ฤทธิ์ด้านการอักเสบของของเหลวที่ได้จากการเพาะเลี้ยงเซลล์เยื่อหุ้มรกมนุษย์ภายหลังจากการใช้เยื่อหุ้มรกมนุษย์เย็บปิดแผลหลุมลึกที่กระจกตาสุนัข

นางสาวทัศวรินทร์ วิชญคุปต์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ANTI-INFLAMMATORY EFFECTS OF TOPICAL SUPERNATANT FROM HUMAN AMNIOTIC MEMBRANE CELL CULTURE AFTER HUMAN AMNIOTIC MEMBRANE TRANSPLANTATION IN CANINE DEEP CORNEAL ULCER

Miss Tasavarin Wichayacoop

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Veterinary Pharmacology
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Thesis Title	ANTI-INFLAMMATORY EFFECTS OF TOPICAL SUPERNATANT FROM HUMAN AMNIOTIC MEMBRANE CELL CULTURE AFTER HUMAN AMNIOTIC MEMBRANE TRANSPLANTATION IN CANINE DEEP CORNEAL ULCER
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ทัศวรินทร์ วิชญคุปต์ : ฤทธิ์ต้านการอักเสบของของเหลวที่ได้จากการเพาะเลี้ยงเซลล์ เยื่อหุ้มรกมนุษย์ภายหลังจากการใช้เยื่อหุ้มรกมนุษย์เย็บปิดแผลหลุมลึกที่กระจกตาสุนัข. (ANTI-INFLAMMATORY EFFECTS OF TOPICAL SUPERNATANT FROM HUMAN AMNIOTIC MEMBRANE CELL CULTURE AFTER HUMAN AMNIOTIC MEMBRANE TRANSPLANTATION IN CANINE DEEP CORNEAL ULCER.) อ. ที่ปรึกษา : รศ.สพ.ญ.ดร. ศิรินทร หยิบโชคอนันต์ , อ. ที่ปรึกษาร่วม : ศ.กิตติคุณ.สพ.ญ.ปราณี ตันติวนิช , 56 หน้า

วัตถุประสงค์ของงานวิจัยครั้งนี้เพื่อศึกษาประสิทธิภาพในการระงับการอักเสบของของเหลวที่ได้จากการเพาะเลี้ยงเซลล์เยื่อหุ้มรกมนุษย์ โดยแบ่งสุนัขจำนวน 25 ตัว ออกเป็น 5 กลุ่ม กลุ่มละ 5 ตัว กลุ่มแรกเป็นกลุ่มสุนัขควบคุมที่มีกระจกตาปกติ และสุนัขที่เหลืออีก 20 ตัวถูกเหนี่ยวนำให้เกิดแผลหลุมที่กระจกตาโดยใช้ trephine ขนาด 0.45 เซนติเมตร จากนั้นจึงใช้เยื่อหุ้มรกมนุษย์ปลูกถ่ายที่กระจกตา แบ่งสุนัขทั้ง 20 ตัวนี้ออกเป็น 4 กลุ่มคือ 1) กลุ่มที่ได้รับยาปฏิชีวนะหยอดตา 2) กลุ่มที่ได้รับยาหยอดตากลุ่มสเตียรอยด์ 3) กลุ่มที่ได้รับอาหารที่ใช้สำหรับเลี้ยงเซลล์ (สารละลาย mock) และ 4) กลุ่มที่ได้รับของเหลวที่ได้จากการเพาะเลี้ยงเซลล์เยื่อหุ้มรกมนุษย์ เริ่มหยอดตาในสุนัขทุกกลุ่มที่ 24 ชั่วโมงหลังการผ่าตัด และหยอดต่อเนื่องกันทุก 4 ชั่วโมงติดต่อกัน 9 วัน เก็บน้ำตาเพื่อนำไปวัดระดับอินเตอร์ลิวคินเบต้า (IL-1ß) โดยใช้ Canine IL-1ß ELISA kit และไนตริกออกไซด์ โดยวิธี Griess assay การเก็บน้ำตาเริ่มเก็บในวันก่อนทำการผ่าตัด (วันที่ 0) 24 ชั่วโมงหลังการผ่าตัดแต่ก่อนการหยอดตาด้วยสารที่ต้องการทดสอบ (วันที่ 1) และในวันที่ 3, 5, 7 และ 9 หลังการผ่าตัด ผลการศึกษาครั้งนี้พบว่า ยาหยอดตากลุ่มสเตียรอยด์ซึ่งใช้เป็นยาอ้างอิง และของเหลวที่ได้จากการเพาะเลี้ยงเซลล์เยื่อหุ้มรกมนุษย์สามารถลดระดับของอินเตอร์ลิวคินเบต้าและไนตริกออกไซด์ได้อย่างมีนัยสำคัญ นอกจากนี้อาการทางคลินิกต่าง ๆ เช่นเยื่อตาขาวอักเสบ และการเกิดเส้นเลือดใหม่ลดลงอย่างชัดเจนในสุนัขทั้ง 2 กลุ่ม ส่วนสารละลาย mock และยาปฏิชีวนะไม่มีผลลดระดับอินเตอร์ลิวคินเบต้าและไนตริกออกไซด์ รวมทั้งไม่มีผลทำให้อาการทางคลินิกดีขึ้นอย่างชัดเจน ดังนั้นจากการศึกษาครั้งนี้สรุปได้ว่า การหยอดตาด้วยของเหลวที่ได้จากการเพาะเลี้ยงเซลล์เยื่อหุ้มรกมนุษย์มีผลลดการอักเสบได้ในสุนัขที่ถูกเหนี่ยวนำให้มีแผลหลุมที่กระจกตา โดยยับยั้งการสร้างอินเตอร์ลิวคินเบต้าและไนตริกออกไซด์จากเซลล์ที่เกี่ยวข้องกับการอักเสบ

ภาควิชา เภสัชวิทยา ลายมือชื่อนิสิต.....

สาขาวิชา เภสัชวิทยาทางสัตวแพทย์ ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2550 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEY WORD : HUMAN AMNIOTIC MEMBRANE / INTERLEUKIN-1 BETA / NITRIC OXIDE

TASAVARIN WICHAYACOOP : ANTI-INFLAMMATORY EFFECTS OF TOPICAL SUPERNATANT FROM HUMAN AMNIOTIC MEMBRANE CELL CULTURE AFTER HUMAN AMNIOTIC MEMBRANE TRANSPLANTATION IN CANINE DEEP CORNEAL ULCER. ASSOC. PROF. SIRINTORN YIBCHOK-ANAN, Ph.D. THESIS CO-ADVISOR PROFESSOR PRANEE TUNTIVANICH, M.S. 56 pp.

The objective of this study was to examine the effect of topically applied human amniotic epithelial cell (HAEC) culture supernatant on corneal inflammatory reaction in dogs. Twenty five dogs were randomly assigned into 5 groups. The control group consisted of 5 dogs with normal cornea. Inductions of corneal ulcer were performed using 0.45 cm trephine and human amniotic membrane were transplanted in 20 dogs. These 20 dogs were assigned into 4 groups and treated with topical antibiotic, topical corticosteroid, topical mock media and topical culture supernatant from HAEC, respectively. Administrations of the testing agents started at 24 h after transplantation and continued every 4 h for 9 consecutive days. Tear was collected before operation (day 0), 24 h after transplantation, but before application of the testing agents (day 1), and day 3, 5, 7 and 9 after transplantation. The concentrations of interleukin-1 beta (IL-1β) and nitric oxide (NO) in tear fluid were measured using canine IL-1 β ELISA kit and Griess assay, respectively. Elevations of IL-1β and NO concentrations are associated with inflammatory conditions within the eyes. IL-1β is a major cytokine involved in ocular inflammation and it may induce NO production from many cell types, such as fibroblasts, macrophages and epithelium of the iris-ciliary body. Corticosteroid, a reference anti-inflammatory drug, and the culture supernatant from HAEC significantly decreased IL-1β and NO concentrations. In addition, the clinical signs such as conjunctivitis and neovascularization were improved in both topical corticosteroid and supernatant from HAEC treated groups. Mock and antibiotic solutions failed to decrease NO and IL-1β concentrations. In conclusion, topical application of the culture supernatant from HAEC alleviated inflammation in induced-corneal ulcer of dogs, possibly via inhibition of IL-1β and NO production.

Department of Pharmocology
Field of Veterinary Pharmocology
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Student.....
Advisor's signature.....
Co-advisor's signature.....

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ABO	Antibiotic
AMT	Amniotic membrane transplantation
APCs	Antigen presenting cells
Cd	Cadmium
cNOS	Constitutive nitric oxide synthase
COX	cyclooxygenase
DMEM	Dulbecco modified Eagle's medium
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
GAG	Glycosaminoglycan
h	Hour
HAEC	Human amniotic epithelial cells
HAMT	Human amniotic membrane transplantation
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IgE	Immunoglobulin E
IL-1	Interleukin-1
IL-1 ra	Interleukin-1 receptor antagonist
IL-6	Interleukin-6
IL-8	Interleukin-8
INFγ	Interferon gamma
iNOS	Inducible nitric oxide synthase
IM	Intramuscular
IV	Intravenous
KCS	Keratoconjunctivitis sicca

kDa	Kilo-Dalton
LAF	Lymphocyte-activating factor
LCs	Langerhans cells
LPS	Lipopolysaccharides
MIF	Migration-inhibitory factor
MIP	Macrophage inflammatory protein
MMP	Matrix Metalloproteinase
NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAIDs	Non-steroidal anti-inflammatory drugs
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
PAF	Platelet activating factor
PDGF	Platelet-derived growth factor
PGE	Prostaglandin E
PGF-2α	Prostaglandin 2 alpha
PGs	Prostaglandins
PLA	Polylactic Acid
PLR	Pupillary light response
PMNL	Polymorphonuclear Leukocyte
S.E	Standard error
S.E.M.	Standard error of mean
STT	Schirmer tear test
TNF-α	Tumor necrosis factor alpha
TMB	3,3'5,5' tetramethyl-benzidine

CHAPTER I

INTRODUCTION

The cornea, a multi-layered transparent part of the front of the eye, plays a vital role in vision. An ulceration of cornea is a cavity in the two surface layers of the cornea and in a variable portion of its deeper layers. Corneal ulcer occurs with a variety of causes including injury, bacterial, fungal, and viral infections, diseases of the eye and eyelid, and a number of other conditions that cause the cornea to ulcerate.

Treatment depends on the depth of the ulcer. Superficial ulcers are usually treated with antibiotics to eliminate or prevent infection and atropine to alleviate ulcer-associated eye spasm. Deep corneal ulcers often require both medical and surgical treatments to prevent possible blindness. Several methods of surgical management in canine corneal ulcer include temporary tarsorrhaphy, third eyelid flap, conjunctival pedical graft, tissue adhesive, corneal-scleral graft and transplantation of natural or synthetic material. Human amniotic membrane has long been used as a surgical material for transplantation in human ophthalmic surgery. It is very effective in promoting healing of corneal ulcer (Fukada et al., 1999). Nowadays, human amniotic membrane transplantation has been performed in treatment of canine corneal ulcer (Tasavarin et al., 2005) and excellent clinical results were also obtained as in human.

Topical anti-inflammatory agent is very important for management of postoperative corneal transplantation to suppress inflammation and to prevent scar and granulation tissue formation. One of the most common anti-inflammatory agents that are used in ophthalmology is corticosteroid. Topical corticosteroid solution is widely used for postoperative anti-inflammation. It is a powerful tool in preventing scarring, maintaining transparency, and treating the immune-mediated inflammation of some forms of keratitis, uveitis, conjunctivitis, scleritis/epicleritis and corneal transplants. However, corticosteroid has been reported to associate with an increase risk of infection and a worse stromal melting. In addition, it also suppresses the repair process and aggravates corneal ulceration.

