The Effects of Topical Nalbuphine on Canine Corneal Fibroblasts In Vitro

Thesis

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By

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Abstract

Assessing the effects of novel topical ophthalmic medications on the viability and wound healing of corneal tissue is essential in ophthalmic pharmacology and therapeutics. The objective of this study was to evaluate the effects of a topical nalbuphine solution on canine corneal fibroblasts in vitro. Nalbuphine is a mixed agonist-antagonist opioid medication that possesses kappa agonist and mu antagonist properties. It has recently been compounded into a 1.2% ophthalmic solution for use for the management of pain associated with diseases of the ocular surface in veterinary patients. Multiple concentrations of nalbuphine were assessed for their effects on viability and migration of cultured canine corneal fibroblasts at various time points. Solutions of 1% and 0.5% nalbuphine proved to be acutely toxic to cultured canine corneal fibroblast, while lower concentrations exhibited less toxicity. The drug also delayed the migration of corneal fibroblasts at a concentration of 0.1%. Future studies are needed to assess the effects of nalbuphine solution on cultured canine corneal epithelial cell viability and migration, as well as in vivo pharmacologic and cytotoxic properties.
Dedication

To my Grandfather Uranio Spatola. Mi manchi Pappa
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Corneal Anatomy and Physiology

The cornea is the transparent anterior one-fifth of the ocular fibrous tunic. When normal, it is responsible for several important functions essential to optimal vision. Anteriorly, the cornea is covered by the precorneal tear film, while posteriorly it is bathed in the aqueous humor of the anterior chamber. The precorneal tear film and superficial cornea create an air-to-liquid interface that is considered the first refractive structure along the visual axis; together they account for approximately two-thirds of the total refractive power of the eye (1,2). Abnormalities in either the precorneal tear film or curvature of the cornea can lead to refractive error and suboptimal vision. The cornea’s lack of blood vessels, low cellular density, and organized structural arrangement allow for the undisturbed transmission of light (3,4). Hallmarks of corneal disease include corneal edema, cellular infiltration, recruitment of blood vessels, thickening and keratinization, pigment deposition and fibrosis, each of which leads to corneal opacity (5). The corneal stroma is composed of precisely arranged collagen bundles, which lend structural integrity and shape to the globe, protecting the internal structures of the eye from mechanical injury. The two-dimensional shape of the mammalian cornea is typically elliptical, measuring longer horizontally than vertically (2,6). This shape can vary considerably among species, with ungulates demonstrating a more pronounced
difference between vertical and horizontal length than carnivores (2,7,8). The difference is minimal in the canine with dimensions of 13-17mm horizontally and 12-16mm vertically, leading to a cornea that is mostly spherical in shape (9). Corneal thickness varies among species, within individuals of the same species, and increases with age. The normal canine cornea averages approximately 560 micrometers in thickness, being thickest at the periphery compared to the center. (10)

The cornea consists of four histologic layers. From anterior to posterior they are the epithelium, stroma, Descemet’s membrane, and the endothelium. The anterior corneal epithelium is a stratified layer of cells derived from surface ectoderm during embryonic development. It is a nonkeratinized, squamous cell layer ranging from 25 – 40 um thick in domestic carnivores (2). In the dog, the corneal epithelium is normally 5 to 7 cell layers thick. It consists of a single layer of columnar shaped basal epithelial cells with an associated basement membrane, a layer of three polyhedral wing cells, and a layer of up to three nonkeratinized squamous cells. Normal turnover of the corneal epithelium is maintained by mitosis and renewal of basal cells (11). Daughter cells simultaneously migrate upwards and differentiate to become wing cells, and eventually squamous cells. The process takes from 7 to 14 days for complete turnover, with subsequent shedding of superficial squamous cells into the tear film (12,13,14).

Together with the precorneal tear film and conjunctival epithelium, the corneal epithelium constitutes the ocular surface. The epithelium has multiple important functions relative to maintaining the health of the cornea. The most superficial layer of squamous cells possesses an abundance of microvilli and microplicae that are thought to help stabilize the mucin layer of the precorneal tear film and increase surface area for
oxygen and nutrient uptake (15,16,17). The hydrophobic nature of the epithelial cell plasma membrane helps prevent the influx of fluid from the precorneal tear film into the deeper corneal tissue. (18). Interdigitation between neighboring superficial epithelial cells is accomplished via tight junctions, gap junctions, and desmosomes, creating a mechanical barrier to chemicals and microorganisms (18,19). An additional component to the barrier function of the cornea is the epithelial basement membrane (BM). The BM is the most basal structure of the corneal epithelium, and provides another barrier to the penetration of microorganisms. (19,20). Ultrastructurally, the basement membrane is a flat, non-undulating, bilaminar structure predominantly composed of type IV collagen similar in composition and function to most epithelial basement membranes (21). It is maintained by secretions from the overlying basal epithelial cells, and helps to anchor the epithelium to the underlying stroma via hemidesmosomes and anchoring fibrils. The epithelial cells and basement membrane also play an integral role in the initiation and coordination of corneal wound healing, and the lack of an intact basement membrane has been associated with prolonged epithelial wound healing and recurrent erosions in canine corneal disease (22).

Directly below the epithelial basement membrane lies the corneal stroma. At its most anterior boundary, the stroma is connected to the overlying corneal epithelial basement membrane through hemidesmosomes. In the non-diseased canine cornea, the stroma accounts for approximately 90% of corneal thickness (2). The precise structural arrangement of the stromal collagen affords the cornea its transparency (23,24). Individual collagen fibrils are synthesized and packaged in a parallel fashion into bundles called lamellae. Type I collagen is the predominant type found in the stroma, and is
synthesized by resident fibroblast-like cells called corneal keratocytes. Collagen fibrils measure approximately 25nm in diameter, and are responsible for the structural integrity of the cornea (18,23). Bundles of collagen fibrils are arranged into approximately 300 to 500 lamellae that span the entire distance of the cornea from limbus to limbus (25). Lamellae are stacked on top of one another to form sheets with an equal distance between lamellae.

The cellularity of the stroma is minimal in the normal cornea, and contains mostly neural-crest derived mesenchymal cells called keratocytes that occupy approximately 2% to 3% of the total volume of the stroma (18,26). Keratocytes play an important role in the maintenance of the corneal stroma through the secretion of collagen and glycosaminoglycans (GAGs), as well as in stromal repair following injury through the secretion of collagen degrading enzymes (27). Collagen degrading enzymes are needed for cellular migration in the early wound healing phases, as well as for the reorganization of collagen in the later remodeling phase. Expression of matrixmetalloproteinases and serine protease are routinely found in wounded corneal stroma, and both of these enzymes have been shown to aid fibroblast and leukocyte migration in acute and remodeling corneal wounds (5,18,27). Stromal turnover rate in the non-diseased cornea is thought to be from 2 to 3 years, and is highly influenced by the health of the surrounding stroma (18,28). Keratocytes are sparsely dispersed, quiescent, non-contractile, fibroblast-like cells imbedded in ground substance between multiple collagen lamellae. In their dormant state, keratocytes are non-migrating and spindle shaped, with clear cytoplasm, thin nuclei, ill-defined borders, low metabolic rate, and minimal mitotic activity (27,29). They tend to occupy distinct layers of the corneal stroma, being most
numerous in the anterior stroma forming networks of interconnected cells (26,30,31). Long dendritic processes connect neighboring cells via gap junctions. Chemical signaling occurs across gap junctions, allowing them to interpret and relay the environment of the surrounding stroma (32). During injury, keratocytes undergo transformation into either active fibroblasts or myofibroblasts, both of which facilitate cellular migration and collagen and extracellular matrix synthesis, events necessary for normal corneal wound healing (27,33). Occasionally, migrating cells of leukocyte origin are also observed in the stromal layers.

