การแก้ไขกระจกตาทะลุในสุนัขโดยใช้เยื่อถุงน้ำคร่ำสุนัขที่เก็บถนอมในกลีเซอรอลเย็น

นางสาวสุขุมาลย์ ลีวิโรจน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาศัลยศาสตร์ทางสัตวแพทย์ ภาควิชาศัลยศาสตร์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

RECONSTRUCTION OF PERFORATING CORNEA USING COLD GLYCEROL PRESERVED CANINE AMNIOTIC MEMBRANE IN DOGS

Miss Sukhumal Leevirojana

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Surgery
Department of Veterinary Surgery
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2014

Thesis Title	RECONSTRUCTION OF PERFORATING CORNEA USING COLD GLYCEROL PRESERVED CANINE AMNIOTIC MEMBRANE IN DOGS
By	Miss Sukhumal Leevirojana
Field of Study	Veterinary Surgery
Thesis Advisor	Assistant Professor Nalinee Tuntivanich, D.V.M., Ph.D.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Veterinary Science
(Professor Roongroje Thanawongnuwech, D.V.M., M.S., Ph.D.)

THESIS COMMITTEE

.....Chairman
(Professor Marissak Kalpravidh, D.V.M., M.S., Ph.D.)

.....Thesis Advisor
(Assistant Professor Nalinee Tuntivanich, D.V.M., Ph.D.)

.....Examiner
(Associate Professor Anudep Rungsipipat, D.V.M., Ph.D.)

.....Examiner
(Professor Emeritus Pranee Tuntivanich, D.V.M., M.S.)

.....External Examiner
(Associate Professor Pinnita Tanthuvanit, M.D., Cert. of Corneal and External Eye Diseases)

สุขุมาลย์ ลีวิโรจน์ : การแก้ไขกระจกตาทะลุในสุนัขโดยใช้เยื่อถุงน้ำคร่ำสุนัขที่เก็บถนอมในกลีเซอรอลเย็น (RECONSTRUCTION OF PERFORATING CORNEA USING COLD GLYCEROL PRESERVED CANINE AMNIOTIC MEMBRANE IN DOGS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. สพ.ญ. ดร.นลินี ตันติวนิช, 64 หน้า.

เยื่อถุงน้ำคร่ำเป็นเยื่อชั้นในสุดของเยื่อหุ้มตัวอ่อนที่มีลักษณะบางและใส ใช้กันอย่างแพร่หลายในการรักษาทางจักษุวิทยาทั้งในคนและสัตว์เพื่อเป็นเนื้อเยื่อปลูกถ่ายทางชีวภาพ การใช้เยื่อถุงน้ำคร่ำชนิดสดทางคลินิกพบไม่มากนักเพราะไม่สะดวกและเสี่ยงต่อการติดโรค งานวิจัยนี้มีวัตถุประสงค์ คือ เพื่อศึกษาผลทางคลินิกของการรักษาสุนัขที่มีภาวะกระจกตาทะลุโดยการใช้เยื่อถุงน้ำคร่ำสุนัขที่เก็บถนอมในกลีเซอรอลเย็นเปรียบเทียบกับการใช้เยื่อถุงน้ำคร่ำมนุษย์ที่เก็บถนอมด้วยวิธีแช่เยือกแข็ง โดยเก็บเยื่อถุงน้ำคร่ำสุนัขภายหลังการผ่าตัดนำลูกออกทางหน้าท้องจากแม่สุนัข 4 ตัว ซึ่งมีสุขภาพแข็งแรงและได้รับวัคซีนที่จำเป็นครบทุกชนิด ทำการเพาะเชื้อเพื่อตรวจหาจุลชีพและประเมินลักษณะทางจุลกายวิภาคของเยื่อถุงน้ำคร่ำสุนัขชนิดสดและชนิดเก็บถนอมในกลีเซอรอลเย็นเป็นเวลา 1 เดือน รวมทั้งทำการประเมินผลทางคลินิกภายหลังการซ่อมแซมกระจกตา ผลการเพาะเชื้อไม่พบจุลชีพในทุกตัวอย่าง ลักษณะทางจุลกายวิภาคของเยื่อถุงน้ำคร่ำสุนัขประกอบด้วย 3 ชั้น ได้แก่ ชั้น epithelium ประกอบด้วยเซลล์รูปสี่เหลี่ยมทรงต่ำที่มีซิเลียเรียงตัวชั้นเดียว ชั้น basement membrane และชั้น stroma ที่มีเส้นใยคอลลาเจน พบว่าเยื่อถุงน้ำคร่ำสุนัขชนิดเก็บถนอมยังคงมีชั้น epithelium อยู่ มีเส้นใยคอลลาเจนที่อัดแน่นมากขึ้นและมีการจัดเรียงตัวที่ไม่เป็นระเบียบ เยื่อถุงน้ำคร่ำสุนัขชนิดเก็บถนอมบางลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับเยื่อชนิดสด ($p<0.05$) ความสำเร็จของการปลูกถ่ายเยื่อถุงน้ำคร่ำสุนัขที่เก็บถนอมในกลีเซอรอลเย็น เท่ากับ 87.5% (7/8 ตา) ส่วนเยื่อถุงน้ำคร่ำมนุษย์ที่เก็บถนอมด้วยวิธีแช่เยือกแข็ง เท่ากับ 83.33% (5/6 ตา) ในขณะที่สุนัข 1 ตัวที่ได้รับการปลูกถ่ายเยื่อถุงน้ำคร่ำสุนัขเกิดการโป่งออกของเยื่อปลูกถ่าย สุนัขอีกตัวที่ได้รับการปลูกถ่ายเยื่อถุงน้ำคร่ำมนุษย์เกิดแผลกระจกตาขึ้นใหม่ สรุป เยื่อถุงน้ำคร่ำสุนัขที่เก็บถนอมในกลีเซอรอลเย็นสามารถใช้เป็นเนื้อเยื่อปลูกถ่ายเอกพันธุ์ทางเลือกสำหรับการรักษากระจกตาทะลุในสุนัข

ภาควิชา ศัลยศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา ศัลยศาสตร์ทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2557

5575322331 : MAJOR VETERINARY SURGERY
KEYWORDS: AMNIOTIC MEMBRANE / CANINE / CORNEAL PERFORATION / GLYCEROL

SUKHUMAL LEEVIROJANA: RECONSTRUCTION OF PERFORATING CORNEA USING COLD GLYCEROL PRESERVED CANINE AMNIOTIC MEMBRANE IN DOGS. ADVISOR: ASST. PROF. .NALINEE TUNTIVANICH, D.V.M., Ph.D., 64 pp.

Amniotic membrane (AM) is the thin, translucent membrane lining at the inner layer of fetal membrane. It has commonly been used as a biological graft in human and veterinary ophthalmology. Clinical application of fresh AM is rare because of inconvenient use and risk of disease transmission. The objective of this study was to evaluate clinical outcomes of canine corneal perforation repair using cold glycerol-preserved canine AM compared to cryopreserved human AM. Canine AM was collected from 4 completely vaccinated, healthy bitches after cesarean sections. Micro-organism culture and histological study using light microscope were assessed in fresh and 1 month-preserved canine AM, as well as clinical outcomes after corneal reconstruction. None of micro-organism was found in all samples. Histologically, canine AM was composed of three layers; single layer ciliated low cuboidal epithelium, basement membrane and stroma containing collagen fibers. Preserved canine AM had intact epithelium and more compact collagen fibers with less organization. Preserved canine AM were significantly thinner than the fresh membrane ($p<0.05$). The success rate using glycerol-preserved canine AM was 87.5% (7/8 eyes) whereas that of using cryopreserved human AM was 83.33% (5/6 eyes). Protrusion of the membrane was found in one case receiving canine AM, while one receiving human AM had recurrent corneal ulcer. In conclusion, cold glycerol-preserved canine AM can be used as an alternative allograft for the treatment of corneal perforation in dogs.

Department: Veterinary Surgery Student's Signature .....
Field of Study: Veterinary Surgery Advisor's Signature .....
Academic Year: 2014

ACKNOWLEDGEMENTS

First, I am especially grateful to my kind advisor Asst. Prof. Nalinee Tuntivanich for her guidance, encouragement, support and the performance for all the surgical procedure in this study. However, this thesis would not have been possible if it had lacked many support from others.

I would like to sincerely thank my thesis committees, Prof. Marissak Kalpravidh, Assoc. Prof. Pinnita Tanthuvanit, Prof. emeritus Pranee Tuntivanich and Assoc. Prof. Anudep Rungsipipat for their valuable suggestions to fulfill this thesis.

I am most appreciative the support from The Thai Red Cross Society for guideline of amniotic membrane preparation.

I am grateful to Ophthalmology Clinic, small animal hospital, Faculty of Veterinary Science, Chulalongkorn University for their support and encouragement. I am also appreciated the assistance of the Department of Veterinary Surgery, Pathology, Obstetrics Gynaecology and Reproduction and Biochemistry Unit, Faculty of Veterinary Science, Chulalongkorn University, on collection, preparation and histological tissue processing of canine amniotic membrane.

I would like to thank the Graduated Student Fund, Faculty of Veterinary Science, Chulalongkorn University for financial support.

Finally, I would like to express my heartfelt thanks to my beloved parents, family and friends for their endless love, constant support, understanding, encouragement and always being there whenever I need. My thesis could not be succeeded without them all.

CONTENTS

Page

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AM	=	amniotic membrane
bFGF	=	basic fibroblast growth factor
cm	=	centimeter
DMEM	=	Dulbecco modified Eagle medium
EGF	=	epidermal growth factor
HLA	=	human leukocyte antigens
HGF	=	hepatocyte growth factor
HIV	=	human immunodeficiency virus
IL-1ra	=	interleukin-1 receptor antagonist
IL-10	=	interleukin-10
KGF	=	keratinocyte growth factor
kg	=	kilogram
mg	=	milligram
ml	=	milliliter
mm	=	millimeter
µg	=	microgram
PEDF	=	pigment epithelium-derived factor
PLR	=	pupillary light response
Psi	=	pounds per square Inch
TGF- β	=	transforming growth factor β
TIMP-1-4	=	tissue inhibitiors of metalloproteinase 1-4
TSP-1	=	thrombospondin-1
U	=	unit

CHAPTER I

INTRODUCTION

Importance and Rationale

Corneal perforation is the condition that part of the entire corneal thickness is lost. It results in leakage of aqueous humor, severe inflammation and intraocular infection. Immediate treatment is required to restore corneal integrity, prevent vision loss and minimize potential complications; such as secondary glaucoma, endophthalmitis and phthisis bulbi (Jhanji et al., 2011). Though therapeutic goals of corneal perforation are a repair of corneal wound, identification of the cause of corneal injury as well as prevention of further damage are very important (Solomon et al., 2002; Maggs, 2013). Among various surgical procedures to correct corneal injury, repairing corneal perforation with biological graft materials allows the graft to be integrated into corneal tissue during wound healing (Ledbetter and Gilger, 2013).

Amniotic membrane is one of the tissue grafts widely used in human and veterinary ophthalmology. It is a thin translucent membrane lining at the inner layer of the fetal membrane (Dua et al., 2006). Human AM has many biological properties scientifically proven to be beneficial as biomaterial in corneal transplantation. It enhances wound healing by promoting corneal epithelialization as well as being a scaffold for cell proliferation and differentiation. Several factors in human AM play a role in lowering inflammation, neovascularization and scar formation (Hao et al., 2000; Woo et al., 2001). In addition, with lack of immunogenicity, AM reduces risk of graft rejection. The success rate of transplantation therefore increases (Godoy-Esteves et al., 2013)

The use of fresh human AM in medical practice is rare. It is due to the fact that time of tissue collection is not well-suited for clinical application and time to evaluation of blood-borne infection is inadequate. Various methods have been developed to preserve AM (Baradaran-Rafii et al., 2008) to maintain histological and biological property comparable to the fresh membrane (Thomasen et al., 2011). Cryopreserved human AM has widely been used to reconstruct corneal surface in several conditions; such as corneal dermoid (Kalpravidh et al., 2009), bullous keratomalacia (Barros et al., 2005) and created deep corneal ulcer (Vongsakul et al., 2009).

The influences of different preservation methods on AM properties depend on media composition, storage temperature and preservation process. Cryopreserved human AM requires deep-freezing storage at -80ºC. Thus, it has become problematic to maintain stable storage temperature during transportation and before use therapeutically (Ravishanker et al., 2003). According to an increase demand for clinical use at present, human AM is commercially available at all times.

Glycerol has long been recognized as one of good preservatives. In addition to protecting cell integrity by replacing most of intracellular water through dehydration mechanism, glycerol stabilizes ionic concentration of preserved cells (Maral et al., 1999). Not only method of preparation can easily be obtained, preservation can be achieved at low temperature over a long period of time. Replacement of cryopreserved human AM by cold glycerol-preserved canine AM may overcome some pitfalls of human AM on availability for corneal reconstruction in dogs.

Research questions

1. Can glycerol be used for the preservation of canine AM?
2. Can cold glycerol-preserved canine AM be clinically used as a transplant for canine corneal perforation?
3. What are macroscopic histological findings of canine AM?

Objectives

1. To evaluate clinical reconstruction of canine corneal perforation with cold glycerol-preserved canine AM compared with cryopreserved human AM
2. To characterize histologically fresh and cold glycerol-preserved canine AM by light microscopy
3. To evaluate technique of canine AM preparation

Advantages of the Study

The cold glycerol-preserved canine AM could be used clinically as an alternative transplant for canine corneal perforation. It is practically prepared and its macroscopic structure remains at least one month of storage at 4°C.

CHAPTER 2

LITERATURE REVIEW

Cornea

Cornea is the outermost fibrous coat of the eye, which is curve, transplant and smooth. With its unique physiology, not only it serves for light refraction, it maintains ocular integrity and protects the eye from external environment. Corneal thickness varies among species. In dogs, the mean thickness was 545.6 21.7 μm (Martin-Suarez et al., 2014). The cornea has four layers, including epithelium, stroma, descemet's membrane and endothelium (Figure 1). Epithelium is the outermost layer consisting of five to seven cell layers in dogs. Non-keratinized stratified squamous cells lie at the surface, while polyhedral cells (wing cells) are located in the intermediate layer, followed by columnar cells (basal cells) at the deepest layer. Basal cells are attached to basement membrane. Corneal stem cells are located at the corneoscleral limbus thus corneal epithelial cells undergo regeneration and maturation throughout life. Stroma is the thickest layer of the cornea, constituting more than 90% of the entire thickness. It composes of keratocytes, collagen, water, and other ground substances. Lamellar arrangement of collagen fibers maintains corneal transparency. Disruption of stromal collagen fibril arrangement is the major cause of loss of transparency. Descemet's membrane is an elastic membrane lining in between stroma and endothelium. It has become thicken throughout life because it is secreted continuously by endothelial cells. Endothelium is the innermost layer of the cornea lining the anterior chamber. It is a one cell layer thick playing a role in transparency via sodium potassium pump. It helps stroma remain relatively dehydrated. (Maggs, 2013)

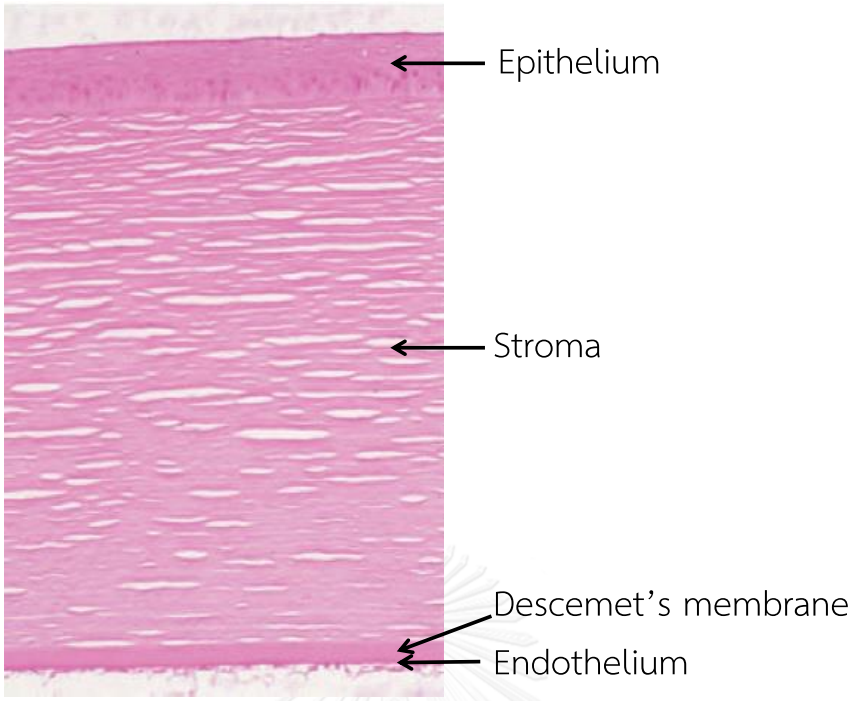

Figure 1. Histological structure of corneal layers
This picture is modified from Maggs, 2013.