Recently, culture supernatant from human amniotic epithelial cell (HAEC) has been reported to express mRNA and protein of anti-inflammatory factors, such as IL-1 receptor antagonist, IL-10, tissue inhibitors of metalloproteinase, collagen XVIII and thrombospondin (Hao et al., 2000). It also plays a role in the suppression of corneal inflammation in human (Kazutaka et al., 2005). However, the use of culture supernatant from HAEC as an anti-inflammatory agent in canine corneal ulcer has never been investigated. Therefore, the objective of this study was to determine the anti-inflammatory effects of the culture supernatant from HAEC in canine corneal ulcer compared with prednisolone, a corticosteroid anti-inflammatory agent that was used as a reference drug. The changes in nitric oxide (NO) and interleukin-1 β (IL-1β) concentrations in tear fluid as well as clinical ophthalmic signs were used as the indicators for this study. It is worth to study the mechanisms underlying its effect if the culture supernatant from HAEC could suppress IL-1β and NO concentrations after transplantation of human amniotic membrane in canine deep cornea ulcer. These data will provide values for further development of the solution from human amniotic epithelial cells to pharmaceutical dosage form.

CHAPTER II

LITERATURE REVIEW

Corneal anatomy

Cornea is the most powerful refractive surface of the eye. It supplies 70% of the eye's refractive or light bending power and it has refractive power equivalent to a + 43 diopter lens. It is transparent so that rays can enter the eye. Transparency is maintained by several anatomic mechanisms such as, lack of blood vessels, lack of pigment, non-keratinized anterior surface epithelium, precise organization of the stromal fibrils, small size of the stromal fibrils and relaively dehydrated. The transparency of the cornea is due to its uniform structure, avascularity and deturgescence or the state of relative dehydration (Maurice, 1963). The state of relative dehydration of the corneal tissue is maintained by the active NA^{+}-K^{+} cell pump of endothelium and epithelium and by their anatomic integrity (Maurice, 1972).

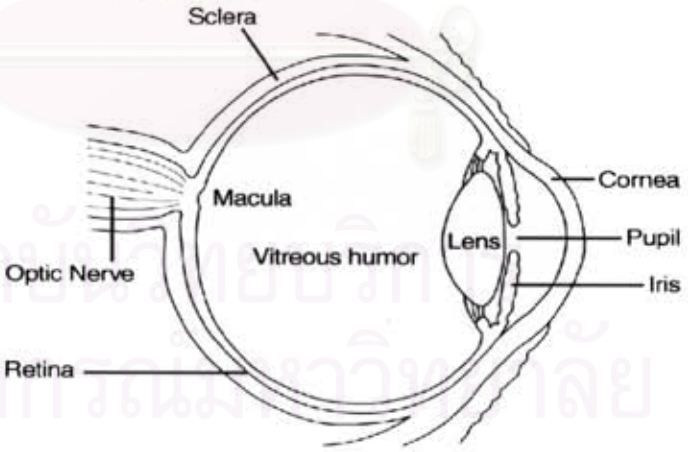

Figure 2.1 Canine eye diagram (Aronson, 2002)

The average diameter of the canine cornea in the horizontal axis is 8.5 mm and varies from 13-17 mm and in the vertical axis is 12-16 mm. The average thickness of the cornea is increased from 0.379 mm at 9 weeks to 0.548 mm at 16 weeks. In the adult dog, the central cornea is 0.56 mm, whereas thickness of the superior and temporal peripheral regions of cornea are 0.612 mm and 0.581 mm, respectively (Slatter, 2003).

Figure 2.2 Histology of the normal canine cornea. a = anterior epithelium; b = basement membrane; c = connective tissue stroma; d = Descemet's membrane; e = endothelium. (Petersen-Jones and Crispin, 2002)

The mature cornea consists of five layers: precorneal tear film, epithelium and basement membrane, stroma, Descemet's membrane (basement membrane of the

endothelium) and endothelium (figure 2.2) (Petersen-Jones and Crispin, 2002; Slatter, 2003) The corneal epithelium in the canine is 5-11 cells thick and has a turnover rate of approximately 7 days. The flattened surface cells are polygonal and have numerous microvillae and microplicae that are coated with a glycocalyx and which function to stabilize the tear film.

The corneal stroma is 90% of the mass of the cornea and consists of parallel lamellae of collagen fibers that extend across the entire diameter of the cornea. While the bundles are parallel to one another, they are not oriented in the same direction. The lamellae are most regular or parallel in the posterior stroma. This arrangement is responsible for the anterior stroma being more reflective. Keratocytes, which are modified fibroblasts with branching processes, are scattered between the collagen lamellae.

The ground substances surrounding the collagen fibers compose of proteoglycans. The proteoglycans are glycosaminoglycans (GAG) that bound to protein chains. The predominant GAG is keratin sulfate. Dermatan sulfate is the other major GAG found in the stroma. The GAGs act as anions and bind to water. Keratin sulfate is the most concentreated in the posterior cornea. The GAGs maintain corneal hydration and help to maintain the critical regular spacing of collagen fibers necessary for corneal transparency.

The posterior layer of cells lining the cornea is usually referred as the endothelium, although it is not a true endothelium. Its single layer of cells is usually hexagonal and lines the inner cornea. The corneal endothelial cell count is about 2,700-2,800 / mm^2 and decreases with age. Healing in the adult is mainly by cellular enlargement and migration, rather than by mitosis. The posterior epithelium shows pleuripotenial capabilities with differentiation into the typical endothelial cell, fibroblasts or an epithelial cell type. The epithelial cell type possesses desmosomal attachment and tonofilaments (Slatter, 1990).

The basilar side of the posterior epithelium lies on the stroma and produces a modified basal lamina and Descemet's membrane. It is produced throughout life and consequently is much thicker in the old animal than in the young animal.

The cornea is richly supplied with sensory innervation from the long ciliary branches of the ophthalmic division of the 5th cranial nerve. The nerves are concentrated in the anterior stroma and penetrate into the epithelium where they terminate as naked nerve endings. The corneal epithelium is the most densely innervated epithelium in the body. The corneas are supplied by 12 nerve trunks. The nerve endings are sensitive to pain, pressure, and temperature. The dense concentration of nerve ending in the epithelium account for the severe pain observed with superficial epithelial loss, whereas a deep ulcer does not exhibit the same degree of pain.

Tear

The physical structure and the chemical composition of tears represent the main part of the nonspecific defense of the ocular surface. There are three layers in tear film structure. The superficial oily layer is a very thin layer that contains mainly waxy and cholesterol esters. Tarsal gland secretes the oily layer to decrease the rate of evaporation. The middle fluid layer is the major part of the tear film, which contains most of the nutrients needed by the cornea, including inorganic salts, glucose, oxygen and protein. This layer is derived primary from the orbital lacrimal gland and the gland of the third eyelid. The deep mucous layer is essential in maintaining the normal wetting of the cornea and it is thinner than the superficial lipid layer. It is secreted by conjunctival golblet cells.

Tears are spread by the blinking movements of the eyelids. A number of complex actions, including capillary action and blinking motions, draw tears into the puncta and propel them through the nasolacrimal duct. The constant movement of tear serves to eliminate any foreign materials, potentially colonizing or infecting microorganisms and metabolic wastes (Van, 1981).

Corneal ulceration

Corneal ulcer is one of common pathological conditions that have been found in dogs (Figure 2.3). The common causes of corneal ulcers are induced by trauma, eyelid foreign bodies and eyelid abnormalities (entropion, distichia, districhiasis, ectopic cilia and trichiasis), which might be followed by secondary bacterial infection (Mary, 1996). Keratoconjunctivitis sicca (KCS) can result in corneal ulceration. This is especially true with acute onset of KSC, in which corneal ulceration occurs rapidly and progress quickly. Ulcers are less common with chronic KCS.

Corneal ulcers are classified according to their depth. If only the outer layer is lost, the lesion is called a superficial corneal ulcer. When more than one half the thickness of the cornea is lost, it is called a deep corneal ulcer or stromal ulcer. Superficial ulcers in healthy eyes heal in a matter of days. Deep ulcers may take several weeks to heal. Deep ulcers require new blood vessel to penetrate the area. They may also leave a corneal scar. A corneal bulge is called a descemetocele. The anterior portion of the eye will collapse and the contents will spill out destroying the eye, if the descemetocele bursts.

If the corneal wound becomes infected before host defenses can mount a protective response, the clinical course is governed by the characteristics of the bacteria and the inflammatory response. The rate of progression of the infected ulcer is modulated largely by more passive components of the cornea's defense mechanism. The integrity of the tear film and normal eyelid function are prime considerations. The progression of corneal ulcer usually is rapid in brachycephalic canine breeds, large eyes and shallow orbits predispose to lagophthalmos, ineffective spreading of tear film, and exposure keratitis (Kern, 1990).

Clinical signs are pain and blepharospasm. Most case has epiphora, a discharge that may accumulate in the cornea of the medial canthus, photophobia and conjunctivitis. Chronic, infected, or progressive corneal ulcers should have an aerobic bacterial and fungal culture and sensitivity taken, and cytology of the cornea should be

collected and examined. This culture procedure will help guide specific medical therapy after surgery.

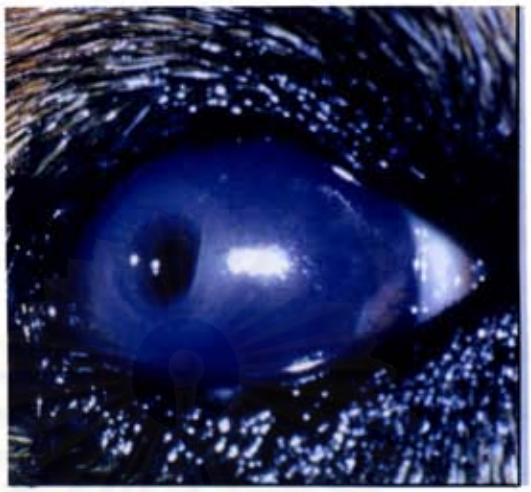

Figure 2.3 Corneal ulceration (Petersen-Jones and Crispin, 2002)

Pathophysiology of corneal ulcer

1. Healing of corneal wounds

1.1. Epithelial healing

Epithelial wound healing is divided into three cellular phases:

1.1.1 Migration: Epithelial cells at the margin of a wound retract and become thicker within the first hour following a trauma. Neutrophils from the tear film and fibrinous material are present in the epithelial defect within 1-3 hours. Hemidesmosomal attachments are dissolved around the edge of the wound and decreased to a single cell layer. After the latent phase of 3-6 hrs, sliding or cell migration occurs. The cells increase their surface area by increasing cell volume with water. Smaller defect are covered by a monolayer in this manner, which eventually thickens by cellular proliferation.

1.1.2. Proliferation: Mitosis occurs after a 24 hours to re-establish the epithelial thickness. The stem cells for the corneal epithelium are located at the limbus. They travel in a centripetal pattern from the limbus. If the healing is not accomplished by

sheets of cells, the small groups of migrating cells form a whorl or vortex pattern. The stem cells are long-live cells that have the ability to undergo mitosis and are responsible for cell replacement and tissue healing. They produce transient amplifying cells that are divided rapidly and eventually differentiated into the terminal differentiated cell of the tissue. The amplifying cell of the corneal epithelium is the basal cell and the superficial cells are the postmitotic and terminally differentiated cells. Removal of the entire epithelium or removal of the limbal epithelium will result in wound healing by conjunctival epithelium migrating over the cornea. Even though lesion of the central corneal epithelium produces an increase in the mitotic rate of the conjunctival epithelium, the conjunctival epithelium also produces goblet cells on the cornea. These goblet cells eventually undergo a process of transdifferentiation into corneal epithelial cells, which takes 6 weeks or more. If the cornea is vascularized, the transdifferentiation is retarded, with the epithelium retaining conjunctival characteristics.

