2017

A novel ex vivo model of equine corneal wound healing

Rita Fay Wehrman
Iowa State University

A novel *ex vivo* model of equine corneal wound healing

by

Rita Fay Wehrman

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Veterinary Clinical Sciences (Veterinary Medicine)

Program of Study Committee:
Gil Ben-Shlomo, Major Professor
Rachel A. Allbaugh
Brett A. Sponseller

Iowa State University

Ames, Iowa

2017

DEDICATION

I dedicate my thesis work to my husband Steve Sanders. You make every day better and I cannot thank you enough for going on this journey with me.

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I would like to thank my committee chair, Gil Ben-Shlomo and my committee members, Rachel Allbaugh and Brett Sponseller, for their guidance and support throughout the course of this research.

In addition, I would also like to thank my parents for their guidance and support. I would not be who I am today without you both.

I would also like to thank my resident mates, Rachel Strauss and Travis Strong, and our additional faculty member Lionel Sebbag. You make work, and learning, fun.

Lastly, I would like to thank the College of Veterinary Medicine at Iowa State University for continuing to make my time here a wonderful and memorable experience.

ABSTRACT

Corneal disease is a significant cause of decreased vision or blindness in the horse worldwide. Numerous topical and systemic drugs over a prolonged period of time may be used to treat an equine corneal ulcer. Despite the high prevalence of corneal disease and ulceration in the horse, and the complexity of corneal wound healing, research is limited and more heavily devoted to *in vivo* studies whose limitations include small sample sizes and ethical considerations. Bench top laboratory *in vitro* studies are limited in number and overlook the complexity of corneal wound healing by assessing each corneal layer separately from its neighbor.

The objective of this study was to develop a novel *ex vivo* model of equine corneal wound healing that maintained structural integrity, and is physiologically relevant, mimicking *in vivo* events.

In this randomized and controlled study, fourteen equine corneas were harvested within two hours of humane euthanasia for reasons unrelated to this study. All donors were screened and corneas were included if they were free of ocular and systemic disease. Corneas were immediately processed and corneoscleral rims excised 2 mm posterior to the limbus. Corneas were randomly assigned to one of the following groups: wounded (n=8) or unwounded (n=6) controls. Each pair of eyes was divided such that corneas from the same horse were assigned to a different group. In the event that only one of the two eyes was included, the eye was assigned to the wounded group. In the 'wounded' group, the axial cornea was wounded for 60 seconds

with a 6 mm disk of filter paper soaked in 1N sodium hydroxide (NaOH). Epithelial ulceration was confirmed employing fluorescein stain. Subsequently, all corneas were cultured using an air-liquid interface model in complete media. The corneal cups were placed on a rocker set at a 6 degree incline and incubated at 37 °C with 5% CO_2 humidity. The media bathed the cornea 8 times per minute to simulate normal horse blinking. Evaluation of corneal healing was performed daily and included fluorescein staining and fluorescein retention (i.e. ulcer measurements). Corneas from both groups were randomly assigned to undergo further processing via histopathology at designated time points of 24 hours (T24; n=5), T48 (n=5), and T72 (n=4) post wounding, and stained with hematoxylin and eosin. Structural integrity and pathologic changes to the corneal epithelium, stroma, Descemet's membrane and endothelium were assessed. Interleukin-6 (IL-6) expression was then evaluated by means of RNAscope *in situ* hybridization.

All wounded corneas healed within 72 hours. Histologically, normal corneal architecture was observed including intact epithelium, minimal stromal edema, and presence of endothelium. Increased IL-6 expression was noted in wounded corneas compared to unwounded controls and was predominantly in the metabolically active basal epithelial cell layer. As the corneal wound healing progressed, IL-6 expression decreased.

The equine air-liquid interface, *ex vivo*, corneal wound healing model is an effective and physiologically relevant model. This model can be utilized to reduce or

eliminate the use of live horses for corneal wound healing studies, while providing an effective platform for corneal wound healing research in this species.

CHAPTER 1. GENERAL INTRODUCTION

Structure of the Equine Cornea

The cornea and sclera are part of the fibrous tunic of the eye and their junction is known as the limbus. The cornea makes up approximately 20% of this outer fibrous tunic and due to its transparency, curvature, and air liquid interface with the outer precorneal tear film (PTF) and the inner ocular media, is the most refractive part of the eye.[1,2,3] Because of its anterior location, its purpose is also to protect the inner contents of the eye.[4] This elliptically shaped structure is comprised of four main layers in the horse, including the epithelium, stroma, Descemet's membrane, and the endothelium (Fig. 1-1). [1,2] In the horse, the average total thickness of the cornea is between 700 and 900 µm.[5,6,7]

Epithelium

The corneal epithelium is the anterior most layer of the cornea and is a continuation of the conjunctival epithelium (Fig. 1-1). It is in contact with the PTF superficially, which also aids in light refraction. The main function of the epithelium is to serve as a barrier against infection, thereby protecting the rest of the eye.[2] As the cornea is maintained in a state of relative dehydration, any loss of this hydrophobic layer (i.e. ulceration) may compromise the cornea's transparency via an influx of water from tears.[1] Clinically this is appreciated as corneal edema and experimental removal of this layer results in a 200% increase in corneal thickness within 24 hours.[8] In the horse, this layer is up to 15 cell layers thick and consists of three cell

types, which from anterior to posterior are known as superficial epithelial cells, wing cells, and basal cells.[9,10,11] These different cell types communicate with themselves and one another using different intercellular junctions including desmosomes, gap junctions, and zonula occludens.[1,12] These modes of communication contribute to expedient wound healing.[13] As the superficial epithelium is constantly being sloughed off, or desquamating, the deeper wing cells proliferate and move towards the corneal surface, while the basal cells then differentiate into wing cells. Additionally, corneal stem cells are housed in the limbus, or corneaoscleral junction, and as the three cell types push anteriorly, the stem cells migrate centripetally to 're-stock' the basal and superficial layers.[14,15]

Stroma

The stroma is deep to the epithelium, comprises about 90% of the total corneal thickness and is 78% water (Fig. 1-1).[1] This layer is made up of collagen fiber bundles known as lamellae which run parallel to, and across the length of the cornea. The stroma is further subdivided into the anterior stroma and posterior stroma. The anterior portion is considered to be less orderly and the lamellae are smaller in both thickness and width compared to the posterior stroma.[1] They are arranged in a more oblique, or woven, orientation. Deep to the anterior stroma is the posterior stroma, whose broad sheets of lamellae are lined up in a more parallel, uniform, nonwoven orientation. In general, the stroma's collagen fibrils are more precisely arranged with fewer spaces between the fibrils in the central zone versus the peripheral. This specific organization of the corneal stroma also plays a large

role in maintaining corneal clarity.[16,17] Distributed in between the lamellae is the predominant stromal cell type, known as the keratocyte, which communicate via tight and gap junctions. Other cells that may reside normally within the stroma include macrophages, dendritic cells and lymphocytes.[1,18]

Descemet's membrane

The next corneal layer is Descemet's membrane, which is the basement membrane of the endothelium (Fig. 1-1). It is also composed of collagen similar to the stroma; however, this collagen is more layered, which gives it an elastic property that is more resistant to trauma. Descemet's membrane is approximately 10 µm thick and it thickens with age. This thin layer is thought to have regenerative properties.[15]

Endothelium

The inner most layer of the cornea is the endothelium (Fig. 1-1). In this delicate, 5 µm monolayer, cells are joined to one another via tight and gap junctions. This layer's function includes actively pumping aqueous humor via Na^+/K^+-ATPase pumps back into the anterior chamber, and it is most responsible for maintaining a state of relative dehydration in the cornea.[1,8] This has been demonstrated experimentally, in which removal of the endothelium resulted in an increase of greater than 500% in overall corneal thickness.[8] Most mammals are born with their total, maximum amount of endothelial cells, which slowly decreases with time. In the

horse, this endothelial cell density has been established at 3155+/-765 cells/mm^2.[7] As this layer cannot generally undergo mitosis, it 'heals' itself by cellular spreading, causing changes in cell shape (pleomorphism) and size (polymegethism). Should the endothelial cellular density decrease by too large an index to pump fluid efficiently, severe, diffuse corneal stromal edema will result.[15] In the dog, this has been established at less than 500 to 800 cells/mm^2 and is known as corneal decompensation.[1] To date, this threshold has not been determined in the horse.

While the endothelium has the most active job in maintaining corneal transparency, compromise of any of the aforementioned layers will result in corneal pathology and an impaired visual axis. Corneal clarity relies upon many factors including the presence of each layer, appropriate architectural integrity, and the individual cell's ability to communicate with itself and its neighbors. These factors are the cornerstone of corneal wound healing.

Corneal Wound Healing

Corneal injuries are vision and eye threatening across species. Corneal healing is a complex process that involves all cellular layers and their ability to communicate via mediators with one another. Each layer responds differently to insult.

Epithelial healing

The corneal epithelium has stem cells located within the limbus of the eye. These corneal limbal stem cells are able to provide the cornea with a lifetime of

epithelium.[19,20-25] The objective of epithelial wound healing is two-fold: first, to trigger migration of the epithelial cells to the wounded area, and second to upregulate cell proliferation and differentiation, all with the end goal of filling the defect and therefore healing the wound. This is done via autocrine and paracrine cellular mediated signaling with a significant amount of 'cross talk' between the corneal epithelium, limbal stem cells, and the underlying stromal cells.[13] Initial factors that are released following epithelial wound healing include interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α)[13] and growth factors,[26] that trigger the epithelial cells migration and then proliferation. Interleukin-6 (IL-6) also plays a large role, and has been shown to significantly increase epithelial wound healing rates in a rabbit *in vivo* model and to stimulate epithelial cell migration in rabbit *in vitro* models.[27,28] Additionally, matrix metalloproteinases are released, which cause the cells to disengage from one another and to release their cell matrix adhesions.[29]

Stromal healing

Due to this layer's thickness, it goes without saying that the organized healing of the stroma is of paramount importance.[15] Should this layer heal without the precision it requires, there will be a reduction in corneal transparency (i.e. fibrosis, or scar, formation), irregular light refraction, and therefore impaired vision. Damage to the stroma causes a large release of cytokines, that trigger apoptosis of underlying keratocytes.[18,30-34] Once cleared of potentially damaged keratocytes, the remaining activated keratocytes transform into migratory fibroblasts and myofibroblasts. Fibroblasts secrete actin, a contractile cytoplasmic element, while myofibroblasts express a contractile cytoskeleton element known as alpha-smooth muscle actin (α-

SMA). It is well known that the myofibroblast is only detected following stromal corneal wounding and its expression of α-SMA is credited with wound contracture.[25,35] However, this spindeloid myofibroblast cell and its activity with the extracellular matrix (ECM), is at the heart of fibrosis formation within the cornea as the myofibroblast deposits new extracellular matrix and aberrant proteins to fill up the defect.[36,37] With time, these proteins can form scar tissue,[38-46] and as the ECM undergoes very slow turnover (years in humans) scar formation may persist.[40-43,47,48] As such, the "well meaning" myofibroblast has been linked to corneal haze and scar formation following wounding.[18] Inflammatory cells including monocytes, macrophages, polymorphonuclear leukocytes, natural killer cells, and T cells also have a role in stromal wound healing. It is thought these cells enter the cornea at the limbus or through the circulatory system and tear film.[18] The remodeling of the stroma is both complex and lengthy as the anterior and posterior stroma will take months to regain their normal, unique collagen fibril orientation.[1]

Descemet's membrane and endothelial healing

Descemet's membrane has regenerative properties, but similar to the endothelium, has not been studied extensively due to its more interior location relative to the outer stromal and epithelial layers. Compared to epithelial and stromal wound healing, relatively less is known about endothelial wound healing, again due to this layer's inner location within the cornea. However, unlike the epithelium, the endothelium undergoes little to no proliferation, instead closing wound gaps by migration and spreading.[49-52] In the rabbit, a transient morphology to

a myofibroblast-type, α-SMA expressing migratory cell has been noted.[25,53,54] To date, this has not been demonstrated in the horse.

Cellular communication

There is a significant amount of 'cross talk' between the corneal layers, and while each layer heals uniquely, the overall tissue architectural organization lends itself to cellular communication.[55] These corneal cells release mediators secondary to injury that are distributed throughout the cornea using gap junctions, tight junctions, and zonula occludens.[1,13,18] Therefore, models of corneal wound healing that include all corneal layers and that maintain normal corneal architecture are of utmost importance.[56] While *in vitro* assessment of cell lines is certainly important and not to be overlooked due to its relatively low cost and high speed, this type of over simplified model is not able to mimic the complexities of the interactions both at the corneal cellular level, nor at the corneal organ level.[55] Further exploration of the interlayer communication in corneal wound healing may further advance our understanding of this complex process.

