THERAPEUTIC EFFICACY OF SUBCONJUNCTIVAL INJECTIONOF CANINE ADIPOSE-DERIVED MESENCHYMAL STEM CELLSON CANINE SPONTANEOUS CHRONIC CORNEAL EPITHELIAL DEFECT

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Veterinary Surgery
Department of Veterinary Surgery
FACULTY OF VETERINARY SCIENCE
Chulalongkorn University
Academic Year 2022

ประสิทธิภาพของเซลล์ต้นกำเนิดชนิดมีเซนไคม์ที่สกัดจากไขมันที่ฉีดเข้าใต้เยื่อบุตาขาวเพื่อรักษาโรคแผลกระจกตาชั้นผิวชนิดเรื้อรังที่เกิดขึ้นเองในสุนัข

น.ส.เพชรชลี เก่งกล้า

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาศัลยศาสตร์ทางสัตวแพทย์ ภาควิชาศัลยศาสตร์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2565

Thesis Title	THERAPEUTIC EFFICACY OF SUBCONJUNCTIVAL INJECTIONOF CANINE ADIPOSE-DERIVED MESENCHYMAL STEM CELLSON CANINE SPONTANEOUS CHRONIC CORNEAL EPITHELIAL DEFECT
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Field of Study	Veterinary Surgery
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เพชรชลี เก่งกล้า : ประสิทธิภาพของเซลล์ต้นกำเนิดชนิดมีเซนไคม์ที่สกัดจากไขมันที่ฉีดเข้าใต้เยื่อบุตาขาวเพื่อรักษาโรคแผลกระจกตาชั้นผิวชนิดเรื้อรังที่เกิดขึ้นเองในสุนัข . (THERAPEUTIC EFFICACY OF SUBCONJUNCTIVAL INJECTIONOF CANINE ADIPOSE-DERIVED MESENCHYMAL STEM CELLSON CANINE SPONTANEOUS CHRONIC CORNEAL EPITHELIAL DEFECT) อ.ที่ปรึกษาหลัก : รศ. ดร.นลินี ตันติวนิช

โรคแผลกระจกตาชั้นผิวชนิดเรื้อรังที่เกิดขึ้นเองในสุนัข เป็นหนึ่งในการบาดเจ็บที่กระจกตาที่สำคัญในสุนัข ปัจจุบัน ในวงการจักษุทางสัตวแพทย์ มีการทดลองใช้เซลล์ต้นกำเนิดชนิดมีเซนไคม์ที่สกัดจากไขมันฉีดเข้าใต้เยื่อบุตาขาวเพื่อรักษาโรคแผลกระจกตาหลายชนิดในสุนัข การศึกษานี้จึงมีวัตถุประสงค์เพื่อประเมินผลทางคลินิก และเพื่อศึกษาปริมาณสารไซโตไคน์ในน้ำตาสุนัขที่เป็นโรคแผลกระจกตาชนิดเรื้อรัง หลังได้รับการรักษาโดยการฉีดเซลล์ต้นกำเนิดชนิดมีเซนไคม์ที่สกัดจากไขมันเข้าใต้เยื่อบุตาขาว ดวงตาของสุนัขทั้งหมด 9 ตาได้รับการฉีดเซลล์ต้นกำเนิดชนิดมีเซนไคม์ที่สกัดจากไขมันเข้าใต้เยื่อบุตาขาวเพียงครั้งเดียว ในปริมาณ $1x10^6$ เซลล์ ตรวจประเมินผลทางคลินิกในวันที่ 7,14 และ 21 หลังการรักษา โดยถ่ายรูปเพื่อนำไปประเมินผลหาพื้นที่แผลที่กระจกตา เส้นเลือดที่กระจกตา และความขุ่นของกระจกตา โดยใช้โปรแกรมซอฟต์แวร์ประมวลผลภาพ การศึกษาหาปริมาณไซโตไคน์ในน้ำตาสุนัข ทำโดยใช้แผ่นดูดซับสำหรับเก็บตัวอย่างน้ำตาเพื่อหาปริมาณสารเนิร์ทโกรทแฟคเตอร์ เอ็นโดทีเลียมโกรทแฟคเตอร์เอ และทูเมอร์เน็คโคสิทแฟคเตอร์แอลฟ่า โดยวิธีมัลติเพรคอิมมูโนเอสเส ผลการศึกษาพบว่าแผลที่กระจกตาของสุนัขเก้าตัว คิดเป็น 100 เปอร์เซ็นต์ ของสุนัขทั้งหมดหายได้ในระยะเวลาเฉลี่ย 10.89 ± 1.7 วัน สุนัขแสดงอาการเจ็บปวดดวงตาลดลง จากการศึกษาเปรียบเทียบกับก่อนการรักษาพบว่าร้อยละของพื้นที่แผลกระจกตา เส้นเลือด และความขุ่นที่กระจกตาลดลง หลังจากได้รับการรักษา ความเข้มข้นของสารทูเมอร์เน็คโคสิทแฟคเตอร์แอลฟ่า และสารเอ็นโดทีเลียมโกรทแฟคเตอร์เอ ลดลงหลังจากได้รับการรักษา ในขณะที่ความเข้มข้นของสารเนิร์ทโกรทแฟคเตอร์ไม่สามารถสรุปได้ จากการศึกษานี้สรุปได้ว่าการฉีดเซลล์ต้นกำเนิดชนิดมีเซนไคม์ที่สกัดจากไขมันเข้าใต้เยื่อบุตาขาวสามารถใช้เป็นทางเลือกในการรักษาโรคแผลกระจกตาชั้นผิวชนิดเรื้อรังที่เกิดขึ้นเองในสุนัข

สาขาวิชา ศัลยศาสตร์ทางสัตวแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2565 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

6270009231 : MAJOR VETERINARY SURGERY

KEYWORD: CANINE ADIPOSE-DERIVED MESENCHYMAL STEM CELL/SPONTANOUS CHRONIC CHORNEAL EPITHELIAL DEFECT

Pechchalee Kengkla : THERAPEUTIC EFFICACY OF SUBCONJUNCTIVAL INJECTIONOF CANINE ADIPOSE-DERIVED MESENCHYMAL STEM CELLSON CANINE SPONTANEOUS CHRONIC CORNEAL EPITHELIAL DEFECT. Advisor: Assoc. Prof. Dr. NALINEE TUNTIVANICH, D.V.M., Ph.D., Dip.AiCVO., D.T.B.V.S.

Spontaneous chronic corneal epithelial defects (SCCEDs) are important corneal injuries in dogs. Canine adipose-derived mesenchymal stem cells (cAD-MSCs) was used for treatment of various types of corneal ulcers in dogs. This study aimed to evaluate clinical outcomes and concentrations of tear fluid cytokine after subconjunctival injection of cAD-MSCs in canine SCCEDs. 9 canine SCCEDs eyes previously treated by debridement twice were included into this study. All eyes received a single subconjunctival injection of $1x10^6$ cAD-MSCs. Ophthalmic examinations were undergone at day 7, 14 and 21 after treatment. Photographs of the cornea were taken to assess corneal epithelial defect, neovascularization and opacification area using image analysis software. Tear samples were collected using ophthalmic sponge for quantification of nerve growth factor- beta (NGF-β), vascular endothelium growth factor-A (VEGF-A), and tumor necrosis factor-alpha (TNF-α) concentration using canine multiplex immunoassay. Complete corneal healing occurred in 9 eyes at the mean healing time of 10.89 ± 1.7 days. Percentage of the three corneal characteristics decreased compared to pretreatment. Concentration of TNF-α and VEGF-A decreased while that of NGF-β was inconclusive. In conclusion, single subconjunctival injection of cAD-MSCs can be used as an alternative treatment of canine SCCEDs without adverse effect.

Field of Study: Veterinary Surgery Student's Signature .....

Academic Year: 2022 Advisor's Signature .....

ACKNOWLEDGEMENTS

First, I would like to express my sincere gratitude to my major advisor, Assoc. Prof. Dr. Nalinee Tuntivanich for her guidance, encouragement and supports throughout the study. My study would not have been accomplished without her caring supervision.

I would like to sincerely thank Assist. Prof. Dr. Yaowalak Panyasing for her advice on laboratory techniques and instructors.

I would like to thank my thesis committee members, Assoc. Prof. Preenun Jitasombuti and Asst. Prof. Dr. Kumpanart Soontornvipart for their valuable suggestions. And I also would like to thank Dr. Saharuetai Jeamsripong for statistical consultant.

I am grateful for great supports and cares from staffs of the Ophthalmology Clinic, Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. I am also appreciated the assistance of the Department of pathology, and Chulalongkorn University Laboratory Animal Center, Faculty of Veterinary Science, Chulalongkorn University, for supporting of laboratory facilities and equipment.

This research was financially supported by the Graduate Student Fund, Faculty of Veterinary Science, Chulalongkorn university.

Finally, I would like to express my profound gratitude to my parents, my graduated colleagues, my friends for their love, supports, understanding, and continuous encouragement through the process of my study. My accomplishment would not have been possible without them.

Pechchalee Kengkla

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LIST OF ABBREVIATIONS

SCCEDs	=	spontaneous chronic corneal epithelial defects
SEM	=	standard error of the mean
NGF-β	=	nerve growth factor- beta
TNF-α	=	tumor necrosis factor-alpha
VEGF-A	=	vascular endothelium growth factor-A
EGF	=	epidermal growth factor
TGF-β	=	transforming growth factor- beta
IL-1	=	interleukin-1
IL-1β	=	interleukin-1beta
IL-6	=	interleukin-6
α-Sma	=	alpha-smooth muscle actin
MMP-2	=	matrix metalloprotinase-2
MMP-8	=	matrix metalloprotinase-8
MMP-9	=	matrix metalloprotinase-9
MMP-13	=	matrix metalloprotinase-13
MCP-1	=	myocyte chemoattractant protein-1
MIP-1α	=	macrophage inflammatory protein-1alpha
TSP-1	=	thrombospondin-1
PEDF	=	pigment epithelium derived factor
bFGF	=	basic fibroblast growth factor
SP	=	substance P
Fas-L	=	Fas ligand
CGRP	=	calcitonin gene-related peptide immunoreactive
PSGAGs	=	Polysulfated Glycosaminoglycan
DBD	=	diamond burr debridement
ESCs	=	embryonic stem cells
iPSCs	=	induced pluripotent stem cells
ASCs	=	adult stem cells

MSCs	=	mesenchymal stem cells
AD-MSCs	=	adipose-derived mesenchymal stem cells
cAD-MSCs	=	canine adipose-derived mesenchymal stem cells
BM-MSCs	=	bone marrow-derived mesenchymal stem cells
HSCs	=	hematopoietic stem cells
CESCs	=	corneal epithelial stem cells
IV	=	Intravenous
p-STAT3	=	phosphorylated signal transducer and activator of transcription 3
PBS	=	phosphate buffer saline
ml	=	milliliter
mm^2	=	square millimeter
µl	=	microliter
pg	=	picogram
STT-1	=	Schirmer tear test-1
IOP	=	intraocular pressure
UAB	=	universal assay buffer
SA-PE	=	streptavidin-PE
KCS	=	keratoconjunctivitis sicca
OD	=	oculus dexter
OS	=	oculus sinister
ELISA	=	enzyme-linked immunosorbent assay
PED	=	persistent epithelial defect

CHAPTER I

INTRODUCTION

Importance and Rationale

Spontaneous chronic corneal epithelial defects (SCCEDs) are chronic corneal epithelial defect characterized by the presence of an epithelial erosion surrounded by nonadherent epithelium. If left untreated, the defect can persist in association with severe ocular pain, leading to reduced quality of life. Many therapeutic methods have been proposed to treat SCCEDs in dogs, both medical treatment (Kirschner et al., 1991; Morgan et al., 1994; Miller et al., 1996; Murphy et al., 2001) and surgical intervention (Morgan et al., 1994; Bentley et al., 2005; Aldave et al., 2009). In general, topical treatments alone are insufficiently capable to achieving healing. Surgical intervention is required to stimulate corneal epithelial regrowth, to which general anesthesia is mostly involved (Bentley, 2005).

Nowadays, stem cell therapies are widely used in human and veterinary regenerative medicine. In veterinary practices, stem cell therapies are currently used for treatment of various diseases including orthopedic injury in horse (Nixon et al., 2008; Wilke et al., 2007) and ophthalmic diseases in several animal models (Almaliotis et al., 2013; Yao et al., 2011; Falcao MSA et al., 2019). Mesenchymal stem cells (MSCs) are multipotent stem cells isolated from various sources such as bone marrow (Wexler et al., 2001) and adipose tissue (Zuk et al., 2001). Recently, several studies using MSCs for corneal epithelial regeneration in animal model have been published. Not only safety, it has ability to promote corneal healing process, reduce corneal inflammation and angiogenesis and recover corneal transparency (Ke et al., 2015; Zhang et al., 2020).

The therapeutic effects of MSCs rely on its properties mainly reduction of inflammation and angiogenesis via paracrine activity, secretion of growth factors for stimulating resident stem cells, migration towards the injured areas and the transdifferentiation ability of the transplanted MSCs into corneal epithelial cells

(Holan et al., 2015). Several works in animal models suggest that subconjunctivally injected MSCs reduce the infiltration of inflammatory cells into the cornea and decrease mainly TNF-α expression at the site of injury, promoting a less inflammatory microenvironment (Ke et al., 2015; Di et al., 2017; Zhang et al., 2020). In ocular alkali injury of rat model, subconjunctivally injected MSCs also reduce corneal angiogenesis through a decrease of pro-angiogenic cytokine as evident by reducing of corneal neovascularization and VEGF expression (Yao et al., 2011). In addition, in vitro study of Dabrowski and others (2016) demonstrated that MSCs has ability to secrete EGF, VEGF-α, TGF-β for promoting of corneal epithelial healing. The ability of MSCs to secrete NGF was reported by Zhang and others (2012). Interestingly, the administration of topical recombinant human-NGF in treatment of persistent epithelial defect (PED) with neurotrophic keratopathy in human has been reported to be effective (Mastropasqua et al., 2020). In canine SCCEDs, the clinical features and histopathological findings have been currently described and shown to share some similarities to persistent epithelial defect (PED) in humans (Bentley et al., 2001; Murphy et al., 2001). Thus, MSCs may provide some beneficial for promoting corneal healing of canine SCCEDs.

Adipose derived mesenchymal stem cells (AD-MSCs) have been used as a source for cell therapy of various corneal diseases in experimental models. Combination of subconjunctival and topical administration of canine AD-MSCs (cAD-MSCs) in various types of corneal ulcers revealed 84.6% of complete corneal wound healing within 14 days after transplantation (Falcao et al., 2019).

According to the ability of MSCs, cAD-MSCs may possibly be used as substitute for surgery. In this study, subconjunctival injection of cAD-MSCs was applied as an alternative treatment for canine SCCEDs.

Research questions

1. Can subconjunctival cAD-MSCs clinically treat canine SCCEDs?
2. Is the concentration of NGF-β in tear fluid different after subconjunctival injection of cAD-MSCs in canine SCCEDs?
3. Is the concentration of TNF-α in tear fluid different after subconjunctival injection of cAD-MSCs in canine SCCEDs?
4. Is the concentration of VEGF-A tear fluid different after subconjunctival injection of cAD-MSCs in canine SCCEDs?

Research objectives

1. To evaluate clinical outcomes including corneal epithelial defect, neovascularization and opacification after subconjunctival injection of cAD-MSCs on canine SCCEDs
2. To quantify concentration of NGF-β, TNF-α, and VEGF-A in tear fluid after subconjunctival injection of cAD-MSCs in canine SCCEDs

Research Hypothesis

1. Subconjunctival application of cAD-MSCs can clinically treat canine SCCEDs.
2. Concentration of NGF-β in canine SCCEDs tear fluid increases after subconjunctival application of cAD-MSCs
3. Concentration of TNF-α in canine SCCEDs tear fluid decreases after subconjunctival application of cAD-MSCs
4. Concentration of VEGF-A in canine SCCEDs tear fluid decreases after subconjunctival application of cAD-MSCs

Advantage of the study

Subconjunctival application of canine adipose-derived mesenchymal stem cells could be used as an alternative treatment of SCCEDs in dogs.

CHAPTER II

REVIEW OF LITERATURES

Spontaneous Chronic Corneal Epithelial Defects

Spontaneous chronic corneal epithelial defects (SCCEDs) in dogs are characterized as nonhealing defects in the corneal epithelial layer. It commonly occurs in middle-aged dogs averaging 7 to 9 years in all dog breeds, especially boxers. Affected dogs exhibit various degrees of discomfort such as blepharospasm, and epiphora. If left untreated, the defect can persist for longer than 6 months (Bentley, 2001). This disease is also known as boxer erosions, indolent ulcers, persistent corneal erosions, canine recurrent erosions, refractory corneal ulcers, recurrent epithelial erosions, nonhealing erosions, and idiopathic persistent corneal erosions (Bentley, 2005).

The typical clinical appearance of SCCEDs is chronic, superficial, noninfected corneal erosion surrounded by nonadherent epithelium called epithelial lip. The epithelium sometimes become thickened and fluorescein dye leaks beneath it, resulting in less intense staining ring around the exposed stroma (Figure 1) (Bentley, 2005). Bentley and other (2001) reported that the SCCEDs lesion can occur at any location of the cornea especially axial and paraxial area. 58% to 64% of eyes exhibit corneal neovascularization that appears at peripheral lesions while lesions at the center may persist for months without vascular response.

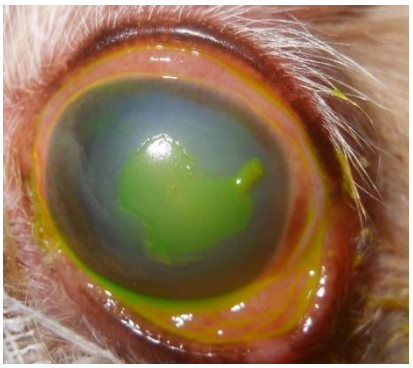


Figure 1. Photograph of typical characteristic of SCCEDs
presented as corneal epithelial lesion surrounded by nonadherent epithelium

Pathophysiology of SCCEDs

Until present, pathophysiology of SCCEDs remains unclear. Possible pathophysiology of this condition has been suggested as followed.

