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A DISSERTATION
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

**Development of Air Assisted Lamellar Keratectomy
for Corneal Haze Model and Deep Anterior
Lamellar Keratoplasty in Dogs**

개에서 각막흐림모델과 심부표층각막이식을 위한
공기주입 각막절제술의 개발

by

Soohyun Kim

MAJOR IN VETERINARY CLINICAL SCIENCES
DEPARTMENT OF VETERINARY MEDICINE
GRADUATE SCHOOL
SEOUL NATIONAL UNIVERSITY

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**Development of Air Assisted Lamellar Keratectomy
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Lamellar Keratoplasty in Dogs**

**by
Soohyun Kim**

**Supervised by
Professor Kangmoon Seo**

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Seoul National University

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Supervised by

Professor Kangmoon Seo

Soohyun Kim

ABSTRACT

The purpose of the present study was development of air assisted lamellar keratectomy (AK) technique for the standardized corneal haze model and deep anterior lamellar keratoplasty in dogs.

The AK was performed to achieve a constant ablation depth and size and to generate the efficient corneal haze. The *ex vivo* porcine corneas were categorized into four groups depending on the following trephined depths: 250, 375, 500 and 750 μm . After 4 ml air was injected to the stroma at the base of the trephination groove, the fuzzy region of the white opaque cornea was removed. No significant differences were observed between the trephined corneal depths for resection and the ablated corneal thickness at depths deeper

than 375 μm . AK and conventional keratectomy (CK) were applied to six beagles and development of corneal haze was evaluated weekly until postoperative day 28. The occurrence of corneal haze and α -smooth muscle actin (SMA) expression following AK were significantly higher than those following CK. Therefore, AK was used to achieve the desired corneal thickness after resection and produce a sufficient corneal haze.

The effect of onion extract ointment on corneal haze suppression was evaluated to verify the experimental utility of AK technique for the evaluation of haze development. After the AK was performed, an artificial tear (group C), prednisolone acetate (group P), onion extract ointment (group O), and transforming growth factor (TGF)- β 1 (group T) were applied to each group. The haze was significantly suppressed in the group P and O compared with the group C from day-14 after the surgery. Also, the total green intensity for α -SMA was significantly less expressed in the group P and O than in the group C. Thus, the onion extract ointment was demonstrated to have the suppression effects for corneal haze development and AK technique was sufficient to evaluate these effects.

The feasibility of deep anterior lamellar keratoplasty (DALK) using big-bubble technique (BBT), upon which the AK technique was based, was evaluated in three dogs. The eyes were examined until 150 days after the operation of DALK. The central portion of the transplanted cornea remained transparent while corneal haze developed around the transplanted margin. The marginal haze was rarely observed between the donor and recipient corneas at 150 days after the operation. A spotted haze developed in the central part of the deep stroma near the Descemet's membrane (DM). Alpha-SMA positive cells were detected at the transplanted margin in which the corneal haze appeared clinically.

Based on the results of the present studies, AK technique could be useful for the evaluation of corneal haze and contribute to improving the standardized corneal haze model.

Keywords: air assisted lamellar keratectomy, big-bubble technique, corneal haze, deep anterior lamellar keratoplasty, dog, onion extract ointment

Student number: 2011-21670

List of Abbreviations

AK	Air assisted lamellar keratectomy
ANOVA	Analysis of variance
BBT	Big-bubble technique
CCT	Central corneal thickness
CK	Conventional keratectomy
DALK	Deep anterior lamellar keratoplasty
DAPI	4',6-diamidino-2-phenylindole
DM	Descemet's membrane
DMEM	Dulbecco's modified Eagle's medium
H&E	Hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IOP	Intraocular pressure
MMC	Mitomycin C
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline
PK	Penetrating keratoplasty
PLR	pupillary light reflex
PRK	Photorefractive keratectomy
ROS	Reactive oxygen species
SD	Standard deviation
SMA	Smooth muscle actin
TGF-β1	Transforming growth factor - β 1

CONTENTS

GENERAL INTRODUCTION	1
CHAPTER I.	
Air assisted lamellar keratectomy for the corneal haze model	
Abstract	6
Introduction	7
Materials and Methods	
1. Experimental design	9
2. Animals	10
3. <i>Ex vivo</i> experiments on porcine eyes	11
4. <i>In vivo</i> experiments on canine eyes	14
5. Clinical grading of corneal haze	15
6. Quantitative corneal haze grading	16
7. Histopathological and immunofluorescence analyses	17
8. Quantification of α -SMA positive cells	18
9. Statistical analyses	19
Result	20
Discussion	30
Conclusions	34

CHAPTER II.

Effect of onion extract on corneal haze suppression after air assisted lamellar keratectomy

Abstract	36
Introduction	37
Materials and Methods	
1. Corneal fibroblast culture and cell viability test for onion extract	40
2. A process of manufacture for the onion extract ointment	42
3. Animals	43
4. Corneal haze generation by the air assisted lamellar keratectomy	44
5. Corneal haze grading	47
6. Immunofluorescence analyses	48
7. Quantification of α -SMA positive cells	49
8. Statistical analyses	50
Results	51
Discussion	59
Conclusions	63

CHAPTER III.

Deep anterior lamellar keratoplasty of dog eyes using the big-bubble technique

Abstract.....	65
Introduction.....	66
Materials and Methods	
1. Animals.....	68
2. Surgical technique.....	69
3. Post-operative care and evaluation.....	72
4. Histopathological evaluation.....	74
5. Immunofluorescence detection of α -SMA.....	75
Results.....	76
Discussion.....	84
Conclusions.....	88
GENERAL CONCLUSIONS.....	89
REFERENCES.....	91
ABSTRACT IN KOREAN.....	103

GENERAL INTRODUCTION

Cornea is a unique transparent structure in the body. Also, the cornea has no blood supply to maintain the transparency. This transparent structure of the cornea is essential to maintain clear vision. Various diseases or corneal surgeries destroy this transparency by developing the corneal haze resulting in the vision loss (Sakimoto *et al.*, 2006). Because the precise mechanisms of the corneal haze formation are unclear, therapies specifically targeting its prevention are limited. Therefore, experimental corneal haze models are needed for the research of the haze developing mechanisms and for the development of newer pharmacological agents.

Several experimental models have been introduced for the experiments to the research of corneal epithelial diseases, stromal fibrosis, and haze.

Experimental methods	References
Mechanical debridement	de Medeiros <i>et al.</i> , 2008
Irregular-phototherapeutic keratectomy (PTK)	Mohan <i>et al.</i> , 2008
Chemical burning	Yang <i>et al.</i> , 2010
Corneal alkali burn	Luo <i>et al.</i> , 2013
<i>ex vivo</i> bovine corneal irritation model	Bhasker <i>et al.</i> , 2015
Photorefractive keratectomy (PRK)	Alcalde <i>et al.</i> , 2015
Novel <i>in vivo</i> corneal model of fibrosis	Gronkiewicz <i>et al.</i> , 2016

However, the resection depth by mechanical debridement (de Medeiros *et al.*, 2008) is not deep enough to develop sufficient corneal haze for the experimental evaluation. For

this reason, this method has been limited to the evaluation of the epithelial healing. The chemical burning methods (Yang *et al.*, 2010; Luo *et al.*, 2013; Bhasker *et al.*, 2015) are difficult to assess an affected depth owing to loss of transparency and induced severe pain postoperatively. Recently, the novel *in vivo* corneal model of fibrosis (Gronkiewicz *et al.*, 2016) was reported using alkali burning. However, fibrosis was accompanied not only by haze but also by severe neovascularization and edema. Thus, this would not be an appropriate method for the evaluation of corneal haze. The delicate stromal ablation could be achieved with the desired depth and size in the PRK (Mohan *et al.*, 2008; Alcalde *et al.*, 2015). But, these methods require expensive specialized laser equipment and the result haze is insufficient for evaluation of treatment effects.

From these aspects, this study was designed to establish AK technique for standardization of corneal haze model by modification of BBT. The stromal structure is deformed to become loose by injecting air when the BBT was applied. In this study, the BBT was modified so that the air injected to the partial stromal layer. This method permitted visualization of the superficial stroma and the loosely deformed stromal structure. Additionally, the occurrence of corneal haze resulting from the AK technique was evaluated by comparing it with the occurrence of corneal haze resulting from the CK technique.

To verify experimental utility of the AK technique for the corneal haze development, the onion extract ointment was used to evaluate the capability of maintaining corneal transparency by suppressing the corneal haze development. Onion extract contains antioxidant phytochemicals, sulfur, and other numerous phenolic compounds (Benkeblia,

2005). These compounds have been reported to possess many biological activities which are effective in various diseases. Especially, onion extract was shown to have fibroblast-inhibiting properties for the reduction of proliferative activity (Ho et al., 2006). Because the key factors of the corneal haze expression are the differentiation of keratocytes to myofibroblasts (Gupta *et al.*, 2011), the fibroblast-inhibiting effect of onion extract could be a good therapeutic candidate as a new medicine for corneal haze suppression.

For the repair of deep corneal defects, various surgical managements using an autograft or preserved biological membranes have been used in veterinary ophthalmology. However, most of these surgical methods for large corneal defects would not be able to recover corneal transparency. Transplantation of various corneal parts is an essential technique for the treatment of severe corneal damage to maintain the corneal transparency and clear vision in human medicine (Bahar *et al.*, 2008). On the contrary, corneal transplantation is very limited in dogs due to insufficient resources of donor cornea and frequent graft rejections. DALK is one of the corneal transplantation methods to remove and replace the pathologic corneal stroma while preserving the host endothelium, which eliminates the risk of endothelial graft rejection (Karimian *et al.*, 2010). Thus, this method could suggest the possibility of successfully performing corneal transplantation to the dog without graft rejection.

The AK is an easy and simple surgical technique for the standardized corneal haze model. The AK could achieve the standardized stromal ablation in the desired resection size and depth without a specialized laser equipment. Furthermore, the degree of stromal haze generation was enough for the experimental evaluation of haze. In this study, the AK

was demonstrated to be a useful experimental model to evaluate the suppressive effect of onion extract on corneal haze. DALK, one of the corneal transplantation techniques injecting an air into the stroma of AK, was performed to confirm the availability of this technique in dogs.

CHAPTER I.

**Air assisted lamellar keratectomy for the corneal haze
model**

Abstract

To standardize the corneal haze model in the resection depth and size for efficient corneal haze development, AK was performed. The *ex vivo* porcine corneas were categorized into four groups depending on the trephined depth: 250 (G1), 375 (G2), 500 (G3) and 750- μm (G4). The stroma was equally ablated at the five measurement sites in all groups. Significant differences were observed between the trephined corneal depths for resection and ablated corneal thickness in G1 ($p < 0.001$). No significant differences were observed between the trephined corneal depth for resection and the ablated corneal thickness in G2, G3, and G4. The resection percentage was similar in all groups after microscopic imaging of corneal sections. AK and CK method were applied to six beagles, after which development of corneal haze was evaluated weekly until postoperative day 28. The occurrence of corneal haze in the AK-group was significantly higher than that in the CK-group beginning 14 days after surgery. Alpha-SMA expression was significantly higher in the AK-group ($p < 0.001$) than the CK-group. AK was considered as a useful method to achieve the desired corneal thickness after resection and produce sufficient corneal haze.

Introduction

Corneal transparency, which is one of the most important factors influencing vision, is a functional translation of the detailed ultrastructure of the stroma that is primarily attributed to the narrow, uniform diameter of collagen fibrils (Meek *et al.*, 2001). Corneal haze is associated with disruption of the collagen fiber array (Møller-Pedersen, 2004) and proliferation of newly formed myofibroblasts during the fibrotic response (Fini, 1999). The development of corneal haze resulting from refractory corneal diseases and PRK has been reported (Sakimoto *et al.*, 2006). Although fibrotic response is an essential component of the normal corneal healing process (Hu *et al.*, 2009), significant corneal opacity can be induced during this process and mediate a decline in visual acuity. Therefore, a crucial aspect of corneal wound healing is minimizing corneal haze.