Interleukin-6 Cytokine Activity

Interleukin-6 is a potent immunologic mediator and it is involved in acute phases of inflammation.[57] It is considered a myokine, or a cytokine produced from muscle, and when released by myocytes, serves a protective, anti-inflammatory role within the musculature.[58,59] However, it is also a proinflammatory cytokine[57] and its secretion from T cells and macrophages leads to inflammation and tissue damage

especially following burn trauma.[58-60] Interleukin-6 has been extensively studied within the context of corneal wound healing and its expression is upregulated following corneal injury in humans, as well as in animal and *in vitro* models.[60-64] While the indirect suppression of IL-6 has been shown to have a negative effect on *in vivo* corneal epithelial cell migration in mice[63] and on *in vitro* immortalized human corneal epithelial cell migration,[61,63] increases in IL-6 expression actually expedite corneal wound closure in scratch assays employing human[62,65] and rabbit corneal epithelial cells,[66] and in *in vivo* rabbit corneal wound healing models.[67] Interleukin-6 has also been shown to increase in equine disease processes such as dermatologic epidermal wound healing,[68] pneumonia,[69] and endometritis.[70] It is likely that IL-6 is one of the more systemically important equine cytokines. Although IL-6 expression has been demonstrated in rabbits,[66,67] mice,[60,62] and humans[61,64,71] post corneal wounding, its role in equine corneal wound healing has not been studied to date.

Chapter 1 References:

1. Samuelson DA. (2013) Ophthalmic Anatomy. In: *Veterinary Ophthalmology* (ed. Gelatt, K.N.), 5th ed., pp. 39–170. Ames, IA: John Wiley and Sons

2. Pepose JS, Ubels JL: The cornea. In Hart WM, editor: *Adler's physiology of the eye*, St Louis, 1992, Mosby, pp 29-70

3. Roberts SM. Equine vision and optics, *Vet Clin North Am Equine Pract* 8:451-457, 1992

4. Gilger, BC. (2005) *Equine Ophthalmology*. 3rd ed. St. Louis: Elsevier Saunders

5. van der Woerdt A, Gilger BC, Wilkie DA, Strauch SM. Effect of auriculopalpebral nerve block and intravenous administration of xylazine on intraocular pressure and corneal thickness in horses, *Am J Vet Res* 56: 155-158, 1995

6. Ramsey DT, Hauptman JG, Petersen-Jones SM: Corneal thickness, intraocular pressure, and optical corneal diameter in Rocky Mountain horses with cornea globosa or clinically normal corneas, *Am J Vet Res* 1999;60:1317-1321, 1999

7. Andrew SE, Ramsey DT, Hauptman JG, Brooks DE. Density of corneal endothelial cells and corneal thickness in eyes of euthanatized horses. *Am J Vet Res*. 2001 Apr;62(4):479-82

8. Watsky, MA, Olsen TW, Edelhauser HF. (1995) Cornea and sclera. In: *Duane's Foundation of Clinical Ophthalmology* (eds Tasman, W. & Jaeger, E.A.), Vol. 2, pp. 1–29. Philadelphia: J.B. Lippincott

9. Gum GG, Gelatt KN, Ofri R: Physiology of the eye. In Gelatt KN, editor: *Veterinary Ophthalmology,* ed 3, Philadelphia, 1999, Lippincott Williams & Wilkins, pp 151-181

10. Andrew SE, Willis AM. Diseases of the cornea and sclera. (2007) In: *Equine Ophthalmology*, 4th edn (ed. Gelatt KN) Blackwell, Ames, IA, pp157-251

11. Nautscher N, Bauer A, Steffl M, Amselgruber WM. Comparative morphological evaluation of domestica animal cornea. *Vet Ophthalmol* 2016;19:297-304

12. Szalay J, Nunziata B, Henkind P. Permeability of iridal blood vessels. *Exp Eye Res* 1975;21:531–543

13. Wilson SE, Liu JJ, Mohan RR. Stromal-epithelial interactions in the cornea. *Prog Retin Eye Res* 1999;18:298-309

14. Cenedella RJ and Fleschnew CR. Kinectics of corneal epithelium turnover in vivo: studies of locastatin. *Invest Ophthal and Vis Sci* 1990;31:1957-1962

15. Ledbetter EC, Gilger B.C. (2013) Diseases and surgery of the canine cornea and sclera. In: *Veterinary Ophthalmology* (ed. Gelatt, K.N.), 5th ed., pp. 976–1049. Ames, IA: John Wiley and Sons

16. Hogan MJ, Alvarado JA, Weddell JE. (1971) *Histology of the Human Eye*. Philadelphia: WB Saunders

17. Hassell JR & Birk DE. The molecular basis of corneal transparency. *Exp Eye Res* 2010; 91, 326–335

18. Wilson SE, Mohan RR, Ambrosia R Jr, Hong J, Lee J. The corneal wound healing response: cytokine-mediated interaction of the epithelium, stroma, and inflammatory cells. *Prog Retin Eye Res* 2001;20:625-637

19. Lehrer MS, Sun TT, Lavker RM. Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. *J Cell Sci* 1998;111, 2867e2875

20. Ahmad S, Kolli S, Lako M, Figueiredo F, Daniels JT. Stem cell therapies for ocular surface disease. *Drug Discov Today* 2010;15,306e313

21. Ordonez P, Di Girolamo N. Limbal epithelial stem cells: role of the niche microenvironment. *Stem Cells* 2012;30, 100e107

22. Amitai-Lange A, Altshuler A, Bubley J, Dbayat N, Tiosano B, Shalom- Feuerstein R. Lineage tracing of stem and progenitor cells of the murine corneal epithelium. *Stem Cells* 2015;33: 230e239

23. Di Girolamo N, Bobba S. Raviraj V, Delic NC, Slapetova I, Nicovich PR. Halliday GM, Wakefield D, Whan R, Lyons GJ. Tracing the fate of limbal epithelial progenitor cells in the murine cornea. *Stem Cells* 2015;33, 157e169

24. West JD, Dora NJ, Collinson JM. Evaluating alternative stem cell hypotheses for adult corneal epithelial maintenance. *World J. Stem Cells* 2015;7,281e299

25. Ljubimov AV and M Saghizadeh. Progress in corneal wound healing. *Prog Retin Eye Res* 2015;49:17-45. doi: 10.1016/j.preteyeres.2015.07.002

26. Tuominen IS, Tervo TM, Teppo AM, Valle TU, Gr€onhagen-Riska C,Vesaluoma MH. Human tear fluid PDGF-BB, TNF-a and TGF-b1 vs corneal haze and regeneration of corneal epithelium and subbasal nerve plexus after PRK. *Exp Eye Res* 2001;72, 631e641

27. Nishida T, Nakamura M, Mishima H, Otori T, Hikida M. Interleukin 6 facilitates corneal epithelial wound closure in vivo. *Arch Ophthalmol* 1992a;110(9):1292-1294

28. Nishida T, Nakamura M, Mishima H. and Otori T. Interleukin 6 promotes epithelial migration by a fibronectin-dependent mechanism. *J Cell Physiol* 1992b;153: 1–5

29. Tuft SJ, Gartry DS, Rawei M, Meek KM. Photorefractive keratectomy: implications of corneal wound healing. *Br J Ophthalmol* 1993;77, 243e247

30. Moller-Pedersen T, Cavanagh HD, Petroll WM, Jester JV. Corneal haze development after PRK is regulated by volume of stromal tissue removal. *Cornea* 1998a; 17: 627–639

31. Moller-Pedersen T, Cavanagh HD, Petroll WM, Jester JV. Neutralizing antibody to TGF beta modulates stromal fibrosis but not regression of photoablative effect following PRK. *Curr Eye Res* 1998b; 17: 736–747

32. Moller-Pedersen T, Li HF, Petroll WM, Cavanagh HD, Jester JV. Confocal microscopic characterization of wound repair after photorefractive keratectomy *Invest Ophthalmol Vis Sci* 1998c; 39: 487–501

33. Fini, M.E. Keratocyte and fibroblast phenotypes in the repairing cornea. *Prog Retin Eye Res* 1999;18,529e551

34. Wilson SE. Analysis of the keratocyte apoptosis, keratocyte proliferation, and myofibroblast transformation responses after photorefractive keratectomy and laser in situ keratomileusis. *Transactions Amer Ophthalmolog Soc* 2002; 100: 411–433

35. Jester JV, Petroll WM, Barry PA, Cavanagh HD. Expression of alpha-smooth muscle (alpha-SM) actin during corneal stromal wound healing. *Invest Ophthalmol Vis Sci* 1995;36: 809–819

36. Zieske JD, Guimaraes SR, Hutcheon AE. Kinetics of keratocyte proliferation in response to epithelial debridement. *Exp Eye Res* 2001; 72: 33–39

37. Torricelli AA., Wilson S.E. Cellular and extracellular matrix modulation of corneal stromal opacity. *Exp. Eye Res*. 2014;129, 151e160

38. Ishizaki M, Shimoda M, Wakamatsu K, Ogro T, Yamanaka N, Kao CW, Kao WW. Stromal fibroblasts are associated with collagen IV in scar tissues of alkali-burned and lacerated corneas. *Curr Eye Res* 1997;16, 339e348

39. Ishizaki M, Zhu G, Haseba T, Shafer SS, Kao WW. Expression of collagen I, smooth muscle alpha-actin, and vimentin during the healing of alkali-burned and lacerated corneas. *Invest Ophthalmol Vis Sci* 1993;34,3320e3328

40. Maguen E, Alba SA, Burgeson RE, Butkowski RJ, Michael AF, Kenney MC, Nesburn AB, Ljubimov AV. Alterations of corneal extracellular matrix after multiple refractive procedures: a clinical and immunohistochemical study. *Cornea* 1997;16, 675e682

41. Maguen E, Zorapapel NC, Zieske JD, Ninomiya Y, Sado Y, Kenney MC, Ljubimov AV. Extracellular matrix and matrix metalloproteinase changes in human corneas after complicated laser-assisted in situ keratomileusis (LASIK). *Cornea* 2002.21, 95e100

42. Maguen E, Maguen B, Regev L, Ljubimov AV. Immunohistochemical evaluation of two corneal buttons with post-LASIK keratectasia. *Cornea* 2007.26, 983e991

43. Ljubimov AV, Alba SA, Burgeson RE, Ninomiya Y, Sado Y, Sun TT, Nesburn AB, Kenney MC, Maguen E. Extracellular matrix changes in human corneas after radial keratotomy. *Exp Eye Res* 1998b;67:265e272

44. Kato T, Chang JH, Azar DT. Expression of type XVIII collagen during healing of corneal incisions and keratectomy wounds. *Invest Ophthalmol Vis Sci* 2003;44, 78e85

45. Kamma-Lorger CS, Boote C, Hayes S, Albon J, Boulton ME, Meek KM. Collagen ultrastructural changes during stromal wound healing in organ cultured bovine corneas. *Exp Eye Res* 2009;88, 953e959

46. Torricelli AA, Wilson SE. Cellular and extracellular matrix modulation of corneal stromal opacity. *Exp Eye Res* 2014;129, 151e160

47. Latvala T, Tervo K, Mustonen R., Tervo T. Expression of cellular fibronectin and tenascin in the rabbit cornea after excimer laser photorefractive keratectomy: a 12 month study. *Br J Ophthalmol* 1995a;79, 65e69

48. Maguen E, Rabinowitz YS, Regev L, Saghizadeh M, Sasaki T, Ljubimov AV. Alterations of extracellular matrix components and proteinases in human corneal buttons with INTACS for post-laser in situ keratomileusis keratectasia and keratoconus. *Cornea* 2008;27:565–573

49. Joyce NC, Meklir B, Neufeld AH. In vitro pharmacologic separation of corneal endothelial migration and spreading responses. *Invest Ophthalmol Vis Sci* 1990;31, 1816e1826

50. Ichijima H, Petroll WM, Barry PA, Andrews PM, Dai M, Cavanagh HD, Jester JV. Actin filament organization during endothelial wound healing in the rabbit cornea: comparison between transcorneal freeze and mechanical scrape injuries. *Invest Ophthalmol Vis Sci* 1993a;34, 2803e2812

51. Gordon SR. Cytological and immunocytochemical approaches to the study of corneal endothelial wound repair. *Prog Histochem Cytochem* 1994;28,1e64

52. Mimura T, Yamagami S, Amano S. Corneal endothelial regeneration and tissue engineering. *Prog Retin Eye Res* 2013;35,1e17

53. Nakano Y, Oyamada M, Dai P, Nakagami T, Kinoshita S, Takamatsu T. Connexin43 knockdown accelerates wound healing but inhibits mesenchymal transition after corneal endothelial injury in vivo. *Invest Ophthalmol Vi. Sci* 2008;49: 93–104

54. Petroll WM, Barry-Lane PA, Cavanagh HD, Jester JV. ZO-1 reorganization and myofibroblast transformation of corneal endothelial cells after freeze injury in the cat. *Exp Eye Res* 1997; 64: 257–267

55. Astashkina A, Mann B, Grainge DW. A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity. *Pharmacology & Therapeutics*. 2012; 134:82-106.

56. Alekseev O, Tran AH, Azizkhan-Clifford J. Ex vivo organotypic corneal model of acute epithelial herpes simplex virus type 1 infection: a video article. *J of Visualized Exp* 2012; 69; e3631.