Abnormal epithelial structure: Bentley and others (2001) studied morphology and immunohistochemistry of SCCEDs in dogs. Common characteristic of SCCEDs was revealed as corneal tissue adjacent to ulcerated area exhibiting abnormal epithelial structure and poor attachment of epithelium to an underlying stroma (Figure 2). Stroma underneath the lesion had varying degrees of leukocytic infiltrates, predominate neutrophils and lymphocytic-plasmocytic infiltration. Basement membrane as well as its components and adhesion complex including laminin, collagen IV, and collagen VII were absent or discontinuous on stromal surface surrounding the ulceration. In contrast, fibronectin, the important factor in the early migration and formation of temporary adhesions, was presented on the surface of the erosion. This finding suggested that early epithelial wound healing occurred, but the reformation of adhesion complexes was delayed. It then resulted in the poorly attachment of epithelium to the underlying stroma. Accordingly, these abnormal structures may play a role in the avoidance of corneal re-epithelialization.

Acellular barrier: Bentley and others (2001) found a superficial hyalinized acellular zone in the superficial corneal stroma of all canine SCCEDs samples (Figure 2). They suggested that this zone becomes a barrier to the reformation of corneal adhesion complexes and normal basement membranes. So, this barrier may play an important role in refractory characteristics of this ulcer.

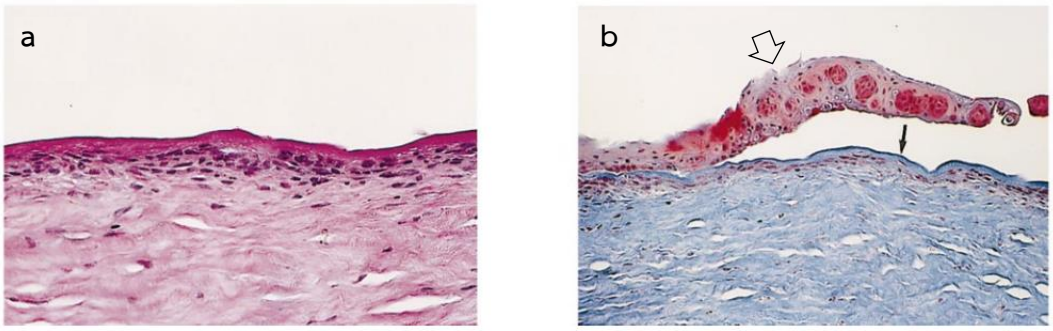


Figure 2. Morphological photographs of (a) canine normal cornea (b) canine SCCEDs cornea demonstrating epithelial dysmaturation and poor attachment of epithelium to the underlying stroma (open arrowhead). Note: superficial stromal hyaline acellular zone (solid arrowhead). (Bentley et al., 2001)

Abnormal plexus nerve fibers containing substance P and CGRP: Canine cornea is densely innervated with sympathetic and sensory nerves particularly substance P-containing sensory nerves. substance P (SP) is neurotransmitter released form canine corneal sensory nerves. It has trophic abilities to promote corneal epithelial healing including promotion of migration and stimulation of proliferation. Murphy and others (2001) studied the alterations of innervation and SP content in canine SCCEDs. Immunohistochemistry results demonstrated dense abnormal plexus nerve fibers containing SP and calcitonin gene-related peptide immunoreactive (CGRP) in the corneal stroma around the epithelial erosion. These characteristics, however, was not found in normal corneas that underwent weekly experimental epithelial debridement for 4 weeks. It is therefore suggested that abnormal hyperinnervation with accumulation of SP content may play a possible role in pathogenesis of SCCEDs.

Treatment of SCCEDs

There are many strategies that have been proposed for the treatment of SCCEDs.

Medical treatment

Substance P with or without Insulin like growth factor-1: Many studies suggest that SP with or without Insulin like growth factor-1 may play an importance role in pathogenesis of SCCEDs (Murphy et al., 2001). According to the previous study of Murphy and others (2001), they studied its efficacy, topically applied in dog with SCCEDs. Treated dogs had healing rate approximately 70% and 75% respectively.

Aprotinin: Aprotinin is proteinase plasmin inhibitor which is associated with refractory corneal ulcer. Proteinases are suggested to play a role in pathogenesis of SCCEDs due to preventing reattachment of basement membrane to an underlying stroma. A study using topical aprotinin in conjunction with epithelial debridement for treatment of SCCEDs in dog reveals a healing rate approximately 38.8% which is not significantly different from untreated dogs. Accordingly, it is currently not recommended for treatment of SCCEDs in dogs (Moore et al., 2003).

Epidermal growth factor (EGF): EGF is growth factor that promotes epithelial cell mitosis and protein synthesis. The one study of using topical EGF administration in conjunction with epithelial debridement for treatment of SCCEDs in dog demonstrated approximately 80% healing rate within 14 days post treatment (Kirschner et al., 1991). However, it is not commercially available for topical use.

Fibronectin: Fibronectin is glycoprotein acting as an adhesive molecule to promote cell epithelial cell migration and adhesion. Topical fibronectin administration in persistent epithelial defects in human demonstrated approximately 92.8% of healing rate (Kim et al., 1992). However, it is not commercially available for topical use.

Serum: Serum is considered beneficial for treatment of SCCEDs in dogs because it contains several factors including EGF, fibronectin, proteinase inhibitors, SP, and vitamin A. However, a study by Eaton and others (2017) with the use of topical heterologous serum on re-epithelialization rate of SCCEDs in dogs demonstrated that median time to re-epithelialization in the treatment group was not significantly different from placebo-treated group.

Polysulfated Glycosaminoglycan (PSGAGs): PSGAGs is proteolytic inhibitors that work by inhibition of plasmin and activation of plasminogen. Several studies reported that plasmin can disrupt epithelial cell adhesion and involve in the development of refractory corneal ulcers in human. One study of using topically applied PSGAGs in conjunction with epithelial debridement for treatment of SCCEDs in dogs reported 83% healing rate with healing times approximately 11.97 days after treatment (Champagne E.S. et al., 1992). However, the previous study reported increased proteolytic activity in tears fluid of only some dogs with SCCEDs. PSGAGs may be therefore not beneficial for the treatment of SCCEDs in all affected dogs (Bentley, 2005).

Almost studies of medical therapies require epithelial debridement as part of treatment. Thus, it is questionable that the complete wound healing occurs either from the epithelial debridement or from the medical treatment. Additionally, many of these topical medications are commercially unavailable and impractical for using by the owner due to high cost and high frequency of application (Moore et al., 2003).

Surgical treatment

Mechanical debridement and chemical debridement: Mechanical debridement with or without chemical debridement is the most commonly conventional technique for treatment of SCCEDs. After application of topical anesthesia, mechanical debridement is performed by using sterile dry cotton swab to remove all loose epithelium and debris from stromal surface. Povidone-iodine are commonly used for chemical debridement in combination with mechanical debridement (Whitley RD. et al., 1999). Corneal defect is debrided until epithelium is not dislodge from the stroma. This technique stimulates corneal healing by removing abnormal epithelium and basement membrane as well as debris from exposed stroma, resulted in promoting of epithelial cell proliferation and adhesion. However, it has low success rate and prolong post-treatment healing times when compared to other techniques (Moore et al., 2003). The resolution rate with a single epithelial debridement alone is approximately 50% (Bentley et al., 2005). Combination of a contact lens bandage or third eyelid flap after epithelial debridement increased healing rates to 58% and 64%, respectively (Morgan et al., 1994).

Diamond burr debridement (DBD): DBD is another common method for manual epithelial debridement. After topical anesthesia, the diamond burr is gently passed over the ulcerated area in a circular motion to remove all loose epithelium. Contact lens bandage may be applied after complete debridement to protect the ulcerated cornea. This technique significantly decreases the thickness of hyalinized acellular zone in anterior stroma (Dawson et al., 2017). Additionally, it may enhance epithelial adhesion by exposing the newly regenerated epithelium to the anterior stroma, inducing the expression of extracellular matrix and reactive fibrosis, and facilitating the reformation of adhesion complexes (Hung et al., 2021). According to Gosling and other (2013), SCCEDs dog receiving single DBD with the placement of contact lens bandage had 70% and 92.5% healing rate at 7 and 15 ± 5 days after intervention respectively. Another study demonstrated increased overall healing rates to 94% after DBD (Aldave et al., 2009).

Grid keratotomy: Grid keratotomy is a minimal invasive surgical procedure for treatment of SCCEDs in dogs. It can be performed under topical anesthesia with sedation or general anesthesia in aggressive animals. Prior to perform grid keratotomy, the loose epithelium is debrided with a sterile cotton swab. After that, hypodermic needle is used to create superficial linear anterior stromal microincisions in a grid pattern. Linear incisions are made at 1-2 mm intervals across the ulcer surface and extended to 1-2 mm beyond the edge of ulcerated area. The depth of linear incision is approximately one fourth of the anterior stroma. This procedure increases healing rate by penetrating the hyalinized acellular zone in anterior stroma, resulting in providing area of healthy stroma for epithelial adhesion. While it increases success rate, it decreases post-treatment healing time compared to debridement technique. According to the study of Stanley and others (1998), treatment of canine SCCEDs with grid keratotomy without third eyelid flap results in 80-85% success rate and approximately 13.4-14 days post-treatment healing time. Whereas the success rate of using grid keratotomy in conjunction with third eyelid flap is 100% with approximately 10 days post-treatment healing times (Stanley et al., 1998).

Superficial keratectomy: Superficial keratectomy is a surgical technique to remove corneal epithelium, Bowman's membrane, and superficial stroma. Since one fourth to one third of stroma is removed, hyalinized acellular zone in anterior stroma in canine SCCEDs is also eliminated, resulting in eliminating the barrier of epithelial healing. Accordingly, superficial keratectomy in conjunction with third eyelid flap technique demonstrates the highest success rate and shortest post-treatment healing time compared to other procedures. A study of SCCEDs dogs treated with superficial keratectomy demonstrates 100% success rate and approximately 9.3 days post-treatment healing time (Stanley et al., 1998). However, it is a costly procedure due to requiring general anesthesia and specific surgical equipment. Additionally, the surgeon requires experience in microsurgery (Moore et al., 2003)

An alternative treatment

There was the study using canine adipose-derived mesenchymal stem cells (cAD-MSCs) for treatment of canine SCCEDs. In canine corneal ulcers at various degree of severity including 2 dogs with SCCEDs, subconjunctival injection of stem cell suspension in transport medium (250,000 cells/0.25 ml), followed by 11 instillations (once per hours) with same numbers of cAD-MSCs cells were administered (Falcao et al., 2019). The results demonstrate that 84.6% of dogs including 2 dogs with SCCEDs had complete corneal wound healing within 14 days after treatment. Degree of corneal ulcer depth and discomfort significantly decreased by 14 days follow-up period. No adverse effect of cAD-MSCs transplantation was reported throughout the experiment.

Stem cells

Stem cells are undifferentiated cells that have specific properties including indefinite self-renewal capacity and the ability to proliferate more differentiated daughter cells for aging replacement throughout life. They can differentiate into various cell types in the body (Lin et al., 2013). Stem cells are mainly classified into 3 types based on their differentiation potential into various cell lineages including totipotent, pluripotent, and multipotent stem cells (Balistreri. C.R., 2017.) (Figure 3).

The totipotent stem cell is only a zygote as it can develop into any specialized cell in the organism (Balistreri. C.R., 2017.). Totipotent stem cells are capable to self-renewing and differentiating into the three primary germ cell layers of the early embryo as well as extra-embryonic tissues while pluripotent stem cells have the same capacity except differentiation to placenta. There are 2 types of pluripotent stem cells including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Embryonic stem cells (ESCs) are isolated from inner cell mass within blastocysts. These cells can proliferate in vitro and differentiate into all cell types of three germ layers including ectoderm, endoderm, and mesoderm (Choo et al., 2008). Although several in vivo and in vitro studies support that ESCs possibly provide high therapeutic potential, they have considerable adverse effects including

tumor formation (e.g., teratoma and teratocarcinoma) and immune rejection of transplanted stem cells (Herberts et al., 2011). Additionally, ethical concerns have raised upon the need of isolation stem cells from pre-implantation embryos.

Induced pluripotent stem cells are pluripotent stem cell derived from adult somatic cells that have been reprogrammed back into an embryonic-liked pluripotent through inducing *Yamanaka* factors (Oct3/4, Sox2, Klf4, c-Myc) (Ye et al., 2013). Several studies supported that iPSCs have high replicative capability in vitro. iPSCs seem to have the greatest promise without ethical and immunologic concerns by using human ESCs. However, iPSCs have considerable adverse effects including the ability to form tumor (e.g., teratoma) in vitro. Moreover, the previous studies demonstrated only the therapeutic potential of human iPSCs -based therapy in vitro and in experimental animal models. Long term of safety data and clinical studies are furtherly required before using human iPSCs for treatment of disease in human and animals.

Multipotent stem cells are undifferentiated adult stem cells (ASCs) that can differentiate into distinct cell types based on their tissue of origin. There are several types of multipotent stem cells including mesenchymal stem cells, intestinal stem cells, hematopoietic stem cells, neuronal stem cells, and stem cells of the epidermis and hair follicles (Prochazkova et al., 2015). In human regenerative medicine, multipotent stem cells have been recently used in the treatment of various diseases such as hematological, neurological, cardiovascular, orthopedic, ophthalmic diseases and skin regeneration. In veterinary medicine, mesenchymal stem cells (MSCs) have been recently used for treatment of orthopedic injuries, such as tendonitis (Nixon et al., 2008), cartilage injury (Wilke et al., 2007) and osteoarthritis (Frisbie et al., 2009) in horses and dogs. In addition, MSCs are also clinically used for treatment of ophthalmic diseases, for example, corneal alkali injuries in rabbits (Almaliotis el al. in 2013), corneal alkali injuries in mice (Shukla et al., 2019), corneal alkali injuries in rats (Yao et al., 2011), corneal ulcer in horses (Marfe et al., 2011), corneal ulcer in dogs (Falcao et al., 2019), and eosinophilic keratitis in cats (Villatoro et al., 2018).

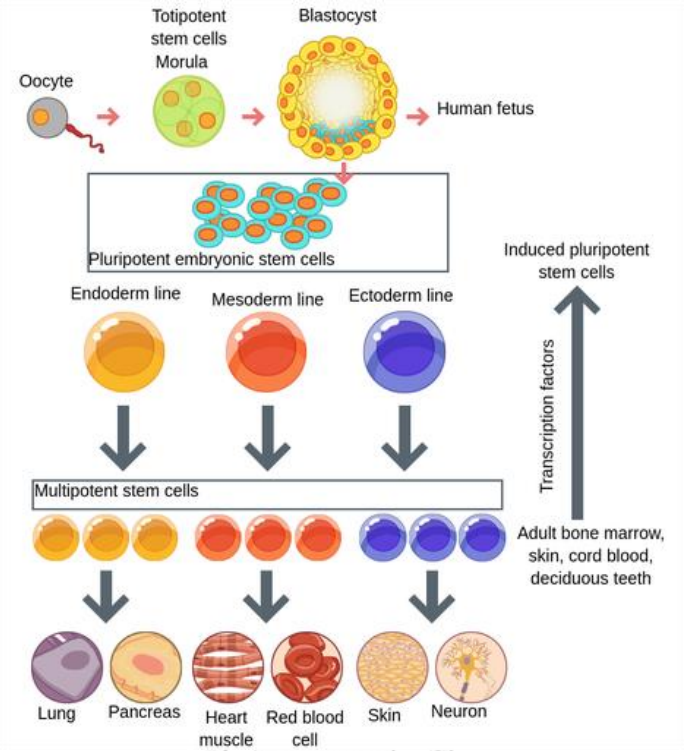


Figure 3. Type of stem cells classified based on their differentiation potential. This picture is modified from Balistreri. C.R., 2017.

Mesenchymal stem cells (MSCs)

Mesenchymal stem cells are multipotent cells isolated from various tissue such as bone marrow (Wexler et al., 2001), adipose tissue (Zuk et al., 2001), muscle (Asakura et al., 2001), umbilical cord (Wang et al., 2004), or oral tissue (Seo et al., 2004).

Isolation of MSCs

After the initial isolation of MSCs from adult human bone marrow, MSCs has been alternatively isolated from other tissues including embryonic tissues, muscle, adipose tissue, periodontal ligament, tendon, synovial membranes, trabecular bone, periosteum, and skin (Orbay et al., 2012). Common isolation method of MSCs is briefly described. MSCs isolated from various tissues are cultured in culture medium including Dulbecco's Modified Eagle Medium (DMEM) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Nonadherent cells are removed 24-72 hours later by changing the medium. This step is repeated until primary cultures reach 70-90% confluence, then adherent cells are sub-cultured and characterized according to minimal criteria defined by the International Society for Cellular Therapy (ISCT) as followed (Dominici M. et al., 2006).

1. MSCs must be adherent to plastic in standard culture condition.
2. MSCs must be able to express surface markers such as CD90, CD73, and CD105, and lack expression of surface markers such as CD14, CD19, CD45, CD34, and HLA-DR.
3. MSCs must have the capacity to differentiate into adipocytes, chondroblasts, and osteoblasts in vitro.

Route of administration of MSCs

Intravenous injection: In general, intravenous injection is the most common route of administration of MSCs. The advantage of this route is that MSCs can be transported through blood circulation to any site of injury in the body, hence, it is suitable for treatment of systemic diseases. However, major limitation of this route is the local penetration in ocular surface. Furthermore, there is a report that most MSCs are trapped in the lung during transportation. Fewer MSCs could be delivered to the site of injury (Lee et al., 2009).