The extracellular matrix of the corneal stroma consists mostly of GAGs, collagen and water (2,18). Glycosaminoglycans are long unbranched polysaccharides that surround a negatively charged protein core to form a structure termed a proteoglycan. Keratin sulfate is the most abundant GAG found in the corneal stroma of mammalian species, with chondroitin sulfate and dermatan being other prominent GAGs (2,18,34). In the normal cornea, GAGs and their associated proteoglycans are found dispersed between and connected to collagen fibrils. They play an integral role in the structural arrangement of stromal collagen lamellae via their precise alignment and aggregation between individual collagen fibrils (35,36). The binding of GAGs to collagen fibrils is thought to aid in the maintenance of the uniform distance found between fibrils in the corneal stroma (36). Glycosaminoglycans also have distinct water binding capabilities allowing them to act as a reservoir for water moving though the corneal stroma, in effect creating a water buffer that works in conjunction with the aqueous regulatory mechanisms of the corneal epithelium and endothelium to help maintain the relative
deturgescence of the corneal tissue (24,37). The anterior corneal stroma is also densely populated with sensory nerve fibers, which is discussed below.

Descemet’s membrane, also referred to as the posterior limiting lamina, is a thin, acellular, collagenous membrane found in the posterior cornea beneath the stroma. It is synthesized by the corneal endothelium and is considered its basement membrane. Ultrastructurally, it is composed of multiple types of collagen, including Type IV and Type VIII (38,39). Descemet’s membrane is continually added to by the corneal endothelium, and thickens considerably with age in most mammalian species (38). Much the same as the corneal epithelial basement membrane, Descemet’s membrane acts as a structural barrier to mechanical trauma and the migration of organisms. It is considered the last mechanical layer of the cornea prior to entry into the anterior chamber of the eye.

The corneal endothelium is a single layer of flat, hexagonal cells whose apical borders come in direct contact with the aqueous humor of the anterior chamber. The salient functions of the corneal endothelium are to maintain the relative deturgescence of the cornea in the presence of direct apposition to an aqueous substance, as well as nutritional and waste transport associated with corneal metabolism (18,40). Interdigitation between neighboring cells is accomplished via zonula occludens and maculae adherens. Dissimilar to the epithelium, the tight junctions of the endothelium are discontinuous, leading to a relatively “leaky” state. Osmotic draw from the hydrophilic corneal stroma, coupled with hydrostatic pressure from the aqueous humor leads to an influx of water and ions across the endothelial lining (40,41). In order to counteract the movement of water into the corneal stroma, the endothelium possesses an abundance of
basolateral ATP dependant ion pumps, which create a concentration gradient favoring movement of water from the corneal stroma to the aqueous humor (40,41).

1.2 Histologic and Ultrastructural Morphology of Corneal Wound Healing

This discussion will be limited to the response of the corneal epithelium and stroma in response to uncomplicated corneal injury. The cornea’s ability to heal after injury is a complex process involving the rapid and coordinated interaction of resident cells, inflammatory cells, cytokines, various growth factors and proteases. These elements are stimulated instantly upon insult, and are employed at the site of injury endogenously as well as from perilimbal blood vessels and lacrimal gland secretions (27,42). The primary purpose of these components in the wound healing response is to reestablish the tissue’s barrier function and architecture in an attempt to prevent further injury, restore normal physiology, and maintain transparency.

Corneal Epithelium:

Morphologically the corneal epithelium responds to injury by characteristic patterns of cellular proliferation, migration, differentiation, and adhesion. In a normal state, limbal stem cells and mitotically active basal cells are responsible for replacing superficial epithelial cells shed into the tear film. This pattern is referred to as the “XYZ” hypothesis of corneal epithelial maintenance, where X is the proliferation of basal epithelial cells migrating superficially, Y is the contribution of limbal stem cells and peripherally located basal cells that proliferate at a higher rate than cells in the axial cornea, and Z is the loss of superficial epithelial cells into the tear film (43). The homeostatic movement of corneal epithelial cells has been shown to occur in a centripetal
fashion due to the location of the stem cells at the limbus, and the higher degree of mitosis found in basal cells at the corneal periphery compared to the center (44).

An hour after an epithelial defect is created, basal cells at the margin of the wound begin to flatten and form numerous cytoplasmic projections termed lamellipodia and pseudopodia. Ultrastructural studies reveal that these projections contain dense populations of actin filaments, which have been reorganized to the periphery of the cell closest to the wound margin (45). Four to six hours after injury, hemidesmosomes have disappeared at the level of the basement membrane up to 70 micrometers from the wound edge (45,46). These structural changes give the epithelial cells at the wound margin a ruffled appearance, and represent the transition from adherent columnar cells to flattened cells preparing for migration. The flattened basal epithelial cells begin to migrate as a single layer across the area of denuded epithelium, pulling the more apical layers of epithelial cells behind them in a sheet-like fashion until a thin layer of epithelium blankets the injured area (2,27,46). The migration of epithelial cells is dependent on their ability to form transient adhesion to the underlying tissue, a process that is aided by plasminogen activator secreted by the migrating cells (42,47). The formation of adhesions between epithelial cells and the surrounding environment helps them resist mechanical detachment from shear stress, and helps migrating cells maintain the formation of lamellipodia and pseudopodia needed for migration (47). The underlying corneal epithelial basement membrane is integral to migrating epithelial cells, and the rate of migration is largely dependent on the condition of this structure (48). When the basement membrane has been significantly damaged or denuded, lacrimal gland and epithelial cell secretions deposit fibronectin, laminin and type-IV collagen over the
exposed surface (49,50). These proteins are key components of the basement membrane, and in the wound healing phase act as a scaffold upon which transient adhesions, termed adhesion plaques, can be formed between migrating epithelial cells (51,52). Additionally, fibronectin, laminin and type-IV collagen are necessary for the construction of a new basement membrane needed once epithelial cells are established over the wounded area (51). If the basement membrane fails to form properly, appropriate adhesions between the epithelium and basement membrane may not form leading to recurrent corneal erosions (53,54).

As epithelial cells are migrating to cover the wounded area, actively mitotic basal cells positioned approximately 3 to 5mm behind the initial wound margin divide and differentiate to replenish cells that have migrated inwards to close the defect (45). Once the defect is completely covered the epithelium begins undergoing the process of restoring its normal form and function. Three weeks after the initial injury basal cells have reformed adhesion complexes to the underlying basement membrane, regained their columnar shape, and the normal thickness of the epithelial layer has been reconstructed (55). In addition, the tight junctions between neighboring epithelial cells have been shown to be reestablished 4 weeks post-injury (50).

**Corneal Stroma:**

The corneal stroma is composed of predominantly type-1 collagen bundles and sparsely dispersed quiescent neural crest derived cells called keratocytes fixed in a proteoglycan rich extracellular matrix. In a normal state the stroma is blanketed anteriorly and posteriorly by the corneal epithelium and endothelium respectively. Damage to either the epithelium or endothelium can lead to the imbibition of fluid into
the corneal stroma causing the tissue to swell, disrupting the precise arrangement of collagen lamellae (24). The result is increased corneal thickness and opacity. Injury to either the overlying corneal epithelium or stromal tissue leads to varying changes in keratocyte metabolic activity, ultrastructural composition, morphology and number along with varying degrees of inflammatory cell infiltration (29,56). Consequently, wound repair mechanisms are aimed at halting and reversing the above processes in an attempt to restore the tissue’s form and function to a pre-injured state.

The cellular response of keratocytes is crucial in stromal wound healing. The predominant resident cell is the keratocyte, which is a cell responsible for collagen turnover and extracellular matrix production in the uninjured cornea (33,57). Injury to the overlying epithelium stimulates release of an array of chemical mediators that switch the normally quiescent keratocyte to a cell with a fibroblast phenotype and genotype suited for migration, extracellular matrix deposition, and wound contraction (33,57,58).

The first recorded change in keratocyte morphology occurs hours following injury to the corneal epithelium. Keratocytes directly below the wounded epithelium undergo apoptosis, leaving a distinct acellular zone 50um to 75um deep in the anterior corneal stroma that parallels the wound margins (59,60). Evidence suggests that Interleukin-1 (IL-1) supplied by the damaged corneal epithelium and tear film may play an important role in the initiation of keratocyte apoptosis seen in corneal wounds (26,61). The proposed mechanism behind the initiation of apoptosis is paracrine mediated stimulation of the Fas/Fas ligand system by IL-1 with resultant activation of cellular caspase enzymes leading to programmed cell death (61). The exact reasons for keratocyte apoptosis in the
corneal wound healing response is not known, although several theories exist including prevention of viral proliferation and induction of the wound healing response (62).