57. Akira S, Hirano T, Taga T, Kishimoto T. Biology of multifunctional cytokines: IL 6 and related molecules (IL 1 and TNF). *FASEB J*. 1990 Aug;4(11):2860-7

58. Dinarello CA. Proinflammatory cytokines. *Chest J.* 2000;118:503–508

59. Febbraio MA, Pedersen BK. Contraction-induced myokine productionand release: is skeletal muscle an endocrine organ? *Exerc Sport Sci Rev* 2005;33:114–119

60. Sotonzono C, He J, Matsumoto Y, Kita M, Imanishi J, Kinoshita S. Cytokine expression in the alkali burned cornea. *Curr Eye Res* 1997;16:670-676

61. Yang H, Wang Z, Capó-Aponte JE, Zhang F, Pan Z, Reinach PS. Epidermal growth factor receptor transactivation by the cannabinoid receptor (CB1) and transient receptor potential vanilloid 1 (TRPV1) induces differential responses in corneal epithelial cells. *Exp Eye Res* 2010;91:462–471

62. Ebihara N, Matsuda A, Nakamura, S, Matsuda H, Murakam A. Role of IL-6 classic and trans-signaling in corneal sterile inflammation and wound. *Invest Ophthalmol Vis Sci* 2011;52:8549–8557

63. Sumioka T, Okada Y, Reinach PS, et al. Impairment of corneal epithelial wound healing in a TRPV1- deficient mouse. *Invest Ophthalmol Vis Sci* 2014;55:3295–3302.

64. Resan M, Stanojević I, Petković A, Pajić B, Vojvodić D. Levels of interleukin-6 in tears before and after excimer laser treatment. *Vojnosanit Pregl.* 2015 Apr;72(4):350-5

65. Arranz-Valsero I, Soriano-Romaní L, García-Posadas L, Lopez-García A, Diebold Y. IL-6 as a corneal wound healing mediator in an in vitro scratch assay. *Exp Eye Res* 2014:25, 183e192

66. Nakamura M, Nishida T. Differential effects of epidermal growth factor and interleukin 6 on corneal epithelial cells and vascular endothelial cells. *Cornea*. 1999;18:452–458

67. Er H, Uzmez E. Effects of transforming growth factor-b2, interleukin 6 and fibronectin on corneal epithelial wound healing. *Eur J Ophthalmol* 1998;8,224e229

68. Spass JH, Gomiero C, Broeckx S, Van Hecke L, Maccatrozzo L, Martens A. Wound-healing markers after autologous and allogeneic epithelial-like stem cell treatment. *Cytotherapy* 2016; 18: 562–569

69. Sandersen C, Bienzle D, Cerri S, Franck T, Derochette S, Neven P, Mouytis-Michalad A, Serteyn D. Effect of inhaled hydrosoluble curcumin on inflammatory markers in broncho-alveolar lavage fluid of horses with LPS-induced lung neutrophilia. *Multidiscip Respir Med.* 2015 Apr 15;10(1):16

70. Siemieniuch MJ, Szóstek AZ, Gajos K, Kozdrowski R, Nowak M, Okuda K. Type of inflammation differentially affects expression of interleukin 1β and 6, tumor necrosis factor-α and toll-like receptors in subclinical endometritis in mares. *PLoS ONE* 2016;11(5): e0154934

71. Malecaze F, Simorre V, Chollet P, Tack JL, Muraine M, Le Guellec D, Vita N, Arne JL, Darbon JM. Interleukin-6 in tear fluid after photorefractive keratectomy and its effects on keratocytes in culture. *Cornea* 1997 Sep;16(5):580-7

Figure for Chapter 1

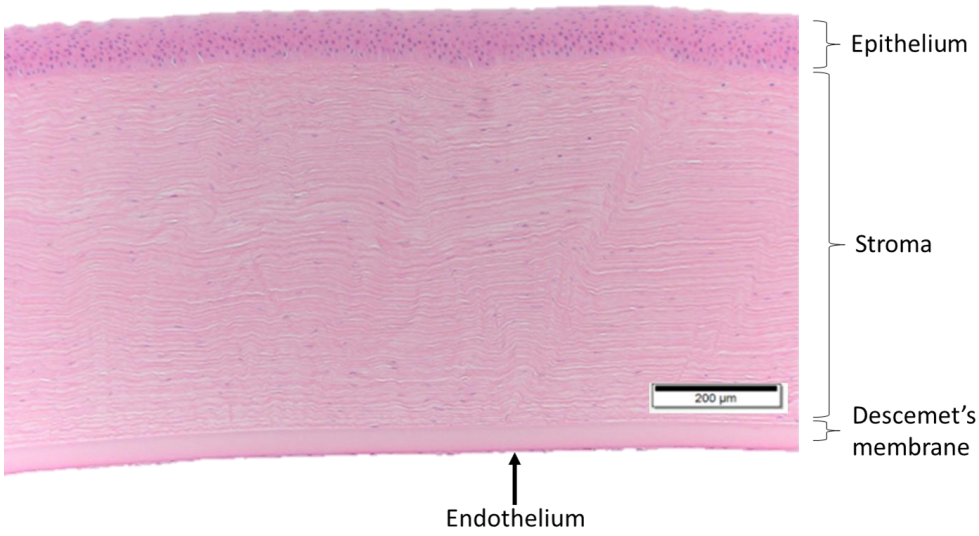

Figure 1-1. Histologic section of a normal equine cornea. Note the superficial epithelial layer, consisting of approximately 15 epithelial cell layers, the well-organized, thick, stromal layer, Descemet's membrane, and the single-cell, inner-most, endothelial layer (black arrow).

CHAPTER 2. LITERATURE REVIEW

In vivo Corneal Wound Healing Studies

Ocular disease is one of the most common ailments of the horse[1] and it is one of the most significant causes of decreased vision or blindness in the horse world-wide.[2] This is likely due to several factors including a prominent globe, the lateral location of the globe within the skull, and the equid's exuberant and head throwing behavior.[1] As such, the horse is predisposed to corneal injury. Although equine corneal disease and the subsequent wound healing cascade is a complex process whose mechanisms are still not fully understood,[3] it is known that inflammatory cells and their secretory products such as cytokines and growth factors are involved, as discussed in Chapter 1. In addition, underlying genetics may also play a role[3] and it has been suggested that the aggressiveness of the corneal destruction may be linked to the equine host's immunophenotype.[4] This includes the local innate response of Pattern and Damage Recognition Receptors (PRRs, DRRs) that reside within the corneal epithelium and their role in cytokine recruitment.[4] Hypothesis driven, *in vivo*, corneal wound healing studies have been conducted extensively in mice, rats, and rabbits,[5-10] and included evaluation of corneal regeneration and healing processes, corneal epithelial cell migration, and IL-6 expression.[11] Additionally, the role of IL-6 in rabbit,[12,13] mouse,[5,14,15] and human[16] corneal wound healing has also been studied *in vivo*.

Despite the high prevalence of corneal disease and ulceration in the horse, the importance of a clear visual axis in this species, and the complexity of corneal

wound healing, few *in vivo* studies are hypothesis driven. Instead, most studies are heavily devoted to retrospective studies, reviewing patients' medical records.[17-24] Only few hypothesis driven corneal wound healing studies have been conducted *in vivo* to date.[25,26] The limitations of such studies include small sample sizes, high cost, ethical concerns, and large variation amongst few subjects which often results in a lack of statistical significance.[27] An *ex vivo* equine corneal wound healing model can overcome these limitations, and provide the platform for hypothesis driven studies of corneal wound healing in the horse.

In vitro Corneal Wound Healing Studies

In the laboratory setting, *in vitro* work possesses its own set of limitations. Cell culture lines that are purchased from commercial sources may be contaminated with other cell lines, may not be validated for the cellular phenotype in question, and may be over-passaged.[28-31] Growing primary corneal cell cultures can prove to be a challenge as well. For example, while Mathes *et al* successfully established a reproducible cell culture model of equine keratocyte and endothelial cells, the epithelial cells grew slowly, did not survive multiple passages and were seen to suffer from keratocyte or fungal organism contamination.[27] Additionally, by experimenting on individual corneal layers separately from neighboring layers,[32,33] *in vitro* corneal research may be an overly simplified model which lacks the dynamic, physiological events of the *in vivo* corneal model.[32-34] *In vitro* cell culture lines are not able to simulate the cornea's complex molecular processes nor the interactions and 'cross talk' amongst the corneal layers, especially the epithelium and stroma. For

example, it is well known that epithelial and stromal interactions are mediated by paracrine expression and that autocrine signaling is likely occurring as well.[35,36] By studying and experimenting on one corneal layer at a time, it is likely the cell to cell and cell to extracellular matrix interactions (the 'cellular microenvironment') are overlooked.[34,37-41] These intercellular interactions are paramount to the normal routine of the cell, and tissue injury can result in disruption of these connections and therefore cellular communication. Therefore, the cells' ability to respond to its local environment is impeded, and cell migration, differentiation and healing are diminished[37-41] as the individual cell is not able to communicate the signaling information on to groups of other cells.[42-44] As such, the *in vitro* researcher may be faced with the challenge of obtaining clinically applicable results.

Current *Ex vivo* Studies

The inherent challenge comes in the desire to learn more about corneal wound healing without either harming healthy animals *in vivo* or struggling with clinical applicability *in vitro*. The *ex vivo* model of corneal wounding has been developed in other species including the human,[45] dog,[46] pig (authors unpublished data), and rabbit,[47,48] and represents a logical stepping stone between the equine *in vitro* ocular bench top models and the *in vivo* clinical studies, all while providing physiologically-based, clinically applicable conclusions.[48] Recent publications have revolutionized the *ex vivo* approach, demonstrating superior epithelial preservation using an air-liquid interface human *ex vivo* model.[45] Deshpande *et al* refined the *ex vivo* corneal wound healing model, by ensuring that media rolled over the cultured

rabbit corneas. The technique resulted in an extended corneal lifespan of up to 4 weeks, versus 2 weeks in static conditions.[47] This modified technique allowed them to evaluate the inflammatory role of the cytokine interleukin-17A on their rabbit corneas over an extended period of time. Previously, Lin *et al* had evaluated the shorter term effects of interleukin-33 in a modified human *ex vivo* model.[49] However, to our knowledge, IL-6 expression has not been studied utilizing the *ex vivo* corneal wound healing model.

A species specific, reproducible model of corneal wound healing is of great importance. To date, no *ex vivo* models of equine corneal wound healing have been established. Therefore, the purpose of this study was two-fold. First, to establish an *ex vivo* model of equine corneal wound healing, and second, to investigate the physiologic relevance of this model. This study is at the heart of improved animal welfare and represents a logical stepping stone between the *in vitro* bench top models and the *in vivo* clinical studies.

The successful development of this model could serve to decrease *in vivo* corneal wound healing studies in the horse, while providing an effective platform for equine corneal wound healing research.

Chapter 2 References:

1. Gilger, BC. (2005) *Equine Ophthalmology*. 3rd ed. St. Louis: Elsevier Saunders

2. Buss DB, Guiliano EA, Sharma A, Mohan RR. Isolation and cultivation of equine corneal keratocytes, fibroblasts and myofibroblasts. *Vet Ophthalmol* 2010; 13: 37–42

3. Maycock NJR, Marshall J. Genomics of corneal wound healing: a review of the literature. *Acta Ophthalmol* 2014; 92:170-184. doi: 10.1111/aos.12227

4. Mathews AG. Clinical ophthalmic challenges in the horse: a retrospective view forward. *Vet Ophthalmol* 2014; 17: 2-5

5. Sotonzono C, He J, Matsumoto Y, Kita M, Imanishi J, Kinoshita S. Cytokine expression in the alkali burned cornea. *Curr Eye Res* 1997;16:670-676

6. Er H, Uzmez E. Effects of transforming growth factor-b2, interleukin 6 and fibronectin on corneal epithelial wound healing. *Eur J Ophthalmol* 1998;8,224e229

7. Nakamura M, Ofuji K, Chikama T, Nishida T. Combined effects of substance P and insulin-like growth factor-1 on corneal epithelial wound closure of rabbit in vivo. *Curr Eye Res* 1997;16:275–278

8. Sosne G, Szliter EA, Barrett R, Kernacki KA, Kleinman H, Hazlett LD. Thymosin β4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. *Exp Eye Res* 2002;74:293–299

9. Ruiz-Ederra J, Verkman AS. Aquaporin-1-facilitated keratocyte migration in cell culture and in vivo corneal wound healing models. *Exp Eye Res* 2009;89:159–165

10. Xu K, Yu FS. Impaired epithelial wound healing and EGFR signaling pathways in the corneas of diabetic rats. *Invest Ophthalmol Vis Sci* 2011;52:3301–3308

11. Nakamura M, Nishida T. Differential effects of epidermal growth factor and interleukin 6 on corneal epithelial cells and vascular endothelial cells. *Cornea* 1999;18:452–458

12. Nishida T, Nakamura M, Mishima H, Otori T, Hikida M. Interleukin 6 facilitates corneal epithelial wound closure in vivo. *Arch Ophthalmol* 1992a;110(9):1292-1294

13. Imanishi J, Kamiyama K, Iguchi I, Kita M, Sotozono C, Kinoshita S. Growth factors: importance in wound healing and maintenance of transparency of the cornea. *Prog Retin Eye Res* 2000;19:113-129

14. Yang H, Wang Z, Capó-Aponte JE, Zhang F, Pan Z, Reinach PS. Epidermal growth factor receptor transactivation by the cannabinoid receptor (CB1) and transient receptor potential vanilloid 1 (TRPV1) induces differential responses in corneal epithelial cells. *Exp Eye Res* 2010;91:462–471

15. Ebihara N, Matsuda A, Nakamura, S, Matsuda H, Murakam A. Role of IL-6 classic and trans-signaling in corneal sterile inflammation and wound. *Invest Ophthalmol Vis Sci* 2011;52:8549–8557