Topical application: In ophthalmology, topical eye drop is the most common route of MSCs administration. Not only the ease of application and less systemic side effects, it also provides high effective concentration directly to the ocular surface. However, approximately 95% of the topical eye drop is eliminated due to tear turnover and reflex blinking. Accordingly, active substances of eye drops may not remain on the ocular surface for efficient contact time (Rafiei et al., 2020).

Cell carriers for MSCs transplantation: Amniotic membrane (AM) has been used as cell carriers for MSCs transplantation to ocular surface, both in experimental models and humans (Galindo et al., 2021). According to the previous study of using human AM as cell carriers for MSCs transplantation in rat by Ma and others (2009), severely damaged corneal surface in rat is reconstructed by inhibition of inflammation and angiogenesis. As compared to other route of administration, the number of stem cells delivered to ocular surface with cell carrier is lower. There is a high risk of disease transmission via AM and limited availability of AM. Moreover, surgical intervention of AM as cell carrier for MSCs transplantation to the ocular surface results in high economic cost (Galindo et al., 2021).

Subconjunctival injection: Subconjunctival injection is a practical alternative route for drug administration to the eye. It has several advantages including minimal invasion, administration of high drug concentration in a small volume and achievable routine care for different disorders. Several studies are reported administration of MSCs via subconjunctiva to treat different ophthalmic diseases in animals, such as corneal alkali injury in rabbits (Almaliotis et al., 2013), corneal ulcer in dogs (Falcao et al., 2019) and bacterial ulcerative keratitis in horses (Marfe et al., 2011). This

procedure does not require intensive surgical facilities or additional post-injection interventions, resulting in reduction of cost and time (Galindo et al., 2021).

Comparison of therapeutic efficacy in different route of MSCs administration

In this regard, the route of MSCs administration for corneal injury is an importance factor influencing its therapeutic efficacy. Currently, Shukla and others (2019) investigated regarding the best route of MSCs administered to the ocular surface for promoting of corneal regeneration. They assessed the therapeutic effects among different routes of BM-MSCs ($5x10^6$ cells) administration including topical, subconjunctival, intravenous (IV) and intraperitoneal (IP) following corneal mechanical injury in mice. The results demonstrated that corneal re-epithelialization was significantly accelerated in subconjunctival and IV administered group as evidence by reducing of corneal epithelial defect following corneal injury compared to untreated group. In term of reducing corneal inflammation, CD45+ cells infiltration in the cornea as well as expression of proinflammatory cytokines including TNF-α and IL-1β were significantly reduced following subconjunctival and IV administration as compared to untreated group. Furthermore, corneal opacification area as well as expression of α-Sma and TGF-β were also significantly reduced in both groups. Taken together, these data suggested that subconjunctival and IV routes of MSCs administration were greatly effective in promoting corneal re-epithelization, reducing corneal inflammation and opacification following corneal injury in mice (Shukla et al., 2019).

Another study comparing the effect of two applications of BM-MSCs including subconjunctival injection and AM transplantation on corneal neovascularization and wound healing in rats with corneal alkali injury. They demonstrated that BM-MSCs via subconjunctival injection appeared more effective than transplantation with the AM. The difference in clinical outcome was predominantly significant for corneal neovascularization, rather than corneal epithelial defect or opacity. Immunohistochemistry results demonstrated inflammatory cells infiltration and area of corneal neovascularization were significantly reduced in subconjunctival injection

group. Additionally, the expression of proangiogenic cytokine including VEGF and MMP-9 were also significantly reduced in the subconjunctival injection group compared to both AM transplantation and untreated group. (Ghazaryan et al., 2016).

Furthermore, El-Din and others (2021) evaluated the therapeutic efficacy of intravenous versus subconjunctival BM-MSCs on experimentally ultraviolet-induced corneal injury in albino rats. The results demonstrated that subconjunctival injection of BM-MSCs had a remarkable regenerative efficacy on the corneal injury as compared to the IV route. This results possibly occurs through the inhibition of neovascularization, suppression of the inflammatory reaction, and degenerative changes of the epithelial cells induced by chronic ultraviolet irradiation exposure (El-Din et al., 2021).

Properties of MSCs

Anti-inflammatory and immunomodulatory effects

The immunomodulatory and anti-inflammatory mechanisms of MSCs are suppressing proinflammatory cytokines production, producing anti-inflammatory cytokines and inhibiting neutrophils, macrophages, and lymphocytes activation.

Several studies have investigated effects of MSCs on corneal epithelial damage in animal models. Di and others (2017) investigated anti-inflammatory effect of subconjunctival injection of mouse BM-MSCs on corneal mechanical damage in mice. The result demonstrated that the secretion of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and myocyte chemoattractant protein-1 (MCP-1), was reduced after intervention. Additionally, other studies on ocular alkali burn in rat model reported that the expression of pro-inflammatory cytokines including TNF-α, IL-6, IL-1, MCP-1, and MIP-1α was significantly reduced in BM-MSCs-subconjunctivally administered rats (Ke et al., 2015; Zhang et al., 2020).

Anti-inflammatory activity of MSCs is present through the inhibition of neutrophil, macrophage, and lymphocyte activation. Investigations in mice with corneal mechanical damage models demonstrated that ocular inflammatory

response and infiltration of macrophages (CD86+ and CD45-positive cells) in the cornea were reduced after subconjunctival injection of BM-MSCs (Ke et al., 2015; Zhang et al., 2020). Another study in mice with mechanical corneal injury demonstrated that neutrophil infiltration in the cornea were reduced after subconjunctival injection of BM-MSCs (Ke et al., 2015; Zhang et al., 2020). Additionally, infiltration of T lymphocytes (CD3+) in the ocular surface of mice was decreased after subconjunctival injection of human BM-MSCs (Martinez-Carrasco R. et al., 2019).

Role of MSCs on Tumor necrosis factor-α (TNF-α)

Tumor necrosis factor-α is the proinflammatory cytokine involving in the corneal inflammatory response and wound healing following corneal injuries. Wang and others (2020) reported the mechanism that proinflammatory cytokines modulate corneal epithelial wound healing via the $p16^{Ink4a}$-STAT3 signaling. They found that after corneal epithelial debridement in mice, persistent treatment with IL-1β or TNF-α suppressed corneal epithelial wound healing through reducing phosphorylated signal transducer and activator of transcription 3 (p-STAT3) level and increasing cell cycle inhibitor $p16^{Ink4a}$ level. It then resulted in the suppression of STAT3 signaling pathway which regulated the genes involved in modulating corneal inflammation and corneal epithelial stem cells (CESCs) proliferation, migration, and differentiation. It was evident that corneal epithelial defects in control mice were healed faster than those mice received topical IL-1β or TNF-α administration. This suggests that persistent elevation of these cytokines is an important cause of corneal epithelial wound healing impairment in persistent inflammation (Wang et al., 2020).

The efficacy of subconjunctival injections of BM-MSCs (2×10^6 cells) combined with polysaccharide hydrogel treatment was investigated after corneal alkali injury in rats. The results demonstrated that TNF-α expression was highly detected in the chemically induced-ulcerated cornea and gradually decreased when the corneal ulcer had healed. Corneal epithelial defect area was highly increased on day 3 and gradually decreased on day 7 after corneal injury. Clinical appearance of epithelial

defect area was correlated with an increase in TNF-α expression on day 3 and gradually decreased from day 7 through day 28 after corneal injury. Furthermore, after subconjunctivally injected BM-MSCs on corneal alkali injury, TNF-α expression as well as corneal epithelial defect were significantly reduced in combined polysaccharide hydrogel and BM-MSCs treated group as compared to control group (Ke et al., 2015). Another study in mechanically corneal injury in mice, subconjunctival injection of BM-MSCs (5×10^5 cells) enhanced corneal epithelial healing. At day 4 post-injury, both corneal epithelial defect and TNF-α expression were significantly reduced compared to untreated group (Shukla et al., 2019).

Anti-angiogenesis effect

The anti-angiogenesis effect of MSCs mainly occurs through the inhibition of inflammation-related angiogenesis in the cornea via paracrine activity. MSCs upregulates the expression of thrombospondin-1 (TSP-1), an anti-angiogenic factor which can downregulate inflammation related proangiogenic factors, such as matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor (VEGF) (Galindo et al., 2021). According to the previous study by Yao and others (2011), corneal neovascularization and VEGF expression were decreased after subconjunctival injection of BM-MSCs on ocular alkali injury in rat model. Moreover, topical application of MSCs on chemical burn of rat corneas revealed that corneal neovascularization and TSP-1 in cornea was upregulated, whereas MMP-2 was significantly downregulated after the administration of MSCs (Oh JY. et al. 2008).

Role of MSCs on Vascular endothelial growth factor (VEGF)

After any corneal insult, corneal neovascularization is pathological process of angiogenesis closely associated with corneal inflammation. It is characterized by the invasion of new blood vessels into the corneal stroma as a result of imbalance between proangiogenic and antiangiogenic factors that preserve corneal transparency. Antiangiogenic factors include angiostatin, endostatin, pigment epithelium derived factor (PEDF), Fas ligand (Fas-L), and thrombospondin-1 (TSP-1).

Proangiogenic factors include VEGF, basic fibroblast growth factor (bFGF), interleukin-1 (IL-1) and matrix metalloproteinases (MMPs) (Brantman et al., 2013). There are four steps required for corneal neovascularization formation including (1) recruitment of leukocytes, (2) degradation of endothelial basement membrane and extracellular matrix (ECM), (3) invasion and proliferation of endothelial cells, and (4) the formation of vascular sprouts and proper lumens (Zhang et al., 2020).

Several studies supported that VEGF is proangiogenic cytokine that plays a key role in corneal neovascularization by inducing endothelial cells proliferation, migration, and tubulogenesis. During corneal insult, epithelial injury induces the release of inflammatory cytokines mainly IL-1, TNF-α and inflammatory cells infiltration such as neutrophils and macrophage can then stimulate VEGF production leading to corneal angiogenesis. In addition, tissue hypoxia also induces producing of VEGF (Brantman et al., 2013).

Ke and others (2015) investigated the efficacy of subconjunctival injections of BM-MSCs combined with polysaccharide hydrogel treatment on corneal alkali burn in rat. The reduction of corneal neovascularization was significantly enhanced by the combined treatment compared to control group. Of note, the control group showed gradually increased area of corneal neovascularization from day 7 through day 28 after corneal injury. In contrast, the areas of corneal neovascularization in polysaccharide and MSCs treatment groups were significantly less than in the control group at all time points. In this combined group, corneal neovascularization area was increased from day 7 to day 14 then gradually decreased through day 28 after corneal injury. Meanwhile, the level of VEGF expression increased untill day 3 and remarkably decreased through day 7 and 14 thereafter in all groups. Additionally, corneal neovascularization area, together with level of VEGF expression were significantly reduced in polysaccharide and MSCs treatment groups as compared to control group (Ke et al., 2015).

Growth factor secretion

MSCs has ability to secrete growth factors which are known to help promoting migration and proliferation of corneal epithelial cells, as well as contributing to the corneal epithelial regeneration**.** According to the study of Dabrowski and others (2016), MSCs secretes several cytokines including IL-10, IL-6, epidermal growth factor (EGF), TNFα, VEGF-α, transforming growth factor-β (TGF-β), and MMP-1, MMP-8 and MMP-13 in vitro. These cytokines are beneficial for corneal epithelial regeneration and anti-inflammatory process. Moreover, another in vitro study found that MSCs could secrete nerve growth factor (NGF). To compare among different sources of MSCs, the mRNA levels of NGF from cell culture supernatant expressed in AD-MSCs were significantly higher than in BM-MSCs at undifferentiated stages (Zhang et al., 2012).

Migration and trans-differentiation

Several evidences suggested that MSCs can migrate to injured areas. According to the previous study of Di and others (2017) in diabetic mice subconjunctivally injected with BM-MSCs, migration of the MSCs to the stroma of the corneal wound edge and limbal stroma was observed after two days of administration. Accordingly, Shukla and others (2019) reported migration of mouse BM-MSCs to the corneal and conjunctival stroma four days after subconjunctival injection in corneal mechanical injury of mouse model. However, there are controversial reports of no migration of MSCs from the injection site to the wound area in rat models of corneal alkali burn after 4 weeks of BM-MSCs subconjunctival injection (Yao et al., 2012; Ghazaryan et al., 2016; Zhang et al., 2020). Therefore, several authors suggested that the transplanted cells enhanced corneal wound healing by trophic factor production, anti-inflammatory and immunomodulatory effect, rather than by direct transdifferentiation into corneal cells.

Adipose-derived mesenchymal stem cells (AD-MSCs)

Efficacy of AD-MSCs for corneal epithelial damage

The efficacy of AD-MSCs for treatment of corneal epithelial damage in human and animals has been studied. There was a case report of using topical application of autologous human AD-MSCs for treatment of neurotrophic persistent corneal epithelial defect (PED) in human. In this study, 35 years old man diagnosed with PED after traumatic corneal injury was refractory to previous treatment; various topical application including antibiotic, anti-fungal as well as combined systemic and topical anti-herpetic therapy. Moreover, conventional medical treatment of neurotrophic PED including patching, unpreserved artificial tears, soft contact lens bandage, autologous serum eyedrops were prescribed. Despite these, no corneal epithelial healing occurred for the following 7 weeks after treatment. Interestingly, after $3x10^6$ AD-MSCs was applied to the bottom of the ulcer using an insulin syringe with a 27-G needle attached, under slit lamp observation, complete corneal epithelial healing was observed by one month (Agorogiannis et al., 2012).

Lin and others (2013) studied effects of cultured human adipose-derived mesenchymal stem cells on corneal alkali injury in rabbits. Eight rabbit eyes received a single subconjunctival injection of a stem cell suspension ($1.3x10^5$ cells/0.2 ml). Corneas were removed after 30 days of injection and sent for histological analysis, corneal haziness grading, and surface marker assessment. The results demonstrated rapid wound healing and less corneal opacity in the experimental group as compared to the control group. Histologically, the corneal epithelial cell layers of the experimental group were five to six layers, whereas the cornea of control group consisted of two to three layers. Additionally, surface marker assessment showed significantly increase of Cx43 and beta catenin which indicated corneal epithelial cells proliferation. Furthermore, in the acute stage of corneal alkali injury in experimental rabbits treated with rabbit AD-MSCs, less angiogenesis, less opacity, and better corneal wound healing rate were evident up to 28 days post-injury, as compared to controls (Almaliotis et al., 2013).

CHAPTER III

MATERIALS AND METHODS

Animals

The study was approved by the Institutional Animal Care and Used Committee (IACUC), Faculty of Veterinary Science, Chulalongkorn University (Approval ID: 2131024). Nine eyes from eight dogs diagnosed with SCCEDs presented at the Ophthalmology Unit, Small Animal Teaching hospital, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand were included in this study. All procedures were performed after the approval by the owner via the informed-consent form. Thorough history including clinical signs, duration of lesions, previous treatment, other ophthalmic or systemic diseases, were taken from the owner.

Inclusion criteria

Dogs included in this study met the following criteria.

1. The presence of SCCEDs in one or both eyes with stromal involvement: presence of nonadherent and loosened epithelium at the margins of ulcer and ophthalmic signs of chronic superficial keratitis
2. The previous treatment history of epithelial debridement for at least twice with no evidence of complete corneal healing
3. Schirmer tear test 1 value not less than 5 mm wetting per *minute*

Exclusion criteria

Dogs with following conditions were excluded from the study.

1. The presence of underlying cause of the persistent corneal defect (e.g., eyelid abnormalities, distichiasis, ectopic cilia, enlarged eyeball)
2. The presence of significant ocular co-morbidity disease (e.g., uveitis, glaucoma)
3. The presence of any systemic diseases affecting corneal healing process (e.g., diabetic mellitus)

The study was divided into 2 parts.

Part I: Evaluation of clinical outcomes after treatment of SCCEDs in dog with subconjunctival injection of cAD-MSCs

Part II. Quantification of NGF-β, TNF-α and VEGF-A concentrations in tear fluid of canine SCCEDs treated with subconjunctival injection of cAD-MSCs

Part I: Evaluation of clinical outcomes after treatment of SCCEDs in dog with subconjunctival injection of cAD-MSCs

Ophthalmic examinations

All participated dogs underwent complete ophthalmic examinations at all examination time points (day 0, day 7, day 14 and day 21). Ophthalmic examinations included neuro-ophthalmic reflexes (menace response, dazzle reflex, pupillary light response; PLR and blink reflex), Schirmer tear test1; STT1, fluorescein staining test, tonometry (TonoVet Plus; Icare, Finland Oy, Helsinki, Finland), and slit lamp biomicroscopy (SL-2 handheld slit lamp; Kowa, Tokyo, Japan).

The degree of ocular discomfort was assessed in all dogs by the same blinded examiner at the same time. Details of ocular discomfort (Table 1) was categorized into four degrees; absent, mild, moderate, and intense.

Table 1. Degree of ocular discomfort in dogs

Degree of discomfort	Clinical signs
absent	- no sign of ocular discomfort
mild	- intermittent blepharospasm - mild conjunctivitis or none
moderate	- continuous blepharospasm - lacrimation - moderate conjunctivitis
intense	- severe blepharospasm - lacrimation - conjunctival hyperemia, chemosis

After subconjunctival injection of cAD-MSCs, adverse effects that might occur were closely monitored throughout the experiment; conjunctival necrosis at the injection site, ocular infection, and systemic complications.