Ulcerative Keratitis

Corneal ulcer is a condition that corneal epithelium is lost exposing underlying stroma or basement membrane. There are many underlying causes of corneal ulcer (Maggs, 2013); inadequate corneal protection such as keratoconjunctivitis sicca, facial nerve paralysis, ectropion, etc or excessive epithelial loss such as trauma, foreign body, abnormal hair growth, entropion, infectious disease, etc. Regardless of cause, depth and degree of corneal damage should also be considered. Blunt trauma is more widespread. It tends to cause globe rupture, intraocular hemorrhage and retinal detachment, while sharp trauma tends to be more localized and associated with lens capsule rupture.

Less than full thickness ulcer has good prognosis with appropriate therapy. Full thickness (perforating) wound with globe rupture have a poorer prognosis as compared to less than full thickness ulcer because of intraocular damage and greater tissue disruption at the wound edge (Ledbetter and Gilger, 2013). Differentiation of full

thickness corneal ulcer requires Seidel's test. It is a test in which fluorescein stain is applied to the cornea without being rinsed off. Positive Seidel's test reveals fluorescein dye within the anterior chamber, an indicative aqueous humor leakage (Ledbetter and Gilger, 2013). Careful examination must be made to evaluate the extent of corneal injury, not only for well plan of therapy but avoidance risk of further ocular damages. Ophthalmic assessment should therefore include menace response, dazzle reflex, pupillary light response, palpebral reflex, quantitative tear production, fluorescein staining test, examination of bright slit beam and thorough examinations of ocular adnexa. Penetrating corneal injury can lead to complications such as uveitis, rupture of lens capsule, endophthalmitis, secondary glaucoma, permanent blindness and phthisis bulbi.

Corneal perforation is a cause of profound vision loss. It is a great deal of discomfort for dogs. Clinical signs (Figure 2) include epiphora, photophobia, blepharospasm, conjunctival hyperemia, corneal edema, miosis, aqueous leak and iris prolapse (Jhanji et al., 2011).

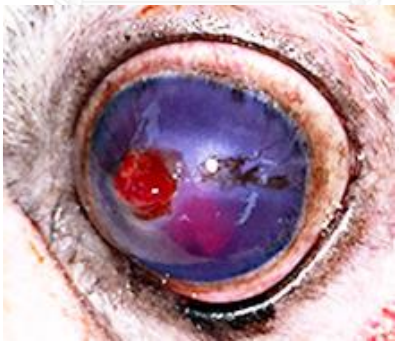

Figure 2. Canine penetrating cornea
Note that fibrin plug and blood clot appears at the corneal wound edge.

Corneal Wound Healing

Corneal wound healing is a complex mechanism, which is part of body reaction to minimize unwanted end results. Many factors play a role in wound healing process, such as causative agents, size and cause of the wound, property of tear film and extra cellular matrix (Ledbetter and Gilger, 2013). Healing process involves all layers when the cornea is fully penetrated. Epithelial wound healing involves three separate phases; latent, cell migration and cell proliferation. During initial latent period, movement of existing basal cells occurs within several hours after injury, followed by association of leukocytes at wound margin. Production of cellular processes on the basal edges of cells occurs toward the final stage of latent phase. The second phase of healing process involves migration of epithelial cells across the wound surface in a centripetal fashion. Extra cellular proteins then provide matrix to migrating cells, thus anchoring to the basement membrane. Tight adhesion between epithelium to stroma replies on hemidesmosomal attachment, which is complete within 36 hours after injury. The final phase of corneal healing process is epithelial cell proliferation originated from corneal stem cells at the limbus.

In stromal healing process, blood vessels may be in growth from the limbus as part of inflammatory reactions. Vascular ingrowth begins 3-4 days following corneal stromal injury. Keratocytes undergo proliferation and migration triggered by cytokines. After 48-72 hours of stromal damage, fibroblasts are transformed and increased in population via cell mitosis. Newly synthesize collagen fibrils result in non-uniform organization of cells; irregularity of corneal spaces, disruption of corneal transparency, and then scar formation. Stromal remodeling however occurs at the late stage to re-organize collagen fibrils arrangement.

When endothelium is damaged, its integrity is crucial to maintain stromal transparency. Loss of endothelial active transport and barrier function causes imbibition of water from the tear film and aqueous humor into stroma leading to severe corneal edema. Endothelial healing process significantly depends on enlargement of cells and cell movement rather than replications. Sliding of a single layer endothelium occurs, in corneal perforation, over stromal and descemet's

membrane. Fibroblast-liked cells, collagen fibrils and membrane proteins are then formed, as a result of endothelial healing process (Steele, 1999).

Treatment of Corneal Perforation

Therapeutic goals of corneal perforation include immediate sealing of aqueous leak, identification and correction of underlying cause, restoration of tectonic integrity of the perforation site, as well as prevention of further damage from self-trauma (Solomon et al., 2002; Maggs, 2013). Other important therapeutic considerations are topical antibiotics and mydriatic agent, systemic anti-inflammatory medication and anti-collagenases. Optimizing epithelial healing can also be achieved by replenishing the eye's moisture.

The treatment of corneal perforation can be attempted in several ways. In cases of small corneal perforation, bandage contact lens may be suitable as temporary management before definitive treatment (Rodríguez-Ares et al., 2004). Bandage contact lens is a noninvasive technique, for which general anesthesia is not required. It helps to promote epithelial resurfacing and reduce ocular pain. As an alternative, conjunctival flap, third eyelid flap or tarsorrhaphy may be considered to stimulate corneal healing with more blood vessels. However these techniques have high risk of aqueous humor leakage from pressuring the eyeball.

Tissue adhesive may be considered primary treatment or adjunctive therapy. The goal of tissue adhesive is to promptly restore globe integrity. It is easy to use and highly effective in non-infective corneal defect. However, it may cause and recurrent leakage because of glue dislodgment or fast degradation (Duchesne et al., 2001), intraocular structure adhesion, worsening of infectious keratitis and glaucoma. Multiple re-applications are not recommended because it may enlarge perforating defect. Furthermore, cyanoacrylate glue potentially has the toxic effects on intraocular structures (Watté et al., 2004). Corneal suturing is another indicative for small corneal perforation for good wound edge alignment. However, caution is taken in direct correction of circular or irregular wound because of astigmatism (Maggs, 2013) especially in humans.

In cases of large corneal perforations, conjunctival grafts, transplantation of natural materials or corneal transplantation are recommended (Barros et al., 1998; Goulle, 2012; Ledbetter and Gilger, 2013; Maggs, 2013). Conjunctival graft brings superficial blood vessel that continuous supplies serum, which contains anti-collagenases and growth factors. Not only drugs systemically administered could also be easily delivered to the corneal ulcer through these initiated vessels (Maggs, 2013), conjunctiva serves as a good source of fibroblasts that would form collagen fibrils for corneal stromal regeneration. Conjunctival graft may be however inappropriate for large perforation because the leakage may continue under the graft. Loosing donor site may cause inadequate healthy conjunctiva for further surgery (Jhanji et al., 2011) and dense connective tissue of conjunctiva causes poor cosmetic.

Biological membranes derived from various tissue types have widely been use as a transplant for full thickness corneal defects; such as porcine small intestinal submucosa (Vanore et al., 2007), equine pericardium (de Moraes KARKOS et al., 1995), equine renal capsule (Laus et al., 1999) and amniotic membrane (Barros et al., 1998). Among these, cornea seems to be a definitive transplant material in terms of reducing scar formation, decreasing corneal surface irregularity and good visual rehabilitation. There is always risk of recurrence of infection especially in the deeper corneal layer (Jhanji et al., 2011; Goulle, 2012). In veterinary ophthalmology, corneal donor is still unavailable.

Amniotic Membrane

Amniotic membrane (AM) or amnion is the thin, translucent membrane lining at the inner layer of fetal membrane. It contains amniotic fluid and fetus. Human AM, which is free of smooth muscle, nerve fiber, blood and lymphatic vessel, is adjacent to the underlying chorion (Dua et al., 2006). On the other hand, canine AM does not attach with the chorion. It is enveloped by allantois (Figure 3). In the early stage, canine AM is avascular, but then become vascularized by blood vessels from allantois in the later stage of pregnancy (Miglino et al., 2006). Dogs have zonary placenta with complete placental girdle while cats have incomplete one. Placental girdle

characterized by extravasation of blood from maternal capillaries, is started to be apparent from 22-25 days of pregnancy. It is visible in between the two green borders of maternal hematomas, which transfers iron to the fetus (Miglino et al., 2006).

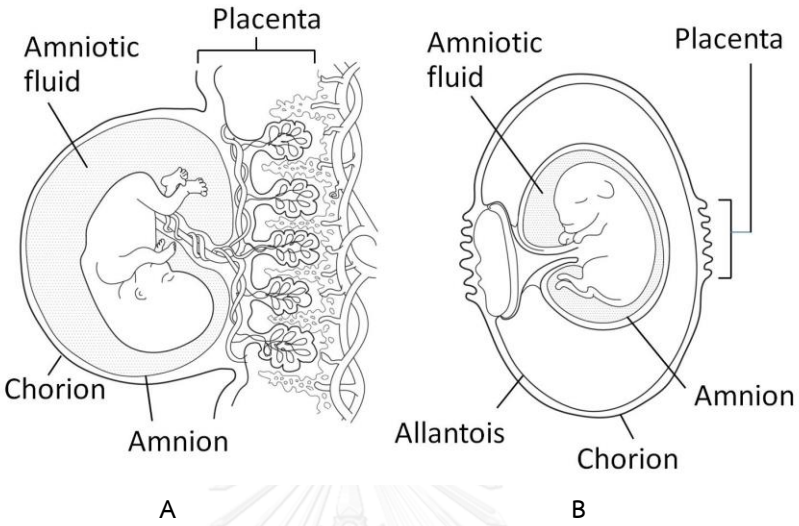

Figure 3. Human (A) and canine (B) fetal membrane.

Human AM has five layers (Figure 4). Epithelium is the innermost layer, laying on the basement membrane connecting to amniotic mesoderm or stroma, and adjacent to amniotic fluid. It consists of a single layer of cuboidal to columnar cells with microvilli and lays. Stroma is composed of three components; compact stromal layer, fibroblast layer and spongy layer. All three layers are difficult to distinguish on histologic section. While compact stromal layer contains collagen and elastic fibers, fibroblast layer consists of collagen, fibroblast and few macrophages. The outermost spongy layer is composed of loose collagen fiber bundles with few fibroblasts. Because spongy layer is incompletely fused with the chorion, human AM is easily separated from it (Baergen, 2005; Baradaran-Rafii et al., 2008; Cirman et al., 2014).

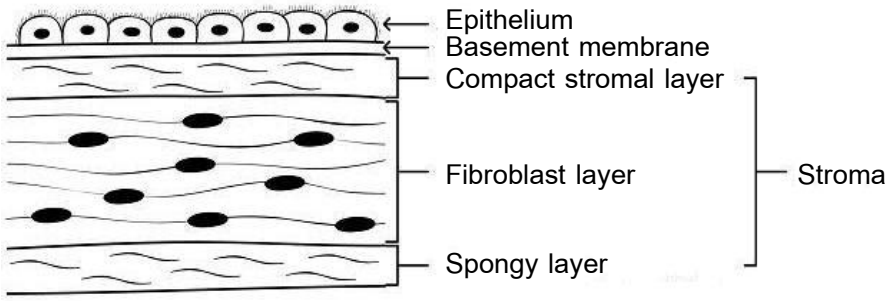

Figure 4. Histological structure of human amniotic membrane.

Amniotic membrane plays many roles during embryologic development. It is essential for structural integrity and junctional permeability of fetal membranes. It plays an important role during the onset of labor including initiation and maintenance of uterine contractions. It also appears to be responsible for resorption, secretion and pH regulation of amniotic fluid (Baergen, 2005).

Biological Property of Human Amniotic Membrane

Human AM has many biological properties that benefit to ophthalmology practice. It promotes epithelialization of the cornea by preventing keratocyte apoptosis and supporting corneal epithelial migration and differentiation (Lee and Tseng, 1997; Woo et al., 2001). Epithelialization is stimulated by many growth factors; such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), keratinocyte growth factor (KGF) and basic fibroblast growth factor (bFGF) (Koizumi et al., 2000). Various types of collagen fibers in human AM serve as substrates for corneal wound healing. Collagen type I, III, V and Vl are present in stroma while collagen type IV and VII are majorly found in basement membrane (Malak et al., 1993; Fukuda et al., 1999). Human AM has extracellular matrix components (e.g. heparan sulfate, fibronectin and laminin) which are good substrates for not only the growth of corneal epithelium but also integration of stroma to the surface of corneal wound (Cooper et al., 2005). In addition, the membrane acts as bandage contact lens to promote corneal wound healing as

well as presents inhibitory effect on many proteinase enzymes, causing persistent corneal destruction (Seitz et al., 2006; Na et al., 1999).

Human AM has many superior benefits to decrease inflammatory reactions. It expresses various anti-inflammatory proteins such as interleukin-1 receptor antagonist (IL-1ra) and interleukin-10 (IL-10) (Hao et al., 2000). With a property to prevent infiltration of polymorphonuclear cells, T and B lymphocytes, it induces these cells to enter apoptosis mechanism (Park and Tseng, 2000; Shimmura et al., 2001; Li et al., 2005). In addition to reduce proinflammatory cytokines such as IL-1α and IL-1β (Solomon et al., 2001) and decrease neovascularization (Cirman et al., 2014), human AM also expresses anti-angiogenic protein such as collagen XVIII, thrombospondin-1 (TSP-1), tissue inhibitiors of metalloproteinase 1-4 (TIMP 1-4), and pigment epithelium-derived factor (PEDF) (Hao et al., 2000; Shao et al., 2004). Furthermore, it inhibits expression of transforming growth factor β (TGF-β) receptors in fibroblasts and suppresses TGF-β signaling that causes less fibrosis and less scar formation in order to maintain corneal transparency (Tseng et al., 1999).

Human AM has antimicrobial and antiviral properties. There was a report that amnion cells express histones H2A and H2B, both of which exhibit antimicrobial and endotoxin-neutralizing activity (Kim et al., 2002). The amnion also expresses antimicrobial molecules such as β-defensin and elafin (King et al., 2007) as well as antiviral molecule; cystatin E, which functions as cysteine proteinase inhibitor (Ni et al., 1997).