16. Resan M, Stanojević I, Petković A, Pajić B, Vojvodić D. Levels of interleukin-6 in tears before and after excimer laser treatment. *Vojnosanit Pregl.* 2015 Apr;72(4):350-5

17. Hendrix DV, Brooks DE, Smith PJ, Gelatt KN, Miller TR, Whittaker C *et al*. Corneal stromal abscesses in the horse: a review of 24 cases. *Equine Vet J* 1995; 27: 440–447

18. Andrew SE, Brooks DE, Smith PJ, Gelatt KN, Chmielewski NT, Whittaker CJG. Equine ulcerative keratomycosis: visual outcome and ocular survival in 39 cases (1987–1996). *Equine Vet J* 1998; 30: 109–116

19. Brooks DE, Andrew SE, Biros DJ, Denis HM, Cutler TJ, Strubbe DT et al. Ulcerative keratitis caused by beta-hemolytic *Streptococcus equi* in 11 horses. *Vet Ophthalmol* 2000a; 3: 121–125

20. Brooks DE, Andrew SE, Denis H, Strubbe DT, Biros DJ, Cutler TJ, Samuelson DA, Gelatt KN. Rose bengal positive epithelial microerosions as a manifestation of equine keratomycosis. *Vet Ophthalmol* 2000b; 3: 83–86

21. Ollivier FJ, Brooks DE, Van Setten GB, Schultz GS, Gelatt KN, Stevens GR, Blalock TD, Andrew SE, Komaromy AM, Lassaline ME, Kallberg ME, Cutler TJ. Profiles of matrix metalloproteinase activity in equine tear fluid during corneal healing in 10 horses with ulcerative keratitis. *Vet Ophthalmol* 2004; 7: 397–405

22. Lassaline ME, Brooks DE, Ollivier FJ, Komaromy AM, Kallberg ME, Gelatt KN. Equine amniotic membrane transplantation for corneal ulceration and keratomalacia in three horses. *Vet Ophthalmol* 2005; 8: 311–317

23. Ollivier FJ, Kallberg ME, Plummer CE, Barrie KP, O'Reilly S, Taylor DP, Gelatt KN, Brooks DE. Amniotic membrane transplantation for corneal surface reconstruction after excision of corneolimbal squamous cell carcinomas in nine horses. *Vet Ophthalmol* 2006; 9: 404–413

24. Brooks DE, Plummer CE, Kallberg ME, Barrie KP, Ollivier FJ, Hendrix DV, Baker A, Scotty NC, Utter ME, Blackwood SE, Nunnery CM, Ben-Shlomo G, Gelatt KN. Corneal transplantation for inflammatory keratopathies in the horse: visual outcome in 206 cases (1993–2007). *Vet Ophthalmol* 2008; 11: 123–133

25. Neaderland MH, Riis RC, Rebhun WC, Erb HN. Healing of experimentally induced corneal ulcers in horses, *Am J Vet Res* 48:427-430, 1987

26. Burling K, Seguin MA, Marsh P, Brinkman K, Madigan J, Thurmond M, Moon-Massat P, Mannis M, Murphy CJ. Effect of topical administration of epidermal growth factor on healing of corneal epithelial defects in horses. *Am J Vet Res* 2000 Sep;61(9):1150-5

27. Mathes RL, Dietrich UM, Krunkosky TM, Hurley DJ, Reber AJ. Establishing a reproducible method for the culture of primary equine corneal cells. *Vet Ophthalmol* 2009; 12: 41-49. DOI: 10.1111/j.1463-5224.2009.00729.x PMID:19891651

28. Masters JR. HeLa cells 50 years on: the good, the bad and the ugly. *Nat Rev Cancer 2* 2002;315–319

29. Hughes P, Marshall D, Reid Y, Parkes H, Gelber C. The costs of using unauthenticated, over-passaged cell lines: how much more data do we need? *Biotechniques* 2007;43(575), 577–578 (581–572 passim)

30. Nardone RM. Eradication of cross-contaminated cell lines: a call for action. *Cell Biol Toxicol* 2007;23, 367–372

31. Capes-Davis A., Theodosopoulos G, Atkin I, Drexler H G, Kohara A, MacLeod RA. Check your cultures! A list of cross-contaminated or misidentified cell lines. *Int J Cancer* 2010;127, 1–8

32. Weinstein WL, Moore PA, Sanchez S, Dietrich UM, Wooley RE, Ritchie BW. In vitro efficacy of a buffered chelating solution as an antimicrobial potentiator for antifungal drugs against fungal pathogens obtained from horses with mycotic keratitis. *Amer J Vet Res* 2006; 67: 562-568

33. Scotty NC, Brooks DE, Schuman Rose CD. In vitro efficacy of an ophthalmic drug combination against corneal pathogens of horses. *Amer J Vet Res* 2008; 69: 101–107

34. Astashkina A, Mann B, Grainge DW. A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity. *Pharmacology & Therapeutics*. 2012; 134:82-106

35. Wilson SE, Walker JWQ, Chwang EL et al. Hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), their receptors, FGF receptor-2, and the cells of the cornea. *Invest Ophthalmol Vis Sci* 1993;34:2544-2561

36. Li DQ and Tseng SC. Differential regulation of cytokine and receptor transcript expression in human corneal and limbal fibroblasts by epidermal growth factor, transforming growth factor-alpha, platelet-derived growth factor B, and interleukin-1 beta. *Invest Ophthalmol Vis Sci* 1996;37:2068-2080

37. Cukierman E, Pankov R, Stevens DR, & Yamada KM. Taking cell–matrix adhesions to the third dimension. *Science* 2001;294, 1708–1712

38. Cukierman E, Pankov R, & Yamada KM. Cell interactions with three dimensional matrices. *Curr Opin Cell Biol* 2002;14, 633–639

39. O'Brien LE, Zegers MM, Mostov KE. Opinion: building epithelial architecture: insights from three-dimensional culture models. *Nat Rev Mol Cell Biol* 2002;3, 531–537

40. Yamada KM, Pankov R, Cukierman E. Dimensions and dynamics in integrin function. *Braz J Med Biol Res* 2003;36, 959–966

41. Larsen M, Artym VV, Green JA, Yamada KM. The matrix reorganized: extracellular matrix remodeling and integrin signaling. *Curr Opin Cell Biol* 2006;18,463–471

42. Ichimura T, Bonventre JV, Bailly V, Wei H, Hession CA, Cate RL. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. *J Biol Chem* 1998;273, 4135–4142

43. Ichimura T, Asseldonk EJ, Humphreys BD, Gunaratnam L, Duffield J S, Bonventre
JV. Kidney injurymolecule-1 is a phosphatidylserine receptor that confers a phagocytic phenotype on epithelial cells. *J Clin Invest* 2008;118, 1657–1668

44. Huo W, Zhang K, Nie Z, Li Q, & Jin F. Kidney injury molecule-1 (KIM-1): a novel kidney-specific injury molecule playing potential double-edged functions in kidney injury. *Transplant Rev* (Orlando) 2010;24, 143–146

45. Janin-Manificat H, Rovere MR, Galiacy SD, Malecaze F, Hulmes DJ, Moali C, Damour O. Development of ex vivo organ culture model to mimic human corneal scarring. *Mol Vis* 2012;18: 2896-2908

46. Proietto LR, Whitley RD, Brooks DE, Schultz GE, Gibson DJ, Berkowski WM Jr, Salute ME, Plummer CE. Development and assessment of a novel canine ex vivo corneal model, *Curr Eye Res* 2017

47. Deshpande P, Ortega I, Sefat F, Sangwan VS, Green N, Claeyssens F. Rocking media over ex vivo corneas improves this model and allows the study of the effect of proinflammatory cytokines on wound healing. *Invest Ophthalmol Vis Sci* 2015; 56: 1553-1561

48. Alekseev O, Tran AH, Azizkhan-Clifford J. Ex vivo organotypic corneal model of acute epithelial herpes simplex virus type 1 infection: a video article. *J of Visualized Exp* 2012; 69; e3631. doi: 10.3791/3631. PMID: 23149439

49. Lin J, Zhang L, Zhao G, Su Z, Deng R, Pflugfelder SC, De-Quan L. A novel interleukin 33/ST2 signaling regulates inflammatory response in human corneal epithelium. *PLOS One* 2013;8(4): e60963

CHAPTER 3. A NOVEL *EX VIVO* MODEL OF EQUINE CORNEAL WOUND HEALING

Rita F. Wehrman, DVM; Gil Ben-Shlomo, DVM, PhD, DACVO, DECVO

Department of Veterinary Clinical Sciences, College of Veterinary Medicine

Iowa State University

Ames, IA, 50011

ABSTRACT

Purpose: To establish a physiologically relevant *ex vivo* equine corneal wound healing model.

Methods: Fourteen globes were harvested from healthy horses within two hours of humane euthanasia for reasons unrelated to this study and immediately processed. Corneas were randomly assigned to one of the following groups: wounded (n=8) or unwounded (n=6) controls. In the wounded group, the axial cornea was ulcerated with a 6 mm filter paper disk soaked in 1 N sodium hydroxide (NaOH) at Time 0. Corneoscleral rims were excised 2 mm posterior to the limbus and corneal cups cultured in complete medium using an air-liquid interface model. The medium bathed the corneal cup eight times per minute to simulate normal horse blinking. Evaluation of corneal healing was performed daily and included fluorescein staining and a modified Hackett-McDonald ocular scoring system which included the calculated ulcer area. Corneas were randomly assigned to undergo further processing via histopathology at 24 hour (T24; n=5), 48 hours (T48; n=5), and 72 hours (T72; n=4) post wounding. Additionally, RNAscope *in situ* hybridization assay was performed according to the manufacturer's instructions to evaluate IL-6 expression.

Results: All wounded corneas healed within 72 hours. Histologically, normal corneal architecture was seen including intact epithelium, minimal stromal edema and the presence of endothelium. Interleukin-6 expression increased in wounded equine corneas compared to unwounded controls. The greatest IL-6 expression was in the basal epithelial layer, close to the wound margin. As the corneal wound healing progressed, IL-6 expression decreased.

Conclusions: The equine air-liquid interface, *ex vivo*, corneal wound healing model is an effective and physiologically relevant model. This model can be utilized to reduce or eliminate the use of live horses for corneal wound healing studies, while providing an effective platform for corneal wound healing research in this species.

INTRODUCTION

Corneal ulcers are very common in horses, and with a lack of appropriate treatment, they present an imminent risk for the eye and for vision.[1] If not identified early or if secondary infection occurs, keratomalacia caused by various proteases acting on corneal collagen, may develop. This condition may lead to perforation of the eye. However, despite the horse being predisposed to corneal injury (large eye size, lateral skull placement, exuberant behavior) and the frequency of ophthalmic presentations to the veterinarian,[1] our knowledge base of equine corneal ulceration resolution is lacking.

Corneal wound healing is a complex process that involves two basic steps – cellular migration to cover the wound followed by mitosis of the epithelial cells to fill in the defect.[2,3] Physical damage to the cornea's outer most layer, the epithelium, is the most common cause of wounding and it is associated with signs of ocular pain including blepharospasm and lacrimation. As the injured eye runs the risk of permanent, vision altering consequences or even perforation, it is not surprising that prompt and aggressive treatment is of paramount importance. Treatment of these corneal disorders can be time consuming, costly and frustrating for both the owner

and the patient, and should a superficial, uncomplicated erosion not be healed within 72 hours, it should be considered complicated and is therefore in danger of perforation.[1] In these cases, painful inflammation is likely involved, and clinical signs of reflex uveitis may be present and include photophobia, corneal edema and aqueous flare.

Despite the high prevalence of corneal disease and ulceration in the horse, the importance of a clear visual axis in this species, and the complexity of corneal wound healing, few *in vivo* studies are hypothesis driven. Instead, most studies are heavily devoted to retrospective studies, reviewing patients' medical records.[4-11] Only a few prospective corneal wound healing studies have been conducted *in vivo* to date.[12,13] The limitations of such studies include small sample size, high cost, ethical concerns, and large variation amongst few subjects that often results in a lack of statistical significance.[14]

In the laboratory setting, *in vitro* work possesses its own set of limitations including obtaining clinically relevant results.[15-17] By experimenting on individual corneal cell types separately from neighboring layers, *in vitro* research may be an overly simplified model that lacks the dynamic, physiologic events of the *in vivo* model. *In vitro* pure cell culture lines are not able to simulate the cornea's complex molecular processes nor the interactions and 'cross talk' amongst the corneal layers, especially the epithelium and stroma. For example, it is well-known that epithelial and stromal interactions are mediated by paracrine expression and that autocrine signaling is likely occurring as well.[18,19] Therefore, the *in vitro* researcher may be faced with the obstacle of obtaining clinically applicable results.

The inherent challenge comes in the desire to learn more about these involved processes without either harming healthy animals *in vivo* or struggling with clinical applicability and physiologic relevance *in vitro*. The *ex vivo* model has been developed in other species,[20,21] and represents a logical stepping stone between the equine *in vitro* ocular bench top models and the *in vivo* clinical studies, all while providing physiologically-based, clinically applicable conclusions.[17,22] While effective, species specific, reproducible models of corneal wound healing are of paramount importance, the development of an equine *ex vivo* model could serve to decease *in vivo* corneal wound healing studies in the horse, all while maintaining the clinical relevance of the results.