Several experimental models have been introduced in the effort to develop treatments to prevent or reduce corneal haze, including mechanical debridement, chemical burning, and PRK (de Medeiros *et al.*, 2008; Soong *et al.*, 2008; Yang *et al.*, 2010). Corneal wound healing is a complex process controlled by various factors (Stepp *et al.*, 2014), and the size and depth of experimental corneal defects are important factors that must be considered for objective experimental modeling. The desired resection depth is not easily attained using experimental methods that include mechanical debridement and chemical burns. Conversely, PRK is better able to achieve the desired ablation thickness, but requires specialized expensive laser equipment. Thus, there is a need for a standardized and easily applied method.

Anwar and Teichmann introduced the BBT to expose DM by injecting air into the deep stroma (Anwar *et al.*, 2002). In this method, the stromal structure is deformed to become loose, and a large bubble is made between the deep stroma and the Descemet's membrane by injecting air. A very small stromal layer resided after applying this method (McKee, 2013). When this technique was applied, the injected air blanched the stroma, resulting in loss of transparency. In this study, the air injection method was applied to the partial stromal layer with slight modification for blanching to acquire the desired depth. This method permitted visualization of the superficial stroma and the loosely deformed stromal structure.

The purpose of this study was to establish a standardized corneal haze model for wound size and depth by using AK, which is a modification of the BBT. Additionally, the occurrence of corneal haze resulting from the AK was evaluated by comparing it with the occurrence of corneal haze resulting from the conventional method.

Materials and Methods

1. Experimental design

Fifty porcine eyes obtained from a slaughterhouse and 12 canine eyes were used in this study. Ten porcine eyes were included in each group according to the trephined corneal depth for resection of 250 (G1), 375 (G2), 500 (G3), and 750 μm (G4). Ten eyes were used to make histopathological sections of normal corneas ($n = 5$) and air-injected corneas ($n = 5$).

Six female beagles were used. One eye of each dog was selected at random for AK group (three right and three left eyes, $n = 6$), while the contralateral eye received CK group (three right and three left eyes, $n = 6$). Basal corneal haze and corneal haze was evaluated *in vivo* at 7, 14, 21, and 28 days after surgery. The dogs were sacrificed 4 weeks postoperatively for Periodic acid Schiff (PAS) and immunofluorescent (anti α -SMA antibody) staining to study the formation of myofibroblasts.

2. Animals

Fifty porcine eyes obtained from a slaughterhouse and six healthy female beagles were used. Prior to the experiment, all dogs underwent an ophthalmic examination including slit-lamp biomicroscopy (SL-D7[®]; Topcon, Tokyo, Japan), indirect ophthalmoscopy (Vantage plus[®]; Keeler, Windsor, UK), rebound tonometry (Tonovet[®], Tiolat, Helsinki, Finland), Schirmer's tear test (Schirmer tear test[®], Intervet, Summit, NJ, USA) and fluorescein staining (Fluorescein paper[®]; Haag Streit AG, Koeniz, Switzerland), and dogs with ocular or systemic diseases were excluded. The animal use and experimental protocols were approved by the Institutional Animal Care and Use committee (SNU-121108-4 and 121123-10; Seoul National University, Korea).

3. *Ex vivo* experiments on porcine eyes (air assisted lamellar keratectomy)

Porcine eyes were placed on a specially designed frame (Fig. 1a). The Intraocular pressure (IOP) was 10–20 mmHg as measured by an applanation tonometer (TonoPen XL[®]; Mentor, FL, USA). The center of the cornea was trephined to 250 (G1), 375 (G2), 500 (G3), and 750 μm (G4) using an 8 mm diameter trephine (Barron radial vacuum trephine[®]; Katena products, Inc., Denville, NJ, USA). The surgical field was kept dry after trephination to minimize stromal edema. A 30 gauge needle was attached to a 4 mL air-filled syringe. The needle was bent 5 mm from its tip so that the terminal segment angled upwards at approximately 60°, while the bevel faced up. The tip was introduced parallel to the corneal surface into the central stroma at the base of the trephination groove (Fig. 1b and f). The plunger of the air-filled syringe was pressed until intrastromal blanching was observed. The fuzzy region of the white opaque cornea was removed using a corneal dissector and blunt-tipped corneal scissors.

Corneal thickness was measured at five places (the central, superior, inferior, nasal, and temporal surface) within the central 8 mm diameter area of the cornea using an ultrasonic pachymeter (PACHMETE DGH 55[®]; DGH Technology Inc., Pennsylvania, USA) before and after applying the AK (Fig. 1g). The pachymetry values are expressed as the average \pm standard deviation (SD) of 25 successive readings. If the SD of a measurement was $> 10 \mu\text{m}$, the value was discarded.

Ablated corneal depth was calculated using the corneal thickness pre- and post-operation, and the calculated thickness and trephined depth were compared.

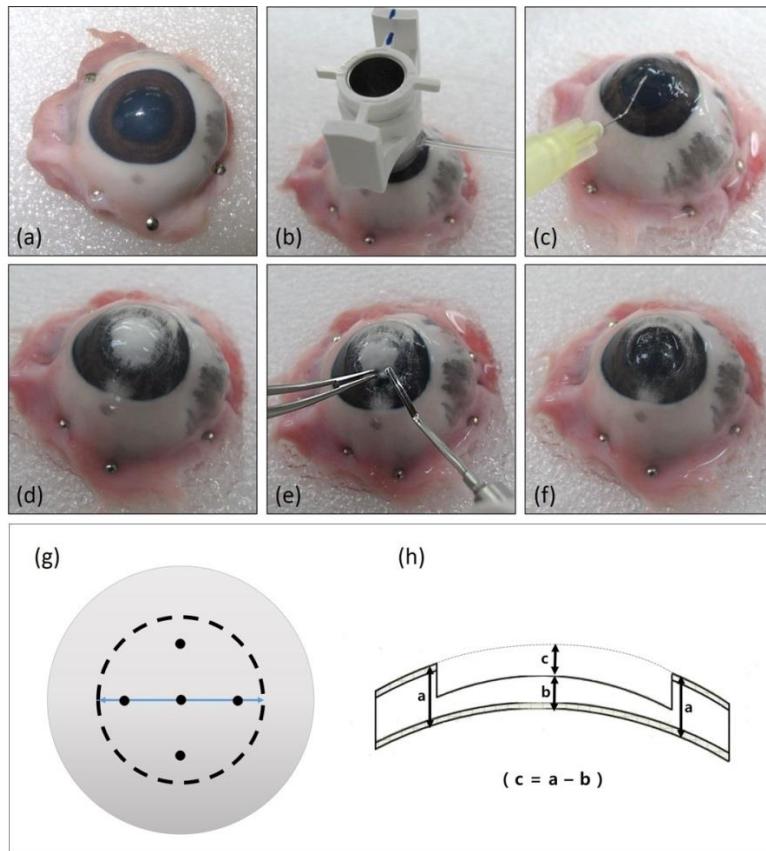


Fig. 1. Procedures of air assisted lamellar keratectomy. (a) The enucleated porcine eye was placed on a specially designed frame. (b) The center of the cornea was trephined using a vacuum trephine. (c) Four mL of air was injected at the base of the trephination gutter into the corneal stroma using a 30-gauge needle attached to a syringe. (d) Intrastromal blanching was observed. (e) The blanched cornea was removed using a corneal dissector and blunt-tipped corneal scissors. (f) Appearance after keratectomy using AK. (g) Schematic diagrams for measuring corneal thickness. The dotted line indicates the ablated corneal area and the blue arrows show an 8 mm diameter. The measurement sites of corneal thickness by ultrasonic pachymeter are marked by black dots. (h) Diagram of the corneal cross section. The thickness of the ablated area “b” was subtracted from the normal corneal thickness “a” to calculate the ablated corneal thickness “c”.

4. *In vivo* experiments on canine eyes (anesthesia and surgical procedures)

Each dog was positioned in dorsal recumbency while under general anesthesia with isoflurane after intubation and induction with tiletamine and zolazepam (Zoletil[®], Virbac, Carros, France; 2.5 mg/kg IV). The head was stabilized with a vacuum pillow, and the ocular surface was disinfected with 0.5% povidone iodine solution. Upper and lower eyelids were braced using an eyelid speculum. Keratectomy was performed in the two groups using either AK or CK after administration of atracurium (ATRA[®], Hanapharm, Seoul, Korea; 0.01 mg/kg IV) for central positioning of the cornea. The center of the cornea was trephined for 375 µm using an 8 mm diameter trephine in both groups. A conventional superficial keratectomy was performed with a #66 lamellar blade. Following surgery, one drop of atropine (1%, Isopto Atropine[®], Alcon, Antwerp, Belgium) was applied only once. Additionally, one drop of levofloxacin (0.5%, Cravit[®], Santen, Osaka, Japan) was applied three times daily until day 7 after surgery in all groups

5. Clinical grading of corneal haze

The level of haze in the cornea was measured by slit lamp biomicroscopy (SL-D7[®]) at 7, 14, 21, and 28 days after surgery and graded as follows (Fantes *et al.*, 1990): Grade 0: completely clear cornea; Grade 0.5: trace haze seen with careful oblique illumination; Grade 1: mild obscuration of iris details; Grade 2: a more prominent haze not interfering with visibility of fine iris details; Grade 3: moderate obscuration of the iris and lens; Grade 4: complete opacification of the stroma in the area of ablation.

Haze grading was performed in a blinded manner by three independent veterinarians.

6. Quantitative corneal haze grading

The slit images were taken under standardized conditions (1 mm wide, 14 mm long slit beam and a 45° angle from the temporal aspect of the cornea without background illumination to evaluate corneal haze preoperatively) at 7, 14, 21, and 28 days post-surgery. Each photograph was converted to an 8 bit gray-scale image using digital image analysis software (ImageJ ver. 1.46r; <http://rsbweb.nih.gov/ij/>). The selected area of the corneal section (100×3 pixels) was isolated, and an intensity of 0–255 was determined by averaging the gray-scale (intensity) indices of individual pixels within the area. Total intensity levels within the selected area were measured.

7. Histopathological and immunofluorescence analyses

Beagle eyes were enucleated by the conventional trans-scleral method after euthanasia with T61 after general anesthesia through administration of tiletamine and zolazepam (Zoletil®; 2.5 mg/kg IV). Porcine and canine corneas were excised from the globe by cutting with a blade and tenotomy scissors 2–3 mm from the limbus. Samples were fixed in 10% buffered formalin and embedded in paraffin. Six tissue sections were obtained at the central corneal part for each eyes. Three sections were stained with PAS according to the standard procedure. A light microscope (BX51®; Olympus, Tokyo, Japan) equipped with a digital camera (DP71®; Olympus) was used for photomicrography. The thickness of the non-ablated and ablated corneas on the histopathological section was determined by digital image analysis and the percentage of the ablated corneal thickness was calculated (Fig. 1h).

Immunofluorescent staining for α-SMA, a marker for myofibroblasts, was performed in three sections for the each eyes using mouse monoclonal antibody for α-SMA (M085101; DAKO, Carpinteria, CA, USA) with Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR, USA). The immunohistochemistry slides were mounted with SlowFade Gold antifade reagent with DAPI (Molecular Probes) and imaged using a fluorescence microscope (BX51®) equipped with a digital camera (DP71®).

8. Quantification of α -SMA positive cells

The green fluorescence of α -SMA positive cells in six randomly selected, non-overlapping, full-thickness central corneal columns, extending from the anterior stromal surface to the posterior stromal surface, were detected from the central ablated cornea of each specimen as previously described (Mohan *et al.*, 2003). The intensity of green fluorescence in each column was evaluated by digital image analysis. The mean green fluorescence intensity for the six column in each samples was used for the final results.

9. Statistical analyses

All measurements were performed in triplicate, and the results are expressed as the mean \pm SD. Statistical analyses were performed using SPSS V20 for Windows (SPSS Inc., Chicago, IL, USA). A student's *t*-test was used to test for significance between the two groups. One-way analysis of variance (ANOVA) with Bonferroni's post-hoc assessment was employed to test for significance when comparing three or more groups. *P* values < 0.05 were considered significant.

Results

The mean IOP before trephination was 13.7 ± 0.2 mmHg (range, 11.3–16.0 mmHg) after fitting the eyes on the specially designed frame. No significant differences were observed between the mean IOP of each group based on one-way ANOVA with Bonferroni's post-hoc test ($p = 0.957$). Mean corneal thickness pre- and post-operation and the ablated cornea for each of the five measurement sites are shown in Table 1. No significant differences were observed among the five measurement sites within each group. The ablated corneal thickness in G1 ($p < 0.001$) was significantly different from the trephined corneal depth (Table 2). No significant differences were detected between ablated corneal thickness and the trephined corneal depth for resection in G2 ($p = 0.214$), G3 ($p = 0.381$) or G4 ($p = 0.439$).