1.1.3 Adhesion: Although the epithelium regenerates rapidly, anchoring units are not formed until the defect is covered. If the basal lamina is intact, hemodesmosomes are formed within 1 week. If the basal lamina is removed, hemodesmosomes are much slower to regenerate. Consequently, the epithelium is susceptible to re-injury for a prolonged period when the basal lamina has been lost, as the epithelial attachment to the basal lamina is important for adhesion to the stroma (Kenyon, 1987)

2. Stromal healing

Stromal healing initially involves the invasion of leukocytes from the tear film or limbus within a few hours. Keratocyte disintegration occurs at the margin of the wound within the first hour. Two to three days after the injury, the wound margin is filled with a mixture of keratocytes and fibroblast type cells. Whether the fibroblasts are all transformed keratocytes or a mixture of transformed keratocytes and monocytes is controversial. After 3-6 days, the fibroblasts invade the fibrin plug of the wound. The initial ground substance of the scar is dermatan sulfate, however, keratin sulfate is detected by

15-30 days after trauma, which indicated a transformation of fibroblasts to keratocytes. This process takes up to 3 months to achieve normal level of keratin sulfate. The area of stromal healing is not transparent due to the disorganization and large collagen fibers. The tensile strength of the wound progress slowly, with only 50% strength achieved by central corneal wounds in 100 days. A lack of epithelium over the stroma healing markedly decreases the tensile strength of the wound.

3. Corneal scar formation and opacification

Generally, epithelial healing occurs without scar formation. However, when the stroma is damaged, the resultant fibroplasia with its disorganized stroma leaves some degree of residual scar. Clinically, scar is often described according to their size and density: a nebula is a very small faint scar or opacity, a macula is a small but distinct white opacity, and a leukoma is dense white scar underlying adhesion of the iris to the cornea.

4. Corneal vascularization

The cornea is normally avascular but whether vascularization is inhibited by a factor or is prevented by the compactness of the tissue is unknown. Hypoxia, neoplasia, and inflammation are initiators of neovascularization by trigger factors such as tumor angiogenic factor, fibroblast growth factor, transforming growth factor-beta, platelet-derived growth factor and vascular endothelial growth factor. Lymphokines produced by activated T-lymphocytes and prostaglandin E_1 are capable of producing corneal neovascularization. Neutrophils are involved to some extent in most forms of corneal neovascularization. While leucopenia animal can develop neovascularization, the reaction is amplified by neutrophils.

5. Corneal immune responses to inflammation

The immune responses in the eye are similar to those in other parts of the body. Initiation of the immune response depends on the local cells such as epithelial cells, fibrocytes and capillary endothelial cells. These resident cells are critical in initiating the early inflammation required for the antigen-presenting cells to activate. For example, in the immune response to local inflammation, it leads to vasodilation, expression of specific adhesion molecule by endothelial cells of associated mucosal capillaries, and release of chemokines and other chemoattractants. As a result of these changes, circulating leukocytes, especially neutrophils, monocytes, dendritic cells and natural killer (NK) cells are recruited to the area of inflammation. The nonspecific immune response releases some toxic chemicals, such as reactive oxygen molecules, nitric oxide and enzyme by neutrophils and macrophages. Several critical cytokines, including interferon, interleukin-1 and interleukin-2 are released by local cells as well as by the recruited inflammatory cells. The immune response of cornea is quite different from the neighboring conjunctiva. Dendritic cells and macrophages extend into the peripheral cornea, but the central cornea is essentially devoid of immune cells including B and T lymphocytes. This lack of intrinsic immune cells and a blood vessel results in the cornea having decreased immune responsiveness (Robert et al, 1999).

Lacrimal glands are a site of interaction between the ocular surface and the immune response. The primary role of lacrimal gland in the specific immune response is delivery of antibody to ocular surface via tear.

5.1 Mediators of inflammation

The mediators released during the inflammatory process perpetuate the inflammatory response and are responsible for the clinical signs associated with inflammation, including pain and fever. They include a variety of chemical groups and exert some redundant activities. In any inflammatory process, leukocyte activation is a prerequisite to mediator release, de novo synthesis of mediators, degranulation, and phagocytosis. Leukocytes are activated by binding to numerous cytokines such as interferon gamma (IFNγ) and tumor necrosis factor (TNF), or other ligands such as the Fc

portion of antibody and complement components. Activated leukocytes are capable of a variety of functions such as adhesion to and migration through endothelial cells, chemotaxis, production of arachidonic acid metabolites, phagocytosis, oxidative burst and degranulation. Mediators such as histamine are preformed and stored in granules. These granules can be released immediately upon activation. Other mediators such as arachidonic acid metabolites require de novo synthesis. Endothelial cell activation results in expression of leukocyte adhesion molecules and, sometimes, procoagulant activity. Endothelial cells are activated by cytokines such as TNF and interleukin-1 (IL-1). Platelets are activated by a variety of mediators to degranulatate and participitate in coagulation, including thombin, adenosine diphosphate and platelet activating factor (PAF). Histamine is found primarily in mast cells that are distributed throughout tissues and it is also present in platelet granules. Histamine is a preformed mediator that is released upon mast cell degranulation. Mast cell degranulation can be stimulated by trauma, cross-linkage of cytophilic IgE with antigen, cytokines IL-1 and IL-8, and binding of anaphylatoxins from the complement cascade. Histamine is the primary mediator of immediate vasodilation and increased vascular permeability in acute inflammation (Boothe, 2001).

5.1.1 Cytokines

Cytokines including tumor necrosis factor-α (TNF-α), gamma interferon and the interleukin (IL), are proteins produced by many cell types that modulate cell function by interaction with cell surface receptor. They are responsible for communication between cells and they can have autocrine, paracrine or endocrine activities. Major cytokines involved in inflammation include IL-1 and TNFα from monocytes and macrophages and INFγ and TNFα from lymphocytes. In general, endothelial cells respond to inflammatory cytokines by expressing leukocyte adhesion molecules (E-selectins) and become more thrombogenic by production of tissue factor. Leukocytes respond by becoming activated and expressing surface molecules that bind to endothelial adhesion molecules. IL-1 and TNFα have many similar activities. Once synthesized and released from macrophages, these cytokines have the following effects (Adams, 2002)

5.1.1.1. Endocrine: resulting in fever, cachexia (by suppressing lipoprotein lipase), acute-phase protein release from the liver, hemodynamic changes (circulatory shock) and neutrophilia.

5.1.1.2. Endothelial cell activator: increased expression of leukocyte adhesion molecules, prostaglandin synthesis, IL-1, IL-8, IL-6 and platelet-derived growth factor (PDGF) synthesis.

5.1.1.3. Fibroblast activation: proliferation of fibroblast, collagen synthesis, PG synthesis.

5.1.1.4. Leukocyte activation: increased cytokine secretion and priming.

Interleukin-1 (IL-1)

IL-1 is a cytokine synthesized by activated mononuclear cells that have been stimulated by ribopolysaccharide or by interaction with $CD4^+$ T lymphocytes. It is a monokine and is a mediator of inflammation, sharing many properties in common with tumor necrosis factors (TNF). IL-1 comprises of two principal polypeptides of 17 kDa, each with isoelectric points of 5 and 7. They are designated IL-1α and IL-1β, respectively. They have the same biological cleavage of 33-kDa precursor molecules. IL-1α acts as a membrane-associated substance, whereas IL-1β is found free in the circulation. IL-1 receptors are present on numerous cell types. IL-1 may either activate adenylate cyclase, elevating cAMP levels and then activating protein kinase A, or it may induce nuclear factors that serve as cellular gene transcriptional activators. IL-1 may induce synthesis of enzymes that generate prostaglandins which may in turn induce fever, a well-known action of IL-1. The actions of IL-1 are different according to whether it is produced in lower or in higher concentrations. At low concentrations, the effects are mainly immunoregulatory. IL-1 acts with polyclonal activators to facilitate $CD4^+$ T lymphocyte proliferation, as well as B lymphocyte growth and differentiation. IL-1 stimulates multiple cells to act as immune or inflammatory response effector cells. It also induces further synthesis of itself, as well as of IL-6, by mononuclear phagocytes and vascular endothelium. It resembles tumor necrosis factor (TNF) in inflammatory properties. IL-1

secreted in greater amounts produces endocrine effects as it courses through the peripheral blood circulation. For example, it produces fever and promotes the formation of acute-phase plasma proteins in the liver. It also induces cachexia. Natural inhibitors of IL-1 may be produced by mononuclear phagocytes activated by immune complexes in humans. The inhibitor is biologically inactive and prevents the action of IL-1 by binding with its receptor, serving as a competitive inhibitor. Corticosteroids and prostaglandins suppress IL-1 secretion. IL-1 was formerly called lymphocyte-activating factor (LAF) (Cruse and Lewis, 2004)

5.1.2 Nitric oxide

The inorganic free radical, NO was first identified as an endothelium-derived endogenous messenger responsible for the regulation of vascular tone (Furchgott and Zawadzki 1980). However, then it has become clear that NO is the signaling molecule responsible for several diverse physiological and pathophysiological processes. NO is synthesized from L-arginine by three isoforms of the nitric oxide synthase (NOS) enzyme. NO is now known to control vascular smooth muscle tone, inhibit platelet and inflammatory cell adhesion and activation, and to be a transmitter at non-adrenergic non-cholinergic (NANC) synapses. Recent studies have revealed that NO can also modulate apoptosis, or programmed cell death, in a variety of cell types, including human inflammatory cells (Taylor **and** Megson, 2003). Current evidence suggests that lower concentrations of NO produced by the constitutive endothelial and neuronal isoforms of NOS (eNOS and nNOS) are cytoprotective, whilst supraphysiological concentrations produced by the inducible NOS isoform (iNOS) trigger cell death (Nicotera and Brune, 1997). This paradox is explained by the free radical nature of NO reacts with other radicals, particularly reactive oxygen species, present in the milieu to form various NO-related species in vivo. For example, NO combines rapidly with inflammatory cell derived superoxide anions ($O_2^{\cdot}$) to form highly cytotoxic peroxynitrite ($ONOO^{\cdot}$) (Maxwell and Lip, 1997).

The main sources of nitric oxide (NO) in ocular surface tissue are corneal epithelium, fibroblast, endothelium and inflammatory cell. NO is an unstable, small, short live, and potential toxic radical, produced by the oxidation of L-arginine by nitric oxide synthase (NOS). NO exhibits a rich biochemistry and a high reactivity and plays an important role as intercellular messenger indiverse physiology processes such as regulation of blood flow, neurotransmitter and platelet aggregation.

NO plays a significant role in the viability of cells in ocular surface tissue, but the role of NO in ocular surface disease is still under investigation (Kim et al., 2002). Prevention of unwanted immune responses is best accomplished early in the initiation process. The goal is not only to decrease the function of effector cells infiltrating the eye and therefore to decrease tissue destruction but also to stop continued immune activation in regional lymphoid tissue, likes non-steroidal anti-inflammatory drugs and corticosteroid are beneficial to reduce local tissue inflammation.