Lack of immunogenicity property of human AM is scientifically known even though expression of human leukocyte antigens (HLA) is still controversial (Cirman et al., 2014). Human AM has ability to suppress T lymphocyte proliferation in allograft limbus cells (Ueta et al., 2002). Number of inflammatory cells and blood vessels in rabbits that respond to amniotic xenografts is significantly higher than the allografts, while no clinically significant difference is evident. Despite presence of viable cells in fresh and 6 month-cryopreserved AM expressing HLA class I antigens (Kubo et al., 2001), the least possible immunogenicity of human AM is still preferred in order to increase success rate of tissue transplantation (Godoy-Esteves et al., 2013).

Amniotic Membrane Preparation

There are several methods of AM preparation and preservation. According to many biological properties, fresh AM is more preferable to preserved membrane. The use of fresh AM in medical practice is however rare. This may be due to the fact of time limitation. Time of tissue collection is not well-suited for clinical requirement. Time from collection to evaluation of blood-borne infection is inadequate. Normally, maternal donors are serologically screened for human immunodeficiency virus (HIV), Hepatitis B and C, and syphilis (Baradaran-Rafii et al., 2008). Serological retest of the donor is also not possible. Time to screen infection that may have occurred during tissue preparation is very important. In addition, amniotic membrane that is freshly prepared but not immediately being used will have to be discarded (Mejia et al., 2000; Adds et al., 2001). Various methods have therefore been developed to preserve AM because preserved membrane relatively maintains histological and biological property compared to fresh membrane (Thomasen et al., 2011). Various preservation methods are used including heat-dried, air-dried, freeze dried, cryopreserved and cold glycerol-preserved.

Heat-dried technique: AM was prepared by drying AM overnight in an oven at 40 ± 2°C, followed by sterilization with the use of gamma radiation. According to this technique, AM loses many biological properties due to the high temperature. Heat-dried AM is therefore often used as a management of burn wound (Bari et al., 2002).

Air-dried technique: after separating and washing, AM is flattened and dried overnight in a laminar flow cabinet. The membrane is later on packed and sterilized by gamma radiation. Although high temperature is not applied in this method, some properties of the AM are still altered due to dehydration (Singh et al., 2003). It is often used for wound dressing.

Lyophilized or freeze-dried technique: AM is cut into pieces prior to being rapidly frozen at -50 to -80°C. After that, the membrane is dried under high vacuum using a freeze drier device. Tissue water is extracted reaching a final water content of 5-10%. The membrane is then packed and sterilized using gamma radiation (Nakamura

et al., 2004). When it is well prepared, it can be used for wound management. This technique is rather complex and more complicated compared to other techniques.

Amniotic membranes that are heat-dried, air-dried and freeze dried can be stored at room temperature for a long period of time without deterioration. They are easy to transportation. These techniques are however considered complicated and required specialized equipment making high cost of the membranes ((Ravishanker et al., 2003; Baradaran-Rafii et al., 2008).

Cryopreserved technique: AM is more commonly cryopreserved as a tissue graft in humans and animals. The membrane is initially processed as described in other preservation techniques. However it is preserved in glycerol which is cryoprotective agent, combined with cell culture freezing media; such as Dulbecco modified Eagle medium (DMEM) at a ratio of 1:1. Antibiotic and antifungal agents are added. Cryopreserved AM is stored at -80°C (Lee and Tseng, 1997). Although this type of preserved AM maintains maximum biological properties compared to other methods, the technique requires extraordinary deep freezing facility (Riau et al., 2010) to maintain stability of storage temperature during transportation and during storage at private practice before use. Cryopreserved human AM is a few available in Thailand.

The use of multilayer cryopreserved human AM for the treatment of corneal perforation in humans revealed complete epithelialization without recurrence within 1 month (Prabhasawat et al. (2001) Leakage of aqueous humor was found in one case. In veterinary ophthalmology, Kalpravidh et al. (2009) reported the use of cryopreserved canine AM as a transplant for corneal dermoids in dogs. Epithelialization was completed within 2 weeks after reconstruction. Corneal neovascularization and scar disappeared by 8 weeks after surgery.

Glycerol preserved technique: glycerol has long been recognized as a preservative. It protects cell integrity by replacing most of intracellular water through dehydration mechanism while stabilizing ionic concentration of cells (Kim and Tseng, 1995). Cold glycerol-preserved AM is usually stored in 85-100% glycerol at 4°C. This technique is simple, easy and low cost requiring (Kim and Tseng, 1995; Maral et al., 1999; von Versen-Hoeynck et al., 2008). Cold glycerol-preserved AM for wound dressing can be kept at 4°C for over a year (Maral et al., 1999). To avoid toxicity from highly

concentrated glycerol, preserved AM should be soaked in isotonic solution prior to transplantation (Thomasen et al., 2011).

The use of glycerol preserved equine AM was reported in created canine corneal perforation with a single layer graft inlay technique (Barros et al. (1998). While leakage of aqueous humor was not found, bulging of AM was noticed in majority of cases. Glycerol preserved equine AM was applied as a single layer graft inlay technique for feline corneal sequestrum (Barachetti et al. (2010). Five of the seven eyes were successful with minimal level of corneal scar and fairly good transparency. However in one cat, one eye showed a high level of bacterial contamination and necrosis of the AM at 2 weeks after reconstruction. The other eye developed corneal perforation under the amniotic membrane.

The Use of Amniotic Membrane

Fetal membranes were firstly used for skin transplantation by Davis (1910). In ophthalmology, De Rotth (1940) was the first one who used fetal membranes for the treatment of conjunctival defect. Air-dried preserved AM firstly applied in ophthalmic surgery was introduced by Sorby and Symons (1946) in patients with ocular chemical burns. Five decades later, the success of repairing corneal defects in rabbits by Kim and Tseng (1995) propelled AM into the ophthalmic practice.

In human, amniotic membrane transplantation can be performed in a variety of corneal conditions; such as deep corneal ulcer, corneal perforation, persistent epithelial defects, acute chemical burn, bullous keratopathy, removal of epithelial or subepithelial corneal lesions, and limbal stem cell deficiency (Meller et al., 2000; Anderson et al., 2001a; Anderson et al., 2001b; Prabhasawat et al., 2001; Solomon et al., 2002; Espana et al., 2003). Besides, it can be used in patients with conjunctival defects; such as symblepharon, pterygium, covering defects after removal of large conjunctival lesions and covering scleral graft (Prabhasawat and Tesavibul, 2001). It is also applied as biological wound dressing for burn injuries instead of skin graft (Ravishanker et al., 2003). Not only it prevents further bacteria contamination but also

reduces loss of water, protein and evaporative heat at the wound surface (Maral et al., 1999; Ravishanker et al., 2003).

In veterinary, AM has been used in variety of ophthalmic conditions, that are corneal ulcer, keratomalacia, corneal perforation, corneal sequestrum, symblepharon, corneal or conjunctival mass and ocular dermoid. Several animals reported using AM included dogs, cats, houses and rabbits. The sources of AM that using in veterinary ophthalmological surgery are from humans (Kim and Tseng, 1995; Plummer, 2009), dogs (Barros et al., 2005; Kalpravidh et al., 2009), horses (Barros et al., 1998; Barachetti et al., 2010), pigs (Tsuzuki et al., 2008), cows (Kim et al., 2009) and rabbits (Godoy-Esteves et al., 2013).

Amniotic membrane was also scientifically used in some other ways. Membranes from human (Pirjali et al., 2013), dog (Park et al., 2012) and horse (Seo et al., 2013) were found to be a rich source of stem cells that could be applied in other surgeries in the future. Human AM was used as a scaffold biomaterial for tissue engineered blood vessel fabrication (Lee et al. (2012). Cultivated corneal or limbal epithelial stem cells using human AM as a carrier were transplanted in several ocular surface disorders (Koizumi et al., 2001; Shimazaki et al., 2002). Topical application of culture supernatant from human amniotic epithelial cell decreases inflammation in canine created-corneal ulcer (Wichayacoop et al. (2009). Human AM was effective in reducing epidural adhesion after laminectomy in canine model (Tao and Fan (2009).

Techniques of Amniotic Membrane Transplantation

There are four basic principles of amniotic membrane transplantation.

- Patching or overlay technique (Figure 5A) is a method that a single layer of AM is spread over the corneal defect or the whole cornea. Edges of the membrane are secured to peripheral cornea or limbus in order to protect the healing ocular surface beneath, like bandage contact lens. Corneal epithelium will later on grow under the AM. This technique is recommended for shallow stromal defect.
- Grafting or inlay technique (Figure 5B) is a method that a single layer of AM is placed on the ocular surface defect with epithelial side up, and then fixed at the

edge of the defect. AM is served as a scaffold for epithelial cell allowing migration and proliferation on it. This technique is recommended for shallow stromal defect. Time of wound healing process is comparable between overlay and single layer inlay technique (Letko et al., 2001).

- Multiple inlay technique (Figure 5C) is similar to a single layer in lay technique but instead, multiple membranes are fixed at the edge of the defect. AM is served as a scaffold but stronger than single inlay technique. This technique is recommended for deep stromal defect or corneal perforation (Seitz et al., 2006; Cirman et al., 2014). Wound healing process from the defect treated with multilayer inlay technique is faster than a single layer inlay technique. Multiple layers of AM would provide more of growth factors required for healing of epithelium (Prabhasawat et al., 2001).
- Combination of overlay and inlay technique with single or multilayer of membranes (sandwich technique) (Figure 5D) is more suitable for very deep corneal defect or corneal perforation with complete endothelial decompensation or intense stromal infiltration (Seitz et al., 2006; Cirman et al., 2014).

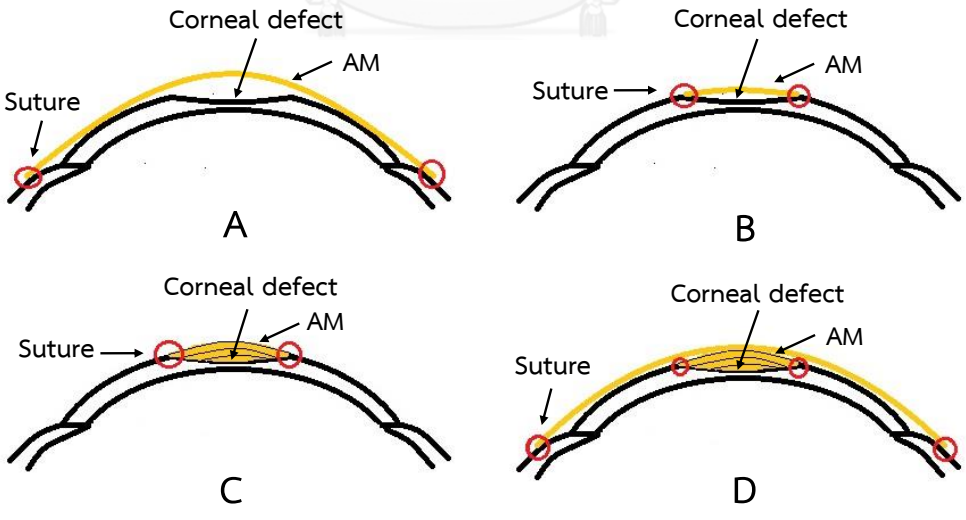

Figure 5. Techniques of amniotic membrane transplantation.

A) Overlay technique; B) Inlay technique; C) Multilayer technique; D) Sandwich technique.

CHAPTER 3

Materials and Methods

This study was composed of 3 parts.

Part I. Preparation of cold-glycerol preserved canine AM

Part II. Light microscopic evaluation of fresh and cold glycerol-preserved canine AM

Part III. Evaluation of clinical outcomes after corneal reconstruction with cold glycerol-preserved canine AM and cryopreserved human AM

Part I. Preparation of cold glycerol-preserved canine AM

Animals:

Canine placenta was collected from four completely vaccinated, healthy bitches, all of which carried the first pregnancy and underwent cesarean sections. Age of dogs was ranged from 1-3 years. The donors were initially evaluated for possibility of blood-borne infection by taking thorough history of previous systemic infection and performing physical examinations. Absolute aseptic technique was applied at all stages of tissue collection.

Preparation of solutions

Equipment and nitrocellulose papers were prepared (Figure 6) and sterilized at 160 psi and 126ºC. Preparation of solutions was performed in a laminar flow hood.

Washing solution:

Ingredients:

- 0.9% normal saline solution
- 40 mg/ml gentamicin sulfate (General Drug House, Bangkok, Thailand)
- 10,000 U/ml penicillin-streptomycin (Biochrom, Berlin, Germany)
- 5 mg/ml amphotericin B (A 9528, SIGMA, Jerusalem, Israel)

Preparation:

1. Mix 0.1 mg/ml gentamicin (2.5 ml), 2.5 µg/ml amphotericin B (0.5 ml) and 50 U/ml penicillin-streptomycin (5 ml) into a beaker containing 1000 ml of 0.9% normal saline solution at room temperature.
2. Stir well.

Storage solution:

Ingredients:

- 99.5% glycerol (VWR International, England)
- 40 mg/ml gentamicin sulfate
- 10,000 U/ml penicillin-streptomycin
- 5 mg/ml amphotericin B

Preparation:

1. Mix 0.1 mg/ml gentamicin (0.25 ml), 2.5 µg/ml amphotericin B (0.05 ml) and 50 U/ml penicillin-streptomycin (0.5 ml) into a flash containing 100 ml of 99.5% glycerol at room temperature.
2. Stir well.

Figure 6. Equipment for canine AM preparation.

Preparation of cold glycerol-preserved canine AM:

Canine AM was immediately separated after parturition from placenta nearby umbilical cord and rinsed with 0.9% normal saline solution to remove blood clots and uteroverdin under sterile condition (Figure 8A). Care was taken to avoid tearing of the membrane. Part of membrane that was highly vascularized was discarded. Epithelial side of the membrane was placed against sterile gauzes soaked with 0.9% normal saline solution to maintain moisture; edges of the membrane were fixed with 26-gauge needle in a sterile tray with cover. The membrane was then transferred to a laminar flow hood (Figure 7) for washing process. The membrane was slightly stretched in an upward position, and then rinsed with the freshly prepared washing solution on both sides for up to 2 minutes (Figure 8B). To inspect contamination during fresh canine AM preparation, membranes from various puppies of the same bitch was randomly selected. Part of the membrane was cut into 6x20 mm^2, immersed in 0.9% normal saline solution in an Eppendorf tube and submitted for fungus and bacterial culture (aerobic and anaerobic bacteria).

After that, the membranes with the epithelial surface up were placed on 2x2 cm^2 nitrocellulose papers (Figure 8C), cut into pieces, and then stored in freshly prepared storage solution at 4°C (Figure 8D) until use. All glass bottles were well labeled with date of tissue collection. Inspection of contamination was repeatedly performed after canine AM had been stored for up to one month. Sample from each

batch of preparation was randomly selected for fungal, aerobic and anaerobic bacterial culture.

Figure 7. Processing of glycerol-preserved canine AM in a laminar flow hood.

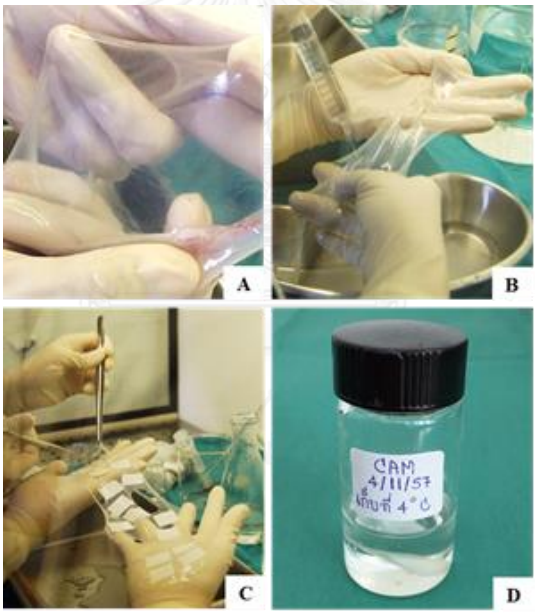

Figure 8. Preparation of canine AM.