Shortly after initiation of apoptosis, approximately six hours after injury, keratocytes adjacent to the wound margin undergo characteristic morphologic changes. Ultrastructurally these cells enlarge to a fusiform shape, show an increased density of rough endoplasmic reticulum, develop a prominent Golgi apparatus, and reorganize their microtubule skeleton into stress fibers (33,55). These changes are characteristic of tissue fibroblasts, and mark the transformation from quiescent keratocytes to activated corneal fibroblasts. The main purpose of these activated fibroblastic cells is migration and tissue repair. Within 24 hours, cells have begun migrating to the wounded area (27,33). Once activated, they increase the expression of genes encoding for extracellular matrix components, proteases and cell surface adhesion proteins such as integrins. The secretion of proteases such as matrix metalloproteinases and collagenases aid in the ability of the cells to migrate through the collagenous stroma, as well as reorganize and repair damaged stromal tissue at the site of injury (63,64). Initial synthesis of collagen lamellae is haphazard compared to the original tissue’s architecture, with the production of collagen bundles of varying sizes and orientations (27). The disorganized production of collagen lamellae in the early stages of stromal wound healing likely contributes to the clinical entity of corneal haze seen weeks to months after significant corneal injury involving the stroma. Activated keratocytes continue producing collagen and GAGs to repopulate the damaged stroma for approximately 3 months to 2 years, during which time remodeling occurs in an attempt to establish pre-injured tissue architecture (65).
The advent of refractive surgery has led to increased investigation into the mechanisms behind corneal wound healing. Particular attention has been paid to a subset of corneal fibroblasts that exhibit many characteristics of previously described tissue myofibroblasts. These cells characteristically stain for alpha smooth muscle actin (α-SMA), an isoform of actin prominent in the contractile apparatus of smooth muscle cells (66,67). The contractile properties of fibroblast cells, later termed myofibroblasts, have long been studied in healing skin wounds after investigators noticed the characteristic wound contraction noted during the mid to late phases of healing (68,69). Myofibroblasts are now a well-described cellular entity in the scientific literature. They are thought to play beneficial roles in wound healing of multiple tissue types, yet have also been implicated in various pathologic processes involving cirrhosis and fibrosis (70,71).

Jester et al. were the first to discover and characterize the corneal myofibroblast in wounded corneal tissue (65). Cells taken from avascular corneal wounds in rabbit eyes demonstrated ultrastructural characteristics similar to skin myofibroblasts including positive staining for alpha smooth muscle actin and dense microfilament bundles located at the cell periphery. These cells were found only in the wounded area, with staining of α-SMA found at the wound margin after 7 days, blanketing the wound at 14 days, and filling the wound to its posterior edge by 28 days (58). When these cells were isolated in culture and stimulated with smooth muscle agonists, such as acetylcholine and vasopressin, they exhibited a measurable contractile force. Activated fibroblasts, lacking the microfilament array and staining for α-SMA were only found adjacent to the wound margin, suggesting that corneal myofibroblasts found in wounded corneal tissue arise
from corneal fibroblasts in the adjacent stroma (58). The importance of the corneal myofibroblasts deserves special attention when discussing wound healing due to recent studies which may suggest a link between this cell and the clinical entity of corneal haze seen after wounds involving the corneal stroma have healed (72,73).

1.3 Corneal Innervation and its Role in Wound Healing

The cornea is the most densely innervated structure in the mammalian body, with studies showing a population of sensory nerve endings per area up to 600 times greater than skin (75). The mammalian cornea is innervated by both sensory and sympathetic nerve fibers. The ophthalmic branch of the trigeminal nerve supplies an abundance of nociceptive C and A fibers, while the superficial cervical ganglion supplies a modest amount of sympathetic fibers (76). Unmyelinated sensory nerve endings far outweigh sympathetic nerve fibers in the cornea suggesting that the predominant role of the corneal neural network is the detection of noxious stimuli (76). The pattern of nervous distribution in the cornea has been described as radially penetrating at the limbus with sequential branching toward the epithelium, forming multiple distinct layers of anastomosing nerves termed plexi (2,27).

The long ciliary nerves, branches of the ophthalmic branch of the trigeminal nerve, send nerve fibers penetrating through the limbus circumferentially to enter the mid corneal stroma. Each bundle divides into sequentially smaller bundles that traverse both centrally and anteriorly through the corneal stroma to form a sparse posterior stroma plexus, a dense mid stromal plexus, and a dense subepithelial plexus (77). From the subepithelial plexus, individual nerve fibers penetrate the epithelial basement membrane,
course between the tight basal epithelial cell intracellular spaces, and eventually terminate in the superficial epithelium (78,79).

Corneal nerves are not only responsible for the detection and relay of potentially noxious stimuli, but play a role in the maintenance and function of corneal epithelial cells in both the uninjured and wounded state (27,76). Both human and canine patients with trigeminal nerve damage can develop characteristic ocular lesions typically manifesting as non-healing corneal ulcers, with potential corneal melting and perforation. This condition has been termed neurotrophic keratitis, and is a well-know clinical entity in patients with corneal nerve pathology (80,81,82). On a microscopic level, the corneal epithelium in denervated eyes is thinner than normal with swelling, vacuolization, and loss of microplicae noted among individual cells (80). Araki et al studied the rate of epithelial wound healing in rabbit corneas after transection of the trigeminal nerve, and found that denervated corneas healed significantly slower, and showed increased rates of desquamation and increased intercellular spacing compared to controls (83).

The exact role that corneal nerves play in the maintenance and healing of the corneal epithelium is not fully known. Decreased corneal sensation may increase the likelihood of mechanical corneal injury as stimulation of afferent corneal nerves initiates the protective reflex mechanisms such as blinking and reflex tearing. Corneal nerve endings also secrete factors such as substance-P and nerve growth factor (NGF), which studies suggest may play a role in the pathology of neurotrophic keratitis. Substance-P has been shown to promote DNA synthesis and stimulate cellular growth in corneal epithelial cells, and denervated corneas show a marked decrease in the level of substance-P (84,85). Both human and canine patients with non-healing corneal ulcers have shown
both decreased levels of corneal NGF and improved wound closure and healing rates when treated with topical NGF (86,87,88). Therefore, it stands to reason that intact corneal innervation is essential to normal corneal epithelial maintenance, function, and wound healing.

1.4 Conventional Topical Ocular Pain Management

The topical management of ocular pain, in particular corneal pain, poses many challenges due to the barrier functions of the ocular surface and flushing action of the lacrimal system. These protective mechanisms decrease drug bioavailability by diluting applied solutions and rapidly decreasing contact time with the ocular surface (89,90). Furthermore, there are limited options to treat corneal pain directly with topical solutions. Canine ocular pain secondary to corneal erosions or ulcerations, infectious or inflammatory keratitis, or trauma, is often quite severe due to the dense sensory innervation of the corneal tissue described above (5). Current approaches to treatment typically aim at removing the offending agent and managing pain and potential infection both topically and systemically.

The external location of the cornea makes it an ideal tissue for the topical application of medication. Commercial topical solutions for the treatment and prevention of ocular disease are the mainstays of clinical ophthalmic pharmacology. The advantage of topically applied medications lies in the ability to directly deliver them to the tissue of interest, reducing the likelihood of systemic side effects. However, any substance that alters physiology can result in undesired side effects, and topical ophthalmic medications are no exception. The ocular surface can be a fickle environment, and any substance added to it has the potential to disrupt normal physiology and damage cells (91,92).
Additionally, the above mentioned flushing action of the tear film and barrier function of the corneal epithelium decrease drug bioavailability and necessitate that many topical ophthalmic solutions be given multiple times a day to achieve therapeutic levels, increasing their concentration on the ocular surface (93,94). It is imperative then that any product designed for topical application to the eye is evaluated for its potential negative impact on the external ocular structures so that appropriate risk assessments can be made when choosing among treatment options.