No significant differences were observed between the ablated percentages measured by ultrasonic pachymetry and digital image analyses of the histopathological sections in any of the groups (Fig. 2). The calculated percentage of corneal resection on the photomicrograph in G1 was significantly different ($p = 0.013$) from the desired percentage of resection. (Fig. 2). The stroma of the air-injected cornea above the needle insertion layer was severely deformed by the small air bubbles relative to the normal cornea (Fig. 3a and b). The most deformed stromal fiber due to air bubbles was removed after resection; hence, the surface of the wounded cornea was smooth following application of AK (Fig. 3c). Several small bubbles inserted into the stroma at the levels of the trephination gutter paralleled the stromal fibril layer on the outside margin of the trephined area.

Table 1. Corneal thickness at each measurement site

Corneal thickness pre-operation (μm) [*]					Mean \pm SD	P value [†]
	Central	Inferior	Superior	Nasal	Temporal	
G1	984.1 \pm 3.1	974.3 \pm 17.7	979.0 \pm 13.0	969.7 \pm 14.2	976.1 \pm 16.4	976.6 \pm 5.4
G2	995.7 \pm 5.8	987.3 \pm 7.7	985.7 \pm 9.0	991.5 \pm 5.8	988.2 \pm 8.3	989.7 \pm 4.0
G3	1021.2 \pm 41.8	1011.4 \pm 47.3	995.1 \pm 14.2	993.4 \pm 14.4	993.7 \pm 15.5	1003.0 \pm 12.7
G4	972.0 \pm 19.9	968.5 \pm 27.1	969.7 \pm 26.6	967.2 \pm 26.4	969.6 \pm 23.9	969.4 \pm 1.8
Corneal thickness post-operation (μm) [*]					Mean \pm SD	P value [†]
	Central	Inferior	Superior	Nasal	Temporal	
G1	616.9 \pm 34.2	612.5 \pm 31.3	622.0 \pm 53.9	616.7 \pm 56.9	617.1 \pm 55.0	617.0 \pm 3.4
G2	621.5 \pm 2.8	613.3 \pm 2.2	610.0 \pm 5.4	617.9 \pm 2.8	613.4 \pm 2.2	615.2 \pm 4.5
G3	503.1 \pm 51.7	500.5 \pm 56.9	504.1 \pm 55.3	500.4 \pm 64.2	501.2 \pm 61.5	501.9 \pm 1.6
G4	250.8 \pm 19.2	251.3 \pm 27.1	257.8 \pm 28.6	253.9 \pm 24.6	250.4 \pm 25.5	252.8 \pm 3.1
Resected corneal thickness (μm) [*]					Mean \pm SD	P value [†]
	Central	Inferior	Superior	Nasal	Temporal	
G1	367.3 \pm 33.8	361.9 \pm 46.5	357 \pm 42.3	352.9 \pm 53.2	359 \pm 42.1	359.6 \pm 5.4
G2	374.2 \pm 5.0	374.0 \pm 9.3	375.7 \pm 13.6	373.6 \pm 3.8	374.8 \pm 8.9	374.5 \pm 0.8
G3	518.1 \pm 57.7	510.9 \pm 69.0	491.1 \pm 58.4	493 \pm 65.2	492.5 \pm 53.3	501.1 \pm 12.5
G4	721.2 \pm 21.4	717.2 \pm 32.7	711.9 \pm 35.5	713.3 \pm 37.8	719.2 \pm 35.2	716.6 \pm 3.9

G1, 250 μm trephined group (n = 10); G2, 375 μm trephined group (n = 10); G3, 500 μm trephined group (n = 10); G4, 750 μm trephined group (n = 10) for resection using air assisted lamellar keratectomy. *The values represent the mean \pm SD. †One-way ANOVA was used to investigate differences between measurement sites in each group followed by a Bonferroni's post-hoc test.

Table 2. Measurement of the mean resected corneal thickness in each group

	Mean corneal thickness (μm) [*]			Trehined corneal depth (μm)	P value [†]
	Pre	Post	Resected		
G1	976.6 ± 5.4	617.0 ± 3.4	359.6 ± 5.4	250	< 0.001 [‡]
G2	989.7 ± 4.0	615.2 ± 4.5	374.5 ± 0.8	375	0.214
G3	1003.0 ± 12.7	501.9 ± 1.6	501.1 ± 12.5	500	0.381
G4	969.4 ± 1.8	252.8 ± 3.1	716.6 ± 3.9	750	0.439

G1, 250 μm trephined group (n = 10); G2, 375 μm trephined group (n = 10); G3, 500 μm trephined group (n = 10); G4, 750 μm trephined group (n = 10) for resection using air assisted lamellar keratectomy. ^{*}Values represent the mean \pm SD. [†]Significant differences within the same groups between the mean ablated corneal thickness and trephined corneal depth for resection were identified by the Student's *t*-test. [‡]Statistically significant.

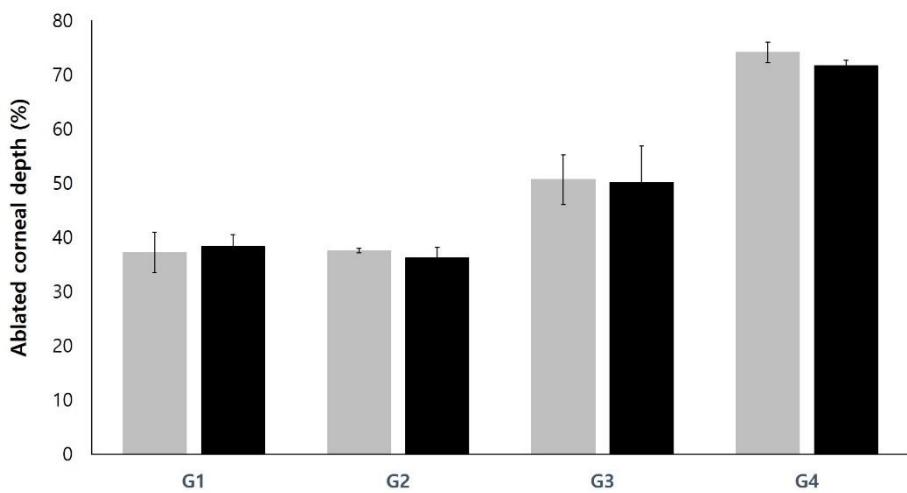


Fig. 2. Ablated corneal thickness (%) calculated based on the pachymetry and photomicrographic image analyses. G1, 250 μm trephined group; G2, 375 μm trephined group; G3, 500 μm trephined group; G4, 750 μm trephined group, ■ ; Pachymetry, and □ ; Digital image-analysis.

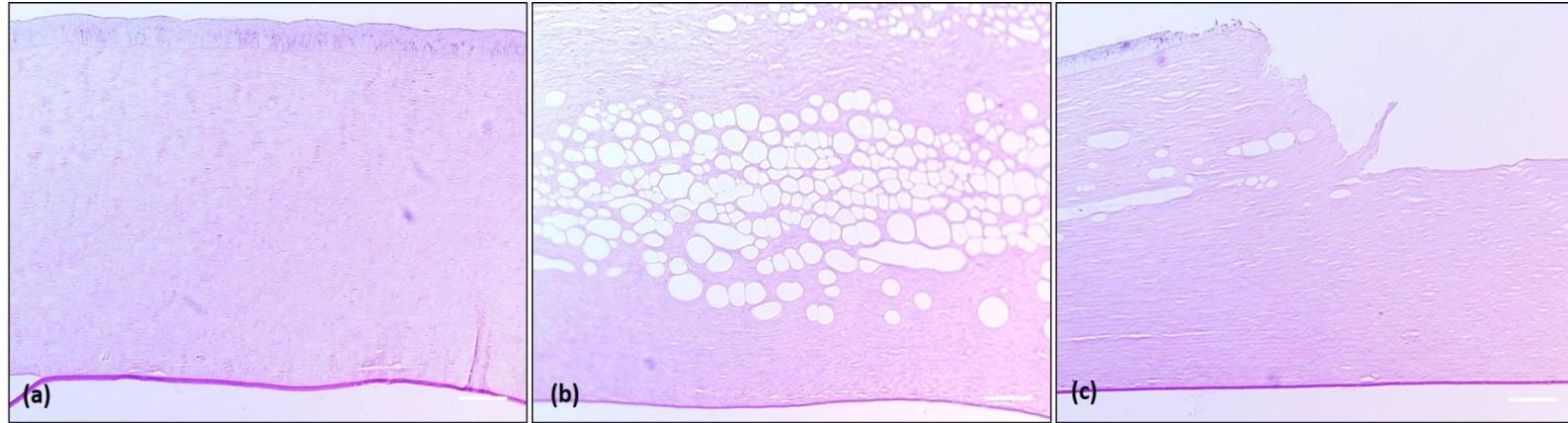


Fig. 3. Photomicrograph of porcine corneas. (a) Normal porcine cornea showing aligned stromal layers. (b) Stroma of the air-injected cornea was deformed by small air bubbles above the layer for needle insertion. (c) After resection by the air assisted lamellar keratectomy showing smooth ablated surface. 200 \times magnification. Scale bar = 50 μm .

Corneal haze was noted at 7 days after surgery and appeared to peak about 21 days after surgery in both groups (Fig. 4). Clinical corneal haze in the AK group was significantly greater than that in the CK group beginning 14 days after surgery (day 14; $p = 0.036$, day 21; $p = 0.044$, day 28; $p = 0.009$) (Fig. 5a). Furthermore, the quantity of corneal haze was more developed in the AK group than in the CK group from 14 days after surgery ($p < 0.001$) (Fig. 5b).

The corneal sections of the canine eyes showed a distinct stromal remodeling pattern in the AK group relative to the normal cornea and the CK group upon PAS staining (Fig. 6a and c). Alpha-SMA-positive cells were selectively detected in the anterior stroma immediately beneath the epithelial basement membrane in the CK and AK groups upon immunohistochemistry (Fig. 6d and f). Total green intensity of the entire stroma was significantly enhanced in the CK ($p < 0.001$) and AK groups ($p < 0.001$) relative to that in the normal cornea. Moreover, total green intensity in the AK group was significantly higher than that in the CK group ($p < 0.001$, Fig. 7).