A) Fresh canine AM was gently stretched and selected for the area with the least blood supplies; B) Canine AM was rinsed with the washing solution; C) The membrane was placed with the epithelial surface up on 2x2 cm^2 nitrocellulose papers and cut into pieces; D) Canine AM was stored in the storage solution.

Part II. Light microscopic evaluation of canine AM

Sample collection:

Fresh canine AM was placed on nitrocellulose paper and 1 month-preserved canine AM were randomly selected per maternal donor. The preserved membranes were rehydrated in 0.9% normal saline for 2 minutes.

Fresh and 1 month-cryopreserved human AM from The Thai Red Cross Society, Bangkok was collect from 1 mother.

Membranes of both species were cut into 6x20 mm^2 for tissue fixation.

Sample preparation:

Samples were fixed in 4% paraformaldehyde in 0.1M phosphate buffer solution at 4°C for 24 hours. They were processed, paraffin embedded, followed by being stained with hematoxylin-eosin (H&E) and Masson's trichrome for evaluation of microscopic appearances.

Part III. Evaluation of clinical outcomes after corneal reconstruction with cold-glycerol preserved canine AM and cryopreserved human AM

Animals:

Fourteen dogs (14 eyes) with 3-6 mm in diameter of corneal perforation were included in this study. All dogs were presented at the Ophthalmology Unit, Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Dogs had never had ophthalmic surgeries prior to participate in this study. All dogs were assigned into 2 groups with randomized single-blinded technique. Dogs in control group (n=6) received corneal reconstruction with the use of cryopreserved human AM, retrieved from The Thai Red Cross Society, Bangkok, Thailand. Dogs in experimental group (n=8) received corneal reconstruction with the use of cold glycerol-preserved canine AM, from part I, that had been stored at 4°C for no longer than one month. All procedures were performed under informed consent of the owners of each dogs. Trial procedures were approved by Chulalongkorn University Animal Care and Use Committee, Bangkok, Thailand. (No. 1431053)

History was well taken the owner of the dogs including cause and duration of injury, previous treatment and clinical signs. Ophthalmic examinations included neuro-ophthalmic responses (menace response, dazzle reflex, pupillary light response; PLR and blink reflex), fluorescein staining test, slit-lamp biomicroscopy, Schirmer tear test I (STT I), and measurement of intraocular pressure by rebound tonometry (Tonovet®, Icare Finland, Helsinki, Finland). Lesions on the cornea were investigated and photographed.

Anesthetic procedures:

Complete blood counts, blood chemistry profiles (serum glutamic pyruvic transaminase; SGPT, alkaline phosphatase; ALP, blood urea nitrogen; BUN, and creatinine) and presence of blood parasites were assessed prior to anesthesia. Dogs were withheld water and food for 6 and 12 hours before anesthesia, respectively. Routine physical examinations were performed to evaluate body temperature, heart rate, respiratory rate, mucous membrane and hydration status. 25 mg/kg cefazolin sodium (Zefa M.H.®; M&H manufacturing Co. Ltd., Samutprakarn, Thailand) and 0.5mg/kg dexamethasone sodium phosphate (Lodexa®; L.B.S.laboratory Ldt., Bangkok, Thailand) were intravenously administered.

Dogs were premedicated intravenously with 0.03 mg/kg acepromazine maleate and 0.3 mg/kg morphine sulfate. General anesthesia was induced intravenously with 4 mg/kg of 1% propofol (Lipuro®; B. Braun, Melsungen, Germany). The anesthesia was maintained with 2% isoflurane (Aerrane Isoflurane USP®; Baxter Healthcare of Puerto Rico, Puerto Rico, U.S.A.) in oxygen. All dogs received lactated ringer's solution intravenously at the rate of 10 ml/kg/h until fully recovered.

Surgical procedure:

The dog was positioned in lateral recumbency with the affected eye upward. By standard aseptic technique, periocular skin, conjunctival fornix and ocular surface were prepared. After surgical drape was placed and secured with towel clamps, eyeball was stably fixed with fixation forceps.

The corneal wound was prepared for surgery. Fibrin plug or necrotic tissue was gently removed (Figure 9A). For concurrent anterior staphyloma, attachment of the iris to the corneal wound edge was separated, and the iris was then repositioned. In the control group, the cryopreserved human AM stored at -80ºC was transplanted to the surgical site. While in the experimental group, canine AM stored at 4 ºC was rehydrated in 0.9% normal saline solution for 2 minutes prior to transplantation.

Transplantation of AM was performed using multilayer graft inlay technique (Prabhasawat et al., 2001). A single piece of AM was initially fixed within the perforating site as a bedding (Figure 9B). Two sheets of AM were trimmed in order to fit to the shape of corneal wound, followed by placing more sheets on with the epithelial side up (Figure 9C). These sheets of AM were anchored to the corneal wound edge by 8-0 polyglactin 910 (Vicryl®, Ethicon, Edinburgh, UK) with interrupted suture pattern. Third eyelid flap was performed using 6-0 silk (Mersilk®, Ethicon, Edinburgh, UK) as secondary support to the wound (Figure 10). All dogs received subconjunctival injection of 4 mg gentamicin sulfate combined with 0.4 mg dexamethasone sodium phosphate.

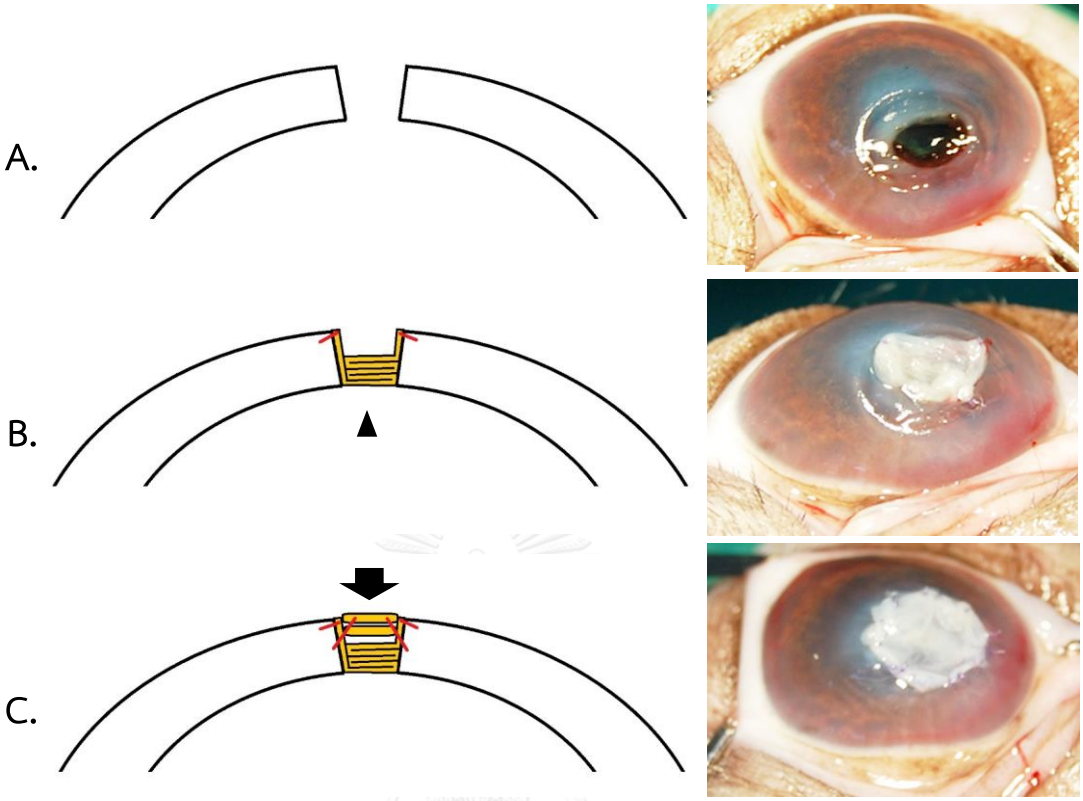

Figure 9. Transplantation of amniotic membrane.
A) Corneal perforation site was prepared; B) A single piece of AM (arrowhead) was initially fixed within the perforating site. C) Two sheets of AM (arrow) were sutured with the epithelial side up to edges of the corneal wound.

Figure 10. Subconjunctival injection and third eyelid flap.

Post-operative care:

Dogs were directed to wear Elizabethan collars at all times after surgery. Systemic administration included 25 mg/kg cephalexin and 0.5 mg/kg prednisolone (orally) for 2 weeks, at which dose of prednisolone was tapered thereafter. Meanwhile, 0.5% levofloxacin (Cravit®, Santen Pharmaceutical, Japan) was topically given every 3 hours, together with artificial tear. Topical 1% atropine sulfate (1% Isopto® atropine, Alcon-Couvreur, Puurs, Belgium) was administered four times a day. The third eyelid flap was removed 1 week after transplantation. As soon as corneal epithelialization was complete, levofloxacin and atropine sulfate were discontinued whereas combination of polymyxin B, neomycin and 0.1% dexamethasone (Maxitrol®, Alcon-Couvreur, Puurs, Belgium) was topically introduced twice daily and 1% oily-based cyclosporine was topically administered at the same frequency. Dogs received artificial tears throughout the study. Ophthalmic examinations were performed at day 7, 14, 21, 28, 42 and 56 post-surgery.

Data collection and analysis

Part I. Preparation of cold glycerol-preserved canine AM

1. Developing techniques of canine AM preparation and preservation were descripted.
2. Micro-organisms in fresh and 1 month-preserved canine AM were identified.

Part II. Light microscopic evaluation of canine AM

1. Fresh and 1 month-preserved canine AMs were examined by light microscopically characterized and histologically compared to fresh and 1 month-cryopreserved human AM. Distribution of collagen in the canine and human membranes specially stained with Masson's trichrome were investigated and compared.

2. Thickness of fresh and preserved AM; epithelium, stroma and the entire membrane, was measured by using i-Solution program (IMT i_Solution Inc., Vancouver, BC, Canada) (Figure 11A). Mean thickness ± SE was calculated from five different

locations (Figure 11B). Dependent t- test was applied to compare the thickness between fresh and preserved AM at the significant level of $p<0.05$ using SPSS program (version 17, IBM Corporation, Armonk, New York, USA).

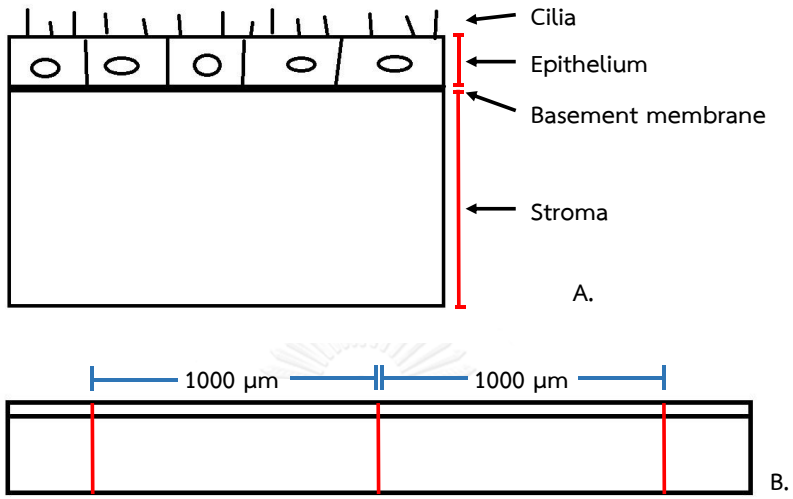

Figure 11. Measurement of amniotic membrane thickness.
A) Measurement of each layer of the membrane; B) Five locations randomly selected for thickness measurement.

Part III. Evaluation of clinical outcomes after corneal reconstruction with cold-glycerol preserved canine AM and cryopreserved human AM

Ophthalmic examinations were examined in all dogs at day 7, 14, 21, 28, 42 and 56 after transplantation.

- Serial photography: to investigate corneal reactions (edema, neovascularization, pigmentation, granulation and scar formation).
- Neuro-ophthalmic examinations: to observe menace response, dazzle reflex, pupillary light response, and blink reflex)
- Fluorescein staining test: to reveal time of complete epithelializatioin
- Seidel test: to reveal leakage of the aqueous humor
- Schirmer tear test I: to investigate changes of tear level among different time points in each group of experiment with the use of repeated measured ANOVA at the

significant level of $p<0.05$ using SPSS program. Difference of STT I level between group of experiment was statistically assessed using independent t- test at the significant level of $p<0.05$ using SPSS program.

- Tonometry: to investigate changes in intraocular pressure among different time points in each group of experiment with the use of repeated measured ANOVA at the significant level of $p<0.05$ using SPSS program. Difference of IOP level between group of experiment was statistically assessed using independent t- test at the significant level of $p<0.05$ using SPSS program.
- Complications: to investigate any ophthalmic complications after transplantation
- Surgical success was determined by three criteria; complete epithelialization, no recurrence of corneal perforation at the same region and no aqueous humor leakage. Percentage of success was determined.

CHAPTER 4

RESULTS

Part I. Preparation of cold-glycerol preserved canine amniotic membrane

1. Developing techniques of canine AM preparation and preservation

1.1 Donors

All AMs used in this study were collected from four healthy Chihuahua, which had 3-6 puppies per litter.

1.2 Separation of canine AM from placenta

After cesarean section, separation of canine AM from placenta was performed in a laminar flow hood to prevent contamination. However, the membrane was permanently stained with uteroverdin, which was unique green secretion of canine placenta. To avoid uteroverdin staining, separation of canine AM was performed in the operation unit immediately after cesarean section. Following separation, amniotic membrane was gently washed several times with 0.9% sterile normal saline solution.

1.3 Identification of canine AM epithelium

Because AM had to immediately be separated, it was problematic to identify epithelium of the amniotic membrane in the operation room. Identification method using very small suture material to keep track of epithelial side was unsuccessful. Method for identification was then adapted by placing the inner side of AM (epithelium)

against sterile gauze well soaked with 0.9% sterile normal saline solution (Figure 12A). Edges of the membrane were stably fixed with needles to avoid slippery and curling. Because the membrane was quite thin and fragile, it was therefore moistened at all time before being transferred to laminar flow hood for further tissue process.

1.4 Washing canine AM

Washing procedure was performed in a laminar flow hood. The membrane could not be rinsed in a flat container because of curling effect. Instead, the membrane was gently stretched in a vertical plane (Figure 12B) and rinsed with washing solution on both sides for up to 2 minutes (Figure 12C). Stroma was later placed against nitrocellulose papers.

1.5 Placement of nitrocellulose papers on canine AM

Since canine AM was very fragile, it was occasionally torn during tissue preparation. It was therefore difficult to flatten the membrane onto large nitrocellulose paper. Moreover, canine AM was generally vascularized, to avoid blood vessels and holes on the membrane could induce more damage. Instead, the nitrocellulose papers were cut into pieces of 2x2 cm^2, and placed on the membrane (Figure 12D). At least fifteen pieces of cold glycerol-preserved canine AM could be retrieved from 1 Chihuahua donor.