Interleukin-6 (IL-6) is a potent immunologic mediator and it is involved in acute phases of inflammation.[23] It is considered a myokine, or a cytokine produced from muscle. Upon its release from myocytes, it serves a protective, anti-inflammatory role within the musculature.[24,25] However, it is also a proinflammatory cytokine and its secretion from T cells and macrophages leads to inflammation and tissue damage especially following burn trauma.[24,25] Additionally, IL-6 has been shown to increase in septic conditions in both humans and horses[26] and high levels of IL-6 are expressed in equine disease processes such as endometritis[27] and pneumonia.[28] Previous equine dermatologic studies have shown significant increases in IL- 6 mRNA levels following pathogen-associated molecular pattern (PAMP) exposure to gram negative bacteria[29] and in an epidermal wound healing model.[30] Equine neutrophils express significantly more IL-6 than other mediators.[31] Given this, it is likely that IL-6 is one of the more systemically important equine

cytokines and may play a large role in equine corneal wound healing as well. Although IL-6 expression has been demonstrated in rabbits,[32,33] mice,[34-36] and humans[37] post corneal wounding, its expression during corneal wound healing in the horse has not been studied to date.

Therefore, the purpose of this study was two-fold. First, to establish an *ex vivo* model of equine corneal wound healing. And second, to investigate the physiologic relevance of this model by evaluating the rate of epithelial wound healing, anatomical structure, and IL-6 expression in wounded and unwounded corneas. This study is at the heart of improved animal welfare and represents a logical stepping stone between the *in vitro* bench top models and the *in vivo* clinical studies.

MATERIALS AND METHODS

Corneas

Corneas were obtained from horses that were euthanized at the Iowa State University College of Veterinary Medicine Lloyd Veterinary Medical Center for reasons unrelated to this study. Fourteen eyes from eight horses were included. The median age and weight of the donors was 19.25 years (range 8-30 years) and 370 kilograms (range 160-540 kgs), respectively. Of these eight horses, three were geldings and five were mares and breeds represented included three Quarter Horses/Quarter Horses crosses, 3 ponies, one Paint and one Peruvian Paso. All

horses were free of systemic and ocular disease. Two eyes (one right and one left) were excluded due to ophthalmic disease.

Corneal wounding

Immediately following humane euthanasia, globes were enucleated, rinsed in 2% betadine (Select Medical Products, Jacksonville, FL, USA), and placed in 1x phosphate buffered saline (PBS; Life Technologies, Grand Island, NY, USA). All further processing occurred within two hours of humane euthanasia. Globes were randomly assigned to wounded (n=8) or unwounded (n=6) control groups. If one of the eyes was excluded the fellow eye was assigned to the wounded group. Wounded corneas included 4 right eyes and 4 left eyes, while unwounded corneas included 3 right eyes and 3 left eyes. During all processing steps, a delicate task wiper (Kimwipes, Kimberly-Clark, Roswell, GA, USA) wet with 1X PBS was used to facilitate holding of the eyeball. For the eight wounded corneas, an alkali burn model was employed in which a 6 mm filter paper disc soaked in 1 N sodium hydroxide (NaOH) was applied to the axial cornea for 60 seconds (Fig. 3-1). After wounding, 1X PBS was used to gently rinse the globe. Fluorescein stain (fluorescein sodium, Akron Inc., Lake Forest, IL, USA) was applied to confirm epithelial erosion and stromal exposure, and baseline ulceration measurements were obtained using a millimeter scale bar (Fig. 3-2). Wounding the cornea with the globe intact allowed for a crisp, well demarcated corneal ulceration. Once wounded, a #20 Bard Parker blade was used to score and then excise the sclera tangentially, approximately 2 mm posterior to the limbus, freeing the corneoscleral (“corneal”) cup (Fig. 3-3). The

non-wounded globes were processed in a similar fashion, excluding the alkaline burn.

Ex vivo cultures

Each corneal cup was placed in an individual 100 mm petri dish (Fischer Scientific, Waltham, MA, USA) along the dishes' edge. Placement of the corneal cup along the side of dish minimized motion of the corneal cup while it was on the rocker. Additionally, great care was taken to ensure that the corneal cup retained its convex shape while introduced to the petri dish as no conformers were used. Once placed in the petri dish, approximately 25 mL of medium was added such that the medium came to the limbus but did not float the corneal cup off the floor of the petri dish. The medium consisted of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/HamF12 + GlutaMAX™, Gibco Life Technologies, Grand Island, NY, USA) in a 1:1 ratio. The medium was supplemented with 10% fetal bovine serum (Fischer Scientific, Waltham, MA, USA), 2.5µg/mL Amphotericin B (Life Technologies, Grand Island, NY, USA), 100 IU/mL penicillin (Life Technologies, Grand Island, NY, USA), 100 µg/mL streptomycin (Life Technologies, Grand Island, NY, USA), 10 ng/mL epidermal growth factor (Gibco Life Technologies, Frederick, MD, USA) and 5 µg/mL bovine pancreatic insulin. The petri dishes were placed on a rocker (Benchmark Scientific, Edison, NJ, USA) that was set at a 6 degree incline and 8 rocks per minute to actively bathe the corneal cup in medium in order to simulate blinking (Fig. 3-4). All corneas were incubated at 37 °C with 5% CO_2 humidity and monitored daily. Petri dishes and medium were changed out daily.

Corneal wound healing assessment

All daily evaluations for ulcer size were performed using slit-lamp biomicroscopy (Keeler SL4 PSL Classic, Keeler Instruments Inc., Windsor, UK) and a modified Hackett-McDonald ocular scoring system.[38] Ulcer size was measured daily utilizing a millimeter scale, following application of fluorescein stain (Fig. 3-5, 3-6), and the ulcer area was calculated $(A = \pi r^2)$ and averaged for each time point. Three and two corneas from the wounded and non-wounded group respectively, were submitted for RNAscope *in situ* hybridization and histopathology analysis 24 (T24) and 48 (T48) hours post wounding, and two corneas from each group were submitted at 72 hours (T72) post wounding.

Modified Hackett-McDonald Ocular Scoring System

The modified Hackett-McDonald scoring system is used frequently in ophthalmology toxicology studies to describe ocular changes,[38] and was employed in this study to describe corneal changes:

a) Fluorescein intensity staining was graded 0-4 with 0=no fluorescein uptake; 0.5=no true uptake/epithelial erosion; 1=faint intensity stain; 2= mild intensity stain; 3= moderate intensity stain; 4= marked intensity stain.

b) Ulcer location and description were noted.

c) Corneal edema was graded 0-4: 0=normal cornea with no edema; 1=slight loss of corneal transparency, all localized around the edge of ulceration; 2=mild loss of corneal transparency, with edema extending outward from the edge of ulceration; 3=moderate loss of corneal transparency with further extension of edema from ulceration's edge; 4= complete loss of corneal transparency with marked edema diffusely.

Histopathology

Corneal histopathology was performed on all corneas to assess structural integrity and pathologic changes to the corneal epithelium, stroma, Descemet's membrane and endothelium. Each sample was fixed in 10% neutral buffered formalin (NBF) for 24 hours and then routinely stained with hematoxylin and eosin. All samples were evaluated using standard bright field microscopy.

IL-6 RNAscope *in situ* hybridization

RNA *in situ* hybridization (RNAscope, Hayward, CA, USA) was used in which single RNA IL-6 molecules within individual cells were visualized. All recommended RNAscope assay procedures were followed according to manufacturer's instructions for formalin fixed paraffin embedded (FFPE) sections using RNAscope FFPE Reagent Kit 2.0 (Advanced Cell Diagnostics, Hayward, CA, USA). Once pulled from culture, corneas were placed in 10% NBF for 24 hours and then paraffin-embedded in preparation for IL-6 gene expression (RNAscope Target Probe C1, 16ZZ Ec-IL6, Advanced Cell Diagnostics, Hayward, CA, USA). The embedded tissue was then cut

into 5 μm sections, mounted on slides and allowed to air dry overnight at room temperature. The following morning, slides were baked for one hour at 60°C, deparaffinized using a xylene immersion twice for 15 minutes and then dehydrated in an ethanol series. Once dry, the slides underwent three pretreatment steps, for 30 minutes each, to ensure access to corneal tissues. After the first pretreatment, the buffer was rinsed off the slides with deionized water and the slides were boiled at 98-104°C for 30 minutes on a hot plate with pretreatment 2 buffer. The slides were again rinsed in deionized water and treated with pretreatment 3 buffer for 30 minutes at 40°C in the HybEZ^{TM} hybridization oven (Advanced Cell Diagnostics, Hayward, CA). This allowed for target probe access to the corneal tissue and blocking of endogenous peroxidase activity. The 16ZZ Ec-IL6 target probe was then applied to each 5 μm tissue section and the slides placed back in the HybEZ^{TM} hybridization oven for 2 hours at 40°C. After washing, a series of amplifiers was applied to each slide and they were then placed back in the oven. This included treatment with Amp 1 solution for 30 minutes, Amp 2 solution for 15 minutes, Amp 3 solution for 30 minutes and Amp 4 solution for 15 minutes. Each of these amplifying steps was baked at 40°C. Amp 5 and 6 solutions were applied for 30 and 15 minutes, respectively, at room temperature. After each of the six amplifying steps, slides were washed with wash buffer for two minutes at room temperature. In order to detect the RNA signal, the red reagent working solution was next applied to the tissue sections and incubated for 10 minutes at room temperature. Once rinsed with deionized water, the slides were counterstained with 50% hematoxylin, rinsed and dried. Positive and negative controls were run simultaneously to confirm the assay's

validity and the corneal samples' RNA quality and included a PPIB housekeeping gene for a positive assay control and a dabP bacterial gene for a negative control. The tissue sections were evaluated using standard bright field microscopy.

RNAscope *in situ* hybridization evaluation criteria

First, all slides from all time submissions were examined utilizing a standard bright field microscopy microscopeunder 4x, 10x, 20x, and 40x magnification for tissue and cell morphology and for 16ZZ Ec-IL6 target probe signal. The number of IL-6 mRNA transcripts was counted using ACD semi-quantitative scoring recommendation guidelines. Ten slides were randomly selected from each submission time point from each cornea and scored. These ten scored slides were used and those slides used to generate an average score for that particular corneal submission. All slides were scored at 400x. Scores were assigned 0-4 as described previously[39] in which a 0 was noted if no staining was seen or <1 dot/10 cells was counted, a score of 1 was denoted if 1-3 dots/cell were seen, a 2 was noted if there were 4-9 dots/cell and few clusters were seen, a 3 was marked if 10-15 dots/cell were seen and <10% of those dots were in clusters, and a 4 was assigned if >15 dots/cell were noted and >10% of dots were in clusters (Advanced Cell Diagnostics). All fields were scored by one investigator who was masked to the wounded or unwounded nature of the cornea and the time period of submission.

Microscopic imaging

Images were acquired using an Olympus BX53 standard bright field microscope (Olympus, Center Valley, PA, USA) and a DP73 microscope camera.

RESULTS

Corneal ulcer resolution

All wounded corneas healed within 72 hours and no unwounded corneas experienced any ulceration over the duration of this study. The area of re-epithelialization ($A = \pi r^2$) via fluorescein stain was documented and averaged over time. The average corneal ulcer areas over time were T0=27.7$\pm$6.1, T24=18.6$\pm$6.3, T48=7.9$\pm$11.1 and T72=0 mm^2 (Fig. 3-7).

Modified Hackett-McDonald Ocular Scoring System

a) Fluorescein intensity (0-4)

All wounded corneas received maximum intensity stain scores at T0 which subsequently decreased until no stain uptake was seen at T72. Specific average intensity scores ($\pm$SD) included T0=3.9 $\pm$0.2, T24=2.9$\pm$0.2, T48=1.6 $\pm$1.0 and T72=0.

b) Fluorescein location and description

All ulcers were located on the axial cornea and were circular in shape.

c) Corneal edema (0-4)

One wounded cornea experienced a slight loss of corneal transparency (score of 1) and paraxial corneal edema immediately adjacent to the ulceration was noted at T0. By T24 the edema had resolved and no other corneas experienced edema at any time point within this study duration.

Histopathology

All corneas displayed normal corneal architecture. Wounded corneas (Fig. 3-8) showed a lack of epithelium but minimal stromal edema and intact endothelium. Unwounded corneas displayed epithelium of normal thickness, minimal stromal edema and adhered, intact endothelium (Fig. 3-9). The endothelium can be seen in both wounded (Fig. 3-10) and unwounded T72 submissions (Fig. 3-11).

RNAscope *in situ* molecular detection analysis

The highest expression of IL-6 was seen in the basal epithelial cell layer adjacent to the wound bed in T24 wounded corneas, while the lowest IL-6 expression was seen nearest the limbus. By T72 all wounded corneas had healed and expressed minimal IL-6. Average RNAscope semi-quantitative scoring for each time submission group was calculated and included wounded RNAscope semi-quantitative scoring for each time point, including wounded T24=1.7 $\pm$0.7, T48=0.9 $\pm$ 0.7, T72= 0.2$\pm$ 0.4 and unwounded T24=0.3 $\pm$ 0.5, T48=0 and T72=0 groups (Figs. 3-12- 3-14).

Decreased IL-6 expression was seen in the wounded corneas nearest the limbus (Fig. 3-15). Minimal diffuse IL-6 expression was seen in T24 unwounded corneas while T48 and T72 unwounded corneas expressed no IL-6 mRNA transcripts (Fig. 3-16).