Treatment

Corneal ulcers usually require medical treatment, surgical treatment or both, which depend on causes, types of complicating factors, degrees of severity and the depth of ulcer. The first step in treating all corneal ulcers involves searching for and removing the inciting cause. In general, treatment should be directed to fulfill several general objectives, where applicable prevention or elimination of contamination or infection, control of anterior uveitis, analgesia, arrest of tissue destruction, preservation of corneal clarity and function and tissue support.

Medical treatment

Topical antibiotic treatment

Topical antibiotics are the main of corneal ulcer management to prevent bacterial infections. Topical antibiotic four times daily is adequate for uncomplicated epithelial ulcer. Progressive corneal ulcers that show signs of melting or have penetrated

more than half the depth of the cornea might require hourly treatment and require surgical treatment.

Subconjunctival injections

Subconjunctival antibiotic injection can be used in combination with topical antibiotic therapy, which provides higher corneal levels of antibiotics than that can be achieved by the topical route. They usually are used for rapidly progressive ulcer.

Systemic treatment

Because of the high tissue levels of antibiotics that can be achieved with topical application and subconjunctival injection, systemic therapy is unnecessary in most cases of ulcerative keratitis. Oral antibiotics might be indicated for severe corneal ulcers or corneal perforation and endopthalmitis is suspected.

Analgesic treatment

Corneal ulcers usually are painful as result of direct sensory nerve stimulation or through the mechanisms of secondary uveal stimulation. Parasympatholytic agent acts on analgesic, which dilates the pupil and stop ciliary spasm by pharmacologically blocking the parasympathetic receptors of the iris and the ciliary body. Mydriasis also helps to prevent a formation of synechiae.

Ocular lubricants

Artificial tears are helpful to reduce irritation from trauma. The indication for artificial tears is obvious in cases in which ulceration results from keratoconjunctivitis sicca. However, artificial tears also are extremely helpful in conjunction with topical antibiotic therapy because artificial tear is the base for antibiotic solutions.

Anti-inflammatory treatment

Glucocorticoids

Glucocorticoids represent the most widely used group of ocular anti-inflammatory agents. The uses of topical glucocorticoids therapy in human have been started more than 50 years ago in patients with uveitis, sclerokeratitis, pemphigus, corneal alkali burns and vernal keratoconjunctivitis.

Pharmacology

Glucocorticoids have shown a great therapeutic advance due to the importance of minimizing scarring in the ocular healing process. They help to avoid ocular catastrophes. Their anti-inflammatory and immunosuppressive effects have made the glucocorticoids a powerful tool in preventing scarring, maintaining transparency, and treating the immune-mediated inflammations of some forms of keratitis, uveitis, conjunctivitis, scleritis/epicleritis and corneal transplants. Glucocorticoids do not eliminate noxious stimuli but appear to only modify the response to the noxious stimuli. Glucocorticoids at therapeutic dose have some actions on every facet of the immune response. Topically applied glucocorticoids have specifically been proven to aggravate *Pseudomonas spp*. Ulcers by increasing the number of viable organisms and delaying healing. Glucocorticoids, therefore, traditionally have been considered to be contraindicated in the presence of corneal ulcer. It has been theorized, however, that glucocorticoids might be beneficial in conjunction with antibiotic therapy to suppress enzymatic tissue destruction and aid in preventing corneal scarring (Aronson and Moore, 1969). Some experimental evidence suggests that concomitant glucocorticoids therapy do not impair and actually might improve antibiotic efficacy. Until the debate is settled, it is advisable to avoid corticosteroids in cases of infected ulcers.

Mechanisms of action

Glucocorticoids are powerful immunosuppressive and anti-inflammatory drugs. They reduce circulating lymphocytes and monocyte, in addition to suppress IL-1

and IL-2 productions. However, their chronic use produces adverse effects, including increased susceptibility to infection, bone fractures, diabetes and cataracts. Glucocorticoids act by binding to the cytoplasmic glucocorticoid receptor which regulates transcription of cytokine genes associated with inflammation. Glucocorticoid receptors are located in the cytoplasm of the target cell associated with heat shock protein and an immunophilin, and intracellular protein that binds other immunosuppressive compounds. The receptor is inactivated until bound to a steroid ligand (Schimmer and Parker, 1995). Glucocorticoids regulate expression of the cytokines TNF-α, glucocorticoids-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6 and IL-8. IL-1, IL-6 and TNF-α induce glucocorticoid release in a prostaglandin-corticotropin-releasing hormone-dependent manner from the hypothalamic-pituitary-adrenal axis. Glucocorticoids are powerful inhibitors of nitric oxide synthase induction and induce the expression of the 37-kDa immunomodulator, lipocortin-1, in PMNL (**Polymorphonuclear Leukocyte**) and macrophages. Lipocortin is a powerful inhibitor of phospholopase A_2, which releases arachidonic acid for proinflammatory eicosanoid synthesis. Glucocorticoids downregulate expression of the eosinophil chemokine RANTES (**Regulated on Activation Normal T Cell Expressed and Secreted**). Endogenous glucocorticoids inhibit neutrophil chemotaxis in inflammatory cholestatis. It is also a diminished neutrophil extravasation and induces a prolong increase in plasma cortisol. (Cruse and Lewis, 2004)

Ocular inflammatory responses are mediated by several factors including cytokines, neuropeptides and lipid-derived substances. Arachidonic acid (AA) is a metabolite of inflammation that found within the phospholipids bilayer of cell membranes and is released by the enzymatic action of phospholipase A_2 in response to mechanical or chemical stimuli. AA is metabolized through two main pathways, the cyclooxygenase and the lipoxygenase. Both of these have been demonstrated within cells of conjunctiva, cornea and uvea. Major products of these pathways include prostaglandins (PGs), thromboxanes, hydroxyl-eicosatetraenoic aids and leukotrienes. These metabolites have been called eicosanoids. Both hydroxyl-eicosatetraenoic acids and leukotrienes act as chemoattractant molecules for inflammatory cells, whereas leukotrienes have an additional direct effect on vascular permeability. Prostaglandins, specifically PGE and

PGF-2α are the predominant metabolites present in ocular tissues during inflammation event and disrupt the integrity of vascular endothelium, resulting in breakdown of the blood-ocular barrier, which is normally maintained by the tight junctions of the ciliary body, nonpigmented epithelium, iris and retinal vascular endothelium and retinal pigmented epithelium. If left untreated, elevated intraocular PG concentrations can lead to a self-perpetuating cycle of inflammation with vision-threatening consequences. Prostaglandins are removed from the eye by active transport by the ciliary body and degradation by PG15-dehydrogenase. Unlike other organs, the eye contains little PG15 dehydrogenase. Active transport by the uvea is a saturable and sodium-dependent system that is impaired with inflammation.

Glucocorticoids are lipophilic 21-carbon proteins derived from cholesterol. Both endogenous and administered corticosteroids bind to intracytoplasmic glucocorticoid receptors. These receptors are almost ubiquitously expressed and regulate cells response to inflammation. Once bound, corticosteroids change the receptor's tertiary conformation so as to release a chaperone protein and reveal a DNA-binding region. The corticosteroid-receptor complex then is translocated to the nucleus, where it binds to specific DNA sequences called glucocortioid response elements interaction with these motifs alters cell gene expression and ultimately, protein synthesis. Some genes are inhibited, although most, including lipocortin are upregulated. Lipocortin inhibits proinflammatory substances, such as platelet-activating factor (PAF) and PLA_2, the enzyme responsible for initiating the AA cascade. Additional evidence suggests that glucocorticoids have a direct negative influence on PGE isomerase, resulting in reduced PGE synthesis and increased vascular stability. At a cellular level, such inhibition of proinflammatory pathways is manifested in the eye as decreased exudation of cells and fibrin, inhibition of fibroblastic and collagen-forming activity, retardation of epithelial and endothelial regeneration, stabilization of lysosomal membranes, reduced neovascularization and stabilization of the blood-ocular barrier (Bradford and David, 2004).

Ophthalmic uses

Glucocorticoids are beneficial in various non-pyogenic inflammatory disorders, but their popular sometimes leads to indiscriminate use. Various topical corticosteroid preparations have been formulated to provide a beneficial therapeutic effect. Even so, one compound may be preferred over another, depending on the location of inflammation in the eye. Dexamethasone sodium phosphate (0.1%) and betamethasone sodium phosphate (0.1%) are poorly absorbed by the cornea but have an anti-inflammatory effect five to seven times that of prednisolone (Mcgee,1992), and these may be the drugs of choice for treatment of superficial ocular inflammations such as blepharitis, conjunctivitis, episcleritis. Prednisolone acetate (1.0%) or dexamethosone alcohol (0.1%) is recommended for treating anterior uveitis because of improved intraocular diffusion. The clinical efficacy of topical glucocorticoid preparations is improved by adjusting the frequency of application to severity of the inflammation. Severe inflammation may require hourly treatment, whereas a mild, external inflammatory condition may be controlled with two or three daily administrations (Pappa, 1993).

Adverse effects and complications

Systemic absorption of topical corticosteroids has been documented in dogs. Adverse reactions resulting from glucocorticoids therapy can occur through either cessation of therapy or prolonged use. Acute adrenal insufficiency results from rapid withdrawal of glucocorticoids after prolonged treatment. Adverse effects of these drugs such as water and salt retention, hypertension, increase corneal mycotic infections, and in all species may worsen a bacterial infection if not covered by appropriate antibiotic, progression of corneal ulceration due to augmentation of MMPs, rapidly developing lipid keratopathy, iatrogenic Cushing's syndrome and adrenal suppression.

Effects on corneal healing

Treatment of certain corneal wounds or ulcers with topical corticosteroids occasionally results in rapid stromal melting in the affected areas. Results of in vitro studies demonstrate that corticosteroids increase the lytic action of corneal collagenase,

thus suggesting this effect might be responsible for corneal destruction in clinical conditions. Therefore, topical corticosteroids are generally considered to be contraindicated in septic and progressive corneal ulcerations except for those that may be immune-mediated. Topical glucocorticoids are routinely used after intraocular surgery to control postoperative inflammation (Wilkie, 1990).

Effects on ocular infections

Glucocoticoids interfere with defense mechanisms by decreasing leukocyte movement from vessels to the site of infection and by depressing macrophage phagocytosis of microbes. Depression of the ingestive ability of monocytes and macrophages might result from changes in the number of receptors on their cell membranes and from decreased activation of macrophages by lymphokines and nitric oxide synthase. As a result, glucocorticoids can potentially activate or exacerbate ocular bacterial, viral or fungal infections, is still debated. For instance, controversy abounds over the role of topical corticosteroids in treatment of bacterial keratitis. Experimental models of bacterial keratitis suggest corticosteroid therapy does not enhance bacterial replication if administered concurrently with an effective bactericidal antibiotic. If these drugs are being considered in clinical settings, they should be used only after the causative agent has been identified and specifically treated.

Other local complications

Posterior subcapsular cataract formation, glaucoma and mydriasis are the most common complications of local or systemic corticosteroid administration in humans. Human corticosteroid-induced glaucoma appears to have a genetic basis and to relate to a decrease in the aqueous humor outflow facility. Such adverse effects have not been documented in small animal, however calcific-band keratopathy has recently been observed as a possible consequence of topical corticosteroid-phosphate preparations in human (Taravella et al., 1994) and the hypertensive effects of glucocorticoids have recently been documented in Beagles with primary open-angle glaucoma (Gelatt,1998). In these animals, topical application of 0.1% dexamethazone four time daily increases the

intraocular pressure by mean 5 mm Hg in the treated eyes within 2 weeks (Gelatt,1998). Steroid-induced cataract or keratopathy has not been documented in small animals.

Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs act on cyclooxygenase (COX) inhibition. The constitutive form of COX is referred to as COX-1 and the inducible form is referred to as COX-2. COX-1 has been found in platelets, kidneys and gastrointestinal tract. COX-2 has been identified in fibroblasts, chondrocytes, endothelial cells, macrophages, and mesengial cells.

COX-2 is induced by exposure to various cytokines, mitogens, endotoxin and it is up-regulated with inflammation. The prostaglandins produced in the gastrointestinal tract and the kidneys that maintain mucosal integrity in the GI tract and renal perfusion appear to be derived from COX-1. Therefore, suppressing COX-1 activity by NSAIDs is believed to be critical to the development of toxicity. It is suggested that COX-2 selective NSAIDs would suppress prostaglandin synthesis at sites of inflammation but would spare constitutive prostaglandin synthesis in the GI tract and kidney. The currently available NSAIDs vary in their potency as inhibitors of COX-2. COX-2 selective NSAIDs, new anti-inflammatory drug are worldwide for the prostaglandins that mediate pain, inflammation and fever. In addition, COX-2 may produce beneficial prostaglandins, therefore highly selective COX-2 inhibitors may produce less adverse reactions. Also, most GI ulceration is associated with significant mucosal inflammation. In these circumstances, it is likely that COX-2 is being expressed and that the derived prostaglandins are responsible for promoting healing, so it is well known that NSAIDs retard the healing of ulcers. (Elizabeth, 2004)

Topical culture supernatant from human amniotic epithelial cells

The placenta comprises of three layers, amnion, chorion and deciduas. The amnion is a thin membrane-lined cavity that fills with fluid and serves, among other things, to cushion the fetus during development and to prevent adhesion of the developing fetus to maternal structures. Human amniotic epithelial cells (HAEC) have been reported to be differentiated to mature neural cells those unique characteristics.

HAEC supernatant significantly inhibits the chemotactic activity of neutrophils and macrophages toward macrophage inflammatory protein (MIP). The supernatant significantly reduces the proliferation of both T and B cells after mitogenic stimulation and induces apoptosis of T and B cells but not those of corneal epithelial and liver cells. In contrast to lymphocytes, macrophages and neutrophils are resistant to apoptosis induced by HAEC supernatant.

In 2004, Kazutaka found that the conditioned medium from HAEC contains small amounts of interleukin-1 receptor antagonist (IL-1 ra) that play roles in the suppression of corneal inflammatory responses. The pro-inflammatory cytokine has a wide range of activities including mediation of the acute-phase response, induction of chemotaxis and activation of inflammatory cells and antigen presenting cells (APCs) and stimulation of neovascularisation that unwanted response. IL-1ra is a naturally occurring IL-1 isoform produced by the same cells that synthesize IL-1. It is a high affinity binding to IL-1 receptors, so that the topical administration of IL-1Ra leads to a profound suppression of inflammation-induced langerhans cells (LCs) migration in the cornea (Niederkorn et al., 1989).

Surgical therapy

The objectives of surgical treatment is to correct underlying abnormalities or anatomical defects, protection for the ulcer, neutralize the destructive effects of collagenolytic enzymes, restoring functional integrity to the damaged cornea and preventing recurrence of ulceration. There are varieties of surgical approaches.

Third eyelid flap

Nictitating membranes (third eyelid) are very useful and practical of therapy for many types of corneal ulcers and chiefly function by protection for the damaged cornea. Flap is sutured to the globe to limit exposure of topically applied medications to the cornea, eye movement causes undesirable friction between the cornea and the nictitating membrane, the bulbar surface of which is frequently rough and irregular and indicated only for the protection of superficial and noninfected ulcer (Blogg et al., 1989).

Conjunctival flaps or grafts

The most commonly used surgical procedure for chronic, infected, or progressive corneal ulcers is a conjunctival flap or graft. Conjunctival flaps provide corneal support, fibrovascular tissue to fill corneal defects, and bring blood supply (and blood-associated immune components, systemic antibiotics, natural anticollagenases (α-2 macroglobulin) to the lesion (Hankanson and Merideth, 1987). Because conjunctival flaps cover only a small area of the normal cornea, they allow visualization of much of the cornea and anterior chamber of the affected eye, which allows continuous examination of these structures to monitor progression of the ulcer and possible anterior uveitis. Having only a small portion of the cornea covered may also allow the animal to continue to be visual.

All types of conjunctival flaps consist of thin bulbar conjunctival tissue transposed onto the cornea to cover the lesion. The flap moves with the eye and thus no

tension is applied to the flap itself. With all types of conjunctival flaps, it is important that the graft bed and ulcer be properly prepared. The recipient bed for the graft is prepared by debriding the lesion, thereby removing loose epithelium and devitalized corneal tissue. Great care should be taken to prevent perforation of the cornea during this debridement.

The hood and pedicle conjunctival grafts are the most versatile and are recommended for most cases of severe corneal ulceration. The hood flap is indicated for peripheral corneal lesions. The conjunctiva adjacent to the lesion is cut from the limbs and undermined. The graft is advanced to cover the lesion and sutured in place, generally with 2 or 4 simple interrupted sutures.

The conjunctival flaps will adhere to the corneal lesion and epithelialization surrounding the flap, but not generally underneath the flap, will occur. Three to 8 weeks after placement of the flaps, the blood supply should be interrupted by cutting the base of the flap at the limbus (Peiffer et al., 1977). This can usually be done with topical anesthesia and Steven tenotomy scissors. Cutting off the blood supply will allow the conjunctival graft to recede and will lessen the resulting corneal scar.

The most common complication of any type of conjunctival grafting procedure is dehiscence of the graft from the corneal lesion. This may occur because the corneal lesion is progressing and damaging the cornea at the points where sutures securing the graft are placed. Excessive tension on the graft or allowing a significant portion of the fibrous Tenon's capsule to remain attached to the graft may result in premature dehiscence of the graft. Proper suture placement in healthy cornea, using a thin conjunctival graft, and concurrent appropriate medical therapy will greatly decrease complications following conjunctival flap surgery.

Corneoscleral or corneoconjunctival transposition

This procedure is a type of autogenous corneo-scleral graft that uses a sliding pedicle of cornea and attached sclera to repair corneal defects. It is indicated in central, deep, or perforated corneal lesions where there is sufficient peripheral healthy cornea present that can be used for the grafting procedure. In general, the distance from the peripheral edge of the lesion to the corneal limbus needs to be at least 1 mm longer than the diameter of the corneal lesion itself to be able to perform the corneal-scleral transposition. Because self tissues are used, the corneal-scleral transposition eliminates the need for corneal tissue donors and decreases immune-mediated inflammation. This may decrease the corneal scarring and allow a clearer postoperative cornea than that seen after conjunctival grafts and some other corneal grafts. A disadvantage, however, that the corneal-scleral transposition damages normal healthy corneal tissue (Parshall, 1973).

Autogenous lamellar corneal grafts

This procedure is indicated for use in corneas with descemetoceles, stromal abscesses, and perforated ulcers. These grafts use adjacent corneal tissue which is slid to cover the corneal defect. Advantages of this procedure are that the autografts are used and therefore graft rejection should be minimal, tissue is usually readily available, and a clear cornea may result after surgery. The main disadvantage of this procedure is that an area of normal cornea is weakened. The surgical procedure is initially similar to the corneal-scleral transposition. Two parallel incisions are made which extend past the lesion toward the limbus. The distance between the incisions is 2 to 3 mm wider than that the diameter of the lesion. The incisions are joined by making a perpendicular incision and a 1/2 thickness keratectomy is performed. The graft should be 0.5 to 1 mm wider and deeper than the lesion. The graft is positioned in the graft bed of the lesion and sutured into place with a continuous or interrupted suture pattern. A conjunctival pedicle flap can be placed over both the graft and lesion to help promote healing, bring in blood supply, and give added strength to the corneal lesions and graft site. Use of the conjunctival flap

may be especially important in infected ulcers or rapidly progressing ulcers; however, increased scarring may subsequently occur (Brightman et al., 1989).

Tectonic corneal grafting

This procedure is using frozen corneal tissue that has also been described for treatment of corneal descemetoceles and perforations (Hacker, 1991). Fresh corneal tissue was collected at the time of euthanasia of donor animals and placed in a bottle of triple antibiotic ophthalmic solution. The tissue was then placed in a standard –20° C freezer and kept suitable for up to 10 months. Others have found tissue suitable for surgery when frozen for over 24 months (Whitley, 1997). When the tissue is needed for surgery, it is thawed in warm water or at room temperature. The grafts were cut and placed over descemetoceles or perforations and sutured into place with 8-0 polyglycolic acid suture. No attempt was made to limit vascularization until the graft was completely vascularized and the corneal fluorescein negative. In Hacker's series of 19 cases, 84% were successful and had vision, despite vascularization and scarring of the graft.

Human amniotic membrane transplantation for corneal ulcer

The first use of amniotic membrane transplantation (AMT) in human ophthalmology was by De Rotth in 1940 who reported partial success in the treatment of conjunctival epithelial defects after symblepharon then, Sorsby and Symons (1946) found that patients with caustic burns of the conjunctiva and cornea can be treated successfully using amniotic membrane. In 1997, Kim and Tseng used AMT for ocular surface reconstruction in persistant epithelial defects with ulceration.

Human amniotic membrane is of embryonic origin and derived from the fetal membranes which consist of the inner amniotic membrane. It consists of single layer of amnion cells fixed to collagen-rich mesenchyme 6 to 8 cells thick loosely attached to chorion. It is composed of three layers: a single epithelial layer, thick basement

membrane and avascular stroma. Human amniotic membrane has been shown to contain collagen types III and V. It also contains collagen types IV and VII similar to corneal epithelial basement membrane as well as fibronectin and laminin. In addition, it contains fibroblast and other growth factors (Fukada et al., 1999). Advantages to the use of human amniotic membrane include its lack of antigenicity and the ease of surgical manipulation of the tissue. There is believed to be nonimmunogenic or low antigenicity (Masato et al., 2001).

Most human ophthalmologists use human amniotic membrane transplantation with tissue preserved following the method of Tseng and colleagues in 1998. The human amniotic membranes are preserved at 80 degree celsius in medium and are flattened onto nitrocellulose paper with the epithelium side up. From the clinical study, they found that human amniotic membrane promotes epithelial healing, reduces inflammation, increases comfort and decreases severity of vascularization. They do not find any infectious, inflammatory or toxic reactions. The exact mechanisms in which human amniotic membrane delivers its beneficial effects on the ocular surface are still being investigated, but human amniotic membrane modulates levels of cytokines and growth factors and has also been shown to have unique properties including pain reducing, fibrosis suppressing, antibacterial and wound protecting.

CHAPTER III

MATERIALS AND METHODS

1. Animal model

Twenty five dogs with various breeds were maintained in animal facility rooms at the Faculty of Veterinary Science, Chulalongkorn University. The protocol was approved from the Ethics Committee of Veterinary Science, Chulalongkorn University. All animals were taken physical and ophthalmologic examinations before the study. They were fed with dog chow (A-Pro®) 2 times a day and water ad libitum.

2. Human amniotic membrane

Human amniotic membranes were obtained from Red Cross Eye Center, Bangkok, Thailand.

3. Group classification

Twenty five dogs were divided into 5 groups; each group consisted of 5 dogs.

Group 1 (Normal control group): Dogs with normal cornea.

Group 2 (ABO alone group): Dogs were induced to have deep corneal ulceration and treated with human amniotic membrane transplantation and topical ABO.

Group 3 (Corticosteroid group): Dogs were induced to have deep corneal ulceration and treated with human amniotic membrane transplantation, topical antibiotic and topical corticosteroid.

Group 4 (Mock group): Dogs were induced to have deep corneal ulceration and treated with human amniotic membrane transplantation, topical antibiotic and topical mock media solution (Dulbecco modified Eagle's medium or DMEM plus0.5 % fetal calf serum or FCS).

Group 5 (HAEC group): Dogs were induced to have deep corneal ulceration and treated with human amniotic membrane transplantation, topical antibiotic and topical supernatant of human amniotic epithelial cell (HAEC).

4. Preparation of topical supernatant of human amniotic epithelial cells (modified from Kazutaka et al., 2005)

Human amniotic membranes were obtained from normal cesarean with approval of the Red Cross Eye Center, Bangkok, Thailand. All placentas were seronegative for human immunodeficiency virus (HIV), hepatitis B and C viruses and syphilis. Sterile human amniotic membrane were transferred into a fresh tissue culture dish and minced with scissors and incubated in 0.5 % trypsin solution. Cells were collected by gentle centrifugation and resuspended in Dulbecco modified Eagle's medium (DMEM). The supernatant was collected 48 hours after incubation, filtered through a 0.22 μm PVDF membrane and stored at 4 °C.

5. Induction of corneal ulceration and transplantation of human amniotic membrane

Surgery was performed under general anesthesia, premedicated with acepromazine (IM) 0.02 mg/kg and morphine (IM) 0.5 mg/kg, induction with propofol (IV) 3 mg/kg, and mainteinanced with isoflurane. First, the induction of deep corneal ulceration was performed by removing corneal epithelium and superficial stroma with a surgical blade. Corneal incision was completely surrounded the lesion by using the corneal trephine and diamond knife. After the incision was made, the edge of the tissue to be removed was grasped by forceps, and corneal dissector. Then, human amniotic

membrane was transplanted on the ulcer with epithelial side up and secured with 10-0 nylon suture 8 stitches (Inlay technique). Lastly, tempolary tarsorhaphy was performed to protect the surgical site.

6. Application of drugs and test agents

The administrations with the agents including ABO, corticosteroid, HAEC and mock media solutions started at 24 h after human amniotic membrane transplantation. The ABO alone group received only topical antibiotic solution (tobramycin 0.3%; Tobrex®) four times daily. The corticosteroid, mock and HAEC groups received topical antibiotic and corticosteroid (prednisolone acetate; Predforte®), mock or HAEC solutions four times daily, respectively.

7. Tear fluid collection

Tear fluid samples were collected from the inferior punctum at the first day before the operation and at every other day by using an insulin syringe 50 IU (0.5 CC) connected with polyester tube. The total volume of collected tear fluid was about 100 µl each time.

8. Cytokine IL-1 assays

The concentration of IL-1 β in tear sample was measured using enzyme-linked immunosorbent assay (ELISA) (Rapidbio, Transhold, China), according to the manufacturer's protocol. Briefly, the samples (50µl) and distilled water (50µl) were added in anti-dog IL-1 beta biotin coated wells plate and incubated at 37°c for 60 minutes; wash the plate 5 times with washing buffer. Then 100 µL of the horseradish peroxidase (HRP) was added into each well and incubated for 30 minutes at room temperature, wash plate 5 times with washing buffer. TMB (3,3'5,5' tetramethyl-benzidine) substrate solution (100µl) was added and incubated for 15 minutes at room temperature, then add 100 µL

stop solution and incubate for another 30 minutes at room temperature. The IL-1 β concentration was determined spectrophotometrically at an absorbence of 450 nm and interpolated with standard curve.

9. Nitric oxide assays

The concentration of nitric oxide in tear sample was assayed by measuring the accumulation of stable degradation products, nitrate and nitrite. In aqueous solution, nitric oxide rapidly degrades to nitrate and nitrite. Spectrophotometric quantitation of nitrite using Griess reagent is straightforward, but does not measure nitrate. Therefore, the metallic cadmium (Cd) was used to convert nitrate to nitrite then, total nitrite concentration was measured using Griess reagents. Briefly, the samples were diluted 20 times with distilled water, mixed with 30% $ZnSO_4$ and incubated at room temperature for 15 minutes. The mixtures were centrifuged at 3,000 rpm for 5 minutes and transferred the resulting supernatant 200 µl to clean microcentrifuge tube and incubated with Cd for 24 hours. After incubation, 100 µl of sample, 50 µl of sulfanilamide (p-aminobenzenesulfonamide) and 50 µl of N-(1-Naphthyl) ethylenediamine dihydrochloride were added on wells plate. Nitrite concentration was determined spectrophotometrically at an absorbence of 540 nm and interpolated with standard curve.

10. Clinical ophthalmic evaluations

The examinations including direct and indirect ophthalmoscopy, Schirmer tear test (STT), papillary light response (PLR), blink reflex, menace reflex, dazzle reflex were performed. Additionally, fluorescein staining of healing ulcer and development of corneal neovascularization were assessed.

11. Statistical analysis

All data were expressed as mean ± standard error of mean (S.E.M.). The data were statistically analyzed using one-way analysis of variance (standard error of mean (S.E.M.) and tukey test. Values of $p<0.05$ were considered to be significant.

CHAPTER IV

RESULTS

1. Influence of proinflammatory cytokines, interleukin-1 β (IL-1 β) and nitric oxide (NO) in tear fluid of normal and inflammatory corneas

In this study, we measured concentrations of proinflammatory cytokines, IL-1β and NO in tear fluid of dogs with normal cornea (n=5) and induced-corneal ulcer (n=20) at 24 hr after human amniotic membrane transplantation (Day 0). The concentrations of IL-1β and NO in tear fluid of the induced-corneal ulcer dogs were significantly increased compared with those of the normal corneal dogs. The average concentrations of IL-1β and NO in tear fluid of normal corneal dogs were 37.29±1.01 mM and 1.49±0.26 μM, respectively, and those of induced-corneal ulcer dogs were 62.80± 0.39 mM and 6.60±0.05 μM, respectively. (Table 4.1, Fig 4.1 and 4.2)

Table 4.1 The concentrations of proinflammatory cytokines, IL-1β and nitric oxide, in tear fluid of dogs with normal cornea and induced-corneal ulcer.

	Concentrations in tear fluid	
	IL-1β (mM)	NO (μM)
Normal corneal dogs (n=5)	37.29±1.01	1.49±0.26
Induced-corneal ulcer dogs (n=20)	62.80±0.39*	6.60±0.05*

All values are expressed as mean ± SE

*Significant at $p < 0.05$, compared to normal dogs

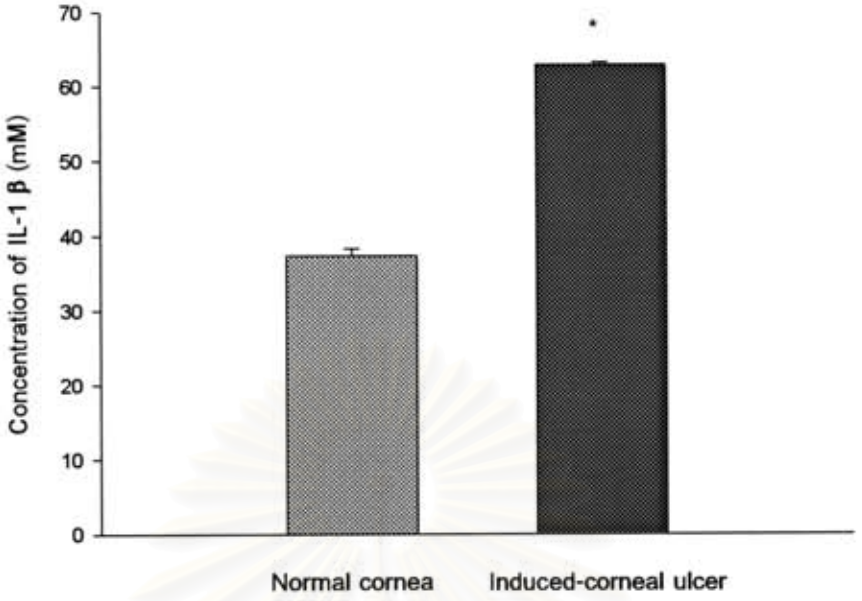

Figure 4.1 The IL-1 β concentration in tear fluid of normal cornea and induced-corneal ulcer dogs. The data were shown as mean ± SE.

* Significant at $p < 0.05$, compared to normal dogs.

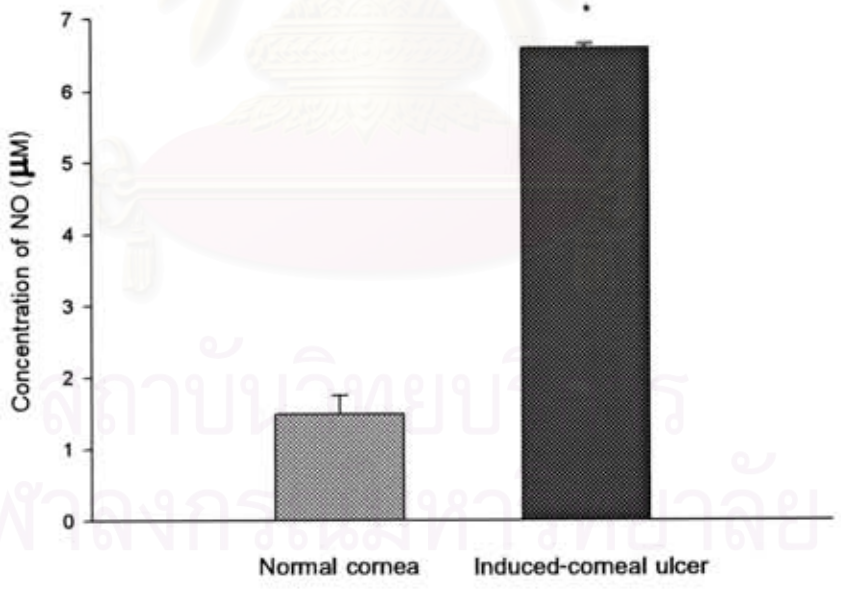

Figure 4.2 The nitric oxide concentration in tear fluid of normal cornea and induced-corneal ulcer dogs. The data were shown as mean ± SE.

* Significant at $p < 0.05$, compared to normal dogs.

2. Effects of topical antibiotic, corticosteroid, mock media and culture supernatant from human amniotic epithelial cell on IL-1 β concentrations in the inflamed corneas

Table 4.2 and figure 4.3 show the concentrations of IL-1 β in tear fluid of induced-corneal ulcer dogs before and after administration of testing agents for 9 consecutive days. In all experimental groups, the IL-1 β concentrations increased significantly after induction of corneal ulcer (day 1) compared with those in day 0 of the same group. In ABO alone group, the IL-1 β concentrations were gradually increased from day 1 to day 5 and then slightly, but not significantly decreased in day 7 and 9 compared to those in day 1. In corticosteroid group, after received topical corticosteroid four times daily, the IL-1 β concentrations were gradually decreased from day 3 to day 9 and statistical significant difference was found in day 9 ($p<0.05$). In mock media group, the IL-1 β concentrations were gradually increased from day 1 to day 9. Similar to corticosteroid group, the IL-1 β concentrations were gradually decreased every day after received topical HAEC solution four times a day but the statistical difference was observed only in day 9, compared to that in day1.

When comparing the IL-1 β concentrations each day between corticosteroid, mock media and HAEC groups, respectively with ABO alone groups, the IL-1 β concentrations in both corticosteroid and HAEC groups were significantly lower than those in ABO alone group at day 7 and day 9 ($p<0.05$). However, in mock media group, the concentrations of IL-1 β each day were higher than those in ABO alone group. In addition, the statistical difference was not found between corticosteroid and HAEC groups.

Table 4.2 The IL-1 β concentrations in tear fluid of induced- corneal ulcer dogs before and after an administration of testing agents for 9 consecutive days.

Groups	Concentrations of IL-1 β (mM)					
	Day					
	0	1	3	5	7	9
ABO alone	45.57±4.55	60.34±1.79*	62.36±1.45	63.57±0.73	62.37±1.51	60.30±1.79
Cortico-steroid	39.02±0.75	60.87±1.39*	53.77±1.36	53.03±1.86	50±0.40$	45.85±1.35#$
mock media	45.46±1.22	70.47±4.93*	71.54±5.53	71.89±5.40	72.98±4.19	74.42±4.53
HAEC	38.14±1.48	60.19±0.94*	58.71±1.37	56.39±1.77	53.95±1.68$	48.88±1.63#$

The data were shown as mean ± SE.

* Significant at $p < 0.05$, compared to day 0 of the same group.

Significant at $p < 0.05$, compared to day 1 of the same group.

$ Significant at $p < 0.05$, compared on the same day to ABO group.

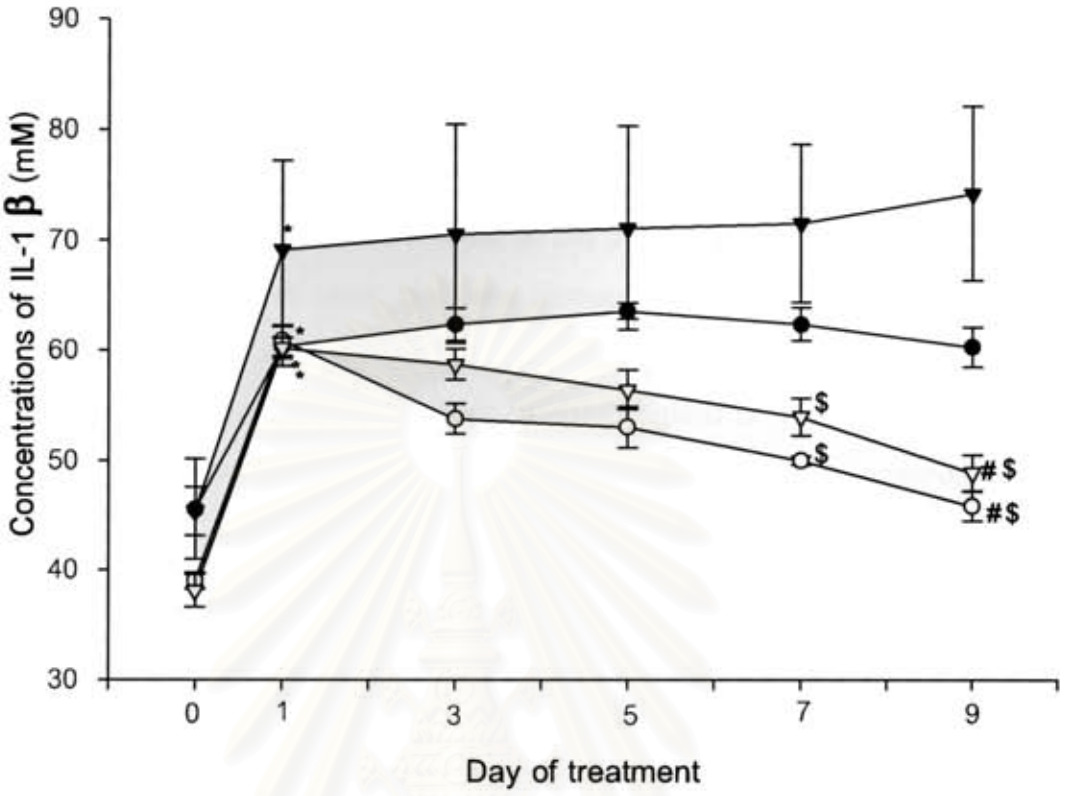

Figure 4.3 The concentrations of IL-1 β in tear fluid of induced-corneal ulcer dogs before and after administration of testing agents including antibiotic (●), corticosteroid (O), mock media (▼) and HAEC (▽) solutions.

*Significant at $p < 0.05$, compared to day 0 of the same group.

#Significant at $p < 0.05$, compared to day 1 of the same group.

$Significant at $p < 0.05$, compared on the same day to ABO group.

3. Effects of topical antibiotic, corticosteroid, mock media and culture supernatant from human amniotic epithelial cell on nitric oxide concentrations in the inflamed corneas

Table 4.3 and figure 4.4 show the NO concentrations in tear fluid of induced ulcer dogs before and after an administration of testing agents for 9 consecutive days. In all experimental groups, the concentrations of NO increased significantly after induction of corneal ulcer (day 1) compared to those on day 0 of the same group. In ABO alone group, the NO concentrations were gradually increased from day 1 to day 7 and slightly decreased on day 9. In corticosteroid group, the NO concentrations were gradually decreased from day 1 to day 9 after receiving topical corticosteroid four times daily. However, the statistical difference ($p<0.05$) compared to day 1 was observed only on day 9. In mock media group, the NO concentrations were increased after induction of corneal ulcer and remained high throughout the period of experiment (from day 1 to 9). In HAEC group, after treated with topical HAEC solution 4 times daily, the NO concentrations on day 3 and 5 were slightly higher than those on day 1, however, at day 7 and 9, they were slightly low, but not significantly compared to those on day 1.

When comparing the NO concentrations collected from the ABO alone-treated group with the other testing agent-treating groups, the NO concentrations in both corticosteroid and HAEC groups were significantly lower than those in ABO alone group at day 7 and 9. However, in mock media group, although the concentrations of NO each day were lower than those in ABO alone group, the statistical difference was not observed. In addition, the statistical difference was not found among steroid, mock media and HAEC groups.

Table 4.3 The nitric oxide concentrations in tear fluid of induced-corneal ulcer dogs before and after an administration of testing agents for 9 consecutive days.

Groups	Concentrations of nitric oxide (μM)					
	Day					
	0	1	3	5	7	9
ABO alone	2.68±0.41	7.69±0.60*	7.73±0.35	8.63±1.69	9.98±1.83	9.26±1.43
Corticosteroid	1.78±0.06	6.95±0.63*	5.2±0.70	5.09±0.55	4.69±0.54$	4.13±0.34#,$
Mock media	2.23±0.13	6.53±0.56*	6.75±0.56	6.55±0.73	6.43±0.56	6.58±0.60
HAEC	1.89±0.44	5.85±0.56*	6.24±0.39	6.11±0.40	5.77±0.31$	5.334±0.37$

The data were shown as mean ± SE.

* Significant at $p < 0.05$, compared to day 0 of the same group.

Significant at $p < 0.05$, compared to day 1 of the same group.

$ Significant at $p < 0.05$, compared on the same day to ABO group.

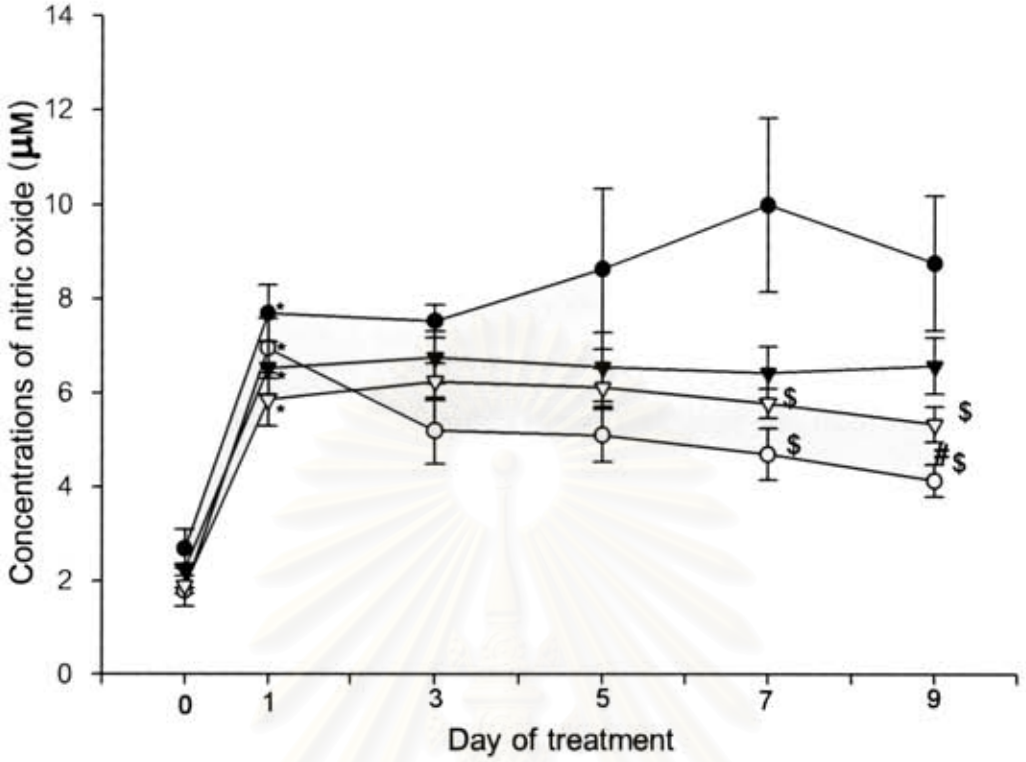

Figure 4.4 The concentrations of nitric oxide in tear fluid of induced-corneal ulcer dogs before and after administration of testing agents including antibiotic (●), corticosteroid (O), mock media (▼) and HAEC (▽) solutions.

*Significant at $p < 0.05$, compared to day 0 of the same group.

#Significant at $p < 0.05$, compared to day 1 of the same group.

$Significant at $p < 0.05$, compared on the same day to ABO group.

4. Clinical evaluations

The examinations including direct and indirect ophthalmoscopy, Schirmer tear test (STT), pupillary light response (PLR), blink reflex, menace reflex, dazzle reflex were normalized in every group.

Clinical evaluations of corneal ulcer, after human amniotic membrane transplantation, the ulcers were completely re-epithelized within 4 days in every group. Conjunctival hyperemia was present in all groups within 24 hours after operation and gradually improved in 48 hours. Purulent ocular discharge was most intense at 24 hours after operation in all groups. After treated with each solution 4 times daily, the purulent ocular discharges in the glucocorticoid, HAEC and ABO alone groups were improved in 48 hours.

Corneal neovascularization was assessed by slit lamp biomicroscope. The corneal neovascularization appeared on day 7 in all groups, except corticosteroid group (Fig. 4.5). In mock (Fig. 4.6) and ABO alone (Fig. 4.7) groups, the neovascularization appeared progressively until the end of our investigation. However, the neovascularization gradually improved and disappeared within 14 days in HAEC group (Fig. 4.8). The number of vessel was considerably lower in HAEC group than ABO alone and mock groups. The degree of corneal neovascularization was significantly less in corticosteroid group than HAEC group.

The corneal scar formation in ABO alone, mock and HAEC groups were intense on day 7 and markedly in ABO alone and mock groups on day 10. In addition, it remain existed throughout the investigation period in ABO alone and mock groups. In HAEC group, the scar did not appear progressively as in ABO and mock groups. However, the degree of scar formation in HAEC group was not significantly improved compared to that in corticosteroid group.

Table 4.4 shows the relationship between concentrations of nitric oxide, IL-1 β and clinical parameters on day 9. In corticosteroid treated group, we found that both NO and IL-1β concentrations were decreased significantly compared to those on day 1. By confirming with fluorescein staining, the ulcers were completely re-epithelized. The signs

of inflammation, such as neovascularization and conjunctivitis were not observed however, scar formation was slightly present (+1). In HAEC group, NO and IL-1β concentrations were also significantly decreased compared to those on day 1. The ulcers were completely re-epithelized and conjunctivitis was not observed, however, both neovascularization and scar formation still appeared in a mild degree (+1). In ABO and mock solution treated groups, NO and IL-1β concentrations were increased compared to those on day 1 and remained in high levels until the end of our investigation. Since the ulcers were transplanted with human amniotic membrane, they were also completely re-epithelized within 4 days, however, the neovascularization and scar formation in the corneas of these two groups still present in moderate (+2) and severe (+3) degree, respectively.

Table 4.4 The relationship between concentrations of nitric oxide, IL-1 β and clinical parameters on day 9.

Group/Parameter	NO	IL-1 β	Fluorescein staining	Neovas-cularization	Conjunctivitis	Scar/ Remnant
Corticosteroid	Decreased*	Decreased*	Negative	0	0	1
HAEC	Decreased*	Decreased*	Negative	1	0	1
Mock	Increased	Increased	Negative	3	0	3
ABO	Increased	Decreased	Negative	2	0	2

*Significant at $p < 0.05$, compared to day 1 of the same group.

The concentrations of NO and IL-1 β increased or decreased, compared to those on day 1 of the same group.

0 = Not found

1 = Mild degree

2 = Moderate degree

3 = Severe degree

Figure 4.5 The cornea of corticosteroid treated group on of day 14.
The granulation tissue was not observed. However, there was slight scar formation.

Figure 4.6 The cornea of mock solution treated group on day 14.
The scar formation and granulation tissue were extended at center of the cornea.

Figure 4.7 The cornea of ABO treated group on day 14.
The scar formation and granulation tissue were extended at center of the cornea.

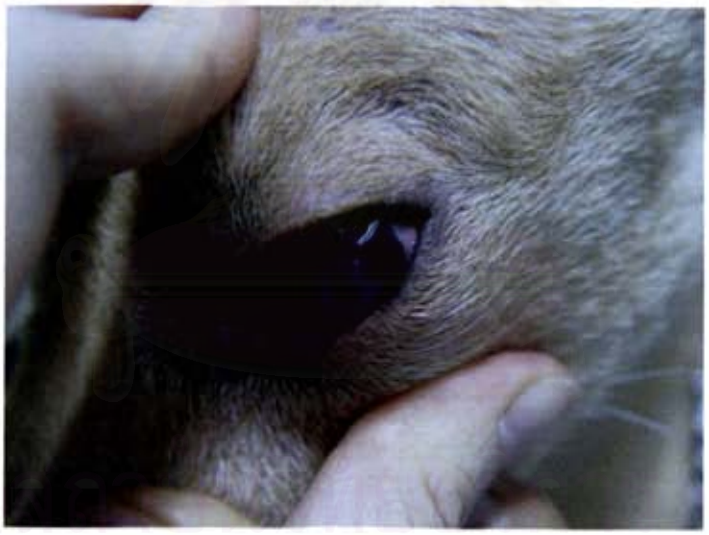

Figure 4.8 The cornea of HAEC treated group on day 14.
Less granulation tissue was found at the area of suture site (4 and 8 o'clock) and slightly corneal opacity from scar was observed.

CHAPTER V

DISCUSSION AND CONCLUSION

Corneal ulceration is a common painful and potentially vision-threatening condition that has been found in dogs. The treatment usually requires medical or surgical treatment or both, depending on the severity of the corneal ulcer, its duration and the suspected underlying cause. Generally, the treatment is focused on treating or preventing infection, controlling pain and inflammation, and minimizing scar formation. For superficial ulcers, the treatment usually begins with appropriate topical antibiotic ointment or drops to prevent infection and atropine solution to reduce painful spasm. Deeper ulcers must be treated aggressively to minimize complications and surgery may be needed to place a protective graft over the ulcer. Several surgical techniques are available, such as third eyelid flap, conjunctival graft, tectonic corneal graft, corneo-conjunctival transposition, autogenous lamellar corneal graft (Paulo et al. 1998) and amniotic membrane transplantation (Tasavarin et al., 2005).

In this study, we induced corneal ulcer in 20 dogs, then transplanted with the human amniotic membrane on the ulcer and investigated anti-inflammatory effects of topical application of culture supernatant from human amniotic epithelial cells (HAEC). Prednisolone, which has been known as a remarkable anti-inflammatory agent, was used as a reference drug. The administrations of topical antibiotic and DMEM culture medium (mock solution) were also investigated as negative control experiments.

The concentrations of IL-1β and NO were quantified along with clinical ophthalmic evaluations. After induction of corneal ulceration, IL-1β and NO concentrations in tear fluid were much higher comparing with those in normal corneal dogs as well as those in the day before the operation (day 0), indicating that an inflammation was occurred during surgical process. Inflammation is the body's response to cellular injury. The chemical mediators that tend to direct the inflammatory response include vasoactive amines (histamine, serotonin), arachadonic acids (prostaglandins, leukotrienes) and cytokines (tumor necrosis factor and IL–1). Our results were similar to the previous reports in which

the production of both IL-1 isoforms, IL-1 α and IL-1 β, are increased in the tear fluid after ocular surface inflammation (Solomon et al., 2001) and NO are expressed during the corneal inflammation (Sennlaub et al.,1999). IL-1 is a potent proinflammatory cytokine that is synthesized by mononuclear cells. It plays an important role in initiating and maintaining inflammation (Kazutaka et al., 2005). The main sources of NO in ocular surface tissue are corneal epithelium, fibroblast, endothelium and inflammatory cell. NO is an unstable free radical that is generated by conversion of L-arginine to citrulline by nitric oxide synthase (NOS), which is expressed in various ocular components, including cornea (Osborne et al., 1993 and Yamamoto et al.,1993). An increase in NO concentration is associated with inflammatory conditions within the eyes, in which inflammatory cells infiltrating the anterior chamber produce NO to the local environment (Yilmaz et al. 2002 and Ohta et al., 2003). NO plays an important role in damaging the endothelium in inflammation (Pervinder et al. 2004) and also, in other physiological functions such as maintaining ocular surface (Kim et al., 2002) and corneal hydration (Yanagiya et al., 1997).

Antibiotic, corticosteroid, culture supernatant from HAEC and mock solution were topically applied four times daily for 9 consecutive days. Both corticosteroid and culture supernatant from HAEC gradually decreased IL-1 β concentrations from day 1 to day 9 and the statistical differences in comparison to day 1 were found in day 9. Although the concentrations of IL-1 β in tear fluid obtained from corticosteroid treated group were slightly lower than those obtained from HAEC group, the statistical difference was not observed. In addition, the concentrations of IL-1 β after treated with corticosteroid and culture supernatant from HAEC were much lower than those treated with ABO and mock medium solutions. Both corticosteroid and culture supernatant from HAEC also suppressed NO production in tear fluid. The concentrations of NO after treated with corticosteroid and culture supernatant from HAEC were significantly lower than those treated with ABO alone group. Corticosteroid is a well-known anti-inflammatory drug, which prevents the formation of both PGs and leukotrienes by inhibiting phospholipase A_2 activity, resulting in reduction of arachidonic acid release. It also inhibits the expression of IL-1 (Bertram, 2001) and decreases leukotrienes that act as chemoattractant molecules for inflammatory cells such as monocytes and macrophages (Ying and Reza, 2001). IL-1 β

is produced primary by monocytes and macrophage and Sennleab et al (1999) has been reported that neutrophil or inflammatory monocytes are the major source of NO in the corneal inflammation. HAEC has been report to exert diverse anti-inflammatory effects by inhibiting IL-1 expression (Solomon, el al., 1999), trapping mononuclear and polymorphonuclear inflammatory cells infiltrating the ocular surface of human eyes (Shimmura et al., 2001), reducing keratocyte apoptosis in rabbit corneas (Wang, et al., 2001), suppressing alloreactive T cells in vitro (Ueta et al., 2002) and expressing mRNA and protein of anti-inflammatory factors, such as IL-1 receptor antagonist and IL-10 (Hao et al., 2000). Therefore, in similar to corticosteroid, the culture supernatant from HAEC exerted an anti-inflammatory action by inhibiting the inflammatory monocytes and macrophages to produce IL-β and NO. In addition, the culture supernatant from HAEC had a tendency to decrease IL-1β concentration better than NO concentration. It is possible that the culture supernatant from HAEC inhibited the production of IL-1β and NO from the inflammatory monocytes and macrophages. However, NO is also produced from other sources that are not involved in inflammatory process such as neuronal and vascular endothelial cells (Nicotera et al., 1997), therefore, the culture supernatant from HAEC worked better in decreasing concentration of IL-1β than NO. Further works are warranted to confirm the molecular mechanisms underlying the anti-inflammatory action of culture supernatant from HAEC in dog.

Topical mock solutions failed to decrease either IL-1 β or NO concentrations. These results confirmed that the mock solution, which was DMEM plus 5% FCS (v/v) alone did not exert an anti-inflammatory effect. The concentrations of IL-1 β in tear fluid obtained from the dogs treated with mock solution were much higher than those from other groups (Fig. 4.4). It is possible that the fetal calf serum, a growth factor, in the mock solution may play a role in stimulating a synthesis of IL- β from monocytes and macrophages (Cruse and Lewis, 2004).

The clinical ophthalmic evaluations have been investigated and we found that the induced-ulcers in corneas of all dogs were entirely re-epithelized within 4 days after transplantation of human amniotic membrane. The human amniotic membrane promotes epithelial healing, reduces inflammation and decrease severity of vascularization (De

Rotth, 1940) The epidermal growth factor present in human amniotic membrane contributes a significant effect to epithelial regrowth (Koizumi et al., 2000).

Neovascularization is a representative of an inflammation in the cornea. It frequently leads to vision loss due to scarring, persistent inflammation and keratopathy. In this study, the corneal neovascularization and corneal scar formation were noticed in all groups, but the degrees of severity were different. The neovascularization and scar formation in mock and ABO alone groups appeared progressively until the end of our investigation, however in HAEC and corticosteroid groups, they gradually improved and disappeared within 14 days. The number of vessel was also considerably lower in HAEC group than those in ABO alone and mock groups. Corticosteroid inhibits vascular and cellular characteristics of inflammation by decreasing vasodilation and reducing capillary permeability. It also suppresses the later stages of inflammation by inhibiting formation of fibroblasts and their collagen-forming activity. These results are in agreement with Kobayashi et al (2002) in which HAEC exerts anti-angiogenic effect that reduces neovascularization by suppression of bFGF-induced angiogenesis (Shinozaki, 1995). Moreover HAEC has expressed four tissue inhibitors of metalloproteases 1, 2, 3 and 4 as well as endothelial cell growth inhibitor endostatin, which contribute to the anti-neovascularization effects (Hao et al., 2000). The anti-scarring mechanism of HAEC has been reported as preventing fibroblast activation into myofibroblasts by reduced expression of α-smooth muscle actin, fibronectin-EDA and intergrin α5 (Tseng et al., 1999).

The results in table 4.4 have shown that the clinical parameters, including neovascularization, conjunctivitis and scar formation were in agreement with the concentrations of NO and IL-1 β, in which the lower the concentrations of NO and IL-1 β, the better the clinical parameters. However, the culture supernatant from HAEC was slightly less effective than corticosteroid in suppressing corneal inflammatory reactions.

Taken together, we concluded that, in similar to corticosteroid, topical application of culture supernantant from human amniotic epithelial cell suppressed inflammatory reactions in induced-corneal ulcer dogs via decrease production of proinflammatory cytokine, IL-1β and nitric oxide.

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