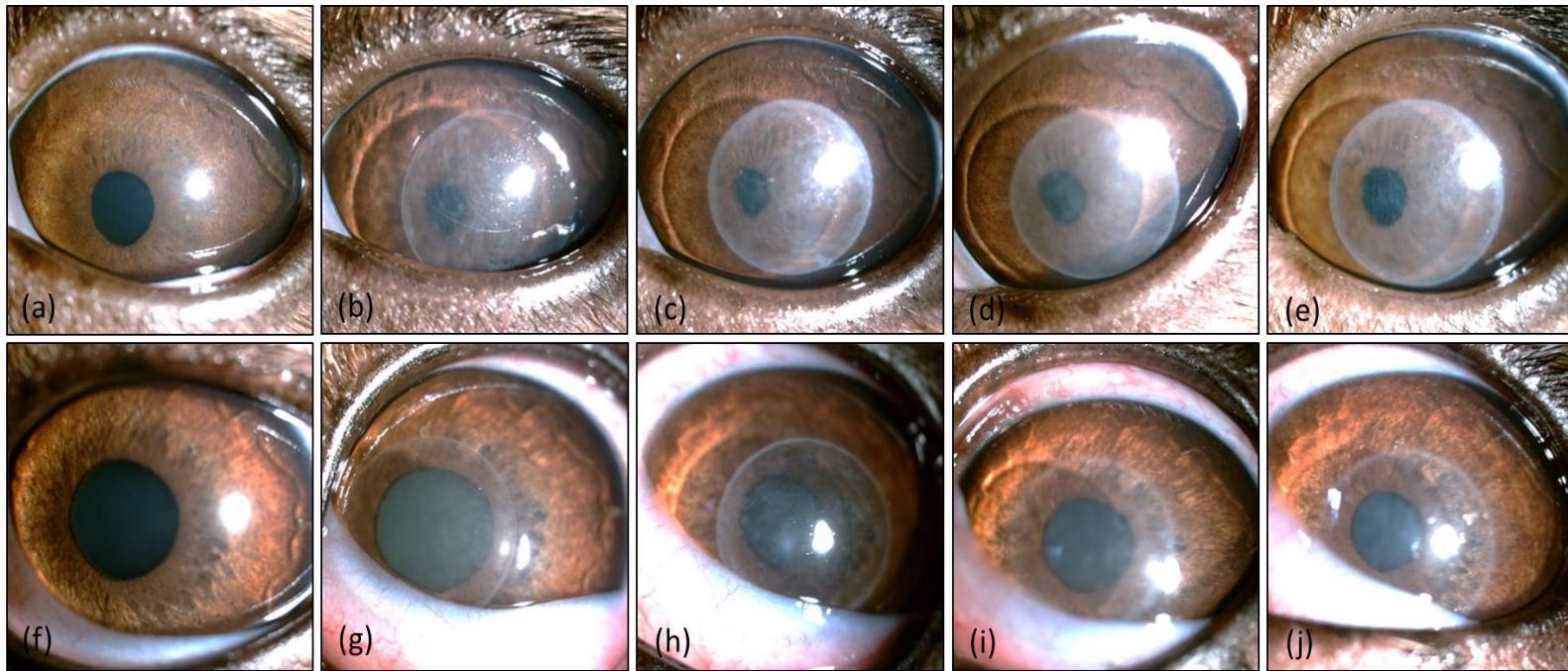


Fig. 4. Slit-lamp biomicroscopy of the corneas with subepithelial haze. Greater corneal haze developed following AK (a-e) than CK (f-j). a and f; pre-operative, b and g; day 7, c and h; day 14, d and I; day 21, e and j; day 28 after the surgery. The size of the captured corneal section was fixed by controlling the magnification of the slit lamp (10 \times magnification) under diffuse illumination (45°).

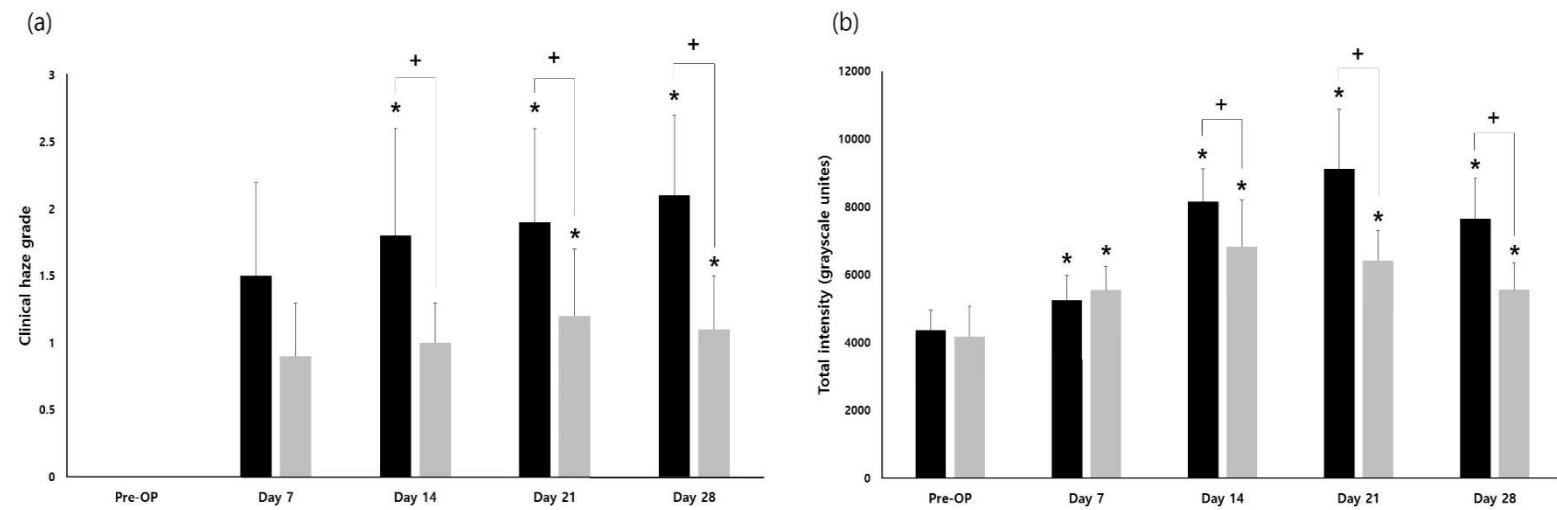


Fig. 5. Corneal haze measurement by clinical haze grading and quantitative method. (a) Clinical corneal haze grading. (b) Quantification of corneal haze. ■ = AK group and □ = CK group. Significant differences are indicated by + Student's *t*-test, $p < 0.05$ and *one-way ANOVA with Bonferroni's post-hoc test, $p < 0.05$.

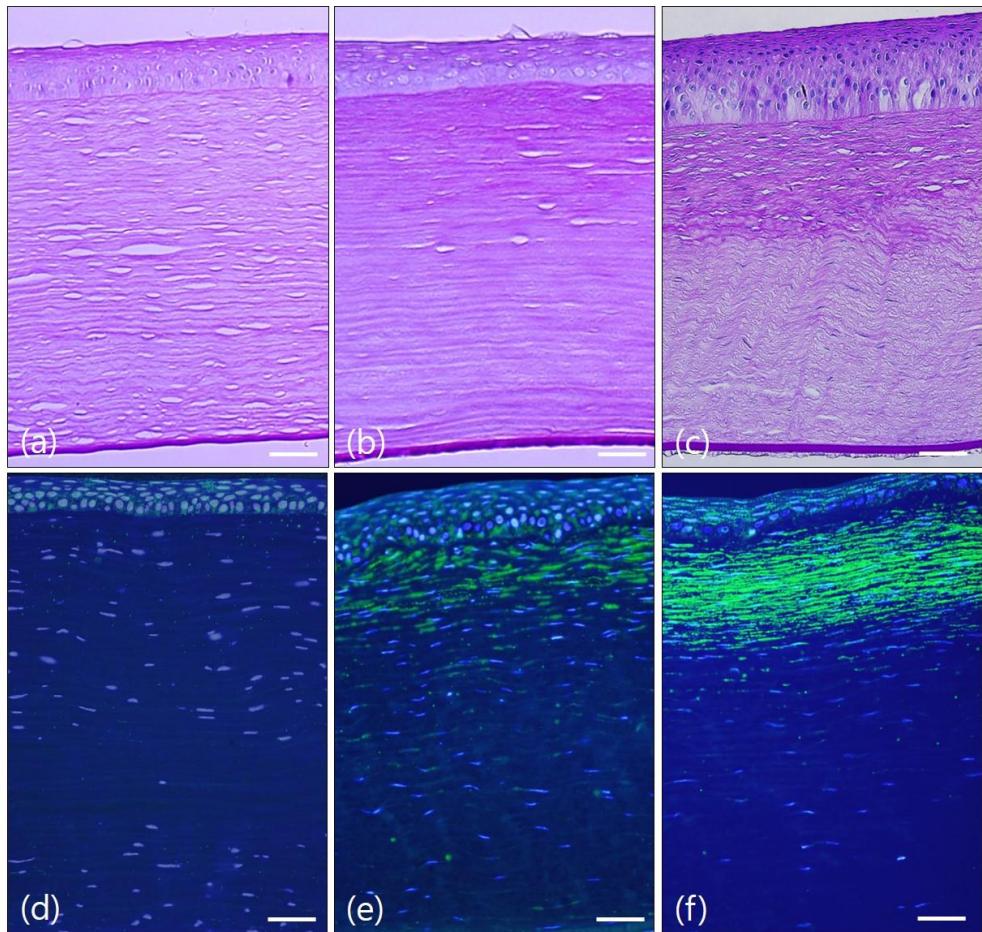


Fig. 6. Histopathology with PAS stain (a–c) and immunohistochemistry for α -SMA (d–f) for the evaluation of corneal haze. DAPI-stained nuclei are shown in blue, SMA-stained cells are shown in green. a and d; normal cornea, b and e; CK group, c and f; AK group. 200 \times magnification. Scale bar = 50 μ m.

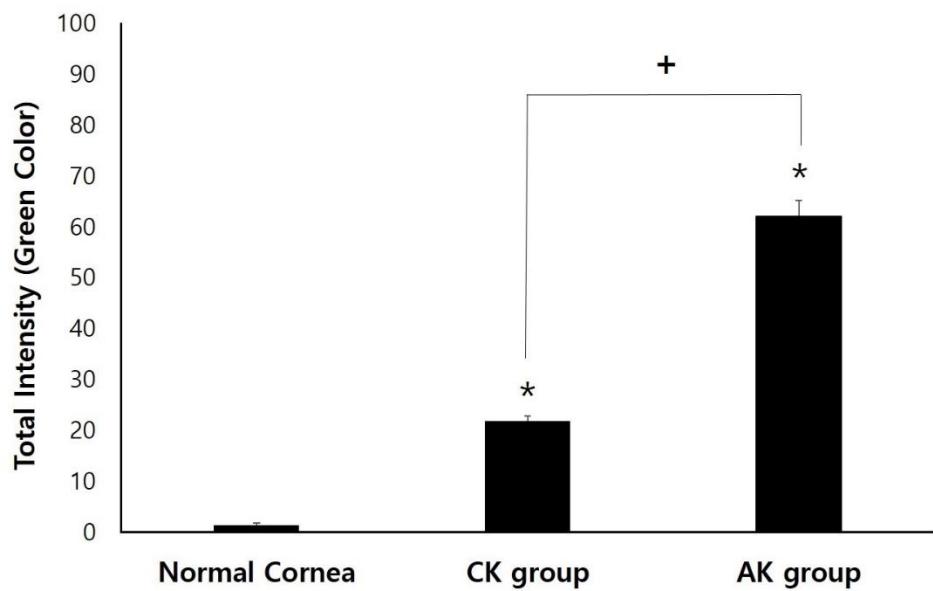


Fig. 7. The quantification of SMA-positive cells by green fluorescence detection.
Significant differences are indicated by + Student's *t*-test, *one-way ANOVA with Bonferroni's post-hoc test, $p < 0.05$.

Discussion

Corneal haze is a common complication following corneal surgery that results in diminished corneal transparency (Sakimoto *et al.*, 2006). Because the precise mechanisms of the formation of corneal haze are unclear, therapies specifically targeting its prevention are limited. Mitomycin C (MMC) has been widely used to prevent corneal haze following surface ablation for myopia (Teus *et al.*, 2009). However, multiple complications of MMC treatment have been reported, including limbal and scleral necrosis, abnormal wound healing, and loss of keratocytes (Safianik *et al.*, 2002). These adverse effects have encouraged the development of newer pharmacological agents that can effectively inhibit the formation of corneal haze without causing serious side effects.

Several experimental methods have been used to induce a corneal wound and haze, including mechanical debridement, chemical burns, and superficial keratectomy (Rieck *et al.*, 1992). Mechanical debridement is primarily used as a model to check the treatment effectiveness of epithelial healing, so the resection is not deep enough to develop sufficient corneal haze. Chemical burning tends to cause loss of keratocytes, further decreasing their density during slow healing injuries (Lin *et al.*, 2004), and resection depth after applying these methods is difficult to assess owing to loss of transparency. PRK is a superficial keratectomy that employs an excimer laser and has been conducted to achieve accurate ablation (Soong *et al.*, 2008). Expensive specialized equipment is needed for this technique, and the haze that develops is grade 1 or less on a scale of four total grades (Mohan *et al.*, 2008), which is insufficient for evaluation of treatment effects. For these reasons, standardized experimental models are needed in this research field.

The first goal of this study was to improve the method of superficial lamellar keratectomy by modifying the BBT to achieve standardized resection size and depth. This study demonstrated that AK resulted in uniform resection depth at each of the five measurement sites without expensive laser equipment. Additionally, ablated corneal thickness using this technique was the same as the trephined corneal depth in G2, G3, and G4. The percentage of ablated corneal thickness on the histopathological section calculated with a digital imaging program was not significantly different from the results obtained using ultrasonic pachymetry. In contrast, the resection thickness of G1 was significantly thicker than the desired depth. The trephined depth was not deep enough to insert a needle into in this group and excess air infiltrated under the stroma at the level of needle insertion. For these reasons, more stroma was ablated than desired. Accordingly, the AK achieved the standardized resection depth at a trephined corneal depth greater than 375 μm .