DISCUSSION

Ex vivo corneal wound healing models are becoming popular in recent years, as they maintain the dynamic, complex, physiological events of the *in vivo* model, while decreasing the need for live animals for this type of studies.[20,40,41] In this study, we established an *ex vivo* model of equine corneal wound healing, and demonstrated its physiological relevance. The anatomical integrity of the different corneal layers was maintained throughout the study period. Apart from the ulcerated site, all corneas maintained a normal epithelial cell layer, and exhibited a minimal amount of stromal edema (Fig. 3-10). In addition, a healthy endothelial cell layer and Descemet's membrane were noted in all corneas in our study (Fig. 3-10). It is noteworthy that in other *ex vivo* corneal wound healing models,[40] as well as in our previous experience (not published), the endothelium is usually damaged or missing where it was contacted by the conformer. Some studies do not mention the integrity of their endothelial layer.[21] Due to the unique anatomical features of the equine cornea (i.e. greater diameter and thickness), a conformer was not needed in our model, leading to a healthy, intact endothelium. As a result, minimal corneal stromal edema was present, compared to models utilizing conformers.[21,40-42]

All corneal ulcers in our study healed within 72 hours from wounding, consistent with previous publications.[1] Corneal epithelial wounds heal by cellular migration and proliferation. Although the mechanism of corneal wound healing is not fully understood, studies have demonstrated the importance of IL-6 in this complex process.[33] Interleukin-6 is upregulated following corneal injury in humans, as well as in animal and *in vitro* models.[34,36,37,43] The increase in IL-6 expression facilitates, and expedites, epithelial wound closure.[36,44,45] Fibronectin has been shown to induce corneal epithelial cells migration, a process that is enhanced by IL-6 *in vivo*.[46] In addition, IL-6 reduces the production of matrix metalloproteinase-2, which may have deleterious effect on wounded cornea.[47] Furthermore, the suppression of IL-6 expression has negative effect on corneal epithelial cell migration, both *in vitro* and *in vivo*.[43]

The results of our study demonstrate increased IL-6 expression in injured corneas, in agreement with other studies. Peak IL-6 expression has seen reported at one day, or 24 hours post corneal surgery in human patients,[37] and 7 days after corneal alkali burn in mice[34] and rabbits.[33] The peak IL-6 expression in our study was noted 24 hours after corneal wounding, similar to the finding of Resan *et al*.[37] However, since in our study the first time point for IL-6 expression was 24 hours post injury, we cannot rule out an earlier peak of IL-6 expression. While we and others[33,34] used an alkali burn model, we used equine corneas, while Sotozono *et al* used mouse corneas[34] and Imanishi *et al* used rabbit corneas.[33] The interspecies differences may account for the discrepancy in IL-6 peak expression time in these studies.

The RNAscope *in situ* hybridization technique was used in our study. This technique is a robust, highly specific and highly sensitive method for analyzing RNA biomarkers, and allows for molecular visualization of gene expression all while maintaining tissue integrity.[48] Utilizing this modality, we showed that IL-6 expression was the greatest adjacent to the wound bed, in the basal epithelial cell layer, similar to the findings of others.[34] Interestingly, Sumioka *at al* found that transient receptor potential cation channel subfamily V member 1 (TRPV1) is present mainly in the basal layer of mouse and rat corneal epithelium, and that TRPV1 is required for the upregulation of IL-6.[43]

This study presents a novel *ex vivo* equine corneal wound healing model. In this model, corneal architecture (including corneal endothelium) was maintained, unlike other *ex vivo* corneal wound healing models. Hence, this model better mimics *in vivo* events as seen by timely wound healing and IL-6 upregulation. In conclusion, this novel *ex vivo* corneal wound healing model is physiologically relevant, and may be utilized for future studies of corneal wound healing, while decreasing or negating the use of live, healthy animals for such studies.

CONCLUSIONS

In summation, this study established an air-liquid interface with medium bathing *ex vivo* model of equine corneal wound healing. Ulcer resolution was noted to be consistent and timely, mimicking the *in vivo* response of an uncomplicated, superficial ulceration that heals within 72 hours. Histologically, intact epithelium,

minimal stromal edema and presence of the endothelium were seen. Further, IL-6 analysis via RNAscope quantification displayed an upregulated response of this pro-inflammatory cytokine in wounded corneas versus unwounded controls. This finding parallels a previous *in vivo* rabbit study which demonstrated that the metabolically active basal epithelial layer expressed the greatest amount of IL-6.[33] The expression and elucidation of this important cytokine, coupled with the timely macroscopic ulceration resolution and the maintenance of corneal architecture histologically, validates the physiologic relevance of this model.

Therefore, this model can be utilized to reduce or eliminate the use of live horses for corneal wound healing studies, while providing an effective platform for corneal wound healing research in this species.

Conflicts of Interest

The authors have no conflicts to declare.

Chapter 3 References:

1. Gilger, BC. (2007) Equine Ophthalmology. In: *Veterinary Ophthalmology*, 5th edn (ed. Gelatt KN) Blackwell, Ames, IA, pp. 1560-1609

2. Andrew SE, Willis AM. (2005) Diseases of the cornea and sclera. In: *Equine Ophthalmology*, 3rd ed (ed. Gilger BC) St. Louis: Elsevier Saunders; pp. 157-251

3. Samuelson DA. (2013) Ophthalmic Anatomy. In: *Veterinary Ophthalmology* (ed. Gelatt, K.N.), 5th ed., pp. 39–170. Ames, IA: John Wiley and Sons

4. Hendrix DV, Brooks DE, Smith PJ, Gelatt KN, Miller TR, Whittaker C, Pellicane C, Chmielewski N. Corneal stromal abscesses in the horse: a review of 24 cases. *Eq Vet J* 1995; 27: 440–447

5. Andrew SE, Brooks DE, Smith PJ, Gelatt KN, Chmielewski NT, Whittaker CJG. Equine ulcerative keratomycosis: visual outcome and ocular survival in 39 cases (1987–1996). *Eq Vet J* 1998; 30: 109–116

6. Brooks DE, Andrew SE, Biros DJ, Denis HM, Cutler TJ, Strubbe DT, Gelatt KN. Ulcerative keratitis caused by beta-hemolytic *Streptococcus equi* in 11 horses. *Vet Ophthalmol* 2000;3: 121–125

7.Brooks DE, Andrew SE, Denis H, Strubbe DT, Biros DJ, Cutler TJ, Samuelson, DA, Gelatt KN. Rose bengal positive epithelial microerosions as a manifestation of equine keratomycosis. *Vet Ophthalmol* 2000; 3: 83–86

8. Ollivier FJ, Brooks DE, Van Setten GB, Schultz GS, Gelatt KN, Stevens GR, Blalock TD, Andrew SE, Komaromy AM, Lassaline ME, Kallberg ME, Cutler TJ. Profiles of matrix metalloproteinase activity in equine tear fluid during corneal healing in 10 horses with ulcerative keratitis. *Vet Ophthalmol* 2004; 7: 397–405

9. Lassaline ME, Brooks DE, Ollivier FJ, Komaromy AM, Kallberg ME, Gelatt KN. Equine amniotic membrane transplantation for corneal ulceration and keratomalacia in three horses. *Vet Ophthalmol* 2005; 8: 311–317

10. Ollivier FJ, Kallberg ME, Plummer CE, Barrie KP, O'Reilly S, Taylor DP, Gelatt KN, Brooks DE. Amniotic membrane transplantation for corneal surface reconstruction after excision of corneolimbal squamous cell carcinomas in nine horses. *Vet Ophthalmol* 2006; 9: 404–413

11. Brooks DE, Plummer CE, Kallberg ME, Barrie KP, Ollivier FJ, Hendrix DV, Baker A, Scotty NC, Utter ME, Blackwood SE, Nunnery CM, Ben-Shlomo G, Gelatt KN. Corneal transplantation for inflammatory keratopathies in the horse: visual outcome in 206 cases (1993–2007). *Vet Ophthalmol* 2008; 11: 123–133. doi: 10.1111/j.1463-5224.2008.00611.x. PMID:18302577

12. Neaderland MH, Riis RC, Rebhun WC, Erb HN. Healing of experimentally induced corneal ulcers in horses, *Am J Vet Res* 48:427-430, 1987

13. Burling K, Seguin MA, Marsh P, Brinkman K, Madigan J, Thurmond M, Moon-Massat P, Mannis M, Murphy CJ. Effect of topical administration of epidermal growth factor on healing of corneal epithelial defects in horses. *Am J Vet Res* 2000 Sep;61(9):1150-5

14. Mathes RL, Dietrich UM, Krunkosky TM, Hurley DJ, Reber AJ. Establishing a reproducible method for the culture of primary equine corneal cells. *Vet Ophthalmol* 2009; 12: 41-49

15. Weinstein WL, Moore PA, Sanchez S, Dietrich UM, Wooley RE, Ritchie BW. 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

16. 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

17. Astashkina A, Mann B, Grainge DW. A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity. *Pharmacology & Therapeutics*. 2012; 134:82-106

18. Wilson SE, Walker JWQ, Chwang EL, He YG. Hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), their receptors, FGF receptor-2, and the cells of the cornea. *Invest Ophthalmol Vis Sci* 1993;34:2544-2561

19. Li DQ and Tseng SC. Differential regulation of cytokine and receptor transcript expression in human corneal and limbal fibroblasts by epidermal growth factor, transforming growth factor-alpha, platelet-derived growth factor B, and interleukin-1 beta. *Invest Ophthalmo. Vis Sci* 1996;37:2068-2080

20. Janin-Manificat H, Rovere MR, Galiacy SD, Malecaze F, Hulmes DJ, Moali C, Damour O. Development of ex vivo organ culture model to mimic human corneal scarring. *Mol Vision* 2012;18: 2896-2908

21. Deshpande P, Ortega I, Sefat F, Sangwan VS, Green N, Claeyssens F, MacNeil S. Rocking media over ex vivo corneas improves this model and allows the study of the effect of proinflammatory cytokines on wound healing. *Invest Ophthalmol Vi. Sci* 2015; 56: 1553-1561

22. Alekseev O, Tran AH, Azizkhan-Clifford J. Ex vivo organotypic corneal model of acute epithelial herpes simplex virus type 1 infection: a video article. *J Vis Exp* 2012; 69; e3631

23. Akira S, Hirano T, Taga T, Kishimoto T. Biology of multifunctional cytokines: IL 6 and related molecules (IL 1 and TNF). *FASEB J* 1990 Aug;4(11):2860-7

24. Dinarello CA. Proinflammatory cytokines. *Chest* 2000;118:503–508

25. Febbraio MA, Pedersen BK. Contraction-induced myokine productionand release: is skeletal muscle an endocrine organ? *Exerc Sport Sci Rev* 2005;33:114–119

26. Toribio RE, Kohn CW, Capen CC, Rosol TJ. Parathyroid hormone (PTH) secretion, PTH mRNA and calcium-sensing receptor mRNA expression in equine parathyroid cells, and effects of interleukin (IL)-1, IL-6, and tumor necrosis factor-alpha on equine parathyroid cell function. *J Mol Endocrinol* 2003 Dec;31(3):609-20

27. Siemieniuch MJ, Szóstek AZ, Gajos K, Kozdrowski R, Nowak M, Okuda K. Type of inflammation differentially affects expression of interleukin 1β and 6, tumor necrosis factor-α and toll-like receptors in subclinical endometritis in mares. *PLoS ONE* 2016;11(5): e0154934

28. Sandersen C, Bienzle D, Cerri S, Franck T, Derochette S, Neven P, Mouytis-Mickalad A, Serteyn D. Effect of inhaled hydrosoluble curcumin on inflammatory markers in broncho-alveolar lavage fluid of horses with LPS-induced lung neutrophilia. *Multidiscip Respir Med* 2015 Apr 15;10(1):16

29. Leise BS, Yin C, Pettigrew A and Belknap JK. Proinflammatory cytokine responses of cultured equine keratinocytes to bacterial pathogen-associated molecular pattern motifs. *Equine Vet J* 2010;42: 294–303

30. Spass JH, Gomiero C, Broeckx S, Van Hecke L, Maccatrozzo L, Martens A. Wound-healing markers after autologous and allogeneic epithelial-like stem cell treatment. *Cytotherapy* 2016; 18: 562–569

31. Joubert P, Silversides DW and Lavoie JP. Equine neutrophils express mRNA for tumour necrosis factor-α, interleukin (IL)-1β, IL-6, IL-8, macrophage-inflammatory-protein-2 but not for IL-4, IL-5 and interferon-γ. *Eq Vet J* 2001;33: 730–733

32. Nishida T, Nakamura M, Mishima H, Otori T, Hikida M. Interleukin 6 facilitates corneal epithelial wound closure in vivo. *Arch Ophthalmol* 1992a;110(9):1292-1294

33. Imanishi J, Kamiyama K, Iguchi I, Kita M, Sotozono C, Kinoshita S. Growth factors: importance in wound healing and maintenance of transparency of the cornea. *Prog Retin Eye Res* 2000;19:113-129

34. Sotonzono C, He J, Matsumoto Y, Kita M, Imanishi J, Kinoshita S. Cytokine expression in the alkali burned cornea. *Curr Eye Res* 1997;16:670-676