Common negative effects of topical medications to the ocular surface include ocular discomfort, direct damage to corneal and conjunctival epithelium, and delayed wound healing (95,96). Various methods, including application of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), are commonly used on cultured corneal cells to determine relative activity of certain cellular enzymes after substance exposure to assess the potential side effects of topical ophthalmic drugs both in vitro and in vivo (97,98). Imaging techniques including laser scanning confocal microscopy and scanning electron microscopy can be used both in vivo and in vitro to assess structural characteristics of cells such as morphology and cell wall integrity (26,99). Wound healing can be evaluated by the use of light microscopy in tissue cultures as well as cellular staining in live patients. Histopathologic evaluation of tissue samples from animal models is also beneficial in evaluating important changes to treated eyes. Each method has its preferred niche, and together they have the ability to provide a collaborative and comprehensive picture of the potential toxicity of topical ophthalmic solutions.
Various classes of drugs have routinely been prepared into topical ophthalmic solutions for use in the treatment of ocular pain. These include corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), sodium channel blocking anesthetics and cycloplegics (100,101,102,103). Each of these medications has both benefits and disadvantages in treating ocular pain. Corticosteroids have long been used for their potent anti-inflammatory and immunosuppressive effects both systemically and locally, yet have been associated with delayed corneal wound healing, increased susceptibility to infection, and collagenase potentiation in both human and veterinary patients (104,105,106), as well as suppression of the hypothalamic pituitary adrenal axis systemically (107,108) when used topically in the eye. Non-steroidal anti-inflammatory drugs have been manufactured into ophthalmic solutions, and in canine patients are routinely used for the prevention and treatment of post-operative inflammation associated with intraocular surgery (109). Reported side effects of topical NSAIDs such as ketorolac, are primarily associated with the cornea and include keratomalacia, punctate keratitis, delayed epithelial wound healing and ocular surface irritation (110,111,112). Topical anesthetic agents are widely used in ophthalmic practice as an effective method of analgesia for diagnostic purposes and minor surgical procedures. However, long-term use of proparacaine and tetracaine has been shown to lead to delayed corneal wound healing, keratomalacia, corneal perforation, ocular surface irritation, and persistent epithelial defects in human patients (113).

1.5 The use of Topical Ophthalmic Opioids in Ocular Analgesia

The systemic administration of opioids for the treatment of severe acute pain is common practice in both human and veterinary medicine. Due to their potency and
efficacy in controlling pain, opioid compounds have been manufactured in multiple forms for administration including oral, parenteral, transmucosal, and rectal routes (114). They primarily mediate the pain response by acting selectively or non-selectively on three classes of receptors termed delta (δ), kappa (κ), and mu (µ) (115). The drugs act as ligands to stimulate the above receptors via G-protein coupled signaling and modulate the transmembrane flow of calcium and potassium, potentially leading to the decreased firing of sensory neurons (116). It has long been known that opioid receptors exist in the central nervous system, primarily the dorsal root ganglion of the spinal cord. However, as recently as 20 years ago the presence of opioid receptors in terminal endings of peripheral sensory nerves was discovered (117). Stein et al were able to demonstrate the upregulation of opioid receptors in the terminal nerve endings, as well as increased production of endogenous opioid peptides by infiltrating immune cells (118). These discoveries have lead to the novel use of these medications locally to control pain associated with inflammation related to surgery, trauma and infection (119,120). Interestingly, results of these studies show that the pain modulating effects of peripherally administered opioids is only effective in inflamed tissue. Theories for this specificity of effect revolve around the potential compromise of the perineurium, the thin connective tissue layer surrounding peripheral nerve bundles, during inflammation. In a normal state the perineurium may provide protection from exposure to chemicals/drugs. Inflammation may cause breakdown of the barrier protection provided by the perineurium with subsequent direct exposure of opioids to nervous tissue.

Topical morphine sulfate, a predominantly µ and δ opioid receptor agonist, has been evaluated for its pain modulating properties and effects on corneal wound healing in
rat, rabbit, canine and human models (121,122,123). Peyman et al. evaluated the effects of topical morphine sulfate on corneal wound healing in rabbit eyes, and found there to be no significant difference in the rate of wound healing in eyes treated with morphine sulfate compared to control eyes treated with topical saline (121). The same study evaluated the analgesic effects of topical morphine sulfate in seven human patients with resultant corneal abrasions following intraocular surgery. Corneal aesthesiometric measurements indicated a significant and relatively rapid, within 10 minutes, onset of corneal analgesia in denuded eyes compared to normal contralateral eyes. The analgesic effect increased up to 20 minutes after application, indicating a time dependant component to topically applied morphine sulfate in eyes with corneal abrasions. Wenk et al. evaluated the effects of topical morphine sulphate on corneal hyperalgesia and inflammation following chemical injury (122). Their study revealed decreased capsaicin blink response in injured eyes treated with topical morphine sulfate at concentrations of 5\(\mu\)M and higher compared to control and normal eyes. Capsaicin blink response involves instilling measured volumes of capsaicin solution, 1 \(\mu\)M in this experiment, into the eyes of sedated rats followed by measuring the number of times the animal blinks. Capsaicin is a known noxious stimulus that leads to reflex blinking when instilled into eyes with intact corneal innervation. Sedation with xylazine was used in this experiment to cease spontaneous blinking. Additionally, the application of topical morphine sulfate at equal concentrations inhibited the infiltration of inflammatory cells into the corneal stroma as measured by evaluating highly magnified hemotoxylin and eosin stained sections of cornea. Stiles et al. evaluated similar properties of morphine sulfate as the above studies in canine eyes with experimentally induced corneal ulcers (123). Results indicated
effective corneal analgesia evidenced by decreased rates of blepherospam and increased corneal aesthesiometric values compared to control eyes treated with topical saline. Additionally, clinical and histopathologic evaluations did not show any difference in the rate of wound healing between treatment and control eyes.

1.6 Nalbuphine: Clinical Use in Human and Veterinary Patients

Nalbuphine hydrochloride is a mixed agonist-antagonist semi-synthetic opioid labeled for the management of moderate to severe pain in humans. Chemically it is a white odorless powder with the name morphinan-3, 6-alpha, 14-triol, 17-(cyclobutylmethyl)-4, 5-alopha-epoxy, hydrochloride. The drug is derived from oxymorphone, and is chemically similar to the opioid antagonist naloxone (124,125). Its primary analgesic action is derived from κ opioid receptor agonism, while it also possesses μ opioid receptor antagonistic effects. Although it possesses narcotic properties, nalbuphine is not currently listed as a controlled substance by the Food and Drug Administration.

The drug has a wide range of clinical applications in human medicine. Multiple studies have found that nalbuphine has analgesic effects similar to morphine in postoperative surgical patients (126,127,128). In addition, intravenous nalbuphine has been found to have a ceiling effect with regards to respiratory depression compared to morphine. Unlike morphine, intravenous doses of nalbuphine higher than 20mg do not produce increased respiratory depression, likely due to its mixed agonist-antagonist properties, making it a more attractive option in patients with respiratory compromise (129). The drug also has minimal effects on the cardiovascular system, making it useful in the anesthetic/analgesic management of obstetric and cardiovascular patients.
undergoing surgical procedures (124,130). Interestingly, at low doses nalbuphine has been shown to demonstrate a sex dependant analgesic response in human patients, being more potent in females than males (131). Due to its $\mu$ receptor antagonistic effects, it can be used to reverse some of the undesired effects of $\mu$ agonist opioids, such as nausea, pruritis and respiratory depression, without totally eliminating analgesia (132,133). Other uses include analgesia in ambulatory medicine and patient controlled analgesia (126,134). To the author’s knowledge there are no published reports documenting the use of topical ophthalmic nalbuphine in association with the treatment of human ocular disease.

Nalbuphine’s use in veterinary medicine has been limited with relatively few studies on its efficacy in animal patients compared to the wider used opioid narcotics morphine, hydromorphone and oxymorphone. One study’s findings suggested that it is less effective at controlling post-operative soft tissue surgical pain in dogs compared to morphine and the non-steroidal anti-inflammatory ketoprofen (135). Various text references on veterinary anesthesia/analgesia describe the potential use of nalbuphine in combination with alpha-2 agonist for standing procedures in equine patients along with its potential application for controlling pain in surgical patients of any species (136,137). Currently, some veterinarians and veterinary ophthalmologists use a compounded 1.2% topical ophthalmic solution of nalbuphine to treat pain associated with disease of the ocular surface such as corneal ulceration and keratitis. To date, there are only two published reports of the drug’s ophthalmic use in veterinary patients. Wotman et al. evaluated the corneal sensitivity of normal horse eyes prior to and shortly after topical application of a 1% nalbuphine solution (138). Results showed no difference in corneal
sensitivity after application of the drug in normal equine eyes between test subjects and matched controls. Clark et al. compared the clinical analgesia of topical 1% ophthalmic nalbuphine vs. oral tramadol in male beagles following experimentally induced corneal ulceration (139). Their results, based on clinical observation of pain and the need for rescue analgesia, indicate that topical nalbuphine is ineffective compared to oral tramadol in regards to analgesia following acute corneal ulceration. To the author’s knowledge, no studies have been conduction evaluating the effects of topical ophthalmic nalbuphine on corneal cell migration and viability.