The corneal stroma is a precisely formed collagen fibril layer that has a unique parallel arrangement in a mucoid matrix scattered with keratocytes (Freund *et al.*, 1995). Because of these stromal structures in the normal cornea, dissection between stromal fibers requires specialized skill. Unlike the normal corneal stroma, the histopathological structure of the air-injected cornea was severely deformed by air bubbles and easily detached using a corneal dissector in this study. Additionally, the injected air bubbles were distributed uniformly over the entire internal area of trephination. These findings indicate that this modified method could be applied more easily and quickly to generate a standardized stromal defect compared to previous models.

Corneal haze developed in all eyes included in this experiment, beginning at day 7 after surgery and appearing to peak about 21 days after surgery. Surface ablation triggers a cascade of physiological events that culminate in mild to severe corneal fibrosis (de Medeiros *et al.*, 2008). Modern technologies that involve mechanical removal of the corneal epithelium have demonstrated upregulation of various pro-inflammatory interleukins that indirectly contributes to corneal fibrosis (Chang *et al.*, 2008). Haze following PRK may result from corneal wound healing, which is likely to be initiated by keratocyte apoptosis and subsequent over-proliferation of cells (Mohan *et al.*, 2003). According to our histopathological results, stromal reorganization was observed after healing from the surface resection represented by differentiation of fibroblasts into myofibroblasts with epithelial cell hyperplasia.

This results show that greater corneal haze developed after applying the AK than after CK. Additionally, α -SMA-positive cells were significantly more numerous in the AK group than those in the CK group upon immunohistochemical evaluation. Expression of α -SMA in fibroblasts is a specific marker of myofibroblast differentiation (Jester *et al.*, 1995). The morphological and immunohistochemical findings suggest that more keratocytes were replaced by smooth muscle-like myofibroblasts during wound healing after the AK. Mechanical tension is an important underlying factor in the molecular mechanisms of tissue repair and fibrosis. Mechanical stress induces myofibroblast differentiation in human corneal fibroblasts (Eckes *et al.*, 2006; Garrett *et al.*, 2004). Thus, it seems reasonable to conclude that the AK could induce more severe corneal haze due to myofibroblasts after the mechanical tension induced by injecting air bubbles. The stromal

structure was severely deformed by the injected air, which is an indication of air bubbles generating mechanical tension on stromal fibers.

Conclusions

In summary, these results suggest that AK can be useful to achieve the desired resection depth of the corneal stroma. The AK induced more corneal haze than the conventional method of superficial keratectomy. Therefore, this technique would contribute to improving standardization of the corneal haze model.

CHAPTER II.

**Effect of onion extract on corneal haze suppression after
air assisted lamellar keratectomy**

Abstract

This study evaluated the effect of onion extract on corneal haze suppression after applying the AK. The AK was performed on 24 canine eyes. They were treated with an artificial tear (group C), prednisolone acetate (group P), onion extract (group O), and TGF- β 1 (group T) three times per day from 7 to 28 days after the surgery. Corneal haze occurred on all eyes and was observed beginning at 7 days after the surgery. The haze was significantly decreased in groups P and O from day 14 compared with the group C using the clinical (group P; $P=0.021$, group O; $P=0.037$) and objective evaluation method (group P; $P=0.021$, group O; $P=0.039$). In contrast, it was significantly increased in group T from day 14 compared with group C based on the clinical ($P=0.002$) and objective evaluation method ($P<0.001$). Subsequently, these eyes were enucleated after euthanasia, and immunohistochemistry with α -SMA antibodies was done. The total green intensity for α -SMA was significantly more expressed in group T and significantly less expressed in groups P and O than in group C. Onion extract could have potential as a therapeutic in preventing corneal haze development by suppressing the differentiation of fibroblasts into myofibroblasts.

Introduction

Many corneal diseases are associated with the development of opacities in the stroma. Corneal haze presents as a superficial opacification of the anterior corneal stroma leading to a transient decrease in corneal transparency after lamellar keratectomy for dermoid, corneal inclusion cyst, and corneal tumor, corneoconjunctival transposition or autologous lamellar keratectomy for deep corneal ulcer in veterinary ophthalmology. Also, it is one of the most important complications of PRK, and its incidence and intensity increase in eyes treated for higher degrees of refractive error in human medicine (Heitzmann *et al.*, 1993). In most transparency disorders, corneal haze may be induced by a combination of two or more predominant factors like corneal edema, scarring, accumulated macromolecules, and reflective keratocytes (Møller-Pedersen *et al.*, 2004). Moreover, the formation of corneal haze involves the apoptosis of keratocytes and the proliferation and transformation of fibroblasts into myofibroblasts (Wilson *et al.*, 2001). Therefore, one of the most crucial aspects of corneal healing from refractive surgery is the minimization of corneal haze.

The efficacy of MMC in reducing the incidence of corneal haze has led to its widespread use in most refractive surgery practices (Teus *et al.*, 2009). However, multiple complications such as limbal/scleral necrosis, abnormal wound healing, and loss of keratocytes are reported with the topical use of MMC (Safianik *et al.*, 2002). These results encourage the development of newer pharmacologic agents that can effectively inhibit the formation of corneal haze without causing serious side effects. Recent research on trichostatin A (TSA), a histone deacetylase inhibitor, reported that it inhibits TGF- β 1-

induced accumulation of the extracellular matrix and myofibroblast formation *in vitro* and markedly decreases haze *in vivo* (Sharma *et al.*, 2009). However, there are no commercially available products for clinical use.

Allium cepa (onion) and onion extract have been reported to be effective in cardiovascular disease, because of their hypolipidemic, anti-hypertensive, anti-diabetic, and antithrombotic effects, and to possess many other biological activities including antimicrobial, antioxidant, anticarcinogenic and immunomodulatory activities (Corzo-Martínez *et al.*, 2007). Especially, flavonoids in onion extract reduce scar formation by inhibiting fibroblast activities (Cho *et al.*, 2010). Recently, commercial products composed of onion extract have been used to reduce hypertrophic scar formation (Ho *et al.*, 2006). Myofibroblasts are an important cell in connective tissue remodeling that differentiates during wound healing and fibrosis development in the pathogenesis of such diseases as hypertrophic scars, liver or pulmonary fibrosis (Desmouliere *et al.*, 2005), and corneal haze formation (Milani *et al.*, 2013). The myofibroblasts could represent an important target for corneal haze treatment like in the treatment for hypertrophic scar formation. Thus, onion extract could be useful as a therapeutic in preventing the development of corneal haze by suppressing the differentiation of fibroblasts into myofibroblasts.

AK is one of the experimental models for the development of corneal haze (Kim *et al.*, 2015). In this method, the wound size and depth were standardized by modification of the bubble technique for corneal transplantation. Also, it could induce more corneal haze than the conventional superficial keratectomy.

The aim of this study was to evaluate the efficacy of onion extract ointment in corneal haze development after applying to the haze model with the AK for canine eyes. In addition, the effect of onion extract ointment in the down-regulation of myofibroblast expression was examined with immunohistochemistry using the α -SMA antibody.

Materials and Methods

1. Corneal fibroblast culture and cell viability test for onion extract

Corneal fibroblasts were cultured from porcine eyes, which obtained from a local slaughterhouse, for the cell viability test of the onion extract. The corneal button which removed by an 8 mm diameter trephine (Barron radial vacuum trephine®, Katena) was obtained. After then, the epithelial cells of the corneal buttons were scrapped off using a # 10 scalpel blade, DMs were peeled off, and the corneal stromas were washed with PBS (pH 7.4) (10010-023; GIBCO™, Grand Island, NY, USA). The corneal buttons were cut into four small pieces and incubated overnight in a humidified CO₂ incubator at 37°C in DMEM (11995-065; GIBCO™) containing 20 mM HEPES (15630-080; GIBCO™) and 1.25 mg/mL collagenase type I (17100-017; GIBCO™). The digested tissues were mixed with media by pipetting and filtered a 100 µm cell strainer (08-771-19; Falcon™, New Jersey, USA). Then, they were centrifuged at 800 g for 5 minutes and resuspended in 2 mL of DMEM containing 20 mM HEPES, 50 µg/mL gentamicin (15750-078; GIBCO™), 1.25 µg/mL amphotericin B (A20678; GIBCO™) and 10% fetal bovine serum (10437-028; GIBCO™). This keratocyte-containing cell suspension was then seeded on 6-well plastic dishes and incubated in a humidified CO₂ incubator at 37°C. Eighty percent confluent cultures of cornea fibroblasts (passage 1-3) were used for experiments.

Trypan blue dye exclusion test was used to evaluate cell viability of corneal fibroblasts after treating onion extract. Onion extract (W281719; Sigma-Aldrich, St. Louis, MO, USA) was treated to the each well at 0, 0.01, 0.1, 1, 10, 50, and 100 μ L/mL concentrations diluted with Dimethyl sulfoxide (DMSO) (AMR-0231-1; Amresco, Solon, OH, USA) for 24 hr. Then, they were resuspended using 0.05% trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA) (25300-054; GIBCOTM) and trypan blue solution (0.4% wt/vol, 15250-061; GIBCOTM) was mixed with the resuspension cells. The suspensions were loaded into a hemocytometer and scored with a light microscope. Cells that stained blue were scored as nonviable.

2. A process of manufacture for the onion extract ointment

A 1% onion extract ointment was made with 10 g white petrolatum (white petrolatum 1 g/g, Sungkwang Pharm., Cheonan, Korea) and 0.1 mL onion extract (W281719, Sigma-Aldrich) mixture in a water bath. The concentration of onion extract ointment depended on the *in vitro* viability test. Before use main study, the onion extract ointment was applied every 12 hours for 2 weeks at the normal cornea of six healthy beagle dogs to test the abnormal allergic reactions, blepharospasm, conjunctival hyperemia, corneal epithelial disorders and other ocular abnormality, by ophthalmic examinations every the other days.

3. Animals

Twenty-four eyes from 12 healthy beagles were used in this study. Before the experiment, all dogs underwent an ophthalmic examination including slit-lamp biomicroscopy (SL-D7[®]), indirect ophthalmoscopy (Vantage plus[®]), rebound tonometry (Tonovet[®]), Schirmer's tear test (Schirmer tear test[®]) and fluorescein staining (Fluorescein paper[®]). Dogs with ocular or systemic diseases were excluded. The animal use and experimental protocols were approved by the Institutional Animal Care and Use committee (SNU-121123-10; Seoul National University, Korea). All dogs were divided into 4 groups consisting of 6 eyes in each group; control (Group C; n = 6), Prednisolone acetate treatment (Group P; n = 6), Onion extract treatment (Group O; n = 6), and TGF-β1 treatment (Group T; n = 6).

4. Corneal haze generation by the air assisted lamellar keratectomy

AK was performed following a method reported previously (Kim *et al.*, 2015). General anesthesia was performed by intravenous injection of tiletamine and zolazepam (Zoletil[®]; 2.5 mg/kg) for induction, and maintained with isoflurane (Ifiran[®], Hana Pharm, Seoul, Korea; MAC 0.5 – 1.5%). Atracurium (ATRA[®]; 0.01 mg/kg IV) was administrated for central positioning of the cornea during the surgery. The AK was performed for all eyes. Briefly, the center of the cornea was trephined 375 µm using an 8 mm diameter trephine (Barron radial vacuum trephine[®], Katena) (Fig. 8a). The surgical field was kept dry after the trephination to minimize stromal edema. Four mL of air were injected at the base of the trephination gutter into the corneal stroma using a 30-gauge needle attached to a syringe. The needle was bent 5 mm from its tip so that the terminal segment angled upwards approximately 60°, while the bevel faces up (Fig. 8b). The tip was introduced parallel to the corneal surface into the central stroma at the base of the trephination groove. The plunger of the air-filled syringe was pressed until intrastromal blanching was observed (Fig. 8c). The fuzzy region of the white opaque cornea was removed using a corneal dissector and blunt-tipped corneal scissors (Fig. 8d).