Ocular application of canine adipose-derived mesenchymal stem cells (cAD-MSCs)

The cAD-MSCs suspension of $1x10^6$ cells in 0.2 ml sterile solution (Kamnerd cells®, Precision Vet, Bangkok Thailand) was prepared and stored in a 1 ml sterile syringe. Before subconjunctival injection, it was thawed at room temperature and then connected to a 30g sterile hypodermic needle. Sedation was induced with acepromazine maleate (0.03 mg/kg) combined with morphine sulfate (0.5 mg/kg) intramuscularly. Topical anesthesia was provided by using 0.5% tetracaine hydrochloride eye drop (tetracaine hydrochloride ophthalmic solution, Alcon *Laboratories Ltd*, Bangkok Thailand). Corneal wound was prepared by gently removing loose corneal epithelium from the wound edge area with a sterile cotton swab (Figure 4a). Corneal wound bedding was undergone gentle dry debridement leaving presence of corneal epithelial wound edge (Figure 4b). cAD-MSCs suspension was subconjunctivally injected at the dorsal bulbar conjunctival area (Figure 5). All eyes were given 0.3% tobramycin eye drops (Tobrex®, Alcon, Bangkok Thailand) and artificial tear containing hydroxypropyl methyl cellulose (Tear naturale® II, Alcon *Laboratories Ltd*, Bangkok Thailand) four times daily throughout the experiment. Dogs were required to wear Elizabethan collars to avoid self-mutilation.

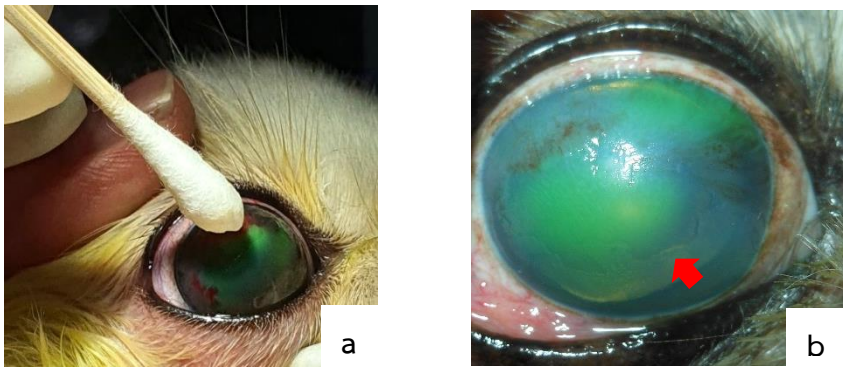


Figure 4. Photographs of the corneal wound; (a) preparation with sterile cotton swab and (b) presence of corneal wound edge after dry debridement (red arrow)

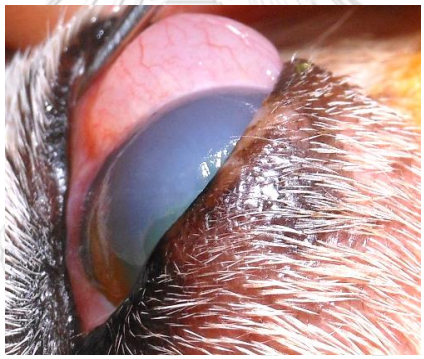


Figure 5. Photograph of subconjunctival injection of cAD-MSCs suspension in a dog

Corneal characteristics

All dogs underwent corneal examinations at day 7, 14 and 21 after cAD-MSCs subconjunctival injection. Corneal photography was performed in a standardized magnification with a digital camera (Canon PowerShot G6, Tokyo, Japan). The three following corneal characteristics were assessed.

1. Corneal epithelial defects: Corneal epithelial defects were assessed by area of fluorescein staining. After the instillation of 0.5% fluorescein dye, corneal epithelial defects were assessed by the area of corneal fluorescein staining with the presence of epithelial lip. Negative corneal fluorescein staining associated with non-epithelial lip characteristic indicated complete corneal re-epithelization. Time to complete re-epithelialization was a SCCEDs healing time. The area of the corneal epithelial defects and the area of total cornea were measured in pixels on digitized photographs (Figure 6a).
2. Corneal neovascularization: Area of corneal neovascularization was measured by tracing the boundaries of all vessel branches. Length of neovascularization includes the distance of all vessels from limbus to the leading edge of vessels. The width of neovascularization includes the diameter of sprouting vessels around the corneal ulcer. The area of the corneal neovascularization and the area of total cornea were measured in pixels on digitized photographs (Figure 6b).
3. Corneal opacification: Area of corneal opacity and the area of total cornea were measured in pixels on digitized photographs (Figure 6c).

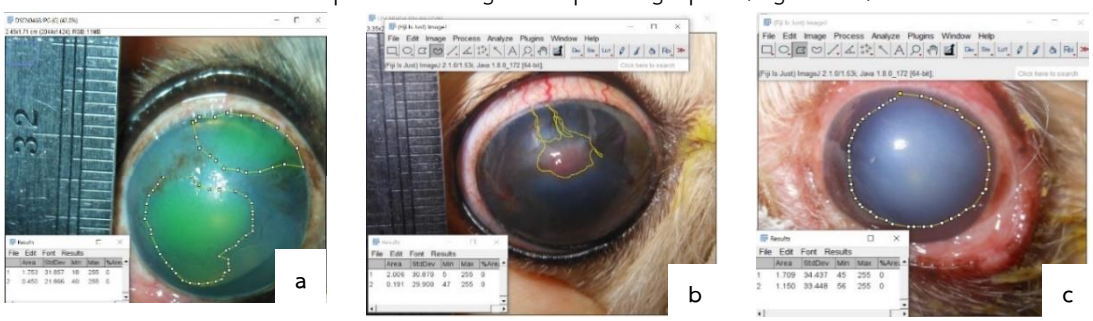


Figure 6. Photographs of corneal characteristics and image analysis (a) corneal epithelial defect (b) corneal neovascularization area (c) corneal opacification area

Data analysis

Degree of ocular discomfort was assessed according to table 1. Adverse effects or clinical complications after subconjunctival injection of cAD-MSCs were assessed.

Corneal photographs were analyzed using image analysis software (ImageJ 1.31v software) in triplicated and the mean value of each corneal characteristics of all time points were evaluated.

1. Corneal epithelial defects

   Area of epithelial defect (mm^2) was calculated as the percentage of corneal epithelial defect area using the following equation previously described by Fernandes-Cunha and others (2019).

$$\text{Corneal epithelial defect area (\%)} = \frac{[\text{corneal epithelial defect area}] \times 100}{[\text{total corneal area}]}$$

2. Corneal neovascularization

   Corneal neovascularization area was calculated as percentage of the total corneal area using the following equation previously described by Kim and other (2013).

$$\text{Corneal neovascularization area (\%)} = \frac{[\text{corneal neovascularization area}] \times 100}{[\text{total corneal area}]}$$

3. Corneal opacity

   The area of corneal opacification was calculated as percentage of the total corneal area using the following equation previously described by Shukla and other (2019).

$$\text{Corneal opacification area (\%)} = \frac{[\text{corneal opacification area}] \times 100}{[\text{total corneal area}]}$$

Normality of data distribution was assessed with the Shapiro-Wilk test. STT1, intra ocular pressures, areas of corneal epithelial defect and corneal neovascularization were assessed by Friedman test with Bonferroni post hoc, while area of corneal opacification was assessed by repeated measures ANOVA with Bonferroni Post hoc. Statistical analysis was performed using SPSS program (IBM SPSS statistic 28.0.0.0). Statistical significance or p-value less than 0.05 was reported. For data illustration, corneal characteristics were expressed in the Box and Whisker plots.

Part II. Quantification of NGF-β, TNF-α, and VEGF-A concentration in tear fluid of SCCEDs treated with subconjunctival injection of cAD-MSCs

Tear fluid collection

Collection of tear fluid was performed in all eyes before and after treatment at day 7, 14 and 21. 100 µl of tear samples were collected from ventral conjunctival sac using cellulose-based ophthalmic sponge (Weck-Cel® Sponge, Points and Strips, Beaver-Visitec International, Inc., Waltham, MA, USA) as previously described by Sebbag and others (2018). The lower eyelid was manually pulled downward. The 4x10 mm^2 strip of cellulose-based ophthalmic sponge was inserted into ventral conjunctival fornix using stainless tweezer (Figure 7) and kept in place for 2 minutes. The wet sponge was transferred into 0.2 ml Eppendorf tube and punctured using an 18-gauge needle to create drainage holes at the bottom of the tube. The 0.2 mL Eppendorf tube containing a wet sponge was then transferred to the 1.5 ml Eppendorf tube to be spun at 6000 round per minute (rpm) for 1 minute in a centrifuge machine. After centrifugation, tear sample from wet sponge were eluted through the drainage hole into 1.5 ml Eppendorf tube. in Finally, the 1.5 ml Eppendorf tube containing tear fluid sample was stored at −80°C for further analysis.

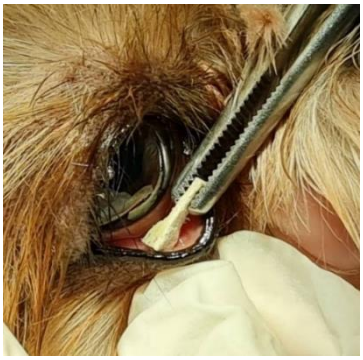


Figure 7. Photograph demonstrating ophthalmic sponge being inserted into the ventral conjunctival fornix using stainless tweezer

Quantification of NGF-β, TNF-α and VEGF-A concentration in tear fluid using multiplex immunoassay

Concentrations of NGF-β, TNF-α and VEGF-A in tear fluid samples obtained from dogs at various time points were quantified by multiplex immunoassay method using canine cytokine magnetic bead panel for NGF-β, TNF-α and VEGF-A (Canine Procarta Plex™ 11-plex immunoassay, Cat No EPX11A-50511-901, Thermo Fischer Scientific, Waltham, MA, USA) then analyzed by a Luminex MAXPIG® analyzer (Thermo Fischer Scientific, Waltham, MA, USA). The estimated minimum detectable concentration for each cytokine provided by the manufacturer was as follows: 4.8 pg/ml for NGF-β; 5.1 pg/ml for TNF-α; and 7.5 pg/ml for VEGF-A.

1. Sample preparation

Frozen tear fluid samples were thawed on ice and mixed well by vortexing. After the samples were centrifuged at 10,000 × g for 10 minutes to pellet out particulates, 10 µl of each tear sample was diluted in 40 µl of 1X Universal Assay Buffer (UAB) to make 50 µl of analyte (1:5 dilution).

2. Preparation of reagents

2.1. Wash Buffer (1X) preparation

The Wash Buffer Concentrate (10X) was brought to room temperature and vortexed for 15 seconds. 20 ml of the Wash Buffer Concentrate was mixed with 180 ml deionized water.

2.2. Standard Mix Preparation and 4-fold serial dilution

The lyophilized Standard Mix was used to generate standard curves. The lyophilized standard mix stock was centrifuged at 2,000 x g for 10 seconds. After the 250 µl of 1X UAB was added to the stock vial, the mixture was vortexed at high speed for 30 seconds and centrifuged at 2,000 x g for 10 seconds to collect contents at the bottom of the vial. The content was incubated on ice for 10 minutes to ensure complete reconstitution. To prepare a 4-fold serial dilution, eight microtubes

were labeled (Figure 8). 200 µl of the reconstituted standard mix was added into Std1 tube, while 150 µl of 1XUAB was added into Std2–Std7 tubes. After that, 50 µl of the reconstituted standard mix was transferred from Std1 tube into Std2 tube and mixed by pipetting 10 times. Next, 50 µl of the reconstituted standard mix from Std2 tube was transferred into Std3 tube using a new pipette tip and mixed well. Step of dilution was repeated for tubes Std4 to Std7 with new pipette tips between each dilution steps. Finally, 150 µl of 1X UAB was added to the last tube served as a background. These standard dilution tubes were kept on ice until use.

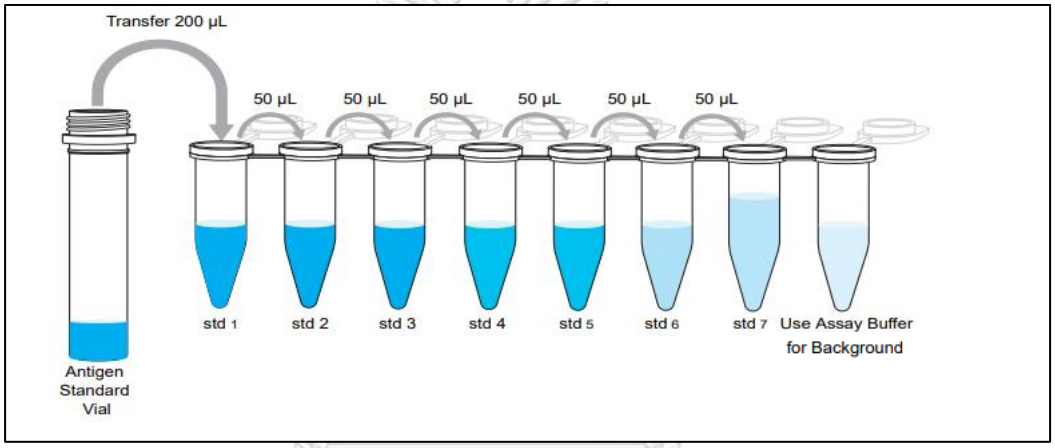


Figure 8. Photograph demonstrating of 4-fold serial standard mix dilution procedure.

3. Assay protocol

3.1. Capture Bead adding

50 μl of 1X Capture Bead Mix of NGF-β, TNF-α and VEGF-A was vortexed for 30 seconds and then added to the 96-well plate using a multichannel pipette (Figure 9a).

3.2. Capture Beads washing

After adding the beads, the plate was washed twice followed by the three following steps. First, the plate was placed on the hand-held magnetic plate washer and waited for 2 minutes to allow the beads to settle at the bottom of each well (Figure 9b). Second, liquid in each well was removed by quickly inverting the washer and plate assembly over a sink. The inverted washer and plate assembly were gently blotted onto several layers of absorbent pad to remove any residual liquid. Third, 150 μl of 1X Wash Buffer was added into each well and waited for 30 seconds. Then, the first and the second step were repeated. Finally, the plate was removed from the plate washer.

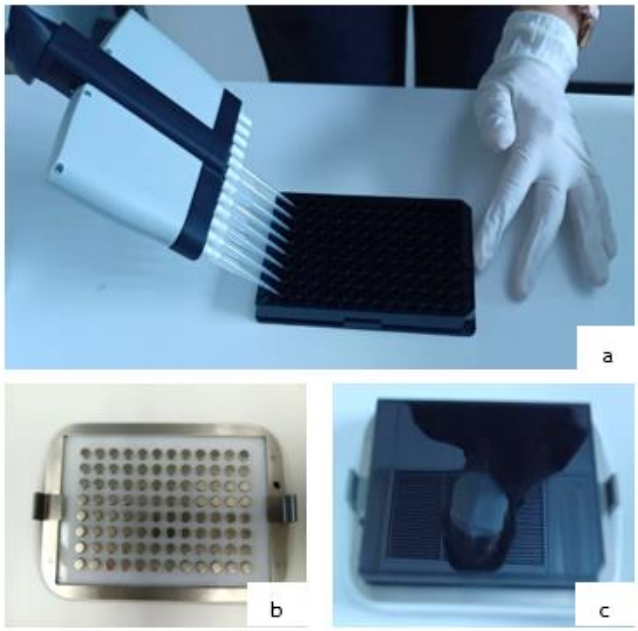


Figure 9. Photographs demonstrating (a) capture bead adding using multichannel pipette and (b and c) capture beads washing

3.3. Tear Samples and standards mix adding

50 µl of prepared standards mix (2.2) or diluted 1:5 Tear samples (1) were added into each 96 well in duplicate. 50 µl of 1X UAB were added to the 2 wells designated as backgrounds. After the plate was sealed and covered with the provided microplate lid, it was shaken at 600 rpm for 2 hours at room temperature. After that the plate was washed twice following step 3.2

3.4. Biotinylated detection Antibody Mix Adding

25 µl of the detection 1X antibody solution was added to each well of the plate using a multichannel pipette. Plate was sealed and covered with the provided microplate lid, and then shaken at 600 rpm for 30 minutes at room temperature. After that the plate was washed twice following step 3.2

3.5. Streptavidin-PE Adding

50 µl of Streptavidin-PE (SA-PE) solution was added to each well using a multichannel pipette. Then, the plate was sealed using new plate seal and cover with the provided Microplate Lid. and it was shake at 600 rpm for 30 minutes at room temperature. After that the plate was washed twice times following step 3.2

3.6. The plate preparation for analysis on Luminex MAXPIG® instrument.

120 µl of 1X UAB was added into each well using a multichannel pipette. Plate was sealed and covered with the provided microplate lid and shaken at 600 rpm for 5 minutes at room temperature. After the plate seal was removed, it was run on a Luminex MAXPIG® instrument (Figure 11).

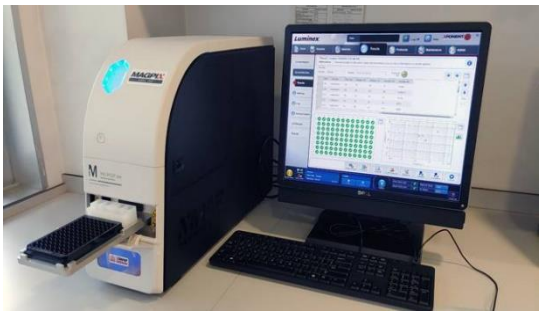


Figure 10. Photograph demonstrating immunoassay analysis for tear cytokines using Luminex MAXPIG® instrument.

Data analysis

Normality of data distribution was assessed with the Shapiro-Wilk test. Comparisons of TNF-α and VEGF-A concentrations in tear samples between before and after treatment different time points were performed using repeated ANOVA with Bonferroni Post hoc. In the meantime, data of NGF- β concentration was compared using a Friedman test with Bonferroni post hoc. All tests were performed using SPSS program (IBM SPSS statistic 28.0.0.0). Statistical significance was defined as the p-value less than 0.05. For result illustration, concentrations of NGF-β, TNF-α and VEGF-A at day 0, 7, 14 and 21 were expressed in Box and Whisker plots

CHAPTER IV

RESULTS

Animals

Ten eyes from nine dogs were included into the study. Mean (± SEM) age of dogs was 10.44 ± 1.65 years with a range of 1 to 15 years of age. Seven of right eyes (OD) were affected while there were three on the left (OS). There were eight females and two males of dogs. Demographic data of canine SCCEDs eyes were described in Table 2.