35. Yang H, Wang Z, Capó-Aponte JE, Zhang F, Pan Z, Reinach PS. Epidermal growth factor receptor transactivation by the cannabinoid receptor (CB1) and transient receptor potential vanilloid 1 (TRPV1) induces differential responses in corneal epithelial cells. *Exp Eye Res* 2010;91:462–471

36. Ebihara N, Matsuda A, Nakamura, S, Matsuda H, Murakam A. Role of IL-6 classic and trans-signaling in corneal sterile inflammation and wound. *Invest Ophthalmol Vis Sci* 2011;52:8549–8557

37. Resan M, Stanojević I, Petković A, Pajić B, Vojvodić D. Levels of interleukin-6 in tears before and after excimer laser treatment. *Vojnosanit Pregl.* 2015 Apr;72(4):350-5

38. Munger RJ. Veterinary ophthalmology in laboratory animal studies. *Vet Ophthalmol* 2002; 5:167-175

39. Warrick JI, Tomlins SA, Carskadon SL, et al. Evaluation of Tissue *PCA3* Expression in Prostate Cancer by RNA in situ Hybridization - A Correlative Study with Urine *PCA3* and *TMPRSS2-ERG*. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc*. 2014;27(4):609-620

40. Castro-Combs J, Noguera G, Cano M, Yew M, Gehlbach PL, Palmer J, Behrens A. 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

41. Proietto LR, Whitley RD, Brooks DE, Schultz GE, Gibson DJ, Berkowski WM Jr, Salute ME & Plummer CE. Development and assessment of a novel canine ex vivo corneal model. *Curr Eye Res* 2017 Jan 27:1-9

42. Marlo TL, Giuliano EA, Sharma A, Mohan RR. Development of a novel ex vivo equine corneal model. *Vet Ophthalmol* 2016 Jul 29. doi: 10.1111/vop.12415

43. Sumioka T, Okada Y, Reinach PS, et al. Impairment of corneal epithelial wound healing in a TRPV1- deficient mouse. *Invest Ophthalmol Vis Sci* 2014;55:3295–3302

44. Er, H., Uzmez, E. Effects of transforming growth factor-b2, interleukin 6 and fibronectin on corneal epithelial wound healing. *Eur Ophthalmol* 1998;8,224e229

45. Arranz-Valsero I, Soriano-Romaní L, García-Posadas L, Lopez-García A., Diebold Y. IL-6 as a corneal wound healing mediator in an in vitro scratch assay. *Exp Eye Res* 2014;25, 183e192

46. Nishida T, Nakamura M, Mishima H. and Otori T. Interleukin 6 promotes epithelial migration by a fibronectin-dependent mechanism. *J Cell Physiol* 1992b;153: 1–5

47. Malecaze F, Simorre V, Chollet P, Tack JL, Muraine M, Le Guellec D, Vita N, Arne JL, Darbon JM. Interleukin-6 in tear fluid after photorefractive keratectomy and its effects on keratocytes in culture. *Cornea* 1997 Sep;16(5):580-7

48. Wang WF, Flanagan J, Su N, Wang LC, Bui S, Nielson A, Wu X, Vo HT, Ma XJ, Luo Y. RNAscope A Novel in Situ RNA Analysis Platform for Formalin-Fixed, Paraffin-Embedded Tissues. *J Mol Diagn* 2012, 14:22–29

Figures for Chapter 3

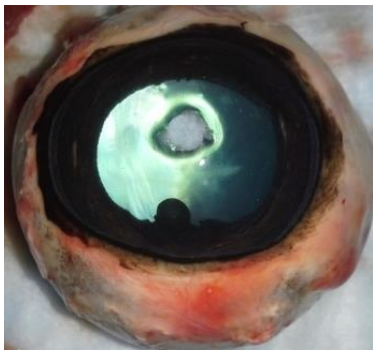

Figure 3-1: Equine globe showing application of the 6 mm filter paper disk soaked in NaOH applied to the axial cornea.

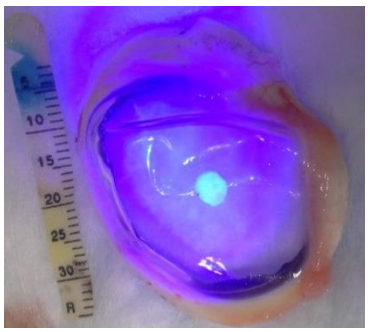

Figure 3-2: Freshly wounded cornea showing circular area of ulceration that is fluorescein positive.

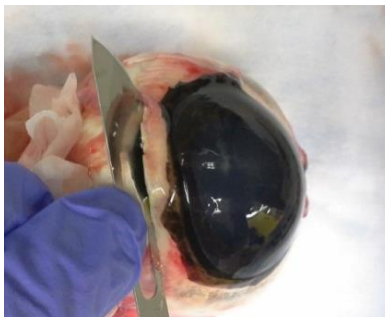

Figure 3-3: Corneoscleral cup excision employing a Bard Parker blade placed approximately 2 mm posterior to the limbus. The anterior portion that contains the cornea (ie “corneal cup”) will then be placed in the incubator cornea side up.

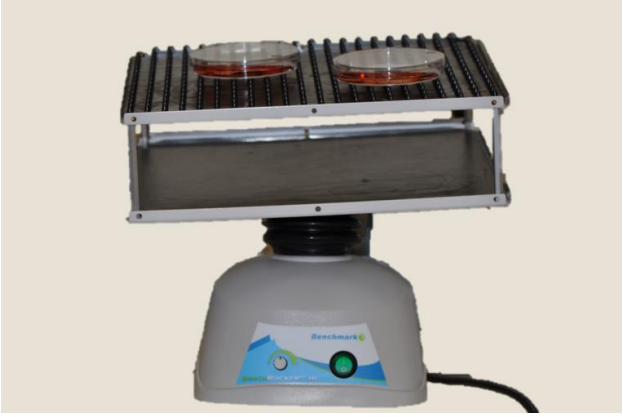

Figure 3-4: Rocker setup including petri dishes with corneas. Note the 6 degree incline that aided in bathing the corneas eight times per minute. The rocker was placed in the CO_2 incubator.

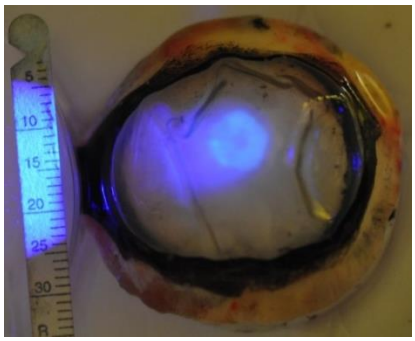

Figure 3-5. Representative wounded cornea at T24. Note the axial, circular fluorescein uptake highlighted by cobalt blue light and the millimeter scale bar.

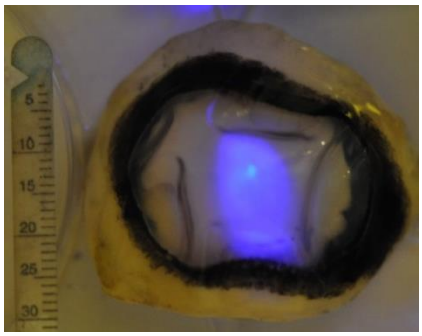

Figure 3-6: Representative wounded cornea at T48. Note the pinpoint axial fluorescein uptake highlighted by cobalt blue light versus the much larger fluorescein positive area shown in the above T24 figure 3-5.

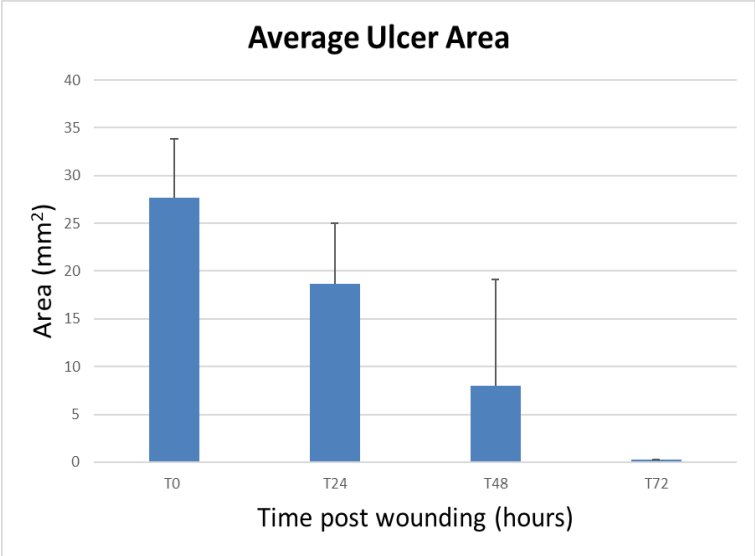

Figure 3-7: Values for the average ulcer area over time including T0=27.7±6.1, T24=18.6±6.3, T48=7.9±11.1 and T72=0 mm^2.

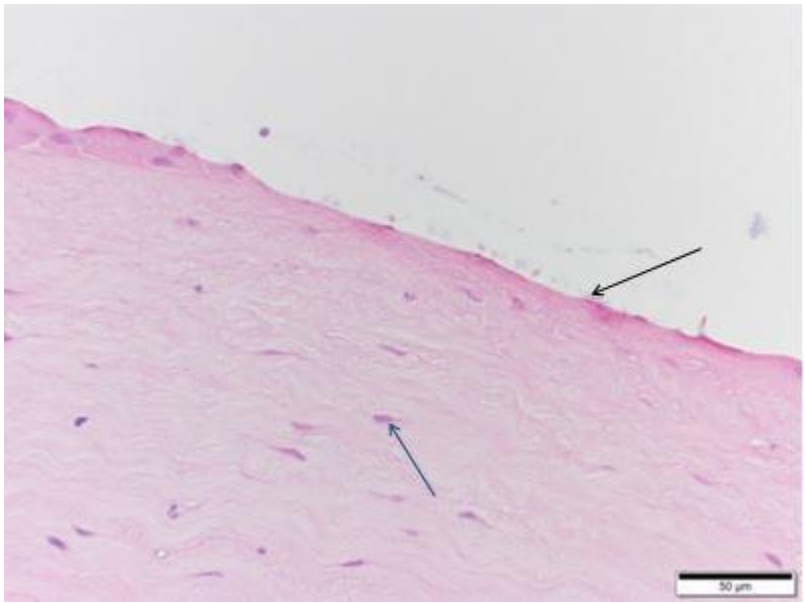

Figure 3-8. Histopathology of representative T48 wounded cornea stained with hematoxylin and eosin. Note the lack of epithelium (black arrow) superficially and the occasional keratocyte (blue arrow) in the stroma. 400x.

Figure 3-9: Histopathology of representative T48 unwounded cornea stained with hematoxylin and eosin. Note the robust 16 cell layer thick epithelium with the basal cell layer (*), wing cell layer (**) and superficial epithelial layer (***). Additionally, note the lack of edema within the stromal layer. 400x.

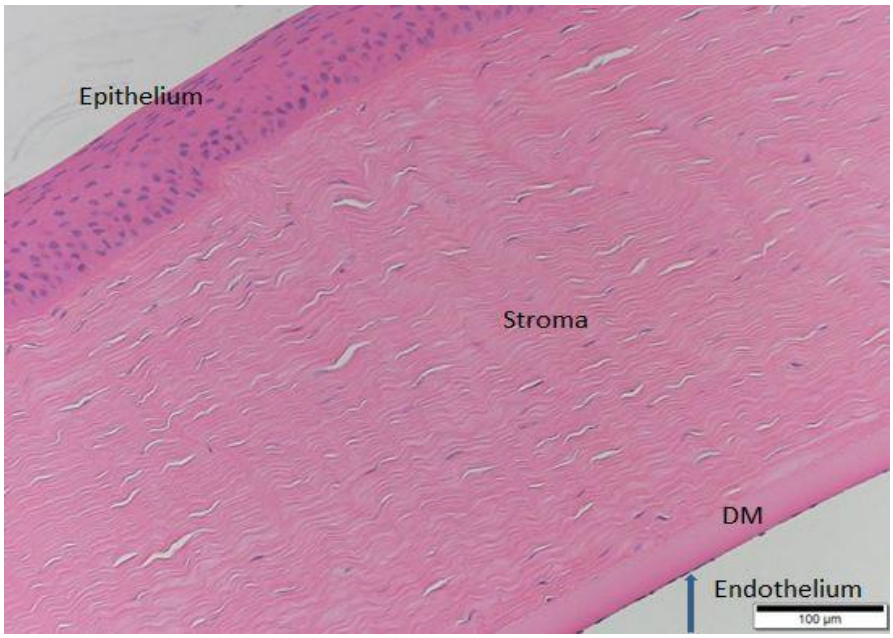

Figure 3-10: Representative wounded corneal submission at T72 displaying intact endothelium in a nonwounded zone. Note the robust epithelial layer, the minimal stromal edema, the thickened Descemet's membrane (abbreviated DM) and the intact endothelial layer (thick blue arrow). 100x.