After surgery, one drop of atropine (1%, Isopto Atropine[®]) was applied for 3 days for the purpose of cycloplegic effect. Levofloxacin (0.5%, Cravit[®]) eye drops were administered three times daily until 7 days after the surgery. After the seventh day, artificial tear eye drops (0.1% sodium hyaluronate; Lacure[®], Samil

Pharm., Seoul, Korea) for group C, prednisolone acetate 1% (Pred-Forte[®], Allergan, Irvine, California, USA) for group P, an onion extract (W281719, Sigma-Aldrich, St. Louis, MO, USA) ointment 10 mL/mg diluted with a white petrolatum (Vaseline[®], SungKwang Pharm, Cheonan, Korea) for group O, and TGF-β1 (T7039, Sigma-Aldrich) 1 ng/mL diluted with artificial tear eye drops for group T were administered three times daily each for 3 weeks.

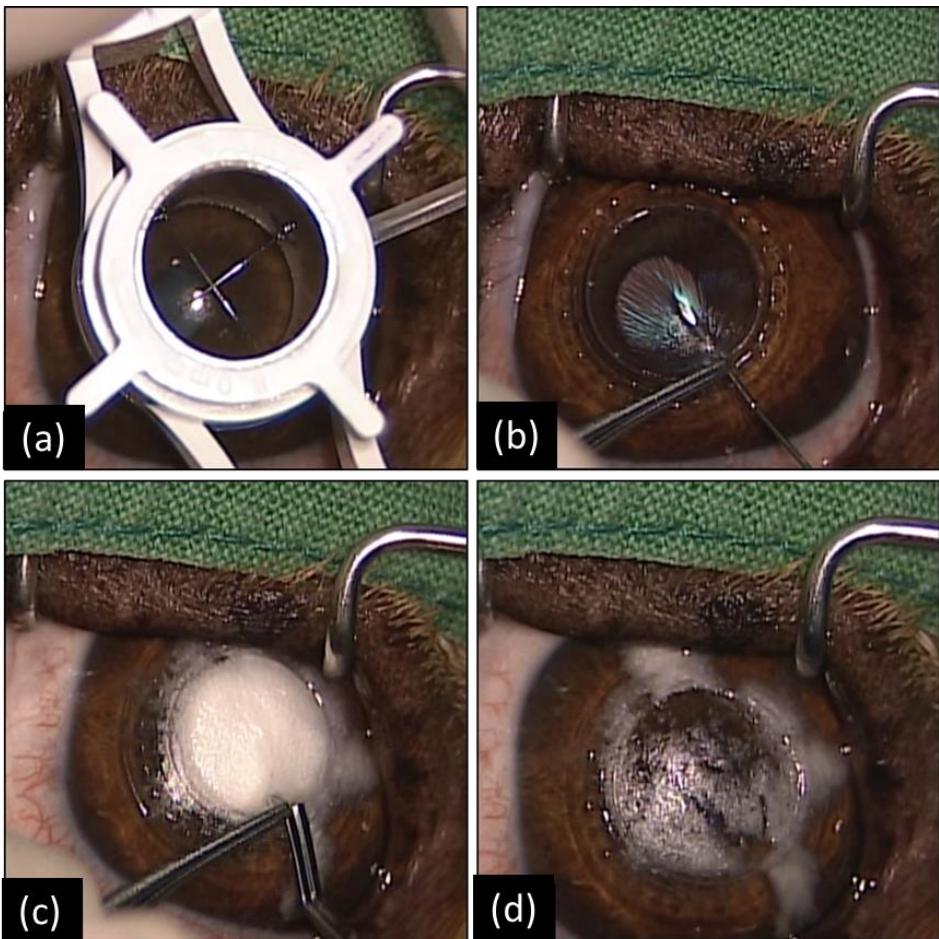


Fig. 8. The procedures of air assisted lamellar keratectomy in dogs. (a) The center of the cornea was trephined $375\text{ }\mu\text{m}$ using an 8 mm vacuum trephine. (b) Four mL of air was injected at the base of the trephination gutter into the corneal stroma using a 30-gauge needle attached to a syringe. (c) Intrastromal blanching was observed, and the blanched cornea was removed using a corneal dissector and blunt-tipped corneal scissors. (d) Appearance after keratectomy using the air assisted lamellar keratectomy.

5. Corneal haze grading

The level of haze in the cornea was evaluated by slit lamp biomicroscopy (SL-D7[®]) at 7, 14, 21, and 28 days after the surgery using two kinds of method: the previously reported clinical grading system (Fantes *et al.*, 1990) and a quantitative method. With the clinical grading, grade 0 was a completely clear cornea; grade 0.5 had a trace amount of haze observed with careful oblique illumination; grade 1 was a mild obscuration of the iris details; grade 2 was a more prominent haze not interfering with the visibility of fine iris details; grade 3 was a moderate obscuration of the iris and lens; and grade 4 was complete opacification of the stroma in the area of the ablation. Haze grading was performed in a blinded manner by three independent veterinarians.

For quantitative haze grading, slit images were taken under standardized conditions: a 1 mm wide, 14 mm long slit beam and a 45° angle from the temporal aspect of the cornea without background illumination. Then, each photograph was converted into an 8 bit gray-scale image using digital image analysis (ImageJ ver. 1.46r). The selected area of the corneal section (100 x 3 pixel) was isolated, and an intensity of 0 to 255 was determined by averaging the gray-scale (intensity) indices of the individual pixels within the area. Total intensity levels within the selected area were measured.

6. Immunofluorescence Analyses

The beagle eyes were enucleated with the conventional trans-scleral method after euthanasia with T61 after general anesthesia through tiletamine and zolazepam (Zoletil[®]; 5.0 mg/kg IV). Corneas were excised 2 – 3 mm from the limbus with forceps and tenotomy scissors. The samples were fixed in 10% buffered formalin and embedded in paraffin. Three tissue sections (4 µm thickness) were obtained at the central corneal part for each eyes. Immunofluorescence staining for α-SMA, a marker for myofibroblasts, was performed. These tissue sections were incubated at room temperature with the monoclonal antibody for α-SMA (M085101, DAKO) at a 1:200 dilution in 1× PBS for 90 minutes and with a secondary antibody (Alexa Fluor 488 goat anti-mouse IgG; Molecular Probes) at a dilution of 1:500 for 1 hour. Tissues were mounted with mounting medium and DAPI (SlowFade[®] Gold antifade reagent, Molecular Probes) to visualize the nuclei in the tissue sections. Sections were viewed and photographed with a fluorescence microscope (BX51[®]) equipped with a digital camera (DP71[®]).

7. Quantification of α -SMA-positive cells

Fluorescence intensity of green color for the α -SMA positive cells was detected using the digital image analyzer (Image J) by comparing size-matched areas from 6 randomly chosen fields of view in the experimental areas. These 6 fields were non-overlapping, full-thickness central corneal columns, extending from the anterior stromal surface to the posterior stromal surface following a method previously reported (Mohan *et al.*, 2003). The mean green fluorescence intensity for the six column in each samples was used for the final results.

8. Statistical analyses

Statistical analysis was performed with SPSS V20 for Windows (SPSS Inc.). Data were expressed as the mean \pm SD, and the level of significance was $P < 0.05$. One-way ANOVA with Bonferroni's post-hoc assessment was used to test for the significance of the objective haze grading and the total green color intensity between the groups. In addition, in one-way repeated measures ANOVA following pairwise comparison, Bonferroni's adjustment was performed to evaluate the objective haze grades against time in the same group. For the clinical corneal haze grading, the values between groups were compared using the Kruskal-Wallis analysis or Friedman test with the Wilcoxon signed rank test.

Results

Cell viability was over 95% at the concentration of 0, 0.01, 0.1, 1, and 10 uL/mL onion extract in vitro evaluation (data not shown). The 1% onion extract ointment were showed no adverse effects and allergic reaction, like blepharospasm, conjunctival hyperemia, corneal epithelial disorders and other ocular abnormalities, when applied every 12 hours to the normal beagle cornea for 2 weeks.

Corneal haze was developed after the AK depending on each treatment group (Fig. 9). Control corneas treated with the artificial tear eye drops (group C) had significant developed corneal haze in clinical grading until 21 days after surgery (day 14; $P = 0.032$, day 21; $P = 0.041$, and day 28; $P = 0.210$) compared with day 7 (Fig. 10). Topical application of 1% prednisolone acetate (group P) and 1% onion extract ointment (group O) caused a statistically significant decrease in corneal haze in group P (day 14; $P = 0.021$, day 21; $P = 0.012$, day 28; $P = 0.001$), and in group O (day 14; $P = 0.037$, day 21; $P = 0.008$, day 28; $P = 0.003$) compared with the same groups for haze grading at day 7. Additionally, corneal haze was significantly increased in the TGF- β 1 treated group (group T) (day 14; $P = 0.002$, day 21; $P < 0.001$, day 28; $P < 0.001$ compared with the haze grading at day 7). For the degree of clinical corneal haze in each group, it decreased in groups P and O from day 14 (day 14; $P = 0.016$ and $P = 0.007$, day 21; $P < 0.001$ and $P = 0.026$, day 28; $P = 0.001$ and $P = 0.011$, respectively) compared with group C. In addition, corneal haze significantly increased in group T at days 21 ($P = 0.022$) and 28 ($P = 0.001$) compared with group C.

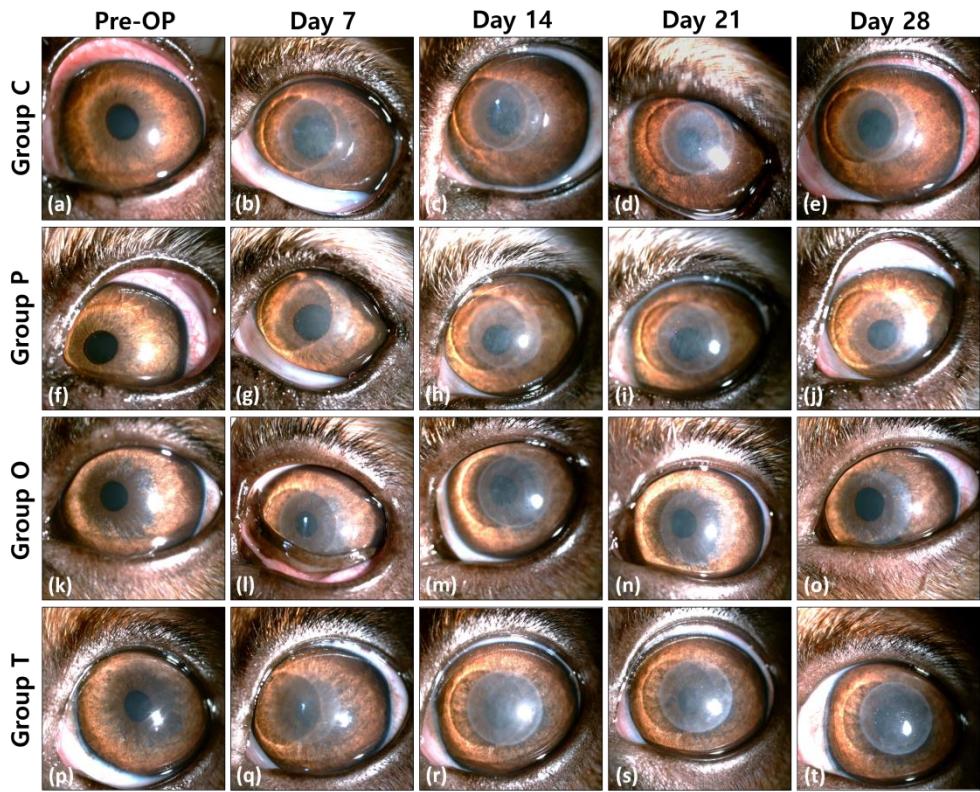


Fig. 9. The evaluation of corneal haze with a slit-lamp biomicroscopy. Group C; artificial tear treatment group (a-e), Group P; prednisolone acetate treatment group (f-j), Group O; onion extract ointment treatment group (k-o), Group T; TGF- β 1 treatment group (p-t). a, f, k and p; pre-operation, b, g, l and q; day 7, c, h, m and r; day 14, d, l, n and s; day 21, e, j, o and t; day 28 after surgery.