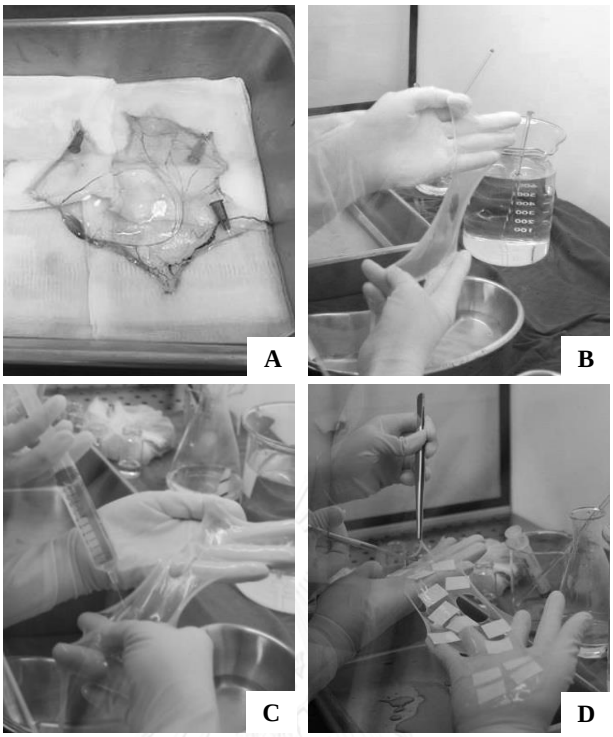

Figure 12. Techniques of canine AM preparation and preservation.
A) Identification of membrane epithelium. B) Thin and fragile canine AM. C) Canine AM being rinsed with 0.9% sterile normal saline solution. D) Placement of small pieces of nitrocellulose papers onto the membrane.

2. Identification of micro-organisms

Culture and identification test revealed no contamination of fungus, aerobic and anaerobic bacteria in all fresh and one-month cold glycerol-preserved canine AM samples.

Part II. Light microscopic evaluation of canine amniotic membrane

1. Light microscopic characterization of AM

Histologically, canine AM was composed of three layers; epithelium, basement membrane and stroma (Figure 13A). The outermost layer of canine AM was single layer of ciliated low cuboidal epithelium attached with basement membrane. Stroma consisted of loose collagen fibers with some small mature fibrocytes, which functioned as a support. One month after preservation in cold glycerol (Figure 13B), epithelium remained attached with the basement membrane and cilia was still intact. However, flatten epithelium with nuclear condensation was evident. Few small mature fibrocytes were observed in the stromal layer though it was more compact with less organized collagen fibers.

Likewise, human AM was composed of three layers similar to canine AM (Figure 13C). There was a single layer cuboidal epithelium without cilium. Stroma was thicker than that of canine AM. It had some small mature fibrocytes and well organized collagen fibers. Following cryopreservation (Figure 13D), epithelium of human AM had nuclear condensation and remained attach with basement membrane. Stroma was more compact but it still had a few small mature fibrocytes.

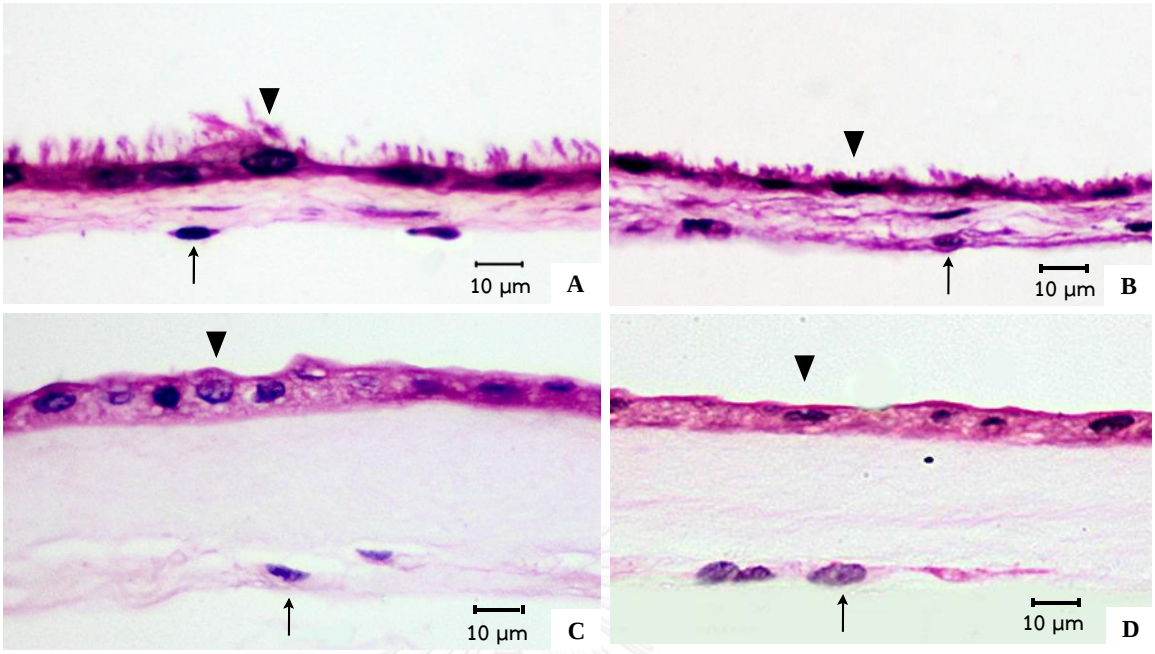

Figure 13. Comparative histological structure (H&E stain, bar = 10 µm) of canine AM and human AM.

A) Fresh canine AM; B) Glycerol-preserved canine AM; C) Fresh human AM; D) Cryopreserved human AM.

Note: arrowheads indicate epithelium; arrows indicate fibrocytes.

Masson's trichrome staining revealed that fresh canine AM had loosely collagen fibers with a few small mature fibrocytes in stroma (Figure 14A). Cold glycerol-preserved canine AM had more compact collagen fibers with less organization, though small mature fibrocytes were present in the stroma (Figure 14B).

In comparison with canine AM, fresh and cryopreserved human AM stained with Masson's trichrome demonstrated thicker epithelial cytoplasm (red) with more number of dense nuclei (black). (Figure 14C). Loose collagen fibers with a few small mature fibrocyte (blue) were evident. Stroma of the cryopreserved human AM was thinner than the fresh one (Figure 14D).

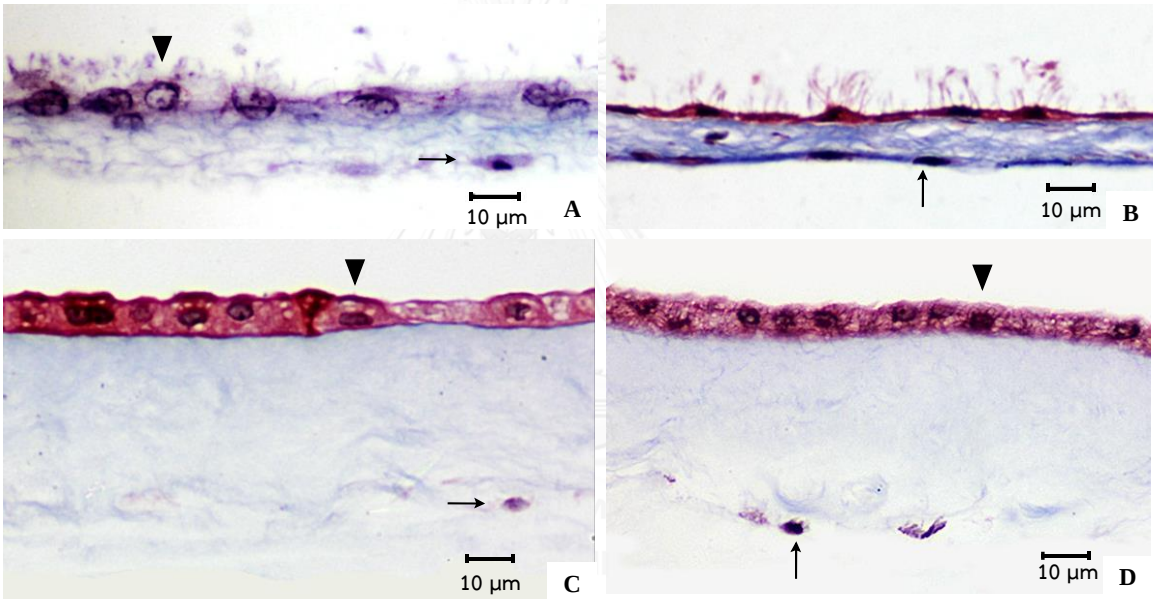

Figure 14. Comparative histological structure (Masson's trichrome stain, bar = 10 µm) of canine AM and human AM.

A) Fresh canine AM; B) Glycerol-preserved canine AM; C) Fresh human AM; D) Cryopreserved human AM.

Note: arrowheads indicate epithelium; arrows indicate fibrocytes.

2. Thickness of canine AM

Thickness of canine AM was consistent throughout the entire sheet. All layers of fresh canine AMs were statistically thicker than those of glycerol-preserved canine AM (*p<0.05*) (Figure 15). Mean full thickness of fresh canine AM was 20.94 ± 1.07 μm while that of glycerol-preserved membrane was 16.04 ± 0.53 μm. In fresh canine AM, mean thickness of stroma was 15.97 ± 1.09 μm while that of epithelium was 4.98 ± 0.34 μm. When preserved, stroma was 12.52 ± 0.60 μm, while epithelium was 3.52 ± 0.24 μm in thickness.

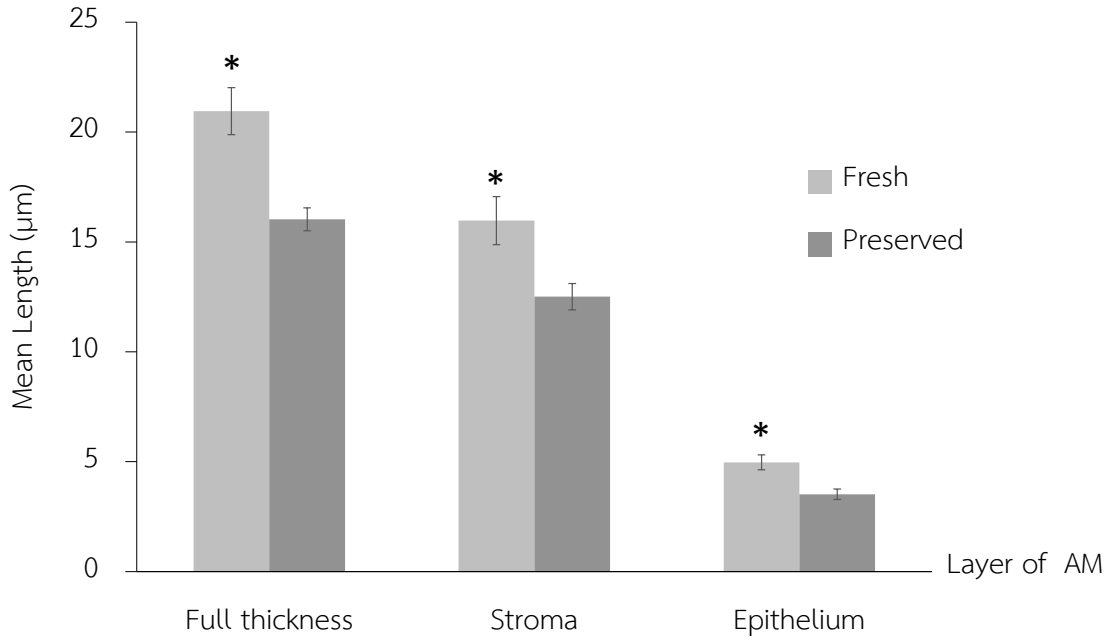

Figure 15. Mean thickness (± SE) of fresh and preserved canine AM.
(Star sign indicates statistical difference at p≤0.05.)

Part III. Evaluation of clinical outcomes after corneal reconstruction with cold-glycerol preserved canine AM and cryopreserved human AM

1. Patients

Fourteen dogs with corneal perforation included 4 Shih Tzus, 3 mixed breeds, 2 Pugs and one of Pekingese, Poodle, Chihuahua, Minature pinscher and Bulldog. Eight dogs were randomly selected to receive transplantation of canine AM (Table 1), whereas six dogs received surgery with the use of human AM (Table 2). There were 7 intact males, 5 intact females and one castrated male and one spayed female. Dogs were 5.7 years of age on average; age ranged from 2.5 months to 13 years. Transplantation of AM was performed in the right eye of six dogs and in the left eye of eight dogs. Perforation located at the central (4/14), paracenter (9/14) and periphery (1/14). Concurrent diseases included blood parasitism, demodicosis, dermatitis, otitis externa, gingivitis, keratoconjunctivitis sicca (KCS), eyelid mass, protrusion of gland of the third eyelid, and proptosis. Other ophthalmic signs associated with corneal perforation were iris prolapse, hyphema, hypopyn, chronic ulcerative keratitis, and rupture of zonular fibers.

All dogs presented severe ocular pain, epiphora, photophobia, blepharospasm, conjunctival hyperemia, corneal edema, and neovascularization. Pigmentary keratitis was found in 4 out of 8 dogs receiving canine AM while 1 out of 6 dogs receiving human AM.

Table 1. Data of patients receiving glycerol-preserved canine AM

Case	Breed	Age (year)	Eye	DBS (day)	Concurrent disease	Ophthalmic sign	Perforation (mm^2)	Location	S/F	Complication
1	Shih Tzu	5	OS	2	BP	Iris prolapse	4x5	Paracenter	S	AS
2	Pekingese	2	OD	7	BP, KCS	Hyphema	3x3	Paracenter	S	-
3	Poodle	9	OD	6	Demodicosis, Bilateral otitis	-	4x3	Paracenter	F	Bulgy membrane
4	Shih Tzu	11	OS	7	BP	Chronic ulcer	3x3	Center	S	Fibrin in AC, AS
5	Pug	3	OS	5	KCS	Iris prolapse	6x4	Paracenter	S	-
6	Mixed	3	OD	1	Proptosis	Hypopyon	6x5	Paracenter	S	AS
7	Mixed	6	OS	3	Dermatitis	-	3x3	Paracenter	S	PS
8	Mixed	0.2	OS	7	-	Iris prolapse	3x4	Periphery	S	-

AS = Anterior synechia, AC = Anterior chamber, BP = Blood parasite, DBS = Day before surgery, F = Failure,

KCS = Keratoconjunctivitis sicca, PS = Posterior synechia, OD = Oculus Dexter, OS = Ocular Sinister, S = Success

Table 2. Data of patients receiving cryopreserved human AM

Case	Breed	Age (year)	Eyes	DBS (day)	Concurrent disease	Ophthalmic sign	Perforation (mm^2)	Location	S/F	Complication
1	Bulldog	1	OS	7	protrusion of gland of the third eyelid	Iris prolapse	3x3	Paracenter	S	-
2	Pug	8	OD	5	KCS	Iris prolapse Hyphema	3x3	Center	S	Glaucoma
3	Shih Tzu	4	OD	1	BP	Iris prolapse	5x4	Paracenter	S	-
4	Chihuahua	3	OS	3	-	Iris prolapse	3x3	Paracenter	S	AS
5	Miniature Pinscher	12	OS	7	Eyelid mass Gingivitis	Hyphema Rupture of lens zonule	3x3	Center	S	Refractory ulcer, PS, Cataract
6	Shih Tzu	13	OD	1	BP	-	3x3	Center	F	Recurrent ulcer

AS = Anterior synechia, BP = Blood parasite, DBS = Day before surgery, F = Failure, KCS = Keratoconjunctivitis sicca,

PS = Posterior synechia, OD = Oculus Dexter, OS = Ocular Sinister, S = Success

2. Clinical reactions following transplantation of AM

After removal of third eyelid flap, both canine and human AMs were securely intact on the corneal surface (Figure 16; C.(b) & H.(b)). Glycerol-preserved canine AM was slight yellow in color whereas cryopreserved human AM was pale grey. Negative fluorescein staining indicated complete epithelialization during 7-14 days after surgery. Majority of dogs; using canine AM (6/8) and human AM (4/6), had complete epithelialization within the first week. Seidel test was negative in all eyes.

Corneal reactions were fairly comparable between transplantation of canine and human AM. Corneal edema was noted in all dogs. It immediately occurred after injury and then gradually decreased from approximately 14 days post-surgery onwards (Figure 16). Corneal vessels, on the other hand, were not initially present in every dogs. They were only manifested in cases with previous corneal irritation (case 5, 6, 7 and 8 receiving canine AM and case 1, 2, 3 and 5 receiving human AM) or prolong duration of perforation (case 2, 4 and 8 receiving canine AM and case 1 and 5 receiving human AM). By the time third eyelid flap was removed, they increased in number and length toward the reconstruction site. They reached the perforation located at the periphery rather faster than at the paracenter or center of the cornea. They became circumferential around the corneal wound edge as soon as they had arrived the edge.

Circumferential vessels were evident at approximately 7-21 days after transplantation and grew into the grafting area, where granulation tissue shortly developed thereafter (Figure 17). In cases of which perforation size was rather big or previous keratitis had involved, extensive granulation tissue was infiltrated to cover corneal defect (Figure 17; H.(a)). It appeared as irregular red plaque over the AM. Regression of the granulation began at approximately day 28 post-surgery, at which topical corticosteroid was administered. Scar tissue started to form while granulation tissue gradually regressed (Figure 16; C.(c) & H.(c) and Figure 17; C.(c) & H.(b)). In one case that received canine AM transplantation, extensive granulation tissue was apparent soon after third eyelid flap was removed, and then rapidly resolved.

Corneal pigmentation was observed along with neovascularization. In four cases (case 2 and 4 receiving canine AM; case 1 and 2 receiving human AM) that had

previous chronic keratitis with pigment, pigmentation following AM transplantation was more extensive. It had been observed since day 14 post-surgery. Newly-formed pigmentation was noted at the locations where corneal vessels were previously located. Once topical corticosteroid was administered, pigment gradually regressed and partly remained on the corneal surface until the end of the study (Figure 16; C.(d) & H.(d) and Figure 17; C.(d) & H.(d)).

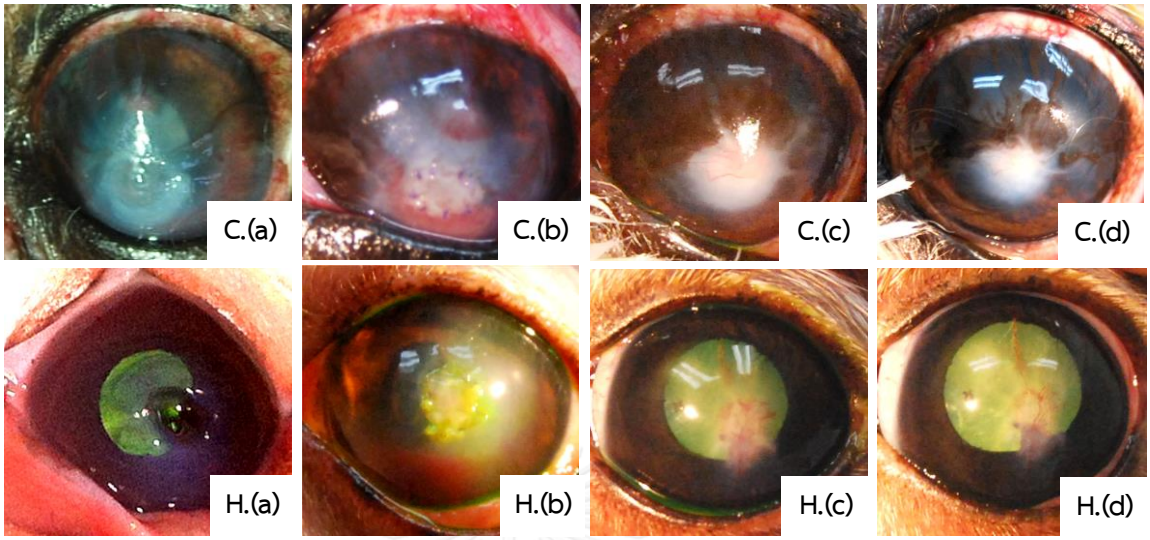

Figure 16. Representative photographs of corneal edema, vascularization and pigmentation following transplantation with canine AM (C) and human AM (H); before surgery (a), and after surgery at day 7 (b), 28 (c) and 56 (d);

C.(a) Canine corneal perforation (case 5) at the center of the cornea;

C.(b) Moderate corneal edema surrounding the surgical site, together with circumferential vessels;

C.(c) Regression of corneal edema and neovascularization, and newly-formed corneal pigmentation where corneal edema and vessels previously found;

C.(d) Regression of corneal pigmentation with remnant corneal vessels;

H.(a) Canine corneal perforation (case 5) at the paracenter of the cornea;

H.(b) Mild corneal edema surrounding the surgical site, together with circumferential vessels moving toward the wound edge;

H.(c) Regression of corneal edema and neovascularization; cornea became clearer;

H.(d) Regression of corneal vessels at the transplanted site.

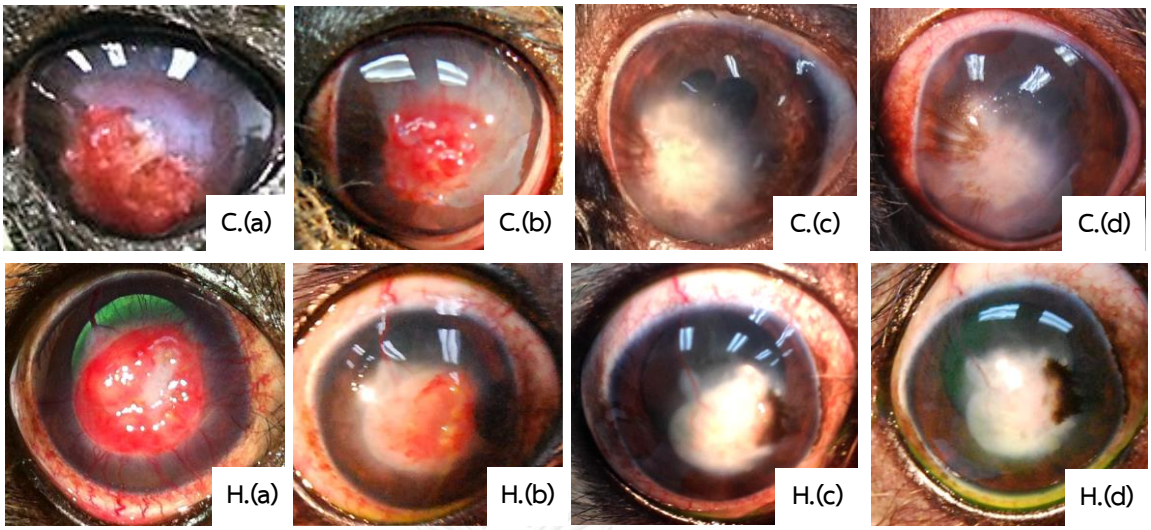

Figure 17. Representative photographs of granulation tissue, corneal scar and corneal pigmentation following transplantation with canine AM (C) and human AM (H); before surgery (a), and after surgery at day 7 (b), 28 (c) and 56 (d);

C.(a) Heavy granulation tissue at the surgical site (case 1) at the paracenter of the cornea;

C.(b) Mild regression of circumferential vessels within the granulation;

C.(c) Scar formation and regression of previous corneal pigmentation;

C.(d) Decrease in scar density continued regression of corneal vessels and pigmentation;

H.(a) Heavy granulation tissue at the surgical site (case 4), at the paracenter of the cornea;

H.(b) Moderate regression of circumferential vessels within the granulation observed in the pale region; corneal pigmentation and the remaining vessels;

H.(c) Scar formation and regression of newly-formed corneal pigmentation;

H.(d) Decrease in scar density; continued regression of corneal vessels and pigmentation.

3. Neuro-ophthalmic results

For the cases that received glycerol-preserved canine AM transplantation (Table 3), six out of eight eyes were positive to menace response prior to surgery (case 1, 3, 4, 5, 7 and 8). While case 2 had negative menace response but positive dazzle reflex, and its menace response returned after corneal reconstruction. In case 6, negative of both responses were found before/after surgery. PLR could not be examined prior to surgery in all dogs due to severe ocular pain. After surgery, it could not be observed in case 2 and case 5, of which the corneas were heavily pigmented. Blink reflexes were positive in all dogs throughout the study.

For the cases that received cryopreserved human AM (Table 4), four out of six dogs positively responded to menace and dazzle examinations. The other two dogs (case 2 & 5) of which menace response and dazzle reflex were both negative, remained negative to both examinations. After surgery, case 2 had glaucoma while case 5 had cataract with rupture of zonular lens fiber. PLR could not be examined prior to surgery in all dogs due to severe ocular pain. The response was positive in all dogs except case 5 of which pupil was fully dilated, and case 2 that could not be examined due to heavy corneal pigmentation. All dogs could normally blink.

Table 3. Neuro-ophthalmic results of dogs with the use of glycerol-preserved canine AM.

Case	Menace response		Dazzle reflex		Pupillary light response		Blink reflex	
	Before	After	Before	After	Before	After	Before	After
1	+	+	+	+	N/A	+	+	+
2	-	+	+	+	N/A	N/A	+	+
3	+	+	+	+	N/A	+	+	+
4	+	+	+	+	N/A	+	+	+
5	+	+	+	+	N/A	N/A	+	+
6	-	-	-	-	N/A	+	+	+
7	+	+	+	+	N/A	+	+	+
8	+	+	+	+	N/A	+	+	+

+ = positive; - = negative

N/A = unable to examine

Table 4. Neuro-ophthalmic results of dogs with the use of cryopreserved human AM.

Case	Menace response		Dazzle reflex		Pupillary light response		Blink reflex	
	Before	After	Before	After	Before	After	Before	After
1	+	+	+	+	N/A	+	+	+
2	-	-	-	-	N/A	N/A	+	+
3	+	+	+	+	N/A	+	+	+
4	+	+	+	+	N/A	+	+	+
5	-	-	-	-	N/A	- #	+	+
6	+	+	+	+	N/A	+	+	+

+ = positive; - = negative

N/A = unable to examine

= pupil dilation

4. Schirmer tear test I

Due to the fact that all dogs had severe ocular pain prior to surgery, STT I could therefore not be examined on the surgical day as well as on day 7 after removal of the third eyelid flap. STT I values were normal distributed. Mean STT I values of both groups showed slight fluctuation, but within the normal limit, throughout the study (Figure 18). It ranged from 12 ± 1.61 to 14.6 ± 1.25 mm in the group receiving glycerol-preserved canine AM while from 14.3 ± 1.97 to 11.16 ± 1.32 mm in the other group. Following day 14 post-surgery, mean STT I values gradually decreased by time. After they had reached the lowest level at day 28 post-surgery, they slowly increased toward the end of the study. Although mean values from the group receiving canine AM were higher than those from human AM, statistical difference was not found between the two groups. Mean STT I values at different times of data collection within the same group of experiment showed no statistical difference (*p>0.05*).

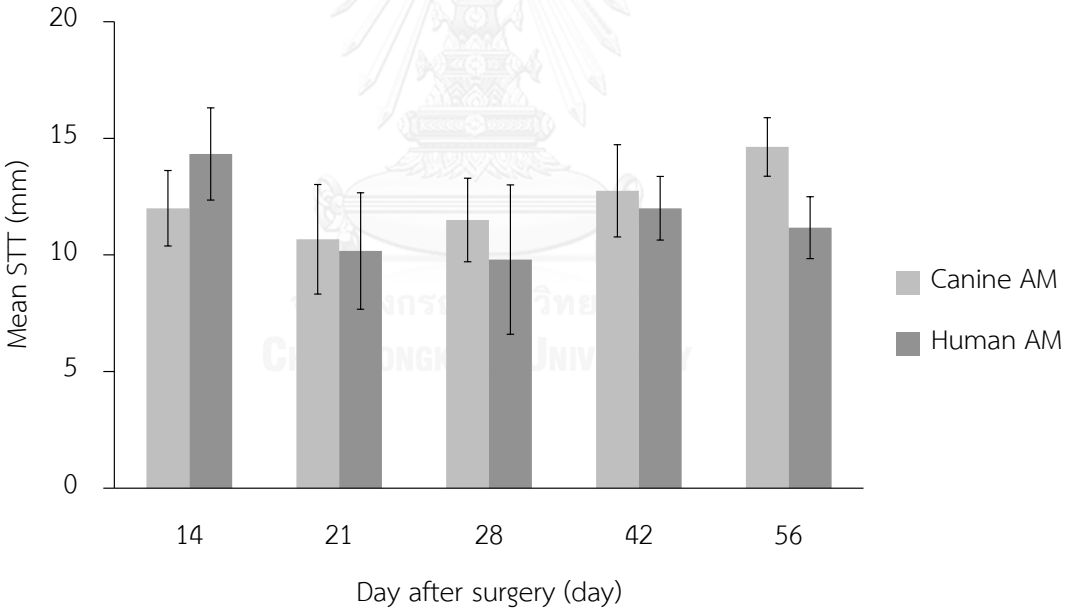

Figure 18. Mean Schirmer tear test I (mm) ± SE at different time points; after transplantation with glycerol-preserved canine AM and cryopreserved human AM.

5. Intraocular pressure

IOP values had normal distribution and ranged within the normal limit (Figure 19). At the day before surgery, the lowest mean value was 7.37 ± 1.33 mmHg in the group transplanted with canine AM and 7 ± 4.35 mmHg in the group transplanted with human AM. After that they gradually increased by time and reached the maximal level at day 42 post-surgery; 13.5 ± 1.76 mmHg in the group transplanted with canine AM and 14 ± 2.17 mmHg in the group transplanted with human AM. Mean IOP value of the eyes receiving human AM tended to be higher than that of the other group. However significant difference was not found between the two groups (*p>0.05*). Significant (*p<0.05*) was on the other hand found among data collected at different times within the group receiving canine AM; between day 42 post-surgery and the very early day of surgery.

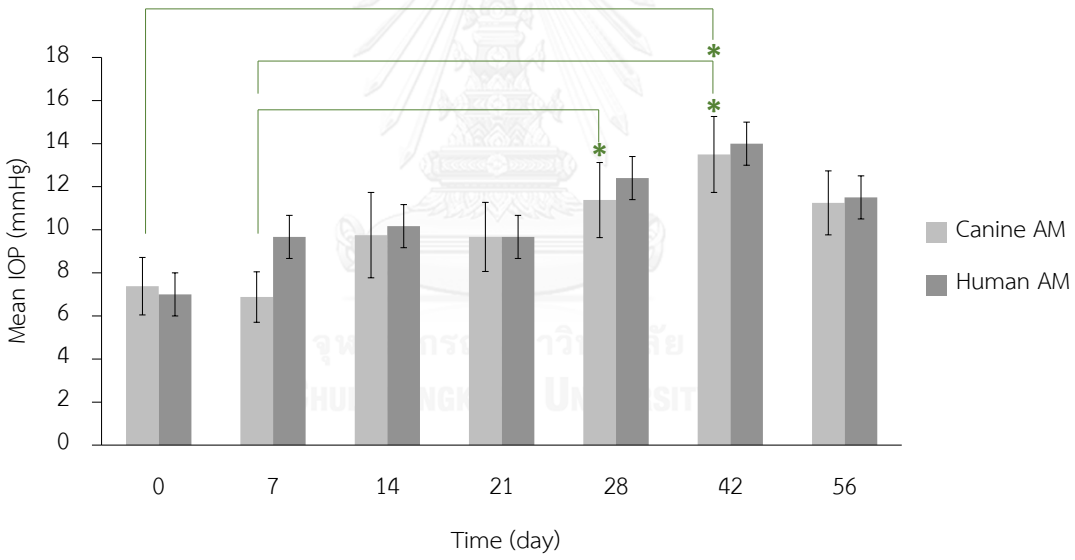

Figure 19. Mean intraocular pressure (mm) ± SE at different time points; pre/post transplantation with glycerol-preserved canine AM and cryopreserved human AM. (Star sign indicates significantly difference at p<0.05.)

6. Complications

Anterior synechia (Figure 20A) was the major compli

cation found in three eyes receiving corneal reconstruction with canine AM and in one eye receiving human AM. Posterior synechia (Figure 20B) was found in one eye from each group. Other complications found only in one eye included refractory corneal ulcer (Figure 20C), mild fibrin formation in the anterior chamber, glaucoma, and cataract.

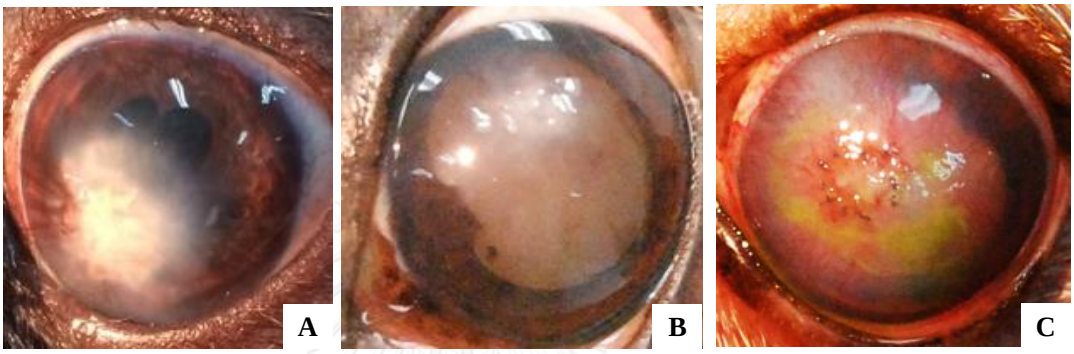

Figure 20. Complications following transplantation with canine and human AM. A) Anterior synechia; B) Posterior synechia; C) Refractory corneal ulcer.

7. Clinical success

As the success was based on three criteria: complete epithelialization, no recurrence of corneal perforation at the same region, and no aqueous humor leakage; the success from transplantation with glycerol-preserved canine AM was 87.5% (7/8 eyes) whereas that of cryopreserved human AM was 83.33% (5/6 eyes). None of the eyes were infected.

Cases that could not achieve three criteria of success were case 3, receiving transplantation with canine AM, and case 6 receiving human AM. AM of case 3 was bulging forward at day 14 post-surgery (Figure 21). The third eyelid flap was therefore reapplied. After removal of the latter flap, epithelialization was complete with accumulation of granulation tissue on the transplanted site. Corneal perforation was finally healed.

Recurrent corneal ulcer was found in case 6 receiving human AM at day 28 post-surgery (Figure 22). Ulcer was at the center of the grafting area and was positive to the fluorescein staining test. By the time that granulation tissue regressed, scar was formed. However, recurrent corneal ulcer persisted but was smaller.

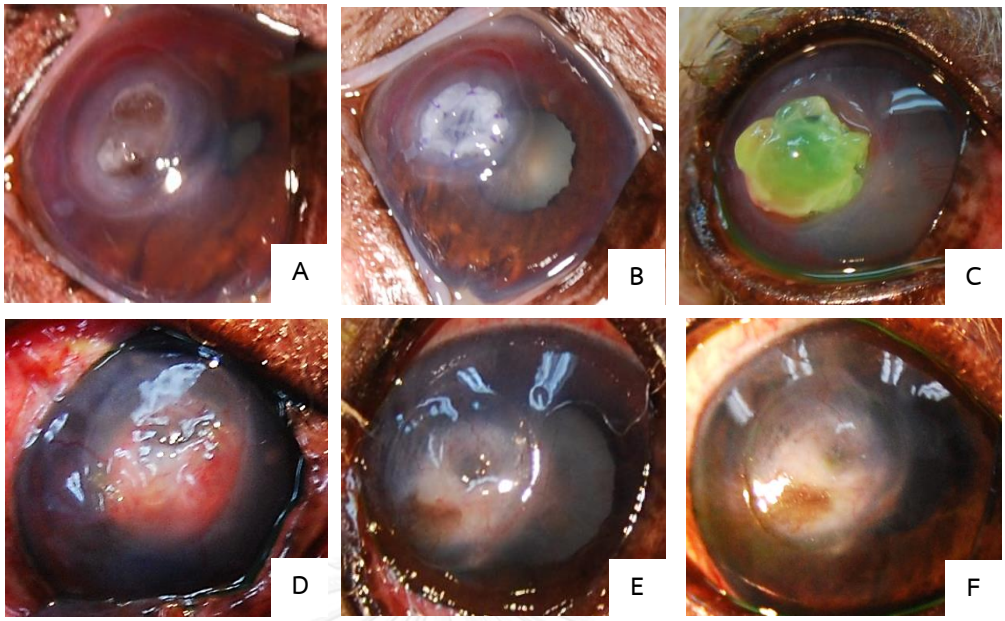

Figure 21. Series of photography taken from the eye of a 9 year-old Poodle treated with glycerol-preserved canine AM transplantation.

A). 4x3 mm^2 corneal perforation at the paracenter of the cornea.

B). Following removal of necrotic tissue from the wound edge, glycerol-preserved canine AM was laid on and fixed to the edge.

C). Canine AM bulging forward with positive fluorescein staining at day 14 post-surgery; third eyelid flap was reapplied.

D). Presence of granulation tissue.

E). Deposition of corneal pigmentation and regression of granulation tissue.

F). Scar formation.

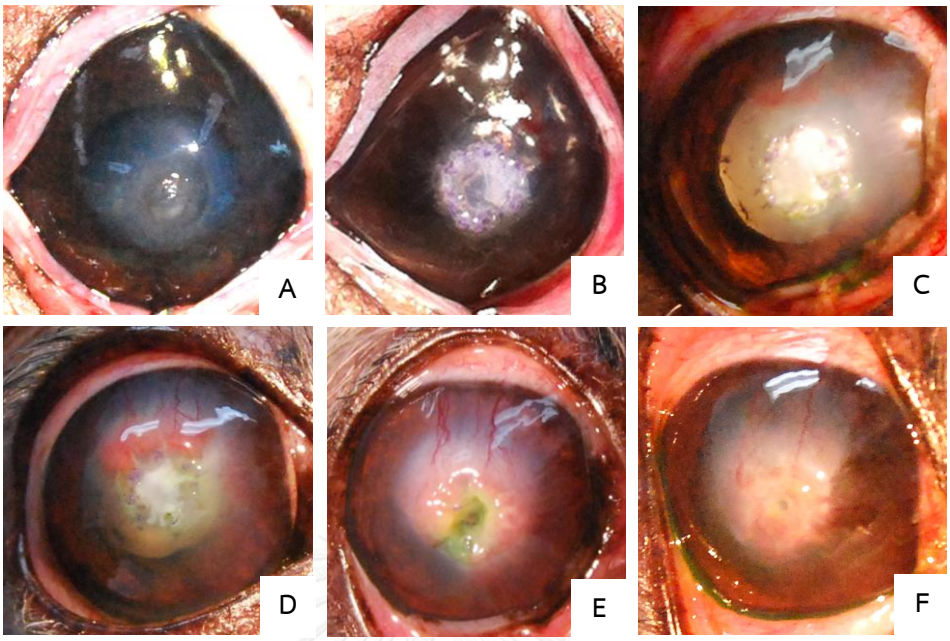

Figure 22. Series of photography taken from the eye of a 13 year-old Shih Tzu treated with cryopreserved human AM transplantation.

A). 3x3 mm^2 corneal perforation at the center of the cornea.

B). Transplantation of human AM. Note membrane excavation at the center.

C). Following removal of the third eyelid flap, there was partial loss of the membrane.

D). Circumferential vessels at the transplanted site.

E). At day 28 post-surgery, fluorescein stained ulcer appeared at the center of the transplanted area.

F). Corneal vessels regressed; ulcer remained but smaller.

CHAPTER 5

DISCUSSION

This study demonstrated the success of using cold glycerol-preserved canine AM as an allograft with third eyelid flap for canine corneal perforation repair. The success rate (87.5%; 7/8) was comparable to that of using cryopreserved human AM (83.33%; 5/6). Following similar criteria of clinical success of Rodríguez-Ares et al. (2004) for corneal perforation repair in humans, our clinical outcomes were comparable to their study.

In our study, time to complete epithelialization occurred within 7-14 days following transplantation. Canine AM may contains many growth factors; such as EGF, HGF and KGF, all of which stimulate epithelialization like humans (Koizumi et al., 2000). Similar duration of epithelialization was reported after the treatment of corneal dermoid in dogs (Kalpravidh et al., 2009) and corneal perforation in humans (Prabhasawat et al., 2001). However it took 2-6 weeks after the use of single layer of cryopreserved human AM in human corneal perforation (less than 3.5 mm in diameter) (Rodríguez-Ares et al., 2004). Numbers of membranes as well as severity of corneal defect are considered part of key factors for time of complete epithelialization. Rapid epithelialization would be achieved in superficial stromal defect with single layer of membrane application, while multilayer of membranes are recommended for deep or perforating cornea (Seitz et al., 2006).

Following transplantation, AMs were well intact in both groups of the study. Hemidesmosomes and desmosomes are adhesive structures in the AM stroma that benefit integration of the membrane into corneal tissue (Resch et al., 2006). Application of the membrane using inlay technique with epithelial side up provides incorporation between the two different tissues (John, 2003). Dehiscence of AM occurred in studies

using overlay technique (Kalpravidh et al., 2009). Instead of incorporating into corneal tissue, AM acts as bandage contact lens, which will slough off when epithelialization has completed. In addition to multilayer graft inlay technique used in this study, third eyelid flap provides mechanical protection to the healing cornea like a bandage contact lens (Ledbetter and Gilger, 2013).

Even though corneal perforation caused severe ocular pain in all dogs, it soon became resolved with gradual reduction of ophthalmic signs toward the end of the treatment. While topical atropine sulfate helps to reduce ciliary spasm, decrease of tear level secondarily occurs (Maggs, 2013). Supplementation of artificial tear together with 1% cyclosporine to quantitatively and qualitatively improve tear property would therefore facilitate corneal wound healing. Increasing of IOP to be within normal limit observed at day 14 after-surgery demonstrated no leakage of the anterior chamber, simultaneously with reduction of intraocular inflammation.

Not only canine AM transplantation facilitates rapid restoration of the perforating cornea together with appropriate technique of graft transplantation and anti-inflammatory property of AM (Hao et al., 2000). Visual status is maintained in most dogs. One dog in canine AM-treated group returned vision. Although pre-surgery blindness was found in a dog receiving canine AM and 2 dogs receiving human AM that remained blind post-surgery. Good reconstruction of the cornea was accomplished. Excluding the two unsuccessful cases, ophthalmic complications occurred in 7 out of 12 eyes in this study. Adhesion of iris to intraocular structure was the major complication despite administration of topical atropine sulfate. Increased frequency of atropine sulfate administration, at the early stage of the disease in particular, would reduce the risk of this complication.

Unsuccessful transplantation occurred in 2 out of 14 eyes. One eye of a 13 year-old Shih Tzu receiving human AM had corneal ulcer at day 28 post-surgery. Partial loss of transplanted membrane was observed immediately after third eyelid flap removal. Several factors such as senile age of dog, the owner's care, as well as technical errors during surgery, may play a role in recurrence of the ulcer. Protrusion of the canine AM observed in the one eye was similar to the dogs receiving a single

layer glycerol-preserved equine AM to repair canine corneal perforation created by penetrating keratectomy (Barros et al., 1998). Application of a single layer membrane may provide inadequate protection of severe corneal defect. Although multiple layer membrane was applied in our study, bulging of the canine AM at the paracenter of the grafting area may be due to slow wound healing in senile dog, other diseases manifested with the dog or poor prevention of self-trauma.

Canine AM is a mild greenish translucent membrane while human AM is colorless and translucent. Green color is derived from uteroverdin, which is substance from maternal hematoma (Lemberg and Barcroft, 1932) located on both sides of placental girdle. If canine AM is left unclean after being separated from placenta, the membrane will appear dark green. This unique structure of canine AM has driven the preparation procedure more complex. Gross comparison, more opaque appearance of glycerol-preserved canine AM may be due to compact characterization of disorganized collagen fibers.

Fresh canine AM is significantly thin compared to fresh human AM. From a report by Baergen (2005), fresh human AM is 35-60 μm. When preserved, AM typically become thin, it is as regards to dehydration mechanism driven by glycerol, which is major component in both cryo and cold glycerol-preservation. Comparison between these two preservation methods revealed that human AM preserved in cold glycerol not only had higher tensile strength, but also became thicker than being cryopreserved (von Versen-Hoeynck et al., 2008). Canine AM collected from Chihuahua donor in our study may have been remarkably thin and lacked of elasticity, leading to membrane protrusion. Moreover, thinning of AM may have been influenced by area of AM collection. Human AM was slight thicker in the region nearby umbilical cord (Baergen, 2005). However, according to limitation of tissue preparation that canine AM should immediately be separated from zonary placenta, identification of the membrane region is therefore difficult. Besides, that area of canine AM heavily contains blood supplies.

Proportion of canine AM prepared from small dog breeds is low compared from large breeds. Fifteen to twenty membranes (2x2 mm^2) could be prepared from one

Chihuahua donor. Dystocia usually occurs in small breed dogs (Reichler and Michel, 2009), of which thin AM is available. With the use of canine AM derived from small dog, the membrane is likely to be torn. Besides, separation of AM from placenta should gently be performed to discard area with heavy vascularization, leaving clear area intact.

Side of canine AM plays a role in corneal healing process (Seitz et al., 2006). By placing epithelial side up, AM serves as a scaffold to support corneal epithelial migration and differentiation (Niknejad et al., 2008). According to specific anatomical structures of canine AM, which is enveloped by allantois and not attached with the chorion (Miglino et al., 2006), identification of the side of the membrane after separation is crucial.

Due to the fact that fresh AM is not practical to collect, time constraint for microorganism screening test and unavailable at time for clinical application, the preserved AM has become more popular in ophthalmology practice. Our glycerol-preserved canine AM stored for one month demonstrated sterility. Though the full thickness membrane was significantly thinner as compared to the non-preserved one, it is more likely that its essential biological properties still remain. Despite the fact that cryopreserved human AM has been proven to be a good transplant for many corneal defects, preservation of the membrane at freezing temperature to maintain its biological properties is still problematic and tis availability is rather limited.

Conclusion

We have herein developed preparation and preservation technique of cold glycerol to preserve canine AM. Application of multiple layer inlay technique using canine AM has become a promising alternative treatment for corneal perforation in dogs.

Suggestions

Success in keeping canine AM epithelium intact with prolong preservation of cold glycerol-preserved membrane would provide more prospect of the treatment of various corneal defects in dogs.

Additional investigation of characterization by electron microscope as well as biological properties of cold glycerol-preserved canine AM would broaden knowledge for improving efficacy of the membrane.

REFERENCES

Adds PJ, Hunt CJ and Dart JK. 2001. Amniotic membrane grafts, "fresh" or frozen? A clinical and in vitro comparison. Br J Ophthalmol. 85(8): 905-907.

Anderson DF, Ellies P, Pires RT and Tseng SC. 2001a. Amniotic membrane transplantation for partial limbal stem cell deficiency. British journal of ophthalmology. 85(5): 567-575.

Anderson DF, Prabhasawat P, Alfonso E and Tseng SC. 2001b. Amniotic membrane transplantation after the primary surgical management of band keratopathy. Cornea. 20(4): 354-361.

Baergen RN. 2005. Histology of the chorionic villi, fetal membranes, and umbilical cord. In: Manual of Benirschke and Kaufmann's pathology of the human placenta. 4 ed. Baergen Rebecca N (ed.). New York: Springer. 80-89.

Barachetti L, Giudice C and Mortellaro CM. 2010. Amniotic membrane transplantation for the treatment of feline corneal sequestrum: pilot study. Vet ophthalmol. 13(5): 326-330.

Baradaran-Rafii A, Aghayan H-R, Arjmand B and Javadi M-A. 2008. Amniotic membrane transplantation. Journal of Ophthalmic & Vision Research. 2(1): 58-75.

Bari M, Choudhury M, Khan A and Nessa A. 2002. Role of human foetal membranes (amniotic membrane) in the management of burn wounds. Annals of Burns and Fire Disasters. 15(4): 183-186.

Barros PS, Garcia JA, Laus J, Ferreira AL and Salles Gomes T. 1998. The use of xenologous amniotic membrane to repair canine corneal perforation created by penetrating keratectomy. Vet ophthalmol. 1(2-3): 119-123.

Cirman T, Beltram M, Schollmayer P, Rožman P and Kreft ME. 2014. Amniotic membrane properties and current practice of amniotic membrane use in ophthalmology in Slovenia. Cell Tissue Bank. 15(2): 177-192.

Cooper LJ, Kinoshita S, German M, Koizumi N, Nakamura T and Fullwood NJ. 2005. An investigation into the composition of amniotic membrane used for ocular surface reconstruction. Cornea. 24(6): 722-729.

Davis JS. 1910. Skin transplantation. Johns Hopkins Hospital Reports. 15: 307-396.

de Moraes KARKOS PS, Angélica de Mendonça V, Malerba TA and JUNIOR MB. 1995. The surgical repair of the cornea of the dog using pericardium as a keratoprosthesis. Braz. J. vet. Res. an tin. Sci. 32(4): 251-255.

De Rotth A. 1940. Plastic repair of conjunctival defects with fetal membranes. Arch Ophthalmol. 23(3): 522.

Dua HS, Maharajan VS and Hopkinson A. 2006. Controversies and limitations of amniotic membrane in ophthalmic surgery. In: Cornea and External Eye Disease. ed. (ed.). Springer. 21-33.

Duchesne B, Tahi H and Galand A. 2001. Use of human fibrin glue and amniotic membrane transplant in corneal perforation. Cornea. 20(2): 230-232.

Espana EM, Grueterich M, Sandoval H, Solomon A, Alfonso E, Karp CL, Fantes F and Tseng SC. 2003. Amniotic membrane transplantation for bullous keratopathy in eyes with poor visual potential. Journal of Cataract & Refractive Surgery. 29(2): 279-284.

Fukuda K, Chikama T-i, Nakamura M and Nishida T. 1999. Differential distribution of subchains of the basement membrane components type IV collagen and laminin among the amniotic membrane, cornea, and conjunctiva. Cornea. 18(1): 73-79.

Godoy-Esteves CA, Gomes JÁP, Yazbek K, Guerra JL and Barros PS. 2013. Lamellar keratoplasty in rabbits using human and rabbit amniotic membrane grafts: a comparative study. Vet ophthalmol. 1-7.

Goulle F. 2012. Use of porcine small intestinal submucosa for corneal reconstruction in dogs and cats: 106 cases. Journal of Small Animal Practice. 53(1): 34-43.

Hao Y, Ma DH-K, Hwang DG, Kim W-S and Zhang F. 2000. Identification of antiangiogenic and antiinflammatory proteins in human amniotic membrane. Cornea. 19(3): 348-352.

Jhanji V, Young AL, Mehta JS, Sharma N, Agarwal T and Vajpayee RB. 2011. Management of corneal perforation. Survey of ophthalmology. 56(6): 522-538.

John T. 2003. Human amniotic membrane transplantation: past, present, and future. Ophthalmol Clin North Am. 16(1): 43-65, vi.

Kalpravidh M, Tuntivanich P, Vongsakul S and Sirivaidyapong S. 2009. Canine amniotic membrane transplantation for corneal reconstruction after the excision of dermoids in dogs. Vet Res Commun. 33(8): 1003-1012.

Kim HS, Cho JH, Park HW, Yoon H, Kim MS and Kim SC. 2002. Endotoxin-neutralizing antimicrobial proteins of the human placenta. The Journal of Immunology. 168(5): 2356-2364.

Kim JC and Tseng SC. 1995. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. Cornea. 14(5): 473-484.

King A, Paltoo A, Kelly R, Sallenave J-M, Bocking A and Challis J. 2007. Expression of natural antimicrobials by human placenta and fetal membranes. Placenta. 28(2): 161-169.

Koizumi N, Inatomi T, Sotozono C, Fullwood NJ, Quantock AJ and Kinoshita S. 2000. Growth factor mRNA and protein in preserved human amniotic membrane. Current eye research. 20(3): 173-177.

Koizumi N, Inatomi T, Suzuki T, Sotozono C and Kinoshita S. 2001. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. Ophthalmology. 108(9): 1569-1574.

Laus J, Tonanni RC and Paulo S. 1999. The use of preserved equine renal capsule to repair lamellar corneal lesions in normal dogs. Veterinary ophthalmology. 2: 79-82.

Ledbetter EC and Gilger BC. 2013. Diseases and surgery of the canine cornea and sclera. In: Veterinary Ophthalmology. ed. Gelatt Kirk N., Gilger Brian C. , and Kern Thomas J. (ed.). Florida: Blackwell Publishing. 976-1049.

Lee PH, Tsai SH, Kuo L, Hwang CY, Kuo CY, Yang VC and Chen JK. 2012. A prototype tissue engineered blood vessel using amniotic membrane as scaffold. Acta Biomater. 8(9): 3342-3348.

Lee S-H and Tseng S. 1997. Amniotic membrane transplantation for persistent epithelial defects with ulceration. American journal of ophthalmology. 123(3): 303-312.

Lemberg R and Barcroft J. 1932. Uteroverdin, the green pigment of the dog's placenta. Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character. 362-372.

Letko E, Stechschulte SU, Kenyon KR, Sadeq N, Romero TR, Samson CM, Nguyen QD, Harper SL, Primack JD, Azar DT, Gruterich M, Dohlman CH, Baltatzis S and Foster CS. 2001. Amniotic membrane inlay and overlay grafting for corneal epithelial defects and stromal ulcers. Arch Ophthalmol. 119(5): 659-663.

Li H, Niederkorn JY, Neelam S, Mayhew E, Word RA, McCulley JP and Alizadeh H. 2005. Immunosuppressive factors secreted by human amniotic epithelial cells. Invest Ophthalmol Vis Sci. 46(3): 900-907.

Maggs DJ. 2013. CORNEA AND SCLERA. In: Slatter's fundamentals of veterinary ophthalmology. 5 ed. Maggs David J, Miller Paul, and Ofri Ron (ed.). Missouri: Elsevier Health Sciences. 184-219.

Malak T, Ockleford C, Bell S, Dalgleish R, Bright N and Macvicar J. 1993. Confocal immunofluorescence localization of collagen types I, III, IV, V and VI and their ultrastructural organization in term human fetal membranes. Placenta. 14(4): 385-406.

Maral T, Borman H, Arslan H, Demirhan B, Akinbingol G and Haberal M. 1999. Effectiveness of human amnion preserved long-term in glycerol as a temporary biological dressing. Burns. 25(7): 625-635.

Martin-Suarez E, Molleda C, Tardon R, Galan A, Gallardo J and Molleda J. 2014. Diurnal variations of central corneal thickness and intraocular pressure in dogs from 8:00 am to 8:00 pm. Can Vet J. 55(4): 361-365.

Mejia LF, Acosta C and Santamaria JP. 2000. Use of nonpreserved human amniotic membrane for the reconstruction of the ocular surface. Cornea. 19(3): 288-291.

Meller D, Pires RT, Mack RJ, Figueiredo F, Heiligenhaus A, Park WC, Prabhasawat P, John T, McLeod SD and Steuhl KP. 2000. Amniotic membrane transplantation for acute chemical or thermal burns. Ophthalmology. 107(5): 980-989.

Miglino MA, Ambrosio CE, dos Santos Martins D, Wenceslau CV, Pfarrer C and Leiser R. 2006. The carnivore pregnancy: the development of the embryo and fetal membranes. Theriogenology. 66(6-7): 1699-1702.

Nakamura T, Yoshitani M, Rigby H, Fullwood NJ, Ito W, Inatomi T, Sotozono C, Nakamura T, Shimizu Y and Kinoshita S. 2004. Sterilized, freeze-dried amniotic membrane: a useful substrate for ocular surface reconstruction. Invest Ophthalmol Vis Sci. 45(1): 93-99.

Ni J, Abrahamson M, Zhang M, Fernandez MA, Grubb A, Su J, Yu G-L, Li Y, Parmelee D and Xing L. 1997. Cystatin E is a novel human cysteine proteinase inhibitor with structural resemblance to family 2 cystatins. Journal of Biological Chemistry. 272(16): 10853-10858.

Niknejad H, Peirovi H, Jorjani M, Ahmadiani A, Ghanavi J and Seifalian AM. 2008. Properties of the amniotic membrane for potential use in tissue engineering. Eur Cell Mater. 15: 88-99.

Park WC and Tseng SC. 2000. Modulation of acute inflammation and keratocyte death by suturing, blood, and amniotic membrane in PRK. Invest Ophthalmol Vis Sci. 41(10): 2906-2914.

Prabhasawat P and Tesavibul N. 2001. Preserved amniotic membrane transplantation for conjunctival surface reconstruction. Cell Tissue Bank. 2(1): 31-39.

Prabhasawat P, Tesavibul N and Komolsuradej W. 2001. Single and multilayer amniotic membrane transplantation for persistent corneal epithelial defect with and without stromal thinning and perforation. British Journal of Ophthalmology. 85(12): 1455-1463.

Ravishanker R, Bath A and Roy R. 2003. “Amnion Bank”—the use of long term glycerol preserved amniotic membranes in the management of superficial and superficial partial thickness burns. Burns. 29(4): 369-374.

Reichler I and Michel E. 2009. Dystocia: recognition and management. Eur J Comp Anim Pract. 19: 165-173.

Resch MD, Schlotzer-Schrehardt U, Hofmann-Rummelt C, Sauer R, Cursiefen C, Kruse FE, Beckmann MW and Seitz B. 2006. Adhesion structures of amniotic membranes integrated into human corneas. Invest Ophthalmol Vis Sci. 47(5): 1853-1861.

Riau AK, Beuerman RW, Lim LS and Mehta JS. 2010. Preservation, sterilization and de-epithelialization of human amniotic membrane for use in ocular surface reconstruction. Biomaterials. 31(2): 216-225.

Rodríguez-Ares MT, Touriño R, López-Valladares MJ and Gude F. 2004. Multilayer amniotic membrane transplantation in the treatment of corneal perforations. Cornea. 23(6): 577-583.

Seitz B, Resch MD, Schlötzer-Schrehardt U, Hofmann-Rummelt C, Sauer R and Kruse FE. 2006. Histopathology and ultrastructure of human corneas after amniotic membrane transplantation. Arch Ophthalmol. 124(10): 1487-1490.

Shao C, Sima J, Zhang SX, Jin J, Reinach P, Wang Z and Ma J-x. 2004. Suppression of corneal neovascularization by PEDF release from human amniotic membranes. Invest Ophthalmol Vis Sci. 45(6): 1758-1762.

Shimazaki J, Aiba M, Goto E, Kato N, Shimmura S and Tsubota K. 2002. Transplantation of human limbal epithelium cultivated on amniotic membrane for the treatment of severe ocular surface disorders. Ophthalmology. 109(7): 1285-1290.

Shimmura S, Shimazaki J, Ohashi Y and Tsubota K. 2001. Antiinflammatory effects of amniotic membrane transplantation in ocular surface disorders. Cornea. 20(4): 408-413.

Singh R, Gupta P, Kumar P, Kumar A and Chacharkar MP. 2003. Properties of Air Dried Radiation Processed Amniotic Membranes under Different Storage Conditions. Cell Tissue Bank. 4(2-4): 95-100.

Solomon A, Meller D, Prabhasawat P, John T, Espana EM, Steuhl K-P and Tseng SC. 2002. Amniotic membrane grafts for nontraumatic corneal perforations, descemetoceles, and deep ulcers. Ophthalmology. 109(4): 694-703.

Solomon A, Rosenblatt M, Monroy D, Ji Z, Pflugfelder SC and Tseng SC. 2001. Suppression of interleukin 1α and interleukin 1β in human limbal epithelial cells cultured on the amniotic membrane stromal matrix. British Journal of Ophthalmology. 85(4): 444-449.

Sorby A and Symons H. 1946. Amniotic membrane graft in caustic burn of the eye. Br J Ophthalmol. 30: 337-345.

Steele C. 1999. Corneal wound healing: a review. Optometry Today. 24: 28-32.

Tao H and Fan H. 2009. Implantation of amniotic membrane to reduce postlaminectomy epidural adhesions. Eur Spine J. 18(8): 1202-1212.

Thomasen H, Pauklin M, Noelle B, Geerling G, Vetter J, Steven P, Steuhl K-P and Meller D. 2011. The effect of long-term storage on the biological and histological properties of cryopreserved amniotic membrane. Curr Eye Res. . 36(3): 247-255.

Tseng SC, Li DQ and Ma X. 1999. Suppression of transforming growth factor-beta isoforms, TGF-β receptor type II, and myofibroblast differentiation in cultured human corneal and limbal fibroblasts by amniotic membrane matrix. Journal of cellular physiology. 179(3): 325-335.

Ueta M, KWEON MN, Sano Y, Sotozono C, Yamada J, Koizumi N, Kiyono H and Kinoshita S. 2002. Immunosuppressive properties of human amniotic membrane for mixed lymphocyte reaction. Clinical & Experimental Immunology. 129(3): 464-470.

Vanore M, Chahory S, Payen G and Clerc B. 2007. Surgical repair of deep melting ulcers with porcine small intestinal submucosa (SIS) graft in dogs and cats. Vet ophthalmol. 10(2): 93-99.

von Versen-Hoeynck F, Steinfeld AP, Becker J, Hermel M, Rath W and Hesselbarth U. 2008. Sterilization and preservation influence the biophysical properties of human amnion grafts. Biologicals. 36(4): 248-255.

Watté CM, Elks R, Moore DL and McLellan GJ. 2004. Clinical experience with butyl-2-cyanoacrylate adhesive in the management of canine and feline corneal disease. Vet ophthalmol. 7(5): 319-326.

Wichayacoop T, Briksawan P, Tuntivanich P and Yibchok-anun S. 2009. Anti-inflammatory effects of topical supernatant from human amniotic membrane cell culture on canine deep corneal ulcer after human amniotic membrane transplantation. Vet ophthalmol. 12(1): 28-35.

Woo H, Kim M, Kweon O, Kim D, Nam T and Kim J. 2001. Effects of amniotic membrane on epithelial wound healing and stromal remodelling after excimer laser keratectomy in rabbit cornea. British journal of ophthalmology. 85(3): 345-349.

VITA

Miss Sukhumal Leevirojana was born on 6 September 1985 in Bangkok, Thailand. She received her bachelor degree, doctor of veterinary medicine (1st class honors) from Faculty of Veterinary Science, Chulalongkorn University in 2009. After graduation, she worked as a veterinarian at Chaengwattana animal hospital in 2010 to 2013. She has attended the Master's Degree Program in Veterinary Surgery of Chulalongkorn University scince 2012.