1.7 Study Objectives and Hypotheses

The goal of this project was to evaluate the effects of topical ophthalmic preparations of nalbuphine hydrochloride on cultured canine corneal fibroblast and epithelial cell morphology, viability and migration in an in vitro model of canine corneal wound healing. We have established two hypotheses relative to this project.

**Hypothesis A:** Topical ophthalmic nalbuphine will not negatively impact canine corneal cell viability in vitro.

**Hypothesis B:** Topical ophthalmic nalbuphine will not negatively impact canine corneal cell migration in vitro.
CHAPTER 2

MATERIALS AND METHODS

2.1 Globe Preparation

All dissections took place in a clean, minimum traffic area that was disinfected immediately prior to and following dissection. Whole, intact globes were obtained by enucleation from adult dogs in good health euthanized for population control reasons. Any eyes with obvious gross ophthalmic abnormalities were not used for culture. Eyes were enucleated within one hour of euthanasia and placed into a 2.0% betadine solution for a minimum of five minutes, then transferred to sterile 1X phosphate buffered solution (PBS; Gibco, Carlsbad, CA) for transport. Within one hour of enucleation, eyes were removed from PBS solution and lavaged with an additional 10mL of sterile PBS. Stevens tenotomy scissors were used to trim the globes of as much adnexal tissue as possible. The trimmed globes were then lavaged with sterile PBS.

2.2 Canine Corneal Stromal Fibroblast Culture

Corneal epithelium was mechanically removed by debridement with a sterile beaver blade (BD Ophthalmic Systems, Waltham, MA). A fresh sterile beaver blade was used to create superficial keratectomy samples (approximately 8mm by 8mm in size) from the axial region of the cornea taking care not to involve the corneoscleral junction. Care was taken during incision to avoid perforation of the cornea. Globes that were perforated during dissection were discarded. Each superficial
keratectomy sample was dissected into quadrants to create four individual cornea explants. Four explants were transferred to an uncoated plastic tissue culture dish using Bishop-Harmon forceps, and manually flattened. The stromal tissue was allowed to adhere to the culture dishes for five minutes at room temperature. Under an ultraviolet sterilized laminar flow hood, 5mL of Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Carlsbad, CA) containing 10% FBS, streptomycin and amphotericin B (Gibco) was added to each culture dish. The culture dishes were then placed in a humidified incubator and maintained at 37°C and 5% CO₂. Dishes were monitored daily via inverted microscopy for cellular growth and proliferation as observed by percent confluence. Culture medium was replenished every four days. After eight days of culture, stromal cells were subcultured. Cells were removed from plates by trypsinization, collected by centrifugation, re-plated, and allowed to grow for an additional four days. Culture media continued to be replenished every four days. Cells were subcultured up to four times, and plated into plastic uncoated 12 well plates (Corning Inc, Corning, NY) prior to experimentation.

2.3 Drug Solution Preparation

Nalbuphine – Nalbuphine Hydrochloride is a white, odorless, fine powder with the chemical structure C₂₁H₂₇NO₄•HCl, and a molecular weight of 393.95. It is hygroscopic, soluble in water to a concentration of 35mg/mL at room temperature, and insoluble in chloroform. A minimum 98% pure nalbuphine hydrochloride powder was obtained from Prescription Center (Fayetteville, NC), courtesy of Gary Newton, FACA. The goal in making the experimental nalbuphine solutions was to replicate the topical ophthalmic nalbuphine preparation distributed by Prescription Center for use in patients.
Preparation of Drug Solution – All test solutions were made immediately prior to experimentation and were not stored for use at a later date. Seventy milligrams of powdered nalbuphine was placed into seven milliliters of room temperature culture media. The mixture was vortexed for five minutes until no powder remained in solution. The resultant mixture was a 1% (weight/volume) nalbuphine solution in culture media. The solution was then filtered through a 0.2 micron syringe filter into a sterile centrifuge tube. Serial dilutions were then prepared using the 1% solution and culture media to obtain solutions with additional concentrations of 0.1%, 0.25% and 0.5%.

The lacrimal system poses one of the many obstacles to the effective delivery of topical medications in ophthalmology. In particular, the concentration of medication in aqueous solutions is often decreased instantly upon administration. Factors such as dilution from mixing with the aqueous tear film, loss from spillage out of the inferior conjunctival cul-de-sac, reflex tearing and blinking, and drainage through the nasolacrimal duct rapidly decreased the concentration of applied solutions (140). The estimated total tear volume in canine eyes is between 2.5µL and 8µL, and the average drop from conventional dispensers is approximately 40µL (140,141). Scintigraphy studies in human eyes have shown that the majority of instilled medication placed into the eye is removed within 30 seconds, and that the total tear turnover rate in normal, undisturbed human eye is approximately 10 minutes (140). Therefore, we elected to test lower concentrations of nalbuphine than the 1% solution to more closely approximate the actual residual concentration the eye would be exposed to over time in vivo. Concentrations of 0.25% and 0.1% more closely approximate concentrations of nalbuphine exposed to the corneal epithelium in vivo.
2.4 Canine Corneal Stromal Fibroblast Morphologic Analysis

Canine corneal fibroblasts were harvested and cultured in uncoated plastic tissue culture dishes following the above protocol. Cells were allowed to grow to 90% confluence prior to treatment. Cultured fibroblasts were treated with a 0.1%, 0.25%, 0.5% and 1.0% prepared nalbuphine solution and evaluated using inverted microscopy and digital photography at 0, 5, 15 and 30 minutes. Three culture wells were used for each treatment (n = 3.) In addition, a dish containing media and saline with no nalbuphine solution (control), and a dish containing media only were also evaluated at an n = 3 for the above time points. The control dish contained all components of the test solution minuse the nalbuphine. The dish containing media only was used to confirm that cells did maintain viability under test circumstances (humidity, temperature) for the duration of the study, and decreases in viability over time were not related to the nature of the cultured cells.

2.5 Canine Corneal Stromal Fibroblast Viability

Stromal Fibroblasts – Cultured stromal fibroblasts were passed into plastic uncoated 12-well plates and allowed to reach 95% monolayer confluence. Cells were treated with 0.1%, 0.25%, 0.5% and 1% nalbuphine solution (n = 3). Stromal fibroblasts were exposed to drug at the above concentrations at room temperature for 0, 5, 15 and 30 minutes. When respective exposure times had been reached for a given group, the nalbuphine solution in culture media was removed from each well. Three hundred microliters of trypsin was then added to each well, and the plate was placed in a humidified incubator at 37°C and 5% CO₂ for 10 minutes. Plates were then removed from the incubator, and 600uL of DMEM containing 10% FBS was added to the wells
and pipetted to create a homogenous suspension. Three hundred microliters of trypan blue stain was then pipetted into each well, and the plate was manually agitated to facilitate dispersion of stain. Trypan blue selectively stains dead cells a dark blue color, which can be visualized and counted microscopically with minimal training and without the need for extra reagents or equipment. Live cells with an intact cell membrane exclude the dye and do not stain. Ten microliters of stained solution was then loaded into a hemocytometer, and both viable and non-viable cells were counted under a light microscope at 40x magnification to obtain a percentage of live cells among 100 counted.

2.6 Canine Corneal Fibroblast Wound Healing Assay

Corneal fibroblast cells were cultured and passed into plastic uncoated 12-well plates and allowed to reach 95% confluence. A 1mm wide defect was created in the cellular monolayer by dragging the end of a sterile 1mL pipette tip across the entire length of the bottom of the well. Cells were then treated with a 0.1% nalbuphine solution (n = 6), incubated in at 37°C and 5% CO₂, and evaluated at zero, four and eight hours with inverted microscopy and digital photography. A concentration of 0.1% was chosen based on stromal fibroblasts viability data obtained via the trypan blue assay. The lowest level of toxicity was chosen to allow cells to remain viable long enough to be evaluated up to eight hours post-treatment. Image analysis software (ImageJ, v 1.43 NIH) was used to calculate the total area of cellular migration over the defect between treatment groups and controls for statistical analysis.