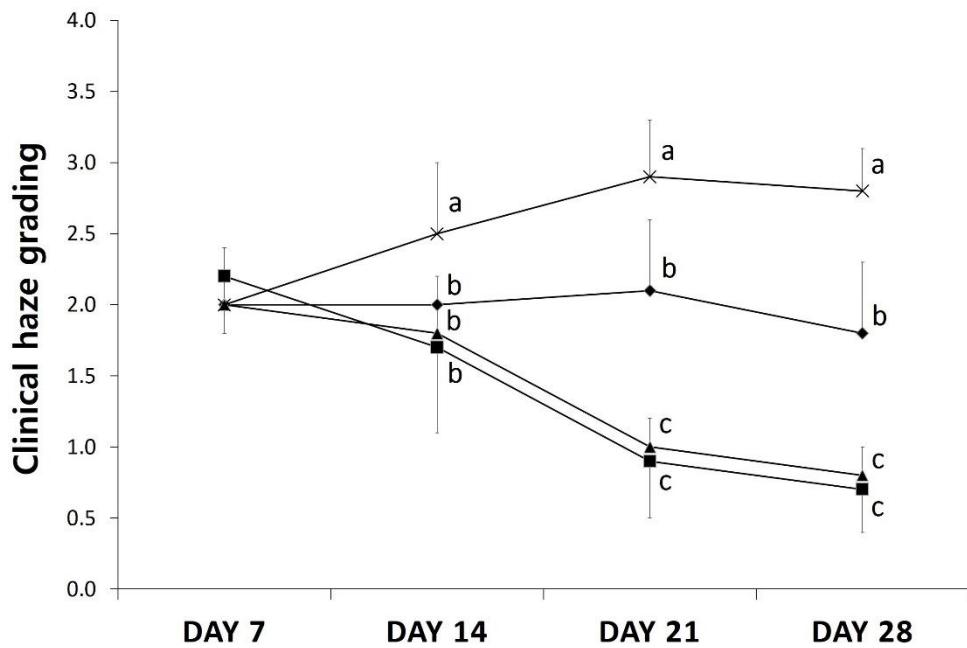


Fig. 10. Clinical corneal haze grading. Mean grades for the clinical evaluation of corneal haze at days 7, 14, 21, and 28 after surgery for each group. ▲ = group C, ■ = group P, ▲ = group O, ✕ = group T. ^{a, b, c} Values with a different superscript were significantly different ($P < 0.05$) between groups in the same evaluation day.

Corneal haze was observed beginning 7 days after surgery in all groups, and appeared to peak about 21 days after surgery in the control group with an objective evaluation method. The total intensity of the grayscale units for the hazed cornea was significantly increased at days 21 ($P < 0.001$) and 28 ($P < 0.001$) compared with day 7 in group C (Fig. 11). The total intensity in groups P (day 14; $P = 0.021$, day 21; $P < 0.001$, day 28; $P < 0.001$) and O (day 14; $P = 0.039$, day 21; $P < 0.001$, day 28; $P < 0.001$) significantly decreased compared with that at day 7. The total intensity of the grayscale units in group T significantly increased at days 14 ($P < 0.001$), 21 ($P < 0.001$), and 28 ($P < 0.001$) compared with that at day 7.

The corneal haze significantly decreased in groups P ($P < 0.001$) and O ($P < 0.001$) at day 21, and in groups P ($P < 0.001$) and O ($P = 0.002$) at day 28 compared with group C. Furthermore, the corneal haze significantly increased in group T at days 14 ($P < 0.001$), 21 ($P < 0.001$) and 28 ($P = 0.003$) compared with group C.

The corneal haze was also evaluated by immunohistochemical staining of myofibroblasts and by quantification of α -SMA-positive cells in tissue sections (Fig. 12). In group C, the corneas exhibited high numbers of α -SMA-positive myofibroblast cells, mostly in the anterior stroma below the epithelium. Topical application of prednisolone acetate (group P) and onion extract (group O) significantly reduced the numbers of α -SMA-positive cells in the stroma. In contrast, TGF- β 1 application (group T) significantly increased the numbers of α -SMA-positive cells compared with that in group C.

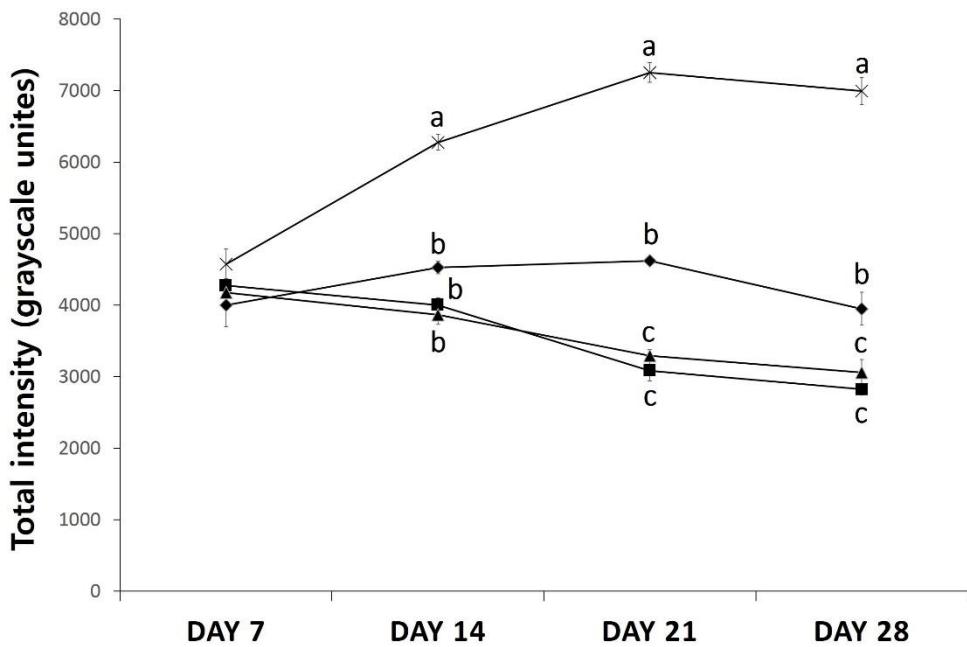


Fig. 11. Total intensity (grayscale units) levels within the corneal section.

◆ = group C, ■ = group P, ▲ = group O, ★ = group T. ^{a, b, c} Values with a different superscript were significantly different ($P < 0.05$) between groups in the same evaluation day.

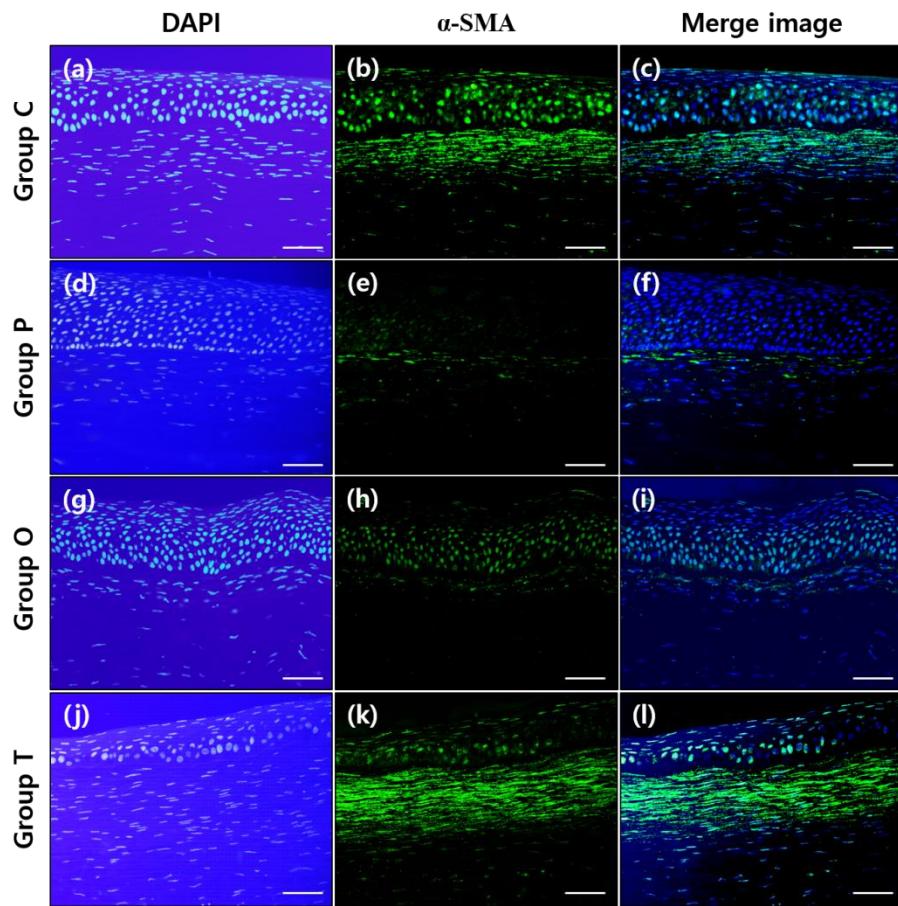


Fig. 12. Immunohistochemistry for α -SMA. (a) stained nucleus with DAPI in control group (group C), (b) α -SMA-positive cells (green color) in the group C, (c) merge image a and b, (d) stained nucleus with DAPI in prednisolone acetate treatment group (group P), (e) α -SMA-positive cells (green color) in the group P, (f) merge image d and e, (g) stained nucleus with DAPI in onion extract treatment group (group O), (h) α -SMA-positive cells (green color) in the group O, (i) merge image g and h, (j) stained nucleus with DAPI in TGF- β 1 treated group group (group T), (k) α -SMA-positive cells (green color) in the group T, (l) merge image j and k. 400 \times magnification, scale bar = 50 μ m.

The total green intensity of the entire stroma was significantly enhanced in group T ($p < 0.001$) compared with that in group C (Fig. 13). The total green intensity in groups P ($p < 0.001$) and O ($p < 0.001$) was significantly lower than that in group C.

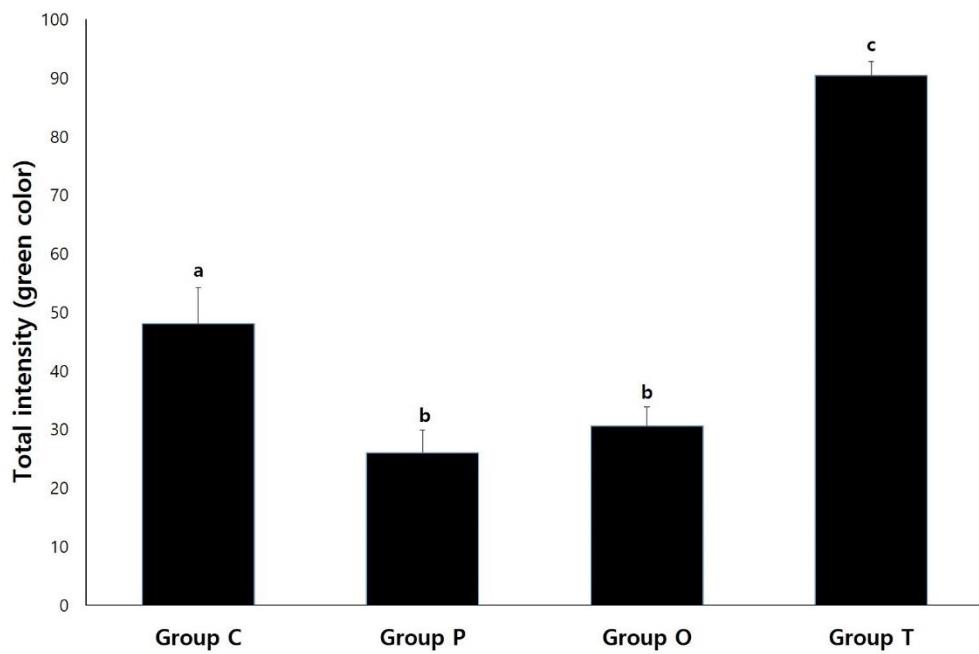


Fig. 13. The total intensity of green fluorescence. Group C; artificial tear treated group, Group P; 1% prednisolone acetate treated group, Group O; onion extract 10 mL/mg treated group, Group T; TGF- β 1 1ng/mL treated group, ^{a, b, c} Values with a different superscript were significantly different ($P < 0.05$).