Table 2. Demographic data of canine SCCEDs eyes

Eye number	*Eye*	*Breed*	*Age (years)*	*Sex*	*Previous treatment*	*Concurrence diseases*
1	OD	Chihuahua	1	F	Mechanical debridement, DBD	-
2	OD	Shih tzu	12	F	Mechanical debridement, DBD	Mild KCS
3	OD	Yorkshire terrier	15	F	DBD 2 times	Mild KCS, Atopic dermatitis
4	OD	Chihuahua	5	M	Mechanical debridement, DBD	-
5	OS	Boston terrier	10	F	Mechanical debridement 2 times	-
6	OD	Shih tzu	13	F	DBD 2 times	Moderate KCS
7	OS	Shih tzu	15	F	Mechanical debridement, DBD	Moderate KCS
8	OD	French bulldog	8	M	DBD 2 times	Atopic dermatitis
9	OS	Yorkshire terrier	15	F	Mechanical debridement, DBD	Mild KCS, Atopic dermatitis

Note: OD = Oculus Dexter, OS = Oculus Sinister, DBD = Diamond burr debridement, KCS = Keratoconjunctivitis Sicca, F = female, M = male

Part I: Evaluation of clinical outcomes after treatment of SCCEDs in dog with subconjunctival injection of cAD-MSCs

1. Clinical outcomes of dogs with SCCEDs after subconjunctival injection of cAD-MSCs

1.1. Ophthalmic examinations

Neuro-ophthalmic reflexes including menace response, dazzle reflex, pupillary light reflex and blink reflex were present in all eyes. STT1 (millimeter of wetness in one minute; mm/min) and IOP (millimeter of Mercury; mmHg) of before and after treatment were described as mean ± SEM (Table 3). Prior to treatment, mean STT1 was high (19.78 ± 0.95 mm/min). It gradually decreased to be within normal limits by the end of the study. Mean IOP was within normal limit throughout the study. No statistical differences of the mean STT1 and the mean IOP were noted at all times of examination.

Table 3. Schirmer tear test1 and intraocular pressure (mean ± SEM) values of canine SCCEDs before and after subconjunctival injection of cAD-MSCs at day 7,14 and 21

Date of treatment	*STT1(mm/min)*	*IOP (mmHg)*
Day 0	19.78 ± 0.95	15.3 ± 0.47
Day 7	16.89 ± 0.74	14.8 ± 0.44
Day 14	15.56 ± 0.52	15.6 ± 0.56
Day 21	14.44 ± 0.4	15.2 ± 0.7

1.2. Degree of discomfort

Before treatment, all eyes had degree of discomfort (Table 4). After treatment, degree of discomfort gradually decreased. It was absent in five eyes at day 7, eight eyes at day 14 and nine eyes at day 21.

Table 4. Degree of discomfort in canine SCCEDs before and after subconjunctival injection of cAD-MSCs at day 7,14 and 21

Degree of discomfort	*Number of eyes*			
	Day 0	*Day 7*	*Day 14*	*Day 21*
Absent	0	5	8	9
Mild	3	4	1	0
Moderate	5	0	0	0
Severe	1	0	0	0

1.3. Ophthalmic complications

After subconjunctival injection of cAD-MSCs, no adverse effects were observed in all eyes throughout the experiment.

2. Effect of subconjunctival injection of cAD-MSCs on corneal characteristics

2.1. Time to complete corneal re-epithelialization

Nine eyes (100%) treated with subconjunctival injection of cAD-MSCs had 10.89 ± 1.7 days of complete corneal re-epithelization by the end of the study (range of 7-21 day). After treatment, 55.56% of eyes achieved complete corneal healing within 7 days, while 88.89% and 100% of eyes had complete corneal healing within 14 days and 21 days, respectively. Epithelial lip or loosened epithelial wound edge was absent according to time of corneal re-epithelialization (Figure 11).

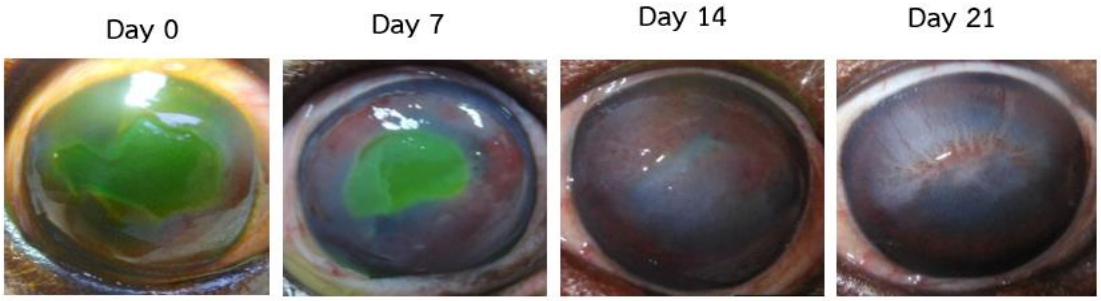


Figure 11. Representative photographs of nonadherent epithelial lip around corneal defect of Eye 8# before and after treatment with subconjunctival injection of cAD-MSCs.

2.2. Corneal epithelial defect

After treatment, strengthening of corneal epithelial wound edge was noticed. Area of corneal epithelial defect obviously decreased on day 7 in some cases, and then continued to decrease by time (Figure 12) . Mean percentage of corneal epithelial defect area before treatment was 22.52 ± 6.02 (4.10-62.37). After treatment at day 7, 14, and 21, it was statistically decreased to 3.24 ± 2.29 (0-21.15), 1.06 ± 1.06 (0-9.53) and 0, respectively (Figure 13).

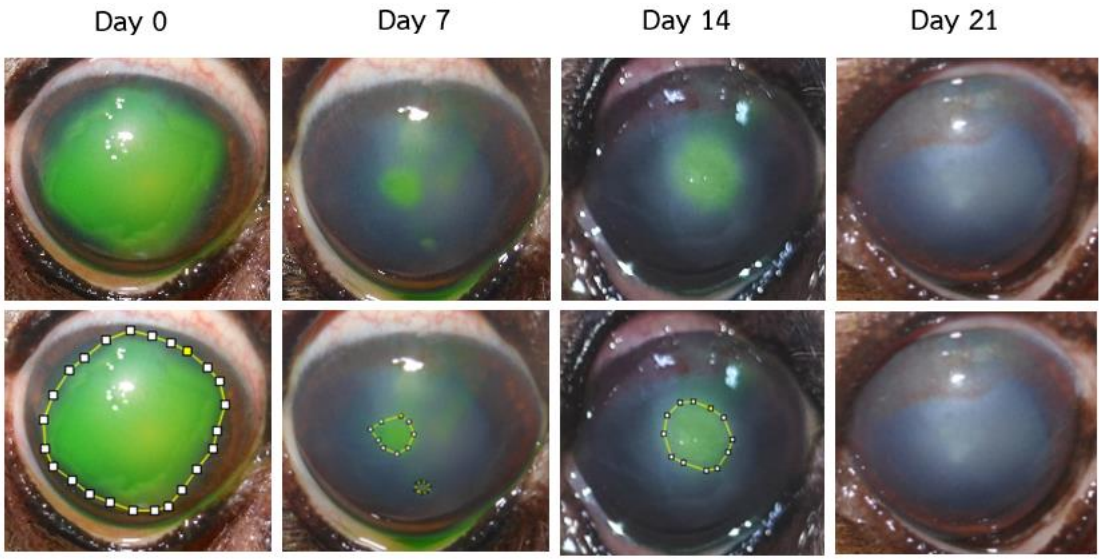


Figure 12. Representative photographs of corneal epithelial defect area (green area) of Eye 3# (top panel) and area measurement (bottom panel), before and after treatment with subconjunctival injection of cAD-MSCs.

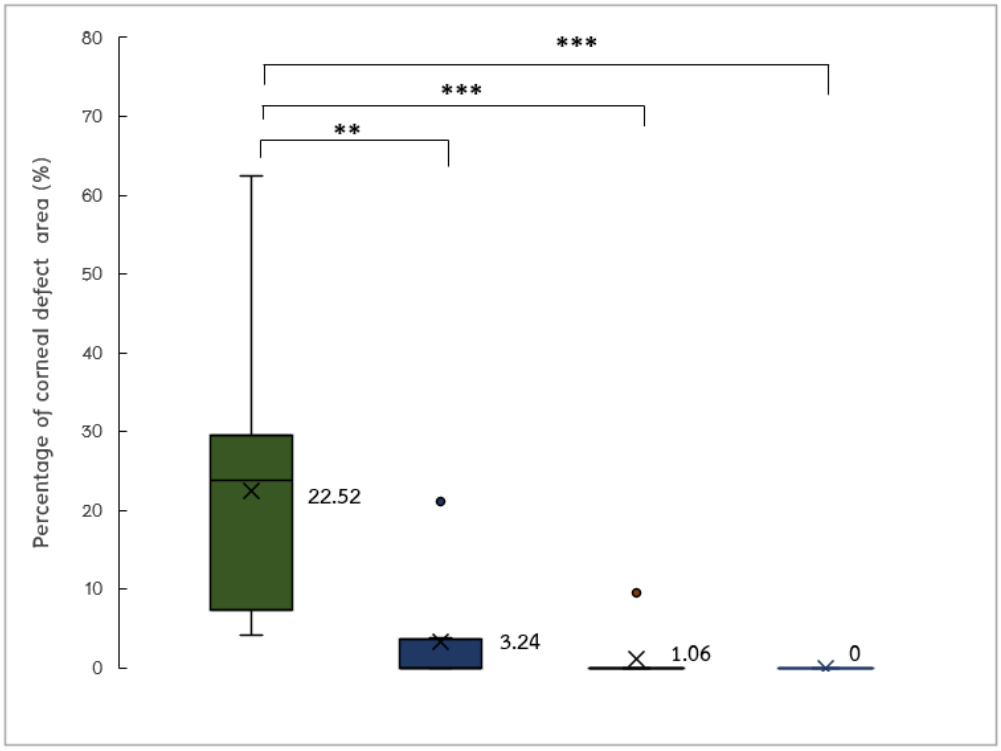


Figure 13. Box and whisker plot illustrating percentage of corneal epithelial defect area before and after treatment with subconjunctival injection of cAD-MSCs.
Note that ** and *** indicate statistically significant difference at $p<0.01$ and $p<0.001$ respectively. X indicates the mean percentage of corneal epithelial defect area.

2.3. Corneal neovascularization

Corneal neovascularization occurred in eight out of nine eyes. New vessels generating from the corneal limbus continued to extend, branch and finally reached the corneal wound edge. Three eyes revealed circumferential vessels on the wound, which markedly regressed thereafter (Figure 14). There was only one avascular cornea at before treatment that remained absence of corneal neovascularization regardless of complete corneal re-epithelialization. Mean percentage of corneal neovascularization area at before treatment was 6.07 ± 2.81 (0-20.58). After treatment, it greatly increased to 14.90 ± 6.05 (0-54.70) at day 7, then slightly decreased to 12.97 ± 4.86 (0-39.29) at day 14 and then to 4.20 ± 1.68 (0-12.78) at day 21 (Figure 15). No statistically significant difference was observed between before and after treatment.

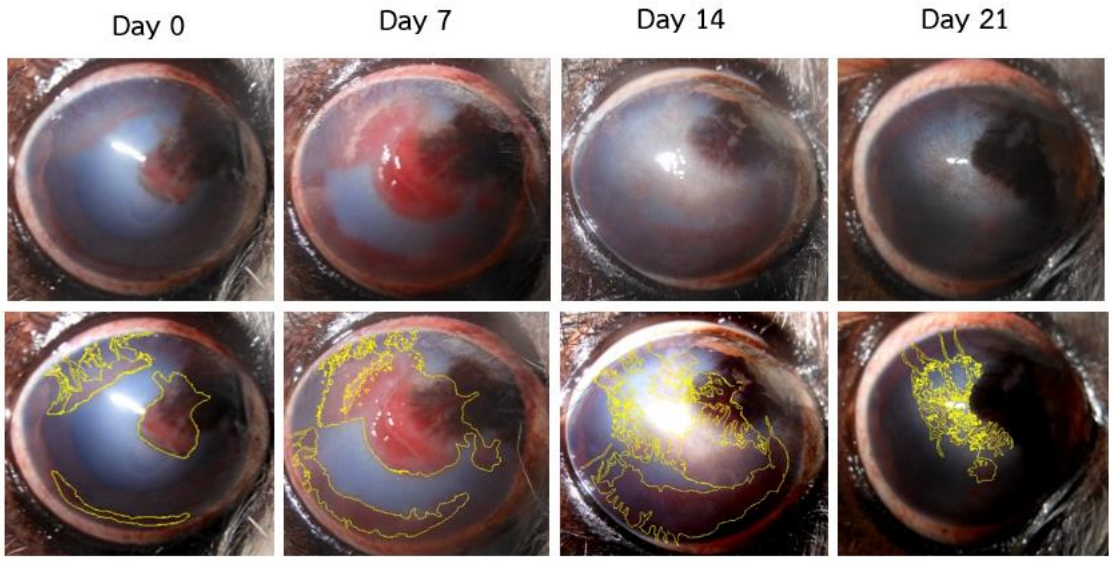


Figure 14. Representative photographs of corneal neovascularization of Eye 6# (top panel) and area measurement (bottom panel), before and after treatment with subconjunctival injection of cAD-MSCs.

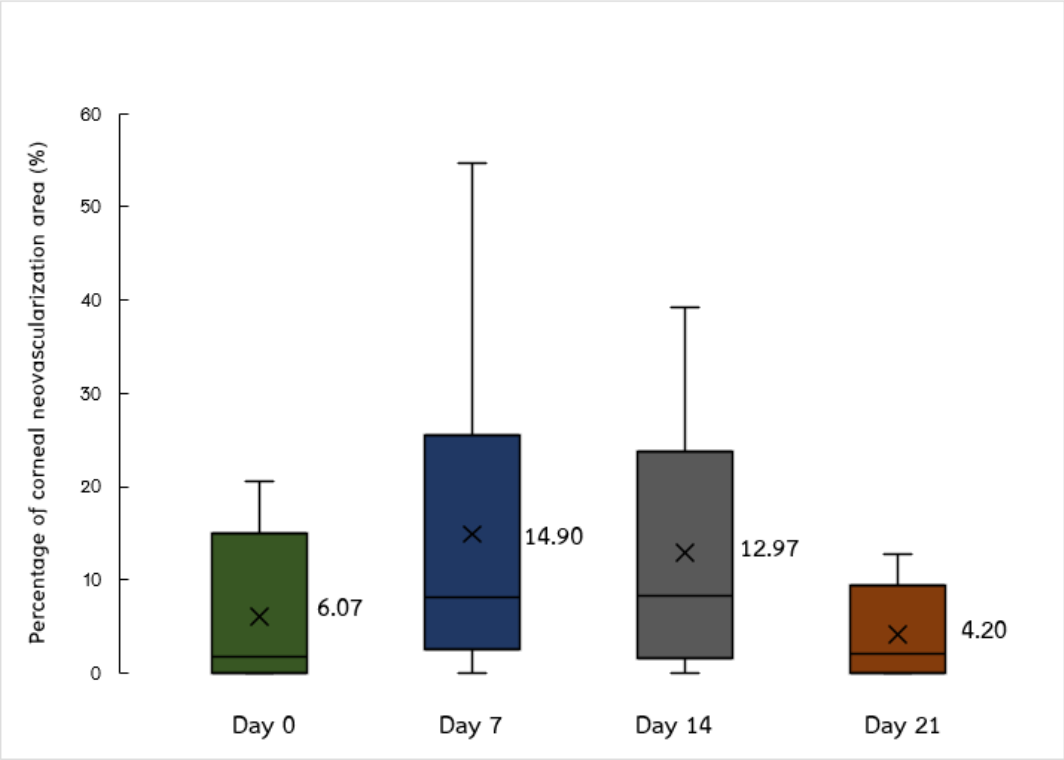


Figure 15. Box and whisker plot illustrating percentage of corneal neovascularization area before and after subconjunctival injection of cAD-MSCs.
Note that X indicates the mean percentage of corneal neovascularization area.

2.4. Corneal opacification

Corneal opacification was observed around the wound in all eyes. Area of corneal opacity continuously decreased after treatment (Figure 16). Mean percentage of corneal opacification area at before treatment was 60.01 ± 6.45 (35.30-80.30). After treatment, it gradually decreased to 48.80 ± 9.22 (19.05-100), 38.06 ± 9.13 (2.98-68.23) and 24.35 ± 7.81 (0-59.40) at day 7, 14, and 21, respectively (Figure 17). Even though corneal opacification remained by the end of the study, its area statistically reduced between post treatment day 21 to before treatment and post treatment day 7 and 14.