2.7 Canine Corneal Epithelial Cell Culture Protocol

Using a sterile scalpel blade held at a 45° angle to the corneal surface, the central corneal epithelium was mechanically removed by debridement. Debridement was
performed over the entire axial cornea, and peripherally approximately 2mm from the limbus. Care was taken not to debride with excessive pressure, and to go over each area of the cornea minimally in an attempt to not breach the basement membrane. Explanted corneal epithelial tissue was then carefully placed in an uncoated plastic tissue culture dish (TPP Techno Plastic Products, Switzerland) and spread out to maximize contact area with the culture dish. Tissue collected from five eyes was placed into one culture dish to maximize cellular yield per dish. The epithelial tissue was allowed to adhere to the culture dish at room temperature for five minutes. Under an ultraviolet sterilized laminar flow hood, 4mL of complete defined corneal epithelial culture media (CELLnTEC Advanced Cell Systems, Switzerland) was added. The culture dishes were then covered, placed in a humidified incubator, and maintained at 37°C and 5% CO₂. Culture dishes were monitored daily via inverted microscopy for cellular growth and proliferation evidenced by percent confluence on the bottom of the culture dish. Culture media was changed every two days, and cells were subcultured on the eighth day via trypsinization (2.5% trypsin EDTA solution, Gibco) and centrifugation, and were subsequently plated into 24 well plastic uncoated tissue culture plates (Corning, NY).

2.8 Statistical Analysis

All statistical analysis was performed using SigmaPlot Version 11 software (Systat Software San Jose, CA). Data was analyzed using two-way analysis of variance (ANOVA) with Holm-Sidak post testing when a significant f-statistic was identified. A p-value of less than 0.05 was considered significant. Comparison graphs of viability and wound healing were generated in Microsoft Excel 2008 for Mac (Microsoft Corporation Redmond, WA).
CHAPTER 3

RESULTS

3.1  Morphologic Analysis of Cultured Canine Corneal Fibroblast

Cultured fibroblasts were deemed to be morphologically viable through the demonstration of adherence to the culture plate, along with lack of evidence of cellular shrinking, rounding, vacuolization, and cytoplasmic retraction. Conversely, cells that did not adhere to the plate and exhibited the above characteristics were considered to have decreased viability. Figure 3.1 is a digital photograph of cultured canine corneal stromal fibroblasts at greater than 90% confluence taken via inverted microscopy at 100x magnification. This sample is representative of the appearance of all cells prior to treatment with the nalbuphine solution. These cells are adhered to the bottom of the culture plate, and are spindloid in shape with long cytoplasmic projections contacting adjacent cells. Cells of this appearance were considered morphologically viable based on similar appearances from previous studies (142,143). Figure 3.2 is a photograph taken via inverted microscopy at 100X magnification of cultured canine corneal fibroblasts after being exposed to a 1.0% ophthalmic nalbuphine solution for 15 minutes. These cells have clumped together and lost their spindloid shape, appear shrunken and rounded, and numerous cells have lifted off the bottom of the culture plate and are in suspension. Cells of this appearance were considered to be morphologically non-viable based on similar appearances from other studies (143,144).
Figure 3.1 – Appearance of cultured canine corneal fibroblasts at >90% monolayer confluence. Cells demonstrate normal morphological appearance of viable cultured fibroblasts including spindloid shape, long cytoplasmic extensions in close proximity to adjacent cells, and adherence to the culture plate.

Figure 3.2 – Appearance of cultured canine corneal fibroblasts after exposure to 1.0 % topical ophthalmic nalbuphine solution for 15 minutes. Cells exhibit multiple morphological characteristics of non-viability (cytotoxicity) including clumping, loss of adherence to the culture plate, shrinkage and rounding.
Cells were treated with five concentrations of prepared nalbuphine ophthalmic solution (0.0%, 0.1%, 0.25%, 0.5% and 1%) along with a control dish of cells maintained in culture media. Digital photographs of treated cells at each time point were subjectively judged to be affected mildly, moderately, severely or not at all in regards to decreased cellular viability. Judgments were based on the apparent total area of cells exhibiting characteristics of decreased cellular viability per digital photo. When less than 25% of the photographic field was subjectively determined to be affected, a score of mild was given; 25% to 50% was given a score of moderate; over 50% was given a score of severe. Cells treated with nalbuphine solutions of 0%, 0.1%, and 0.25% concentrations exhibited no signs of decreased cellular viability over a thirty-minute exposure period compared to control wells containing culture media only (Figure 3.3). Cells treated with a 0.5% solution demonstrated moderate signs of decreased cellular viability starting five minutes after exposure; this progressed to a severe decrease in cellular viability by thirty-minutes of exposure (Figure 3.4a). Cells treated with a 1.0% nalbuphine solution demonstrated severe signs of decreased cellular viability after five minutes of exposure (Figure 3.4b).

3.2 Effects of Nalbuphine Solution on Stromal Fibroblast Viability

Figure 3.5 is a graph representing the average percent of stromal fibroblasts deemed alive via trypan blue staining after exposure to multiple concentrations of nalbuphine solution at four different time points. Statistical analysis revealed a trend for reduced percentage of live cells over some time periods; however, this trend did not achieve statistical significance (p = 0.076). Significant differences in viability scores were found (p = 0.013) between concentrations, indicating that the concentration of
Figure 3.3 – Image “a” represents the appearance of stromal fibroblasts at 100x magnification treated with a 0.1% nalbuphine solution for thirty minutes. Image “b” represents the appearance of stromal fibroblasts at 100x magnification treated with a 0.25% nalbuphine solution for thirty minutes. Both images show cells that are morphologically consistent with cells in Figure 3.1, and were deemed viable.

Figure 3.4 – Image “a” represents the appearance of stromal fibroblasts at 100x magnification treated with a 0.5% nalbuphine solution for five minutes. Image “b” represents the appearance of stromal fibroblasts at 100x magnification treated with a 1% nalbuphine solution for five minutes. Both images show cells that are exhibiting morphological characteristics of decreased viability, including cell shrinkage and rounding. Cells in Image “b” are more affected than those in Image “a”.

The nalbuphine solution used has a significant effect on viability. Comparison analysis revealed that a significant difference existed between cells treated with a 1% solution compared to 0.1% and 0.25% solutions. Control and vehicle solutions were measured for percent viability at thirty minutes along with test solutions to ensure that cells
would remain alive in culture for a full thirty minutes. Graphical representation is provided in figure 3.5. The viability of the test and control solutions at thirty minutes appears to be similar to the viability of all cells at time zero confirming that cells not exposed to nalbuphine were able to live for thirty minutes under similar environmental conditions to treated cells. This helps remove the confounding issue of cells simply losing viability as a function of time. Figure 3.6 is a table representing the difference in mean percentage of live cells between concentrations of nalbuphine solution. Stromal fibroblasts treated with a 1% nalbuphine solution had a 41% reduction in viability compared to those treated with a 0.1% solution (45% vs 86% respectively, \( p = 0.006 \)). Likewise, stromal fibroblasts treated with a 1% nalbuphine solution showed a 42% decrease in viability across time points compared to stromal fibroblasts treated with a 0.25% solution (45% vs 87% respectively, \( p = 0.005 \)).

![Stromal Fibroblast Viability (Trypan Blue Assay)](image)

**Figure 3.5** – Bar graph representing the average percentage of live cells treated with multiple concentrations of nalbuphine ophthalmic solution at four different time points. Determination of live cells was accomplished via trypan blue staining.
<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Difference of Mean % Alive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 vs. 0.1</td>
<td>41</td>
</tr>
<tr>
<td>1.0 vs. 0.25</td>
<td>42</td>
</tr>
<tr>
<td>1.0 vs. 0.5</td>
<td>16</td>
</tr>
<tr>
<td>0.5 vs. 0.25</td>
<td>26</td>
</tr>
<tr>
<td>0.5 vs. 0.1</td>
<td>25</td>
</tr>
<tr>
<td>0.25 vs. 0.1</td>
<td>2</td>
</tr>
</tbody>
</table>

Figure 3.6 – Table showing the difference in viability scores between concentrations.

3.3 Effects of Nalbuphine Solution on Stromal Fibroblast Migration

Figure 3.7 represents the expected progression of normal untreated cultured stromal fibroblast migration and proliferation across a defect over an eight-hour period. Image analysis software was used to measure the area of cellular in-growth which is represented as percent healed. Average percent healed was compared between nalbuphine treatment and control groups. Figure 3.8 represents the percent healing for stromal fibroblasts treated with a 0.1% nalbuphine solution, 0% nalbuphine solution (vehicle), and control containing culture media only. While graphical representation shows a trend for lower rates of cellular migration and proliferation in the nalbuphine treated group compared to the vehicle and control group, no statistically significant difference was found ($p = 0.135$). A statistically significant difference was found between cellular in-growth relative to time ($p = 0.019$), indicating that cells in all groups showed significant migration from zero to four hours, and zero to eight hours.
Figure 3.7 – Three images of normal untreated cultured canine corneal stromal fibroblasts taken at 100x magnification. Image A was taken immediately after a uniform 1mm defect was created in the cellular monolayer. Image B was taken four hours after image A, and shows cellular in-growth progressing from the defect edge inward. Image C was taken eight hours after the defect was created, and shows further progression of cellular in-growth. Image C is showing almost complete closure of the defect, with migrating and proliferating cells from each wound margin beginning to contact one another in the center of the defect.
Figure 3.8 – 0.1% Nalbuphine Wound Healing Assay: Bar graph representing the percentage of area repopulated by corneal fibroblasts after creation of a uniform defect in the cellular monolayer at four and eight hours.
Chapter 4

Discussion

Cultured corneal epithelial and stromal fibroblast cells are commonly used in scientific research to evaluate the effects of compounds on cellular viability and wound healing (97, 98, 112, 144, 145, 146). While caution must be used in translating the results of in vitro studies to in vivo conditions, inferences can be drawn to help guide the use of test compounds in clinical situations. Advantages of using cultured cells in place of in vivo models include the isolation of specific cell types, control of the physiochemical environment, the need for lesser quantities of test compounds, and avoidance of ethical and moral issues associated with live animal testing (147). These advantages are evident in the study of ophthalmic physiology and pharmacology, where the cost, limited tissue availability, and ethical issues of animal testing hinder the progress of studying ocular disease (148, 149). Specific to the cornea, the progressive development of novel drug therapies and surgical procedures such as laser-assisted in situ keratomileusis (LASIK) further highlights the need for efficient and cost-effective methods of testing.

The culture of stromal fibroblasts is well established for most mammalian tissues. Standard protocols revolve around the explanted tissue method, where excised stromal tissue consisting of quiescent keratocytes is placed directly onto a sterile culture apparatus and bathed in serum supplemented culture media (147). Keratocytes then transform into activated fibroblasts, which migrate out of the tissue explants onto the culture dish. The protocol used for culturing canine corneal stromal fibroblasts described in section 2.2 is similar to that used in earlier studies (145, 150). This method consistently
yielded a high number of activated fibroblasts, which maintained appropriate morphology through five passages.

Exposure of 0.5% and 1.0% nalbuphine solutions to cultured canine corneal fibroblasts proved to be acutely toxic with a 31% and 51% decrease in viability respectively compared to controls after thirty minutes of exposure. Statistical analysis showed significant differences in viability between stromal fibroblasts stained with trypan blue following treatment with 1.0% and 0.5% nalbuphine solutions throughout the duration of treatment when compared to cells treated with a 0.1% solution, where 1.0% and 0.5% proved to significantly decrease cellular viability. This indicates that lower concentrations of the drug have less of a negative impact on cell viability, conferring a dose-dependant response to cytotoxicity. This effect was also observed morphologically, where treatment of cells with 0.5% and 1.0% solutions lead to cellular shrinkage, rounding and lifting from the bottom of the culture dish after five minutes of exposure, further indicating marked decrease in cellular viability.

A statistically significant difference was not found when evaluating the effect of time on viability. This would imply that the actual length of exposure has no bearing on potential toxicity of nalbuphine regarding stromal fibroblasts. We believe there are two reasons a significant difference was not found. Analysis of variance takes into account all treatment groups when evaluating time and concentration. If a significant difference is not found in one variable (time or concentration), further evaluation of individual groups is not performed. Lack of sufficient statistical power is the most likely reason for failing to find a difference regarding time across all treatment groups. Statistical power is the likelihood that a test will reject the null hypothesis when the null hypothesis is
actually false. One of the main influential factors affecting statistical power is sample size. For our study, a sample size of three likely limited the studies statistical power to a degree that it was much less likely to find a true difference between all groups regarding time. Also, the limitations of trypan blue staining, more accurately described below, may have overestimated the viability of cells at later time points, further decreasing the likelihood that a true difference would be found with respect to time.

There are several conceivable clinical implications related to stromal fibroblast cytotoxicity. Studies show that infiltration of corneal wounds with activated stromal fibroblasts occurs when the corneal epithelium and underlying stroma are damaged (27, 57,58). The infiltration of activated stromal fibroblasts into the area of damaged corneal stroma is essential to the production of new collagen fibrils and extracellular matrix needed to regain structural integrity and translucency compatible with appropriate ocular function (57,58,65). Topical solutions toxic to corneal fibroblasts, and which are intended to be applied multiple times, theoretically have the potential to reduce the population of stromal fibroblasts below that necessary for adequate tissue repair. The result would be corneal wounds that fail to heal properly, leaving the eye vulnerable to further insult. Canine eyes suffering from various disease conditions including spontaneous chronic corneal epithelial defects and keratoconjunctivitis sicca (KCS), where the overlying corneal epithelium is either absent or diseased for a prolonged period of time, are possibly at greater risk of experiencing cytotoxic side effects from topically applied medications such as nalbuphine.

Activated corneal fibroblasts have also been shown to play an integral role in the migration, contraction, and subsequent closure of corneal wounds (55,57,58). Our
corneal wound healing model revealed no statistically significant difference in the rates of cellular migration between a 0.1% nalbuphine solution and controls. However, photographs and graphical analysis did show a noticeable trend for decreased cellular migration between treatment and control groups after eight hours of exposure. The lack of a statistically significant difference between groups is most likely due to low statistical power, as a small number of study groups were used. The potential clinical in vivo relevance of decreased stromal fibroblasts viability and proliferation is a delay in corneal wound healing and closure. Canine eyes affected by conditions that lead to abnormal corneal wound healing, such as neurotrophic keratitis and spontaneous chronic corneal epithelial defects, are at an increased risk for developing infectious keratitis with resultant corneal perforation (5). It is therefore essential that measures be taken to select therapies that interfere with corneal cell viability and wound healing to the least possible degree. To the author’s knowledge there are no published reports evaluating the density of activated stromal fibroblasts in relation to the strength and rate of corneal wound healing.

On the other hand, decreased viability of activated stromal fibroblasts may actually have a beneficial role in the healing of corneal wounds, specifically related to corneal scarring post-injury. The past decade has seen an increase into the investigation of a group of proteins called corneal crystallins. Corneal crystallins are cytoplasmic, water-soluble, enzymatic proteins expressed by stromal keratocytes. They are thought to play a role in the transparency of corneal tissue due to impacting the refractive property of light passing though the cornea (151). Evidence for this theory comes in light of different refractive properties seen between quiescent corneal keratocytes and activated corneal fibroblasts found in normal vs. wounded corneas. Quiescent corneal keratocytes
have been shown to express higher levels of corneal crystallins compared to activated stromal fibroblasts (72,73,152). Increased numbers of activated corneal fibroblasts have been associated with a condition termed “corneal haze”, which is an opacity seen in the area of a corneal wound after healing has taken place. In effect, corneal haze represents a scarring of the corneal stroma. Various compounds, including mitomycin C, corticosteroids, and heparin have been investigated for the ability to decrease the migration and proliferation of activated stromal fibroblasts associated with corneal wounds (153,154,155). These studies, and others of similar nature, have indeed shown that topical application of anti-proliferative and ant-migratory agents to corneal wounds can reduce the incidence of corneal haze. Therefore, it is possible that an agent such as nalbuphine hydrochloride, which our research indicates has similar affects on fibroblasts proliferation and migration, could have beneficial effects on the development of corneal haze if applied during the duration of the wound healing process.

With regards to topically applied ophthalmic medication, it is essential to study any compounds effects on migration and proliferation of the corneal epithelium. The corneal epithelium is in direct contact with the environment, and is almost always the first structure damaged in corneal injury. Traditionally, animal models have been used to study the effects of test compounds on the ocular surface. This has been met with challenges in experimental cost and sample size, as well as ethical issues revolving around animal welfare (149). Primary cell culture of corneal epithelial cells (CECs) from various species has been successfully executed (97,156,157,158,159). Common applications for the use of CECs include measuring migration, proliferation, viability, absorbance, and pharmacokinetiic properties of experimental solutions. The use of CECs
in experimental testing allows for the reproduction of high quantities of tissue that can be isolated and tested at reduced experimental cost, and without the need to sacrifice large numbers of live animals.

There are numerous reported methods for culturing CECs. Preparation of corneal tissue typically involves the use of either full thickness corneal explants, or sheets of epithelial tissue enzymatically separated from the corneal stroma. R. Ian Freshney in *Culture of Animal Cells, A Manuel of Basic Technique*, advises a protocol for the culture of primary CECs using 2mm full thickness corneal explants (147). After careful dissection, explanted tissue is placed epithelial side down onto collagen coated tissue plates. A single drop of serum-free, enriched culture media is placed directly onto explants daily while CECs migrate onto the bottom of the culture dish. Cells are allowed to proliferate for approximately 14 days, at which point there are passed into subsequent tissue plates for propagation. Hendrix et al. successfully cultured primary canine CECs by enzymatically separating the corneal epithelium form the underlying stroma in preparation for cellular propagation (112,158). This method involves the incubation of full thickness excised corneas in a solution of Dispase, a bacterial derived collagenase shown to separate epithelium from stromal tissue (160). Epithelial sheets are then mechanically removed from the stroma in preparation for cell culture. Sheets of epithelial tissue are then either suspended in culture media and seeded into culture wells, or placed directly onto culture plates as an intact sheet for growth.

An additional component in the culture of CECs is the use of collagen and protein coated culture dishes. Collagen, specifically type-IV collagen, and laminin are fundamental components of the corneal epithelial basement membrane onto which the
corneal epithelium is attached \textit{in vivo}. Their addition to the CEC culture protocol is thought to aid in the propagation of CECs by providing a substrate for cellular attachment and proliferation similar to the intact basement membrane the structurally normal cornea (160). The culture of CECs without the use of collagen or laminin coated plates has been reported, as \textit{in vitro} CECs have been shown to produce both of these components endogenously; however, their addition to the culture dish prior to the seeding of cells has shown to increase the rate and overall success of CEC culture (161). Various studies, in which CECs were successfully cultured, employed the use of collagen-coated plates in the protocols (112,150,158).

Contamination with tissue fibroblasts remains one of the many challenges in establishing pure epithelial cell cultures (147). Most attempts at eliminating fibroblasts contamination involve using serum free media with low levels of calcium and magnesium. Media containing serum typically contains various growth factors such as fibroblast growth factor and platelet derived growth factor, which are stimulatory to fibroblasts cells (147). These growth factors not only enhance the proliferation of fibroblasts, but may also alter the expression of desired cell types, adding confounding elements to study results. The development of serum free media with cell specific supplements has been important in the culture of corneal epithelial cells, and various companies such as Invitrogen and CellNtec offer CEC specific media devoid of serum. Other supplements including recumbent human insulin, and cholera toxin are also commonly added to media when culturing CEC cells. Insulin is essential for glucose uptake and energy production needed in cellular proliferation. Cholera toxin is also commonly added to media used for epithelia cell culture, as it has been shown to decrease
both DNA and RNA synthesis in fibroblasts cells, helping to control for fibroblast contamination (162).

Our attempt at establishing primary cultures of canine CECs was met with multiple challenges, and ultimately we were unable to routinely establish primary cultures beyond the first passage of cells. Initial attempts at culturing CECs involved full thickness removal of the cornea posterior to the limbus, followed by the use of Dispase (Sigma-Aldrich, MO) to enzymatically separate the canine corneal epithelium from the stroma. While we were successful in isolating the corneal epithelium, the vast majority of resultant cultures were contaminated with stromal fibroblasts, which eventually became the dominant cell population in the culture dish. Since Dispase is a collagenase that leads to breakdown of the epithelial basement membrane, the underlying stromal tissue was most likely exposed while manually removing sheets of epithelium. It is highly possible that manipulation of the corneal epithelium during its removal lead to the inclusion of stromal keratocytes during transfer to the culture dish. It is also possible that areas of intact basement membrane were present after incubation with Dispase, and removal of the corneal epithelial layer also included microscopic portions of the anterior stromal tissue containing keratocytes. Stromal contamination is encountered less frequently in the culture of human CECs, and this may be due to the existence of Bowman’s membrane, and thin acellular layer of collagen separating the corneal epithelium from the anterior stroma containing keratocytes.

The protocol presented in section 2.7 was decided upon in an attempt to both reduce any incisions near to or including the corneal tissue, and leave the epithelial basement membrane intact, reducing exposure of the stroma. Achieving both of these
goals reduces the potential for stromal cell inclusion during tissue handling. Consistent success was achieved in establishing primary canine CECs devoid of stromal fibroblast contamination. However, cells failed to both reach the appropriate confluence of 90% needed to carry out viability and wound healing assays, and survive passage in adequate amounts required for sufficient experimental trials. Possible reasons for the failure to establish primary canine CEC cultures capable of cellular passage include the decision to not use collagen coated plates, inappropriate media selection and delivery intervals, and species differences inherent to canine CECs.

There were several limitations to this study. A sample size of three was used to assess stromal cell viability and wound healing for each experimental concentration. This study design limited statistical power, increasing the likelihood of generating false negative results by not generating enough statistics to find a difference between treatment groups when one actually exists. The generation of false positive results is typically linked to statistical significance, and is not generally related to statistical power, but more often the type of statistics applied to a given set of data. Also, a balanced experimental design using control cells at each time point was not used. This made it impossible to detect statistically significant differences between the viability of stromal fibroblasts treated with nalbuphine compared to controls cells treated with no drug. Instead, we had to rely on graphical analysis of treatment groups compared to control groups at a single time point (thirty minutes). Graphical analysis lacks in its ability to accurately generate usable p-values, which is critical for determining true difference between treatment groups. A linear mixed model comparison was attempted to account for the absence of a control group at all time points, however this lowered the statistical power of the study.
and subsequently failed to show the real difference between groups regarding concentration of nalbuphine used. As a result, we were unable to determine statistically if there was a significant difference between control and treatment groups at earlier time points, which made it impossible to assess potential subtle toxicity of lower concentrations on cell viability.

Additionally, there are several limitations to the use of trypan blue staining for the assessment of viability. Trypan blue is a diazole dye that is excluded by intact cell membranes. Non-viable cells are therefore stained blue when exposed to solutions containing trypan blue. Measurements are taken via microscopy, and there can be a great deal of subjectivity when determining viable vs non-viable. We attempted to correct for this by having a single individual conduct all measurements on stained cells. Trypan blue assays have also shown a tendency to overestimate viability compared to fluorometric assays (163). This is thought to be from fragmented non-viable cells being excluded from counting if they are in such a state that they are not recognizable as cells. This could explain the increase in viability seen from 15 minutes to 30 minutes in cells treated with 0.5% and 1% nalbuphine, as physiologically, viability should remain the same or decrease with time, not increase.

Finally, the inability to successfully culture canine CECs inhibited our ability to draw conclusions of nalbuphine hydrochloride’s effects on the ocular surface, as only its effects on stromal fibroblasts could be discussed. The ability to tests nalbuphine’s effects on CEC viability and wound healing would have given our research a more complete picture regarding the drug’s potential cytotoxic effects associated with topical application in canine eyes.
Future investigation is needed to further characterize nalbuphine hydrochloride’s use in clinical veterinary ophthalmology. As stated above, its effects on the corneal epithelium pertaining to viability, migration, proliferation, absorbance and metabolism should be evaluated both in vitro and in vivo to obtain the most comprehensive picture of pharmacodynamics and pharmacokinetics. The next logical step is the evaluation of the drug’s effects on the cornea in vivo, to determine if any side effects are seen with its use.
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