Discussion

Formation of corneal haze involves the apoptosis of keratocytes (Wilson *et al.*, 2001) and transdifferentiation of keratocytes into myofibroblasts in response to endogenous epithelial derived cytokines (Jester *et al.*, 2003). TGF- β 1 directly activates keratocytes and leads to the formation of myofibroblasts as well as the subsequent reformation of subepithelial stromal tissue (Saika, 2006). Myofibroblasts scatter more light than that of undifferentiated fibroblasts or keratocytes, not only from their nuclei, but also from their cell bodies and dendritic processes (Møller-Pedersen, 2004). In addition, this population of cells participates in extracellular matrix remodeling, resulting in a denser and more disorganized extracellular matrix (Jester *et al.*, 1999). Intracellular microfilament fibers such as F-actin and α -SMA were expressed much higher in myofibroblasts than in keratocytes. These cellular components were enabled myofibroblasts to contract and close wounds, but also rendered the cornea less translucent (Meek *et al.*, 2004). Collectively, these changes lead to a loss of corneal transparency.

For a clear cornea, MMC is widely used intraoperatively by clinicians to prevent PRK-induced corneal haze although there are several complications reported with its topical use (Camellin, 2004). There are no effective medicines to control corneal haze except for MMC treatments. Because the application of steroid eye drop occasionally results in rapid corneal stromal melting, use of these drugs for achieving better corneal transparency is restricted. Thus, this study have shown the effects of onion extract ointment in corneal haze prevention and suppression of myofibroblasts from stromal ablation using the AK for the development of new treatment and prevention strategies.

Corneal fibroblasts were viable in the 10 uL/mL concentration of the onion extract. There were no adverse effects or allergic reactions for the 1% onion extract ointments. Therefore, this concentration of onion extract ointments was used in this study to evaluate efficacy of onion extract. According to these results, corneal haze grading and expression of α -SMA-positive cells significantly decreased in the onion extract treated group compared with the control group. Because α -SMA is a specific marker for myofibroblasts, these results suggest that onion extract ointment have suppressive effects on corneal haze.

Treatment with prednisolone acetate showed significant suppression of corneal haze compared with the artificial tear treatment in this paper. Postoperative use of topical corticosteroids has been controversial after PRK. Topically applied steroids, acting as an anti-inflammatory agent, have effectively suppressed corneal haze formation after excimer laser keratectomy in experimental studies (Kaji *et al.*, 2000; Nien *et al.*, 2011). But, this reduction in haze appears to be due in part to a delay in the wound-healing response (Nien *et al.*, 2011). Also, glucocorticoids increase the lytic action of corneal collagenase, suggesting that this effect might be responsible for the corneal destruction in clinical conditions (Brown *et al.*, 1970). Accordingly, the onion extract ointment could use more safely than steroid eye drops, which could induce corneal melting.

Onion (*Allium cepae*) extract contains a great amount of antioxidant phytochemicals, sulfur and other numerous phenolic compounds (Benkeblia, 2005). These compounds have been reported to be effective in cardiovascular diseases because of their hypolipidemic, anti-hypertensive, anti-diabetic, and antithrombotic effects, and to possess many other biological activities including antimicrobial, antioxidant, anticarcinogenic,

antimutagenic, antiasthmatic, immunomodulatory, and probiotic activities (Corzo-Martínez *et al.*, 2007). Especially, onion extract was shown to have fibroblast-inhibiting properties, to reduce proliferative activity, and to produce substances in the extracellular matrix (Ho *et al.*, 2006). Recently, commercial products composed of onion extract have been used to reduce scar formation on the skin (Ho *et al.*, 2006). According to the results of this study, onion extract suppressed the differentiation of myofibroblasts, and as a result, corneal haze developed significantly less than that of the control. Onion extract would be a good therapeutic candidate as a new medicine for corneal haze suppression.

Mechanical removal of the corneal epithelium and PRK up-regulate TGF- β 1 (Gupta *et al.*, 2011). TGF- β 1, a potent profibrotic cytokine, is a key regulator for the differentiation of myofibroblasts during corneal wound healing. It directly activates keratocytes and leads to the formation of myofibroblasts as well as the subsequent reformation of the subepithelial stroma (Gupta *et al.*, 2011). Consequently, these mechanisms could promote the clinical expression of corneal haze after corneal surgery. In these results, corneal haze significantly increased by treatments of additional TGF- β 1 compared with the control. Furthermore, one study showed the prevention of PRK-induced haze through the use of anti-TGF- β 1 antibodies (Møller-Pedersen *et al.*, 1998). Thus, the suppression of TGF- β 1 expression is critical in the prevention of corneal haze.

Fibroblasts differentiate into myofibroblasts through a Smad 2/3 signaling pathway and enhance NADPH oxidases (Nox) 4-derived ROS signaling cascades (Cucoranu *et al.*, 2005). Depletion of Nox4, an essential mediator of Smad 2/3 transcription factor activation in response to TGF- β 1, down-regulates α -SMA mRNA, and overexpression of

Nox4 induces α -SMA expression (Clempus *et al.*, 2007). The precise mechanisms of onion extract have not yet been completely elucidated. The corneal haze grade was significantly lower in the onion extract treated group than in the control group, and the expression of α -SMA was also down-regulated by the onion extract treatments shown in the results of this study. Among the many flavonoid compounds, quercetin is a major component of onion extract (Sellappan *et al.*, 2002), and it has been shown to have powerful antioxidative activity with metal ion binding properties and radical scavenging abilities (Erden *et al.*, 2000). In addition, quercetin has a scavenging effect on superoxide anions and hydroxyl radicals, and it prevents lipid peroxidation by blocking the action of xanthine oxidase and chelating iron (Hwang *et al.*, 2009). These effects could have important roles in the suppressive effect of onion extract against corneal haze formation. These results suggest that onion extract could block TGF- β 1 signaling cascades by scavenging ROS to reduce α -SMA expression and subsequently corneal haze development. Further experiments are needed to understand the exact mechanisms of onion extract.

The limitation of this study is that the evaluation time was short and there were not enough to prove exact mechanism of onion extract ointment against corneal haze formation. Therefore, more studies will be needed to understand the mechanisms.

Conclusions

In summary, onion extract ointment could be useful as a therapeutic in the suppression of corneal haze development after apply the AK through the down-regulation of fibroblast transdifferentiation into myofibroblasts. This effect could be from the scavenging ability of the onion extract.

CHAPTER III.

**Deep anterior lamellar keratoplasty of dog eyes using the
big-bubble technique**

Abstract

The aim of this study was to establish the feasibility of corneal transplantation using BBT for performing DALK in three dogs. After the cornea was trephined 750 µm, 4 mL of air was injected, and the blanched stroma was removed to expose DM. The donor corneal button, which was gently stripped off the DM, was sutured onto the bare DM of the recipient cornea. The dogs received topical antibiotics every 6 hours for 7 days and 2% cyclosporine ointment every 12 hours for 1 month. The eyes were examined post-operatively at 7, 14, 21, 28 and 150 days. The central portion of the transplanted cornea stayed transparent while corneal haze developed around the transplanted margin. Menace response was normal even though the transplanted cornea was edematous until 3 weeks after surgery. A marginal haze was rarely observed between the donor and recipient corneas at 150 days after the operation. A spotted haze developed in the central part of the deep stroma near the DM. In the histopathological examination, the stroma and epithelium of the donor cornea had normal structures. Corneal transplantation using DALK with BBT can be performed in dogs preserving the healthy endothelium.

Introduction

Corneal ulceration is a common and clinically important ocular disease in dogs. Especially in the case of large and deep corneal defects, various surgical managements have been tried to promptly and effectively repair the cornea, including conjunctival pedicle graft (Soontornvipart *et al.*, 2003), corneal-scleral transposition (Parshall *et al.*, 1973) and autogenous corneal graft (Brightman *et al.*, 1989). In addition, preserved biological membranes including pericardium (Alio *et al.*, 2013), intestinal submucosa (Bussieres *et al.*, 2004), amniotic membrane (Barros *et al.*, 1998) and renal capsule (Andrade *et al.*, 1999) are used in medicine and in veterinary general surgery for deep corneal defects and perforations. However, these surgical methods for large corneal defects will not be able to recover corneal transparency. Additionally, the indicated cases are restricted to certain types of techniques.

Transplantation of various corneal parts is an essential technique for the treatment of severe corneal damage in humans (Bahar *et al.*, 2008), and it has been performed successfully in horses for therapeutic and tectonic reasons (Brooks *et al.*, 2005). Furthermore, Penetrating keratoplasty (PK), one of the transplantation methods, has been performed in experimental models in the rabbit cornea (Niederer *et al.*, 2007). However, corneal transplantation is very limited in dogs due to insufficient resources of donor cornea and frequent graft rejections characterized by corneal vascularization, graft failure, and subsequent corneal edema. Therefore, there are few reports on canine corneal transplantation (McEntyre *et al.*, 1968).

DALK removes and replaces the pathologic corneal stroma while preserving the host endothelium, which eliminates the risk of endothelial graft rejection and has a reduced effect on the endothelial cell count (Karimian *et al.*, 2010). Thus, DALK is indicated for a patient with a healthy endothelium to achieve a high success rate for corneal transplantation as an alternative procedure to PK (Karimian *et al.*, 2010). Several surgical methods were used to bare the DM during DALK including layer-by-layer manual dissection (Tsubota *et al.*, 1998), hydro-delamination (Sugita *et al.*, 1997), viscoelastic dissection (Melles *et al.*, 1999) and air injection (Anwar *et al.*, 2002). Especially, the BBT introduced by Anwar and Teichmann is a method that injects air which forms a large bubble in the stroma to detach DM during DALK (Anwar *et al.*, 2002).

The purpose of this study was to establish the feasibility of corneal transplantation with BBT when performing DALK in dogs.

Materials and Methods

1. Animals

Three eyes from 3 healthy male beagles with normal corneas were used in this study. The donor corneas were obtained from dogs scarified in other experiments unrelated to this study. The animal use and experimental protocols were approved by the Institutional Animal Care and Use Committee (SNU-140520-1; Seoul National University, Korea).

Complete ophthalmic examinations were performed before the experiment with a rebound tonometry (Tonovet[®]), Schirmer's tear test (Schirmer tear test[®]), slit-lamp biomicroscopy (SL-D7[®]) and indirect ophthalmoscopy (Vantage plus[®]) with a 30-diopter indirect lens (Classic BIO Lens[®], Volk Optical Inc., Mentor, OH, U.S.A.). None of the beagles had any corneal diseases.

2. Surgical technique

The surgical procedure was performed under general anesthesia with isoflurane after induction with tiletamine and zolazepam (Zoletil[®]; 2.5 mg/kg IV) with the BBT as described by Anwar and Teichmann (2002). Before the surgery, the central corneal thickness (CCT) was measured with an ultrasonic pachymeter (PACHMETE DGH 55[®]). The central axial cornea was trephined 750 µm with an 8 mm diameter trephine (Barron radial vacuum trephine[®], Katena) to create an incision of approximately 80% thickness (Fig. 14a). A partial-thickness superficial anterior keratectomy was performed by dissection with a #66 lamellar blade (Katena products) (Fig. 14b). The introduction of a small amount of air into the anterior chamber by intracameral injection at the limbus was done with a 26 G needle. A 30 G needle attached to a 4 mL filled syringe with the tip manually bent to approximately 30 degrees was introduced with its bevel down into the cornea stroma through the trephination groove and advanced to the center of the cornea (Fig. 14c). At this point, air was injected gently, being forced through the posterior stromal lamella along the path of least resistance, causing the DM to separate from the deep stroma (Fig. 14d). A blanched corneal stroma was incised with a 15° slit-knife (Alcon Laboratories, Fort Worth, Texas, USA) to let the air escape and to collapse the bubble (Fig. 14e). A corneal dissector was carefully inserted and advanced into the cleavage plane that was created until its tip approached the opposite trephination groove. Corneal scissors were used to remove the remaining corneal tissue and expose the DM (Fig. 14f). The donor

cornea was gently stripped off the DM and endothelium with a cellulose sponge or forceps. Then, the donor cornea was punched from the endothelial side with an 8.5 mm diameter Barron punch (Katena, Denville, New Jersey, USA) (Fig. 14g). This prepared donor corneal button was initially sutured onto the bare DM with 4 cