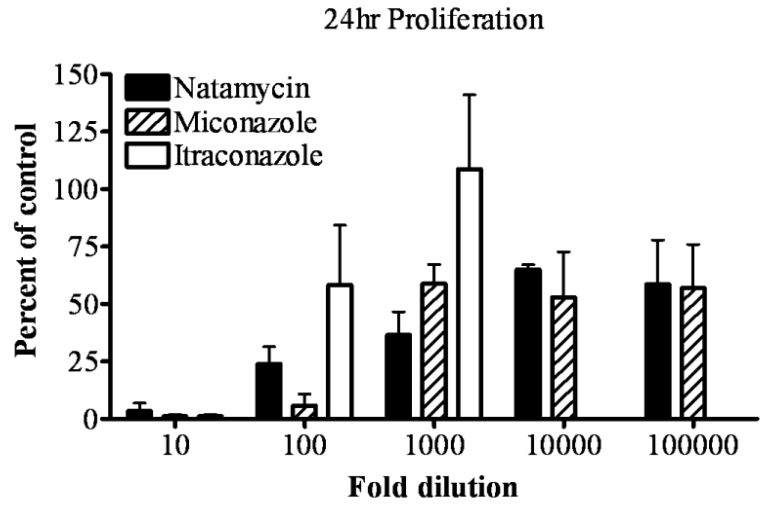


Fig 4.4: Cellular proliferation of equine keratocytes was assessed 24hr, 48hr and 72hr after application of natamycin, miconazole or itraconazole at clinically relevant concentrations, accounting for tear film dilution. Antifungal drugs were compared to each other to assess effects on cellular proliferation. Cellular proliferation is expressed as a percentage of control (100 %). At 24hr, itraconazole inhibited cellular proliferation less than miconazole or natamycin at the 10-fold dilution; however, these results are not significant. At 48hr and 72hr, itraconazole had a significantly lower effect on cellular proliferation than did natamycin and miconazole at 100-fold dilutions. At 1,000-fold dilutions, itraconazole had no effect on cellular proliferation at 48 hours while natamycin significantly inhibited proliferation. At 72hr, itraconazole and miconazole had minimal effect on cellular proliferation while natamycin completely inhibited cellular proliferation at 1,000-fold dilutions. Statistical significance is denoted by the following: \*  $p < 0.001$ , !  $p < 0.01$ , +  $p \leq 0.05$ .

## CHAPTER 5

### DISCUSSION AND CONCLUSION

Most equine corneal research has been performed *in vivo*, creating marked limitations for research advancement due, in part, to expense, time and patient variables. By effectively culturing all three types of equine corneal cells, cryopreserving the cells for later recovery, and using the cultured corneal cells to assess the effects of antifungal drugs, this study presents data in support of all three of the hypotheses of this research program. Therefore, this work provides an important addition to *in vitro* equine corneal research.

Although equine corneal epithelial cells and keratocytes have been cultured previously, the cells had not been fully characterized in culture, based on morphologic descriptions and immunocytochemical staining.<sup>1</sup> In fact, no methods were available for the establishment of all three types of equine corneal cells in culture. This work was effective in describing reproducible methods for obtaining each of the three equine corneal cell types in primary cultures, freezing stocks of these cells for future study and utilizing these cells for drug testing. Aseptic collection of equine corneas was performed and all three types of corneal cells were grown from these processed corneas. It was also clearly demonstrated that equine corneal epithelial cells, keratocytes and endothelial cells can be grown in the same medium formulation under similar culture conditions. This

medium offered major advantages in that all ingredients are available commercially and can be obtained without special permits for their use.

An important part of this study was to establish a set of clear criteria for identifying each of the three corneal cell types. Because corneal cells in other species have specific cytoplasmic components that may be identified with immunocytochemistry, similar methods were utilized to characterize equine corneal cells.<sup>2</sup> Our findings indicate that equine keratocytes and endothelial cells are both positive for vimentin. However, these two types of cells have different growth patterns and are morphologically different in culture, making it easy to distinguish them from one another. Epithelial cells are positive for cytokeratin and have distinct morphological features in culture. The epithelial cells grown in the present study were contaminated by keratocytes, a problem encountered commonly in other studies with primary epithelial cell cultures.<sup>3-6</sup>

Of the three cell types, the epithelial cells were the most difficult cells to obtain in primary culture, a finding that has been previously reported for other species.<sup>2</sup> This may be, in part, due to the lack of a complete basement membrane between the stromal and epithelial layers. Canine corneal epithelial cells have been obtained using explant methods in which sections of stroma and epithelium are placed epithelial surface down in tissue-culture plates and the epithelium is allowed to grow out from the explant.<sup>7</sup> This method has been reported to be less effective at capturing epithelial cells in culture when compared to enzymatic and mechanical removal of the epithelial layer.<sup>2</sup> In our study, the latter approach resulted in good primary epithelial cell growth. However, after multiple passages, there was evidence of keratocyte contamination. In an effort to selectively inhibit keratocyte binding to the culture plates, we exposed the cells to various

concentrations of EDTA for a variety of incubation times. This has been reported to be effective for removing contaminating fibroblasts in some primary epithelial cell cultures.<sup>3</sup> Unfortunately, this approach was not an effective way to eliminate the keratocytes in our cultures.

In addition to definitive identification of each of the three corneal cell types, we were able to cryopreserve and recover equine keratocytes and endothelial cells. This is a technique has not been previously used for veterinary cell culture studies. As primary cell cultures tend to develop altered cellular characteristics and stop proliferating with multiple passages, it is important that early-passage cells be used in research to most closely reflect *in vivo* conditions.<sup>3</sup> Based on the results from the current study, early passage equine keratocytes and endothelial cells may be preserved for long periods of time in liquid nitrogen for later use. Furthermore, they maintain their morphologic and cytoskeletal properties after recovery from the frozen state, and may be used in experiments to determine the responses of corneal cells to pathogens or treatments. We were unable to demonstrate a clear freezing and recovery protocol for corneal epithelial cells, in large part due to their contamination with keratocytes and their slow growth rate.

The ultimate goal of establishing equine corneal cells in culture is to utilize them for *in vitro* corneal research studies. This has been done with canine corneal epithelial cells to measure cellular changes in response to applied anti-inflammatory drugs and antibiotics.<sup>7,8</sup> In the current project using primary cultures of equine keratocytes, we monitored two parameters, namely cell morphology and cell proliferation, before and after application of three different antifungal drugs and four different drug vehicles. We determined that incorporation of 5'-bromodeoxyuridine into keratocytes was an effective

way to evaluate cell proliferation utilizing a commercially available ELISA kit, and that itraconazole affected cell morphology and proliferation the least, while natamycin most severely affected the cells.

The effects of topical ophthalmic drugs depend on a variety of factors *in vivo* including tear film properties, drug permeability, host cell health and the presence of pathogens. While every *in vivo* condition may not be replicated in the *in vitro* setting, evaluating specific cellular responses to drugs that are used topically provides valuable information that clinicians can use to select specific drugs under certain conditions. In human medicine, there is evidence that corneal epithelial cell layers *in vitro* and epithelial cells *in vivo* respond similarly to topically applied substances.<sup>9</sup> These findings have expanded use of *in vitro* cell culture for application to preliminary drug-testing in human medicine. Using methods that have been used successfully with corneal cells from humans and rabbits, it may become possible to test the effects of topically applied ophthalmic drugs in equine cell cultures to avoid toxic or ineffective drug candidates before they are used *in vivo*.

This thesis provides a construct for further *in vitro* equine corneal cell research and expands current knowledge in this field. Because *in vitro* work is not limited by patient variables, high costs, low numbers and poor reproducibility, this work provides a valuable addition to *in vivo* equine corneal research. By demonstrating that each of the three types of equine corneal cells can be cultured, that two of these cell types can be stored frozen and recovered to fully functional cells, and that cultured corneal cells may be used to measure responses to topically applied substances, this thesis enhances our

knowledge of equine corneal cells and suggests that significant progress can be made in equine corneal research through utilization of *in vitro* modalities.

## REFERENCES

1. Haber M, Cao Z, Panjwani N, et al. Effects of growth factors (EGF, PDGF-BB and TGF-beta 1) on cultured equine epithelial cells and keratocytes: implications for wound healing. *Vet Ophthalmol* 2003;6:211-217.
2. Werner A, Braun M, Kietzmann M. Isolation and cultivation of canine corneal cells for in vitro studies on the anti-inflammatory effects of dexamethasone. *Vet Ophthalmol* 2008;11:67-74.
3. Freshney RI. *Culture of animal cells : a manual of basic technique*. 5th ed. Hoboken, N.J.: Wiley-Liss, 2005.
4. Jensen PK, Therkelsen AJ. Cultivation at low temperature as a measure to prevent contamination with fibroblasts in epithelial cultures from human skin. *J Invest Dermatol* 1981;77:210-212.
5. Linge C, Green MR, Brooks RF. A method for removal of fibroblasts from human tissue culture systems. *Exp Cell Res* 1989;185:519-528.
6. Halaban R, Alfano FD. Selective elimination of fibroblasts from cultures of normal human melanocytes. *In Vitro* 1984;20:447-450.
7. Hendrix DV, Ward DA, Barnhill MA. Effects of antibiotics on morphologic characteristics and migration of canine corneal epithelial cells in tissue culture. *Am J Vet Res* 2001;62:1664-1669.

8. Hendrix DV, Ward DA, Barnhill MA. Effects of anti-inflammatory drugs and preservatives on morphologic characteristics and migration of canine corneal epithelial cells in tissue culture. *Vet Ophthalmol* 2002;5:127-135.
9. Castro-Munozledo F. Corneal epithelial cell cultures as a tool for research, drug screening and testing. *Exp Eye Res* 2008;86:459-469.