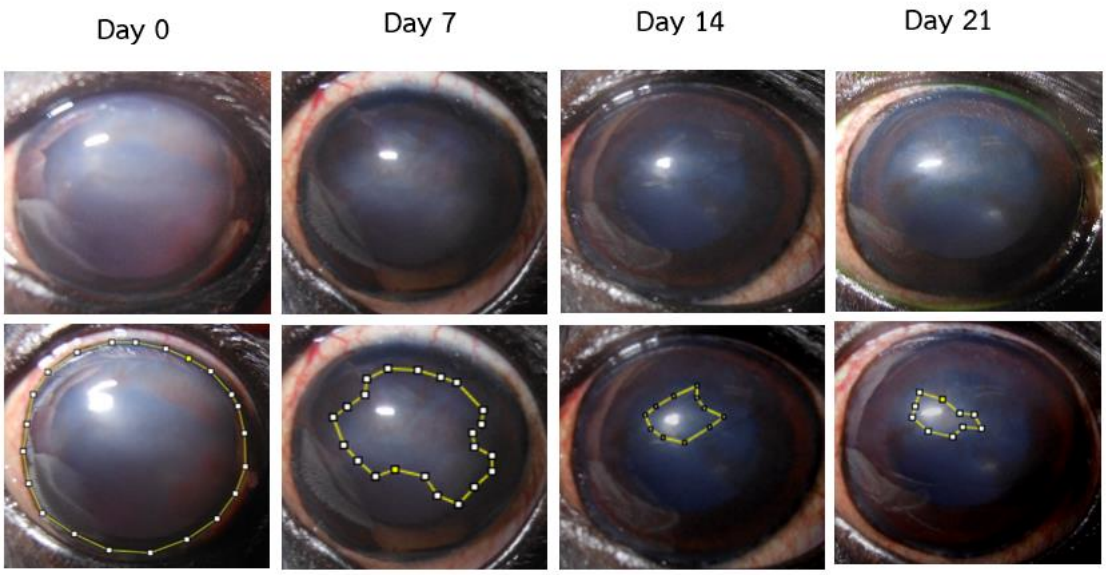


Figure 16. Representative photographs of corneal opacification area of Eye 4# (top panel) and area measurement (bottom panel), before and after subconjunctival injection of cAD-MSCs

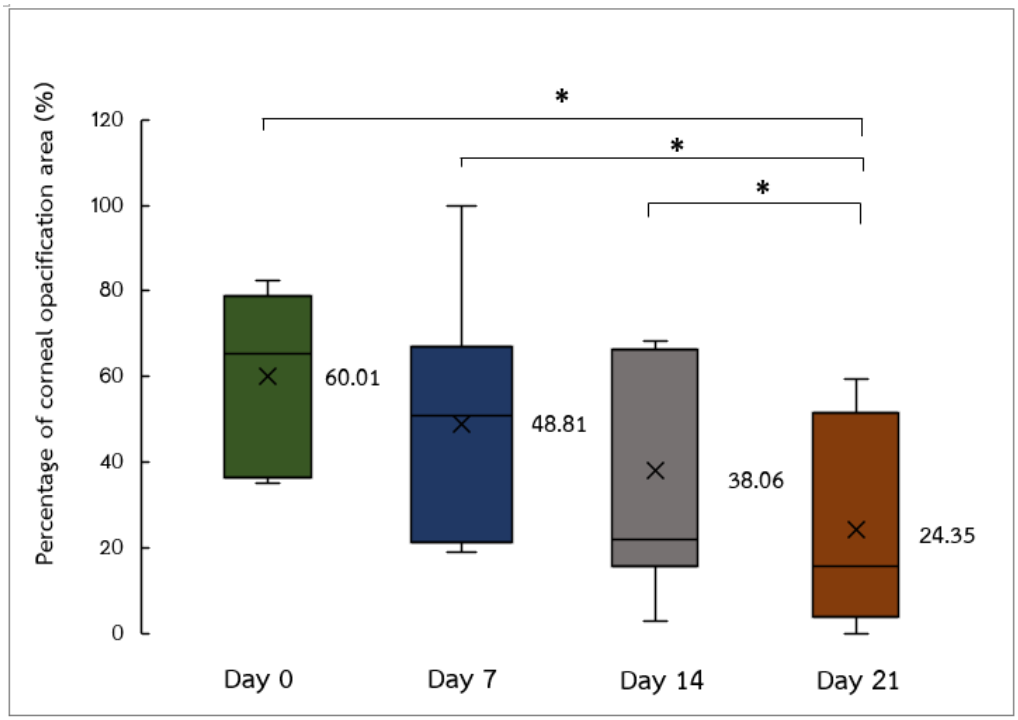


Figure 17. Box and whisker plot illustrating percentage of corneal opacification area before and after subconjunctival injection of cAD-MSCs.

Note that * indicates statistically significant difference at $p<0.05$. X indicates the mean percentage of corneal opacification area.

Part II. Quantification of NGF-β, TNF-α, and VEGF-A concentration in tear fluid of SCCEDs treated with subconjunctival injection of cAD-MSCs

1. NGF-β concentration

NGF-β was detected in 77.5% of tear fluid samples. Mean concentration of NGF-β at before treatment was 0.39 ± 0.04 pg/ml. After treatment, it was 0.37 ± 0.03, 0.39 ± 0.04 and 0.42 ± 0.09 pg/ml at day 7, 14, and 21, respectively (Figure 18). No statistically significant difference of NGF-β concentration was observed between before and after treatment.

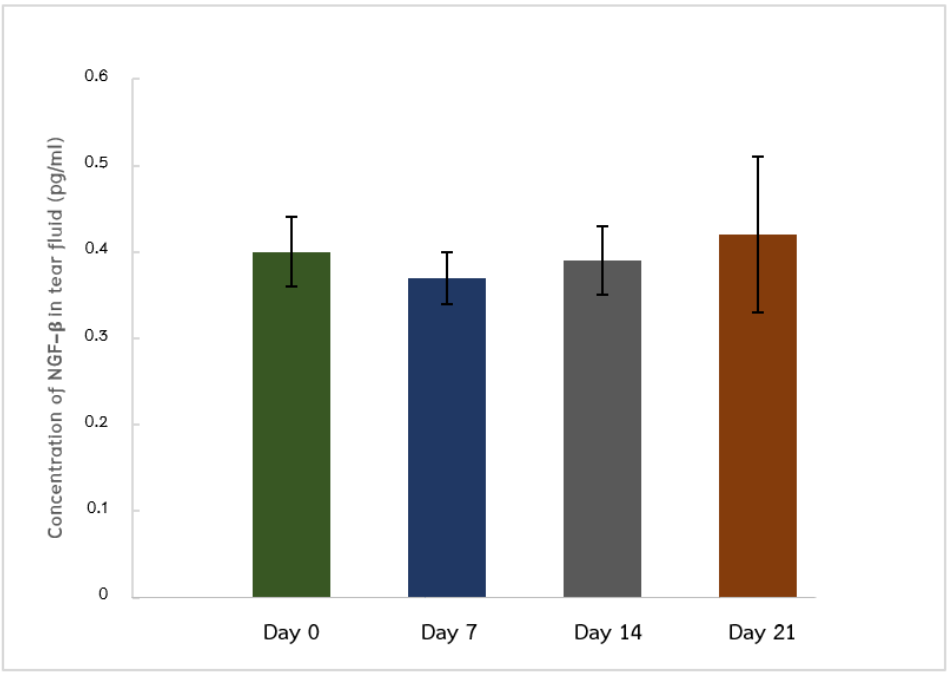


Figure 18. Bar graph illustrating concentration of NGF-β (pg/ml) in tear fluid before and after subconjunctival injection of cAD-MSCs

2. TNF-α concentration

TNF-α was detected in 97.5% of tear fluid samples. Mean concentration of TNF-α at before treatment was 4.33 ± 1.05 pg/ml. After treatment, it was 3.28 ± 0.58, 3.08 ± 0.59 and 2.60 ± 0.31 pg/ml at day 7, 14, and 21, respectively (Figure 19). Decrease of TNF-α concentrations in tear fluid was observed toward the end of the study. However, there was no statistically significant difference between before and after treatment.

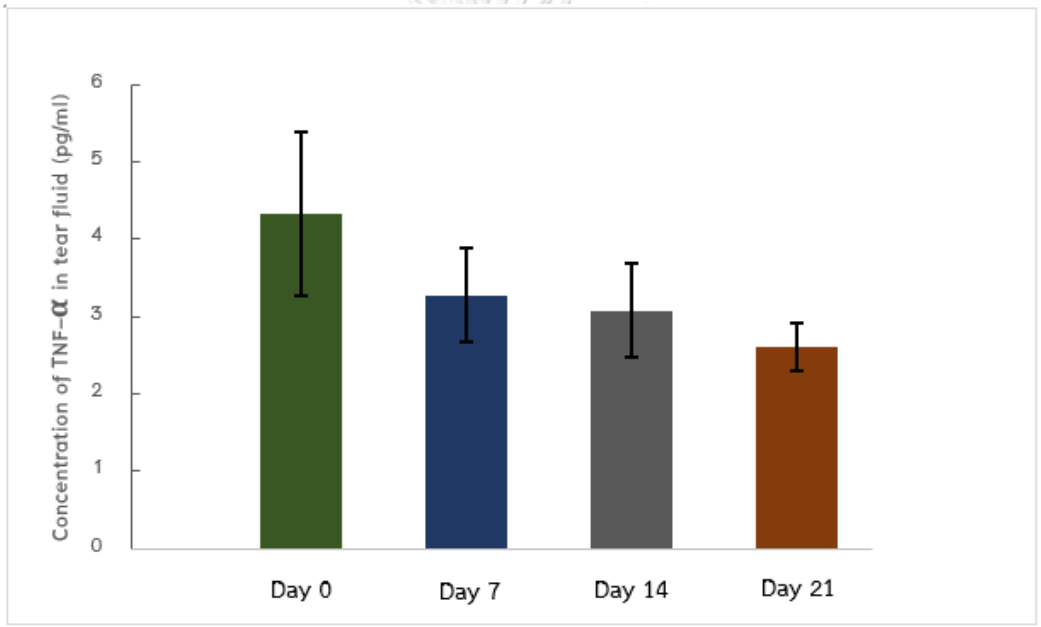


Figure 19. Bar graph illustrating concentration of TNF-α (pg/ml) in tear fluid before and after subconjunctival injection of cAD-MSCs

3. VEGF-A concentration

VEGF-A was detected in all tear fluid samples. Mean concentration of VEGF-A at before treatment was 4371.20 ± 448.96 pg/ml. After treatment, it was 3226.51 ± 316.31, 3401.26 ± 345.68 and 2953.85 ± 341.95 pg/ml at day 7, 14, and 21, respectively (Figure 20). Reduction of VEGF-A concentrations in tear fluid was noticed. Statistically significant difference was revealed between before treatment and post treatment day 21.

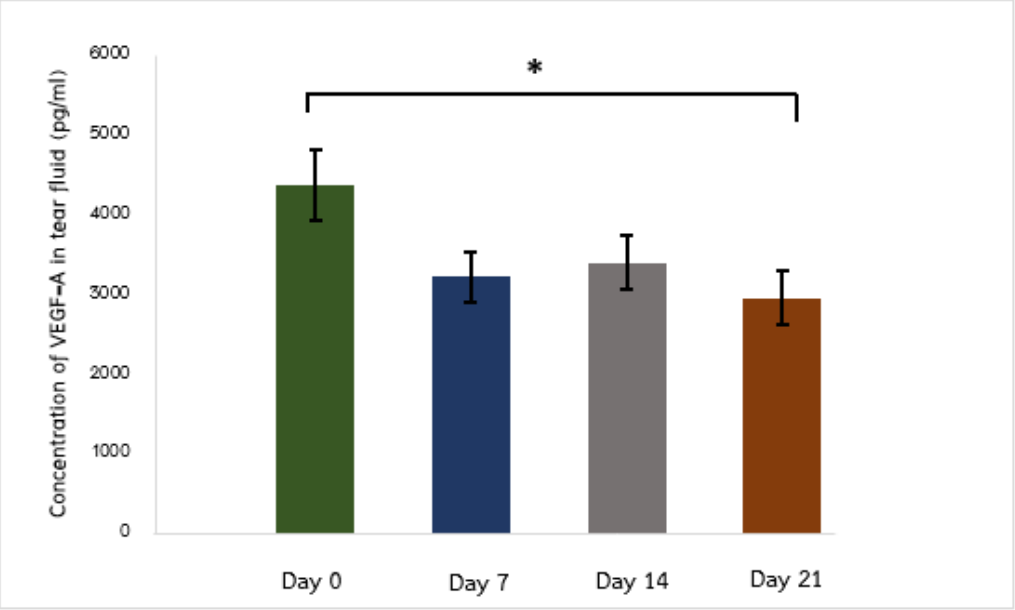


Figure 20. Bar graph illustrating concentration of VEGF-A (pg/ml) in tear fluid before and after subconjunctival injection of cAD-MSCs.

Note that * indicates statistically significant difference at $p<0.05$.

CHAPTER V
DISCUSSION

To date, there are no published studies evaluating therapeutic effect of cAD-MSCs on corneal epithelial defect, corneal neovascularization, corneal opacification in canine SCCEDs. We demonstrated 100% of complete corneal wound healing of canine SCCEDs after a single subconjunctival injection 1 x 10^6 cAD-MSCs. The degree of ocular discomfort decreased after treatment. Adverse effect from subconjunctival injection of cAD-MSCs was not evident, which is similar to other studies in animal model (Villatoro et al., 2018; Falcao et al., 2018). Our mean corneal healing time was 10.89 ± 1.7 days, corresponded to the mean corneal healing time of canine corneal ulcers reported by Falcao and others (2018). Their mean corneal healing time was 12.73 ± 0.59 days after a combination of subconjunctival and topical administration of total 3 x 10^6 cAD-MSCs in various types of canine corneal ulcers. Two out of 26 dogs in their study were diagnosed with SCCEDs and received single subconjunctival injection of 2.5 x 10^5 cAD-MSCs followed by 11 topical instillations of 2.5 x 10^5 cAD-MSCs. Following combined treatment, dogs showed a decreased corneal discomfort while complete corneal wound healing occurred by day 14. Interestingly, despite using lesser number of cAD-MSCs and one route of administration, mean healing time in our study was comparable to their study. However, various ulcer characteristics and severity may influence the healing time in their study (Kim et all, 2009). We demonstrated a significant decrease of corneal epithelial defect at day 7 after cAD-MSCs treatment, which led to complete wound healing in 5 out of 9 eyes. According to subconjunctival injection of BM-MSCs rats with corneal alkali injury reported by Yao and others (2012), progress of corneal epithelial defects to complete corneal reepithelization occurred day 3 and day 7 after treatment. They additionally reported a complete corneal re-epithelialization by day 7, observed in all corneas (Yao et al., 2012).

In practice, topical application is the most common route of MSCs administration due to an ease of application. However, according to the study of Shukla and other (2019), they investigated the therapeutic effects among different routes of BM-MSCs administration including topical, subconjunctival, intravenous, and intraperitoneal following corneal mechanical injury in mice. The results demonstrated that subconjunctival and intravenous routes of MSC administrations were greatly effective in promoting corneal re-epithelization by percentage of corneal defect area. Moreover, histopathological results revealed a reduction of inflammatory cells in the cornea as compared to other routes (Shukla et al., 2019). As canine SCCEDs is considered a chronic superficial corneal ulcer, we suggest that 1 x 10^6 of cAD-MSCs introduced via subconjunctival injection is a suitable route of administration for this type of canine ulcerative keratitis.

According to corneal morphology of canine SCCEDs reported by Bentley and others (2001), it is interesting that all corneas in this study finally attained complete corneal healing after a simple wound preparation, followed by cAD-MSCs treatment. Even though 2 out of ten corneas still had nonadherent wound edges around the defects after cAD-MSCs injection, corneal epithelium appeared to be more tightly reattaching to the underlying stroma. Complete corneal wound healing was thereafter achieved in two eyes by day 14. Despite the fact that all eyes previously received at least twice intensive corneal debridement including mechanical by sterile cotton swab, chemical by concentrated povidone-iodine, followed by diamond burr debridement, SCCEDs characteristics were still present. Dawson and others (2017) demonstrated significantly reduced thickness of stromal hyaline acellular zone in canine SCCEDs receiving diamond burr debridement. It is therefore worthy to note that subconjunctival cAD-MSCs may possibly play a role in promoting of corneal wound healing in canine SCCEDs. To gain a better understanding of corneal cell adhesion, transmission electron microscopy of the canine SCCEDs cornea after cAD-MSCs treatment should be investigated.

Wang and others (2020) reported the mechanism that TNF-α and IL-1β modulated corneal epithelial wound healing via the p16Ink4a-STAT3 signaling. After

corneal epithelial debridement in mice, persistent treatment with IL-1β or TNF-α suppressed corneal epithelial wound healing through two substances, phosphorylated signal transducer and activator of transcription 3 level (p-STAT3) and cell cycle inhibitor p16Ink4a, in the signal transducer and activator of transcription 3 signaling pathway (STAT3). STAT3 regulates the genes involved in modulating corneal inflammation and corneal epithelial stem cells (CESCs) proliferation, migration, and differentiation. The reduction of p-STAT3 and the increase of cell cycle inhibitor p16Ink4a resulted in a suppression of STAT3 signaling pathway. It was evident that corneal epithelial defect in the control mice healed faster than those mice receiving topical IL-1β or TNF-α administration. Therefore, it is suggested from Wang and others (2020) that persistent elevation of these two pro-inflammatory cytokines is an important etiology of corneal epithelial wound healing impairment in mice (Wang et al., 2020).

Several studies suggested that MSCs promote corneal epithelial healing via reducing pro-inflammatory cytokines, TNF-α, in particular (Di et al., 2017; Shukla et al., 2019). Ke and others (2015) demonstrated that TNF-α expression in the cornea was highly detected in the chemically induced rat's cornea, after which gradually decreased after BM-MSCs subconjunctival injection during corneal healing process, as compared to controls. Similarly, after subconjunctival injection of BM-MSCs in mice with mechanical injured cornea, expression of TNF-α in the cornea was suppressed (Di et al., 2017; Shukla et al., 2019). We also demonstrated continuous reduction of TNF-α level, accordingly, but in tear fluid. Though different level TNF-α was not statistically significant between before and after treatment, amount of tear fluids obtained from canine SCCEDs eyes is sufficient for TNF-α detection.