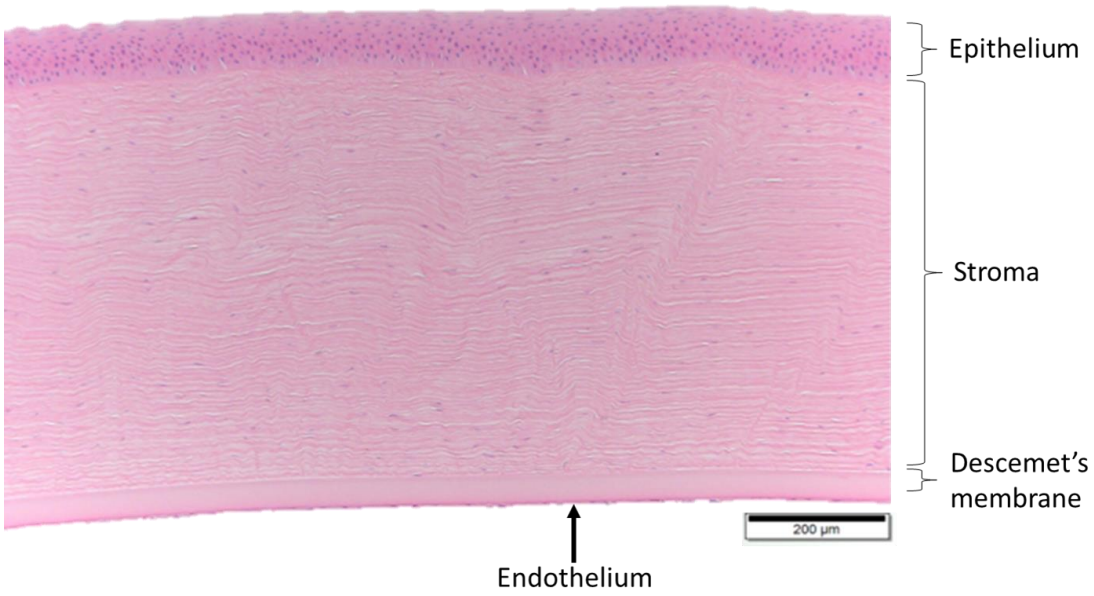

Figure 3-11. Representative unwounded corneal submission at T72 displaying intact endothelium. Note the robust superficial epithelial layer, the minimal stromal edema, the thickened Descemet's membrane, and the intact endothelial layer (black arrow). 40x.

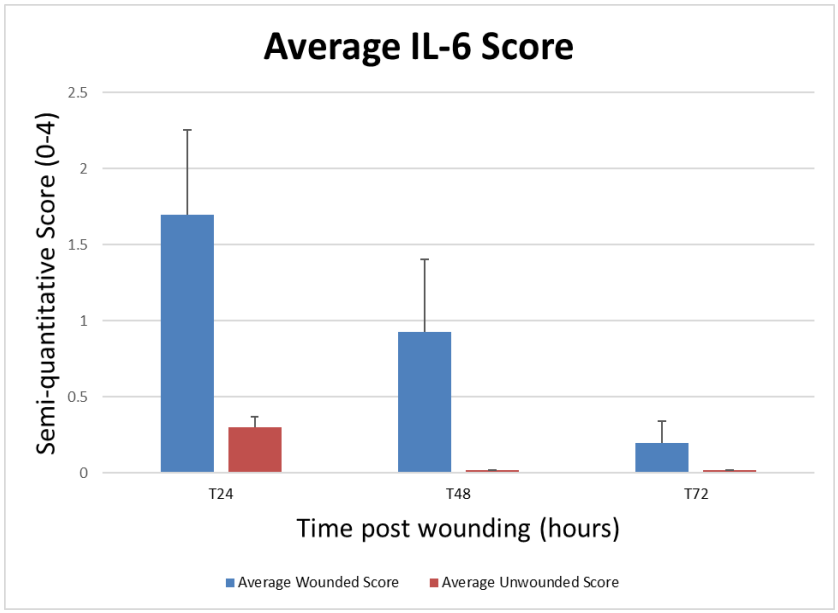

Figure 3-12: RNAscope semi-quantitative scoring for each time point including wounded T24=1.7 ±0.7, T48=0.9 ± 0.7, T72= 0.2± 0.4 and unwounded T24=0.3 ± 0.5, T48=0 and T72=0 groups.

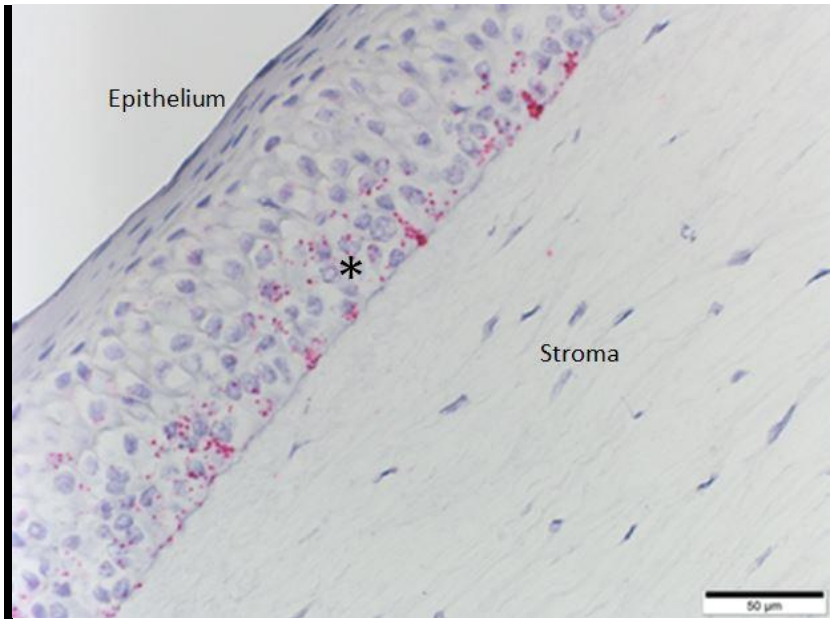

Figure 3-13: RNAscope *in situ* hybridization analysis of a representative T24 wounded cornea immediately adjacent to the alkaline burn. Note the increased IL-6 expression in the basal epithelial cell layer (*). 400x.

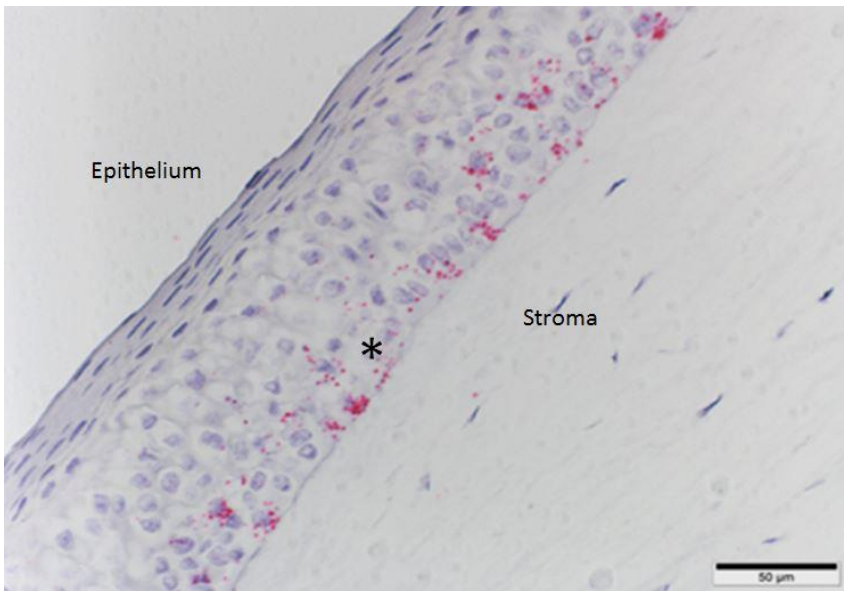

Figure 3-14: RNAscope *in situ* hybridization analysis of paraxial corneal section of a representative T24 wounded cornea. Note the increased IL-6 expression in the basal epithelial cell layer (*) but that the number of IL6 mRNA transcripts are fewer than the above Figure 3-13 as this image is closer to the limbus. 400x.

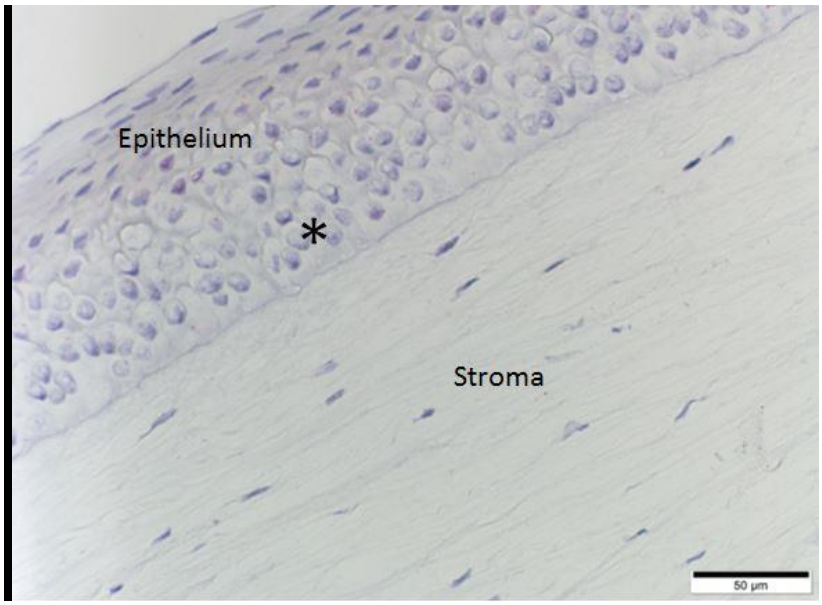

Figure 3-15: RNAscope *in situ* hybridization analysis of a representative, perilimbal area of T24 wounded cornea. Note the lack of IL-6 expression in the basal epithelial layer (*) as this is the farthest point from the wound. 400x.

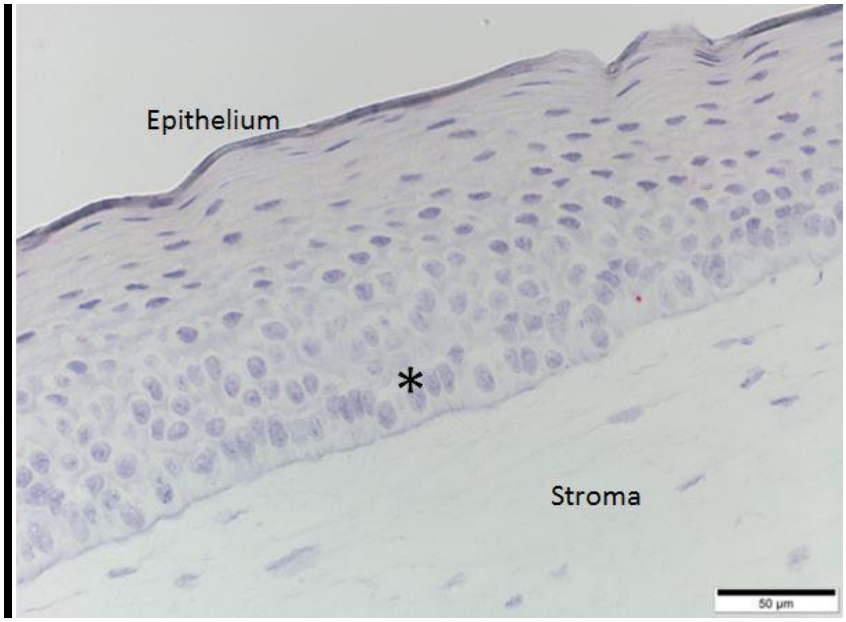

Figure 3-16: RNAscope *in situ* hybridization analysis of a representative, axial area of T72 unwounded cornea. No IL-6 expression is seen in the basal epithelial layer (*). 400x.

CHAPTER 4. GENERAL CONCLUSIONS

Summary

Corneal disease is one of the most significant causes of impaired vision in the horse world-wide. This is likely due to several factors including a prominent globe, the lateral location of the globe within the skull and the equines' exuberant and head throwing behavior. As such, the horse is predisposed to corneal injury. Although equine corneal disease and the subsequent wound healing cascade is a complex process whose mechanisms are still not fully understood, it is well known that inflammatory cells and their secretory products such as cytokines and growth factors are involved. However, despite the high prevalence of corneal disease and ulceration in the horse, the importance of a clear visual axis in this species, and the complexity of corneal wound healing, research is limited and more heavily devoted to *in vivo* studies. Limitations of these *in vivo* studies include ethical concerns, cost, small sample sizes, and large variation amongst subjects. *In vitro* research may be an overly simplified model that lacks the dynamic, physiologic events of the *in vivo* model as these cell culture lines are not able to replicate 'cross talk' across corneal layers. This novel *ex vivo* model bridges the gap between *in vivo* and *in vitro* research and displayed physiologic relevance via timely ulcer resolution, histologically intact corneal layers and IL-6 expression in wounded versus unwounded controls. As such, this model can be utilized to reduce or eliminate the use of live horses for corneal wound healing studies, while providing an effective platform for corneal wound healing research in this species, including, but not limited

to, investigation of corneal wound healing physiology, and evaluation of different treatment modalities.

Concluding Remarks

The *ex vivo* equine corneal wound healing model that was described is not only a novel model but one that has shown physiologic relevance when comparing results to *in vivo* ulcer resolution healing times, maintenance of anatomic structure as seen histologically, and IL-6 expression that mimics previous *in vivo* studies. Hence, this model can be utilized in order to improve our knowledge of equine corneal wound healing while reducing the use of live horses for corneal wound healing studies.