MSCs is widely known to promote angiogenesis in skin wound healing (Lee et al., 2016), brain injury (Muhammad, 2019) and myocardial infarction (Lee et al., 2009). In contrast to other organs, MSCs has anti-angiogenesis effect in the cornea via direct cell-to-cell interaction or to paracrine activity (Li et al., 2014). According to the previous study, corneal vascularization appeared in 58% to 64% of canine SCCEDs (Bentley et al., 2000). Prior to treatment, corneal vascularization was observed in 5

eyes. Area of corneal vascularization was increasingly greatest on day 7 after treatment, then gradually decreased until the end of the study. VEGF-A is the cytokine known to involve in promoting corneal angiogenesis. We demonstrated a constant reduction of VEGF-A concentrations in tear fluid of canine SCCEDs after treatment. Our result is consistent with the study of Ke and others (2015), which investigated the efficacy of subconjunctival injections of BM-MSCs on corneal alkali burn in rats. The reduction of corneal neovascularization was significantly enhanced in experimental group as compared to control group receiving phosphate buffered saline. Meanwhile, control rats showed gradually increasing area of corneal vascularization from day 7 through day 28 after corneal injury. Subconjunctival injection of cAD-MSCs may provide beneficial in reducing corneal neovascularization in canine SCCEDs possibly through downregulation of VEGF. Further study should be focused on VEGF-A concentration in canine SCCEDs without cAD-MSCs treatment.

Interestingly, based on our results, area of corneal vascularization increased at day 7 after treatment whereas concentration of tear film VEGF decreased. These findings are consistent with previously mentioned study of Ke and others (2015). VEGF was upregulated during pathological corneal angiogenesis in rats until day 3 after corneal injury, then started to decline whereas corneal vascularization continued to increase throughout follow up period. It is worthy to note that corneal vascularization did not exist in four eyes prior to treatment but exhibited in three eyes after treatment. Regeneration of corneal vessels after cAD-MSCs injection may possibly correlate with the previous report that investigated secretory activity of AD-MSCs in vitro (Dabrowski et al., 2017). They demonstrated that AD-MSCs could secrete VEGF at 48 hours after cultured. VEGF concentrations in canine SCCEDs may be sufficient to initiate an imbalance of pro-angiogenic and anti-angiogenic cytokines resulted in corneal neovascularization during the initial phase of treatment. However, the pathophysiology of MSCs promoting corneal angiogenesis via secretion of VEGF is still unclear. In future study, investigation of VEGF concentrations at very early stage after cAD-MSCs treatment may provide more knowledge on this matter. Furthermore, previous study suggested that corneal angiogenesis was also stimulated by other cytokines such as basic fibroblast growth factor (bFGF), and matrix metalloproteinase-

2 (MMP-2) (Brantman, Karen R. 2013). Evaluation of other cytokines stimulated angiogenesis such as MMP-2, bFGF are furtherly suggested.

In this study, we demonstrated that corneal opacification area gradually reduced after cAD-MSCs injection, which is consistent with the study by Shukla and others (2019). They demonstrated that after subconjunctival injection of BM-MSCs on corneal injury in mice, percentage of corneal opacification area decreased, together with significantly reduced expression of α-Sma and TGF-β as compared to the untreated group (Shukla et al., 2019). Following corneal injury, upregulation of TGF-β promotes the conversion of quiescent stromal keratocytes into α-Sma-containing myofibroblasts. The excessive accumulation myofibroblast and its activity result in development of corneal opacity and scarring. In addition, corneal opacity was mainly affected by corneal edema at the early phase of corneal injury, followed by fibrosis at the later phase (Labelle. P. 2017). Corneal edema occurs by osmotic absorption of fluid from the tear film during corneal epithelial damage. Thus, the excessive fluid should be removed by the actions of the endothelium after corneal re-epithelialization. Consequently, this mechanism may contribute to reducing of mean corneal opacification area after cAD-MSCs treatment in our study. However, corneal opacity partially remained in some eyes by the end of the study. This remaining corneal opacity may be related to corneal fibrosis from chronic stage of canine SCCEDs, which takes time for corneal remodeling.

MSCs has ability to secrete growth factors, which are known to help promoting migration and proliferation of corneal epithelial cells, important for corneal epithelial regeneration (Galindo et al., 2021). NGF-β is one of the growth factors secreted by MSCs in vitro (Zhang et al., 2012). In our study, concentrations of NGF-β in tear fluid were inconclusive. Concentration levels of NGF-β were very low and some fell below minimum detectable concentration, estimated by the manufacturer. Our result is parallel to studies by Sebbag and others (2019) assessing tear NGF-β concentration by multiplex immunoassay in normal dogs. They demonstrated that NGF-β was below detection limit in all tear fluid samples. In contrast, Woo and others (2005) were able to detect high levels of NGF-β in normal

canine tear fluid using commercially ELISA kit (15.4 ± 4.6 ng/ml). Unfortunately, this specific ELISA test kit is no longer commercially available. Moreover, the ELISA assay guidelines discourage the use of this kit for samples high in IgG (such as serum and tears) because they may cause cross-reactivity with NGF-β and falsely increase absorbance readings.

Two possible reasons of inconclusive result of NGF-β are speculated. First, NGF-β may not play a role in corneal wound healing of canine SCCEDs. The mechanism by which growth factor promoting corneal healing of canine SCCEDs may be possibly related to the upregulation of other growth factors. Interestingly, a study by Kirschner and others (1991) revealed approximately 80% healing rate in canine SCCEDs treated with topical EGF administration in conjunction with epithelial debridement. Further evaluation of another growth factor such as EGF is therefore suggested. The second possibility is the dilution of tear samples. Optimization of tear flid dilution should have previously been performed to select proper tear dilution. Due to the fact that small volume of tear fluids was obtained from dogs, the amount of tear was insufficient for an assay optimization. We consequently selected 1:5 tear dilution which was the same dilution performed with human multiplex immunoassay (Gao et al., 2014). It is still skeptical to assume that this selected dilution may render the cytokine level below detection limit. Therefore, evaluation of NGF-β concentration in canine SCCEDs in undilute tear samples may be considered in the future.

Conclusion

Subconjunctival injection of cAD-MSCs could be used an alternative treatment for canine SCCEDs. It can be administered noninvasively without general anesthesia. This study demonstrated that single subconjunctival injection of 1×10^6 cAD-MSCs is effective in promoting the corneal healing of canine SCCEDs. Not only cAD-MSCs accelerate corneal epithelial healing while reducing corneal neovascularization and opacification, it has no adverse effects. Anti-inflammatory activity through downregulation of TNF-α which is a pro-inflammatory cytokine may be related to the mechanism of cAD-MSCs to promote corneal epithelial healing in canine SCCEDs.

Suggestion

Further studies to investigate effect of cAD-MSCs on morphology of canine SCCEDs which transmission electron microscopy should be considered. To gain a better understanding of the effect of cAD-MSCs on corneal angiogenesis, investigation of VEGF-A concentrations at the very early stage after treatment, VEGF-A concentration of canine SCCEDs without cAD-MSCs treatment, as well as other cytokines stimulated angiogenesis such as MMP-2, bFGF are furtherly suggested. To gain a better understanding of the effect of cAD-MSCs on corneal opacification, investigation of TGF-β concentration in tear fluid of canine SCCEDs may be considered. Investigation of cAD-MSCs on corneal regeneration of canine SCCEDs such as investigation of NGF-β concentration in canine SCCEDs in undilute tear samples along with concentration of MSCs secreted growth factor associated corneal regeneration such as EGF is furtherly suggested.

REFERENCES

Agorogiannis GI, Alexaki V-I, Castana O and Kymionis GD 2012. Topical application of autologous adipose-derived mesenchymal stem cells (MSCs) for persistent sterile corneal epithelial defect. Graefes Arch Clin Exp Ophthalmol. 250(3): 455-457.

Aldave AJ, Kamal KM, Vo RC and Yu F 2009. Epithelial debridement and Bowman's layer polishing for visually significant epithelial irregularity and recurrent corneal erosions. Cornea. 28(10): 1085-1090.

Almaliotis D, Koliakos G, Papakonstantinou E, Komnenou A, Thomas A, Petrakis S, Nakos I, Gounari E and Karampatakis V 2015. Mesenchymal stem cells improve healing of the cornea after alkali injury. Graefes Arch Clin Exp Ophthalmol. 253(7): 1121-1135.

Asakura A, Rudnicki MA and Komaki M 2001. Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. Differentiation. 68(4-5): 245-253.

Balistreri CR 2017. Endothelial Progenitor Cells. In: UNIPA Springer Series.

Bentley E 2005. Spontaneous chronic corneal epithelial defects in dogs: a review. J Am Anim Hosp Assoc. 41(3): 158-165.

Bentley E, Abrams GA, Covitz D, Cook CS, Fischer CA, Hacker D, Stuhr CM, Reid TW and Murphy CJ 2001. Morphology and immunohistochemistry of spontaneous chronic corneal epithelial defects (SCCED) in dogs. Invest Ophthalmol Vis Sci. 42(10): 2262-2269.

Bentley E, Campbell S, Woo HM and Murphy CJ 2002. The effect of chronic corneal epithelial debridement on epithelial and stromal morphology in dogs. Invest Ophthalmol Vis Sci. 43(7): 2136-2142.

Brantman KR 2013. Tear film VEGF in dogs with vascularizing corneal disease. Virginia Tech.

Champagne E and Munger R 1992. Multiple punctate keratotomy for the treatment of recurrent epithelial erosions in dogs. J Am Anim Hosp Assoc.

Choo A, Ngo AS, Ding V, Oh S and Kiang LS 2008. Autogeneic feeders for the culture of undifferentiated human embryonic stem cells in feeder and feeder-free conditions. Methods Cell Biol. 86: 15-28.

Dabrowski FA, Burdzinska A, Kulesza A, Sladowska A, Zolocinska A, Gala K, Paczek L and Wielgos M 2017. Comparison of the paracrine activity of mesenchymal stem cells derived from human umbilical cord, amniotic membrane and adipose tissue. J Obstet Gynaecol Res. 43(11): 1758-1768.

Dawson C, Naranjo C, Sanchez-Maldonado B, Fricker GV, Linn-Pearl RN, Escanilla N, Kafarnik C, Gould DJ, Sanchez RF and Matas-Riera M 2017. Immediate effects of diamond burr debridement in patients with spontaneous chronic corneal epithelial defects, light and electron microscopic evaluation. Vet Ophthalmol. 20(1): 11-15.

Di G, Du X, Qi X, Zhao X, Duan H, Li S, Xie L and Zhou Q 2017. Mesenchymal stem cells promote diabetic corneal epithelial wound healing through TSG-6–dependent stem cell activation and macrophage switch. Invest Ophthalmol Vis Sci. 58(10): 4344-4354.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D and Horwitz E 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 8(4): 315-317.

Eaton JS, Hollingsworth SR, Holmberg BJ, Brown MH, Smith PJ and Maggs DJ 2017. Effects of topically applied heterologous serum on reepithelialization rate of superficial chronic corneal epithelial defects in dogs. J Am Vet Med Assoc. 250(9): 1014-1022.

El-Din WN, Nooreldin N and Essawy A 2021. The potential therapeutic efficacy of intravenous versus subconjunctival mesenchymal stem cells on experimentally ultraviolet-induced corneal injury in adult male albino rats. Folia Morphologica.

Falcão MSA, Brunel HdSS, Peixer MA, Dallago BS, Costa FF, Queiroz LM, Campbell P and Malard PF 2020. Effect of allogeneic mesenchymal stem cells (MSCs) on corneal wound healing in dogs. J Tradit Complement Med. 10(5): 440.

Fernandes-Cunha GM, Na K-S, Putra I, Lee HJ, Hull S, Cheng Y-C, Blanco IJ, Eslani M, Djalilian AR and Myung D 2019. Corneal wound healing effects of mesenchymal stem cell secretome delivered within a viscoelastic gel carrier. Stem Cells Transl Med. 8(5): 478-489.

Frisbie DD, Kisiday JD, Kawcak CE, Werpy NM and McIlwraith CW 2009. Evaluation of adipose-derived stromal vascular fraction or bone marrow-derived mesenchymal stem cells for treatment of osteoarthritis. J Orthop Res. 27(12): 1675-1680.

Galindo S, de la Mata A, López-Paniagua M, Herreras JM, Pérez I, Calonge M and Nieto-Miguel T 2021. Subconjunctival injection of mesenchymal stem cells for corneal failure due to limbal stem cell deficiency: state of the art. Stem Cell Res Ther. 12(1): 1-12.

Ghazaryan E, Zhang Y, He Y, Liu X, Li Y, Xie J and Su G 2016. Mesenchymal stem cells in corneal neovascularization: Comparison of different application routes. Mol Med Rep. 14(4): 3104-3112.

Gosling AA, Labelle AL and Breaux CB 2013. Management of spontaneous chronic corneal epithelial defects (SCCEDs) in dogs with diamond burr debridement and placement of a bandage contact lens. Vet. Ophthalmol. 16(2): 83-88.

Herberts CA, Kwa MS and Hermsen HP 2011. Risk factors in the development of stem cell therapy. J Transl Med. 9(1): 1-14.

Holan V, Trosan P, Cejka C, Javorkova E, Zajicova A, Hermankova B, Chudickova M and Cejkova J 2015. A comparative study of the therapeutic potential of mesenchymal stem cells and limbal epithelial stem cells for ocular surface reconstruction. Stem Cells Transl Med. 4(9): 1052-1063.

Hung JH, Leidreiter K, White JS and Bernays ME 2020. Clinical characteristics and treatment of spontaneous chronic corneal epithelial defects (SCCEDs) with diamond burr debridement. Vet Ophthalmol. 23(4): 764-769.

Ke Y, Wu Y, Cui X, Liu X, Yu M, Yang C and Li X 2015. Polysaccharide hydrogel combined with mesenchymal stem cells promotes the healing of corneal alkali burn in rats. PloS one. 10(3): e0119725.

Kim J-H, Seo H-W, Han H-C, Lee J-H, Choi S-K and Lee D 2013. The effect of bevacizumab versus ranibizumab in the treatment of corneal neovascularization: a preliminary study. Korean J Ophthalmol. 27(4): 235-242.

Kim JY, Won H-J and Jeong S 2009. A retrospective study of ulcerative keratitis in 32 dogs. Intern J Appl Res Vet Med. 7(1): 27-31.

Kim KS, Oh JS, Kim IS and Jo JS 1992. Clinical efficacy of topical homologous fibronectin in persistent corneal epithelial disorders. Korean J Ophthalmol. 6(1): 12-18.

Kirschner S, Brazzell R, Stern M and Baird L 1991. The use of topical epidermal growth factor for treatment of nonhealing corneal erosions in dogs. J Am Anim Hosp Assoc.

Lee DE, Ayoub N and Agrawal DK 2016. Mesenchymal stem cells and cutaneous wound healing: novel methods to increase cell delivery and therapeutic efficacy. Stem Cell Res. Ther. 7(1): 1-8.

Lee RH, Pulin AA, Seo MJ, Kota DJ, Ylostalo J, Larson BL, Semprun-Prieto L, Delafontaine P and Prockop DJ 2009. Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. Cell stem cell. 5(1): 54-63.

Li F and Zhao S-Z 2014. Mesenchymal stem cells: potential role in corneal wound repair and transplantation. World J Stem Cells. 6(3): 296.

Lin HF, Lai YC, Tai CF, Tsai JL, Hsu HC, Hsu RF, Lu SN, Feng NH, Chai CY and Lee CH 2013. Effects of cultured human adipose-derived stem cells transplantation on rabbit cornea regeneration after alkaline chemical burn. Kaohsiung J Med Sci. 29(1): 14-18.

Ma Y, Xu Y, Xiao Z, Yang W, Zhang C, Song E, Du Y and Li L 2006. Reconstruction of chemically burned rat corneal surface by bone marrow–derived human mesenchymal stem cells. Stem cells. 24(2): 315-321.

Marfe G, Massaro-Giordano M, Ranalli M, Cozzoli E, Di Stefano C, Malafoglia V, Polettini M and Gambacurta A 2012. Blood derived stem cells: an ameliorative therapy in veterinary ophthalmology. J Cell Physiol. 227(3): 1250-1256.

Martínez-Carrasco R, Sánchez-Abarca LI, Nieto-Gómez C, García EM, Sánchez-Guijo F,

Argüeso P, Aijón J, Hernández-Galilea E and Velasco A 2019. Subconjunctival injection of mesenchymal stromal cells protects the cornea in an experimental model of GVHD. Ocul Surf. 17(2): 285-294.

Mastropasqua L, Lanzini M, Dua HS, D'Uffizi A, Di Nicola M, Calienno R, Bondì J, Said DG and Nubile M 2020. In vivo evaluation of corneal nerves and epithelial healing after treatment with recombinant nerve growth factor for neurotrophic keratopathy. Am. J. Ophthalmol. 217: 278-286.

Miller W 1996. Using polysulfted glycosaminoglycan to treat persistent corneal erosions in dogs. Vet Med. 91(10): 916-922.

Moore PA 2003. Diagnosis and management of chronic corneal epithelial defects (indolent corneal ulcerations). Clin Tech Small Anim Pract. 18(3): 168-177.

Morgan R and Abrams K 1994. A comparison of six different therapies for persistent corneal erosions in dogs and cats. Vet Comp Ophthalmol.

Muhammad SA 2019. Mesenchymal stromal cell secretome as a therapeutic strategy for traumatic brain injury. BioFactors. 45(6): 880-891.

Murphy CJ, Marfurt CF, McDermott A, Bentley E, Abrams GA, Reid TW and Campbell S 2001. Spontaneous chronic corneal epithelial defects (SCCED) in dogs: clinical features, innervation, and effect of topical SP, with or without IGF-1. Invest Opthalmol Vis Sci. 42(10): 2252-2261.

Nixon AJ, Dahlgren LA, Haupt JL, Yeager AE and Ward DL 2008. Effect of adipose-derived nucleated cell fractions on tendon repair in horses with collagenase-induced tendinitis. Am J Vet Res. 69(7): 928-937.

Oh JY, Kim MK, Shin MS, Lee HJ, Ko JH, Wee WR and Lee JH 2008. The anti-inflammatory and anti-angiogenic role of mesenchymal stem cells in corneal wound healing following chemical injury. Stem cells. 26(4): 1047-1055.

Orbay H, Tobita M and Mizuno H 2012. Mesenchymal stem cells isolated from adipose and other tissues: basic biological properties and clinical applications. Stem cells international. 2012(2012): 461718.

Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R and De Luca M 1997. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. Lancet. 349(9057): 990-993.

Prochazkova M, Chavez MG, Prochazka J, Felfy H, Mushegyan V and Klein OD 2015. Embryonic versus adult stem cells. In: Stem cell biology and tissue engineering in dental sciences. 1st ed. A. Vishwakarma ed. Amsterdam: Elsevier. 249-262.

Rafiei F, Tabesh H and Farzad F 2020. Sustained subconjunctival drug delivery systems: current trends and future perspectives. Int. Ophthalmol. 40(9): 2385-2401.

Rama P, Matuska S, Paganoni G, Spinelli A, De Luca M and Pellegrini G 2010. Limbal stem-cell therapy and long-term corneal regeneration. N Engl J Med. 363(2): 147-155.

Sebbag L, Harrington DM and Mochel JP 2018. Tear fluid collection in dogs and cats using ophthalmic sponges. Vet Ophthalmol. 21(3): 249-254.

Seo B-M, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY and Shi S 2004. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet. 364(9429): 149-155.

Shukla S, Mittal SK, Foulsham W, Elbasiony E, Singhania D, Sahu SK and Chauhan SK 2019. Therapeutic efficacy of different routes of mesenchymal stem cell administration in corneal injury. Ocul Surf. 17(4): 729-736.

Stanley R, Hardman C and Johnson B 1998. Results of grid keratotomy, superficial keratectomy and debridement for the management of persistent corneal erosions in 92 dogs. Vet Ophthalmol. 1(4): 233-238.

Villatoro AJ, Claros S, Fernández V, Alcoholado C, Fariñas F, Moreno A, Becerra J and Andrades JA 2018. Safety and efficacy of the mesenchymal stem cell in feline eosinophilic keratitis treatment. BMC Vet Res. 14(1): 1-13.

Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, Fu YS, Lai MC and Chen CC 2004. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem cells. 22(7): 1330-1337.

Wang X, Zhang S, Dong M, Li Y, Zhou Q and Yang L 2020. The proinflammatory cytokines IL-1β and TNF-α modulate corneal epithelial wound healing through p16Ink4a suppressing STAT3 activity. J Cell Physiol. 235(12): 10081-10093.

Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B and Hows JM 2003. Adult bone marrow is a rich source of human mesenchymal 'stem'cells but

umbilical cord and mobilized adult blood are not. Br J Haematol. 121(2): 368-374.

Whitley R 1999. Diseases of canine cornea and sclera. In: Vet Ophthalmol. Philadelphia. 635-673.

Wilke MM, Nydam DV and Nixon AJ 2007. Enhanced early chondrogenesis in articular defects following arthroscopic mesenchymal stem cell implantation in an equine model. J Orthop Res. 25(7): 913-925.

Yao L, Li Z-r, Su W-r, Li Y-p, Lin M-l, Zhang W-x, Liu Y, Wan Q and Liang D 2012. Role of mesenchymal stem cells on cornea wound healing induced by acute alkali burn. PLoS one. 7(2): e30842.

Ye L, Swingen C and Zhang J 2013. Induced pluripotent stem cells and their potential for basic and clinical sciences. Curr Cardiol Rev. 9(1): 63-72.

Yin Z 2018. The experimental research on the treatment of rabbits with acute cornea alkali burn by taking bone marrow mesenchymal stem cell. J Acute Dis. 7(2): 78.

Zhang N, Luo X, Zhang S, Liu R, Liang L, Su W and Liang D 2021. Subconjunctival injection of tumor necrosis factor-α pre-stimulated bone marrow-derived mesenchymal stem cells enhances anti-inflammation and anti-fibrosis in ocular alkali burns. Graefes Arch Clin Exp Ophthalmol. 259(4): 929-940.

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP and Hedrick MH 2001. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 7(2): 211-228.

APPENDICES

Appendix 1. Corneal characteristics (mean ± SEM) before and after subconjunctival injection of cAD-MSCs

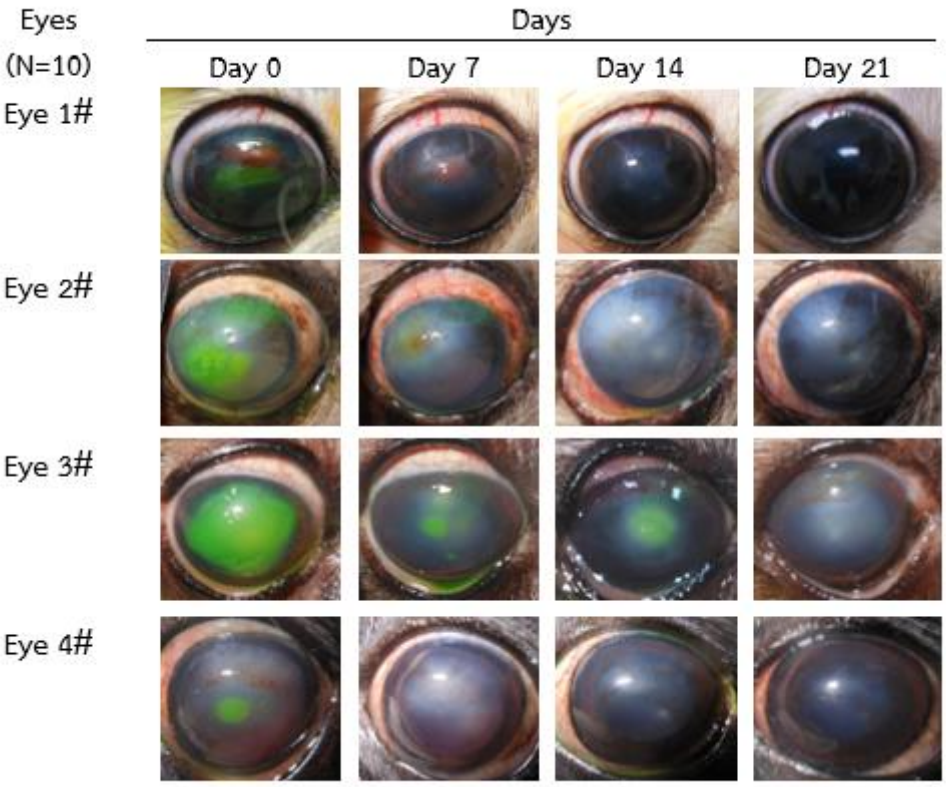
Corneal characteristic area (%)

Day	Corneal epithelial defect	Corneal neovascularization	Corneal opacification
Day 0	22.52 ± 6.02	6.07 ± 2.81	60.01 ± 6.45
Day 7	3.23 ± 2.29	14.90 ± 6.05	48.80 ± 9.22
Day 14	1.06 ± 1.06	12.97 ± 4.86	38.06 ± 9.13
Day 21	0	4.20 ± 1.68	24.35 ± 7.81

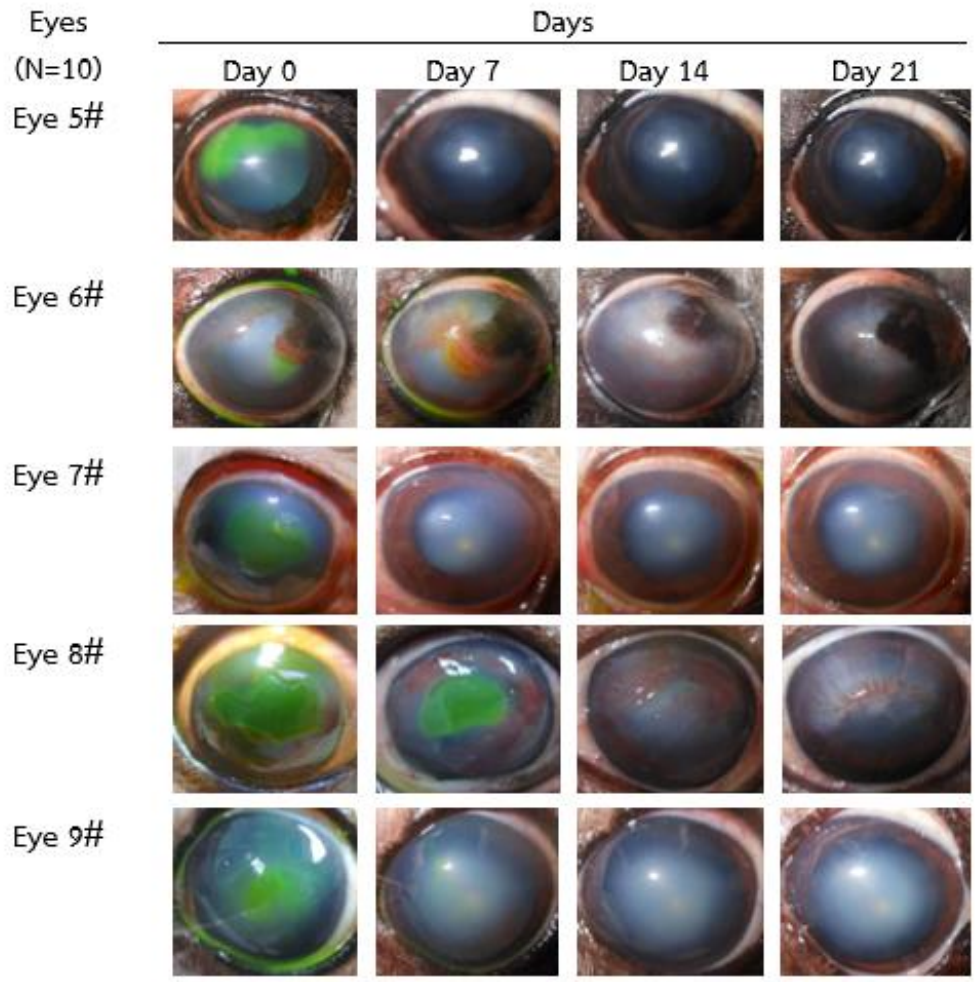
Appendix 2. Concentration of TNF-α,NGF-β and VEGF-A (mean ± SEM) in tear fluid before and after subconjunctival injection of cAD-MSCs

Concentration of cytokine in tear fluid (pg/ml)

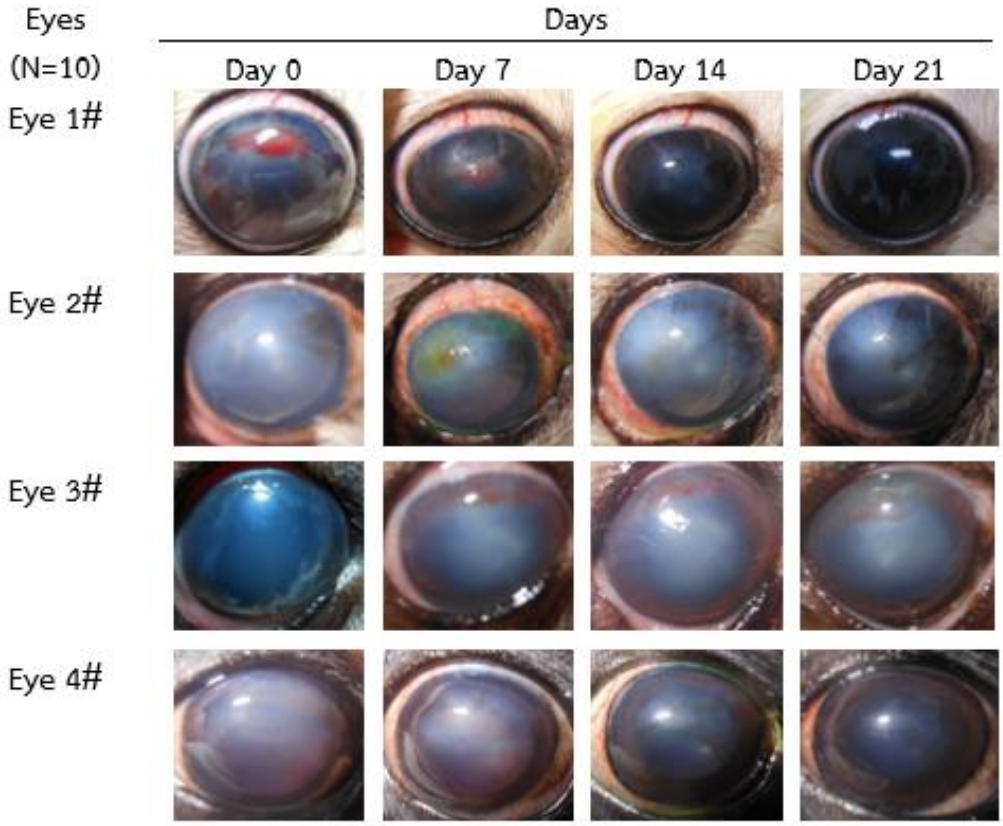
Day	TNF-α	NGF-β	VEGF-A
Day 0	4.33 ± 1.05	0.39 ± 0.04	4371.20 ± 448.96
Day 7	3.28 ± 0.58	0.37 ± 0.03	3226.51 ± 316.31
Day 14	3.08 ± 0.59	0.39 ± 0.04	3401.26 ± 345.68
Day 21	2.60 ± 0.31	0.42 ± 0.09	2953.85 ± 341.95



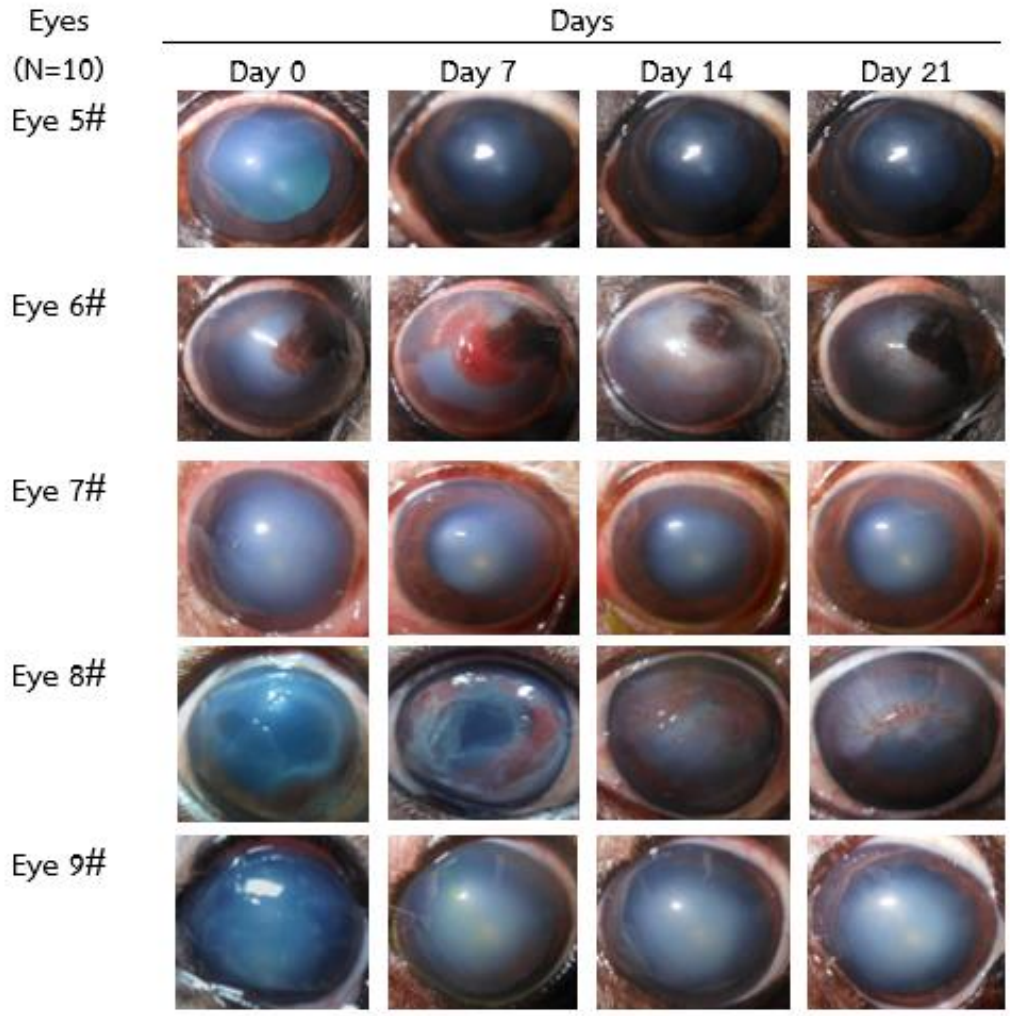
Appendix 3. Photographs of corneal epithelial defect of Eye1# - Eye4# before and after subconjunctival injection of cAD-MSCs at day 7,14 and 21



Appendix 4. Photographs of corneal epithelial defect of Eye5# - Eye10# before and after subconjunctival injection of cAD-MSCs at day 7,14 and 21



Appendix 5. Photographs of corneal neovascularization and opacification of Eye1# - Eye4# before and after subconjunctival injection of cAD-MSCs at day 7,14 and 21



Appendix 6. Photographs of corneal neovascularization and opacification of Eye5# - Eye10# before and after subconjunctival injection of cAD-MSCs at day 7,14 and 21

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HOME ADDRESS	60 M.9 Bangphud, Pakkred, Nonthaburi, 11120
PUBLICATION	Chulalongkorn University Veterinary Conference 2022
AWARD RECEIVED	72th Anniversary of Chulalongkorn University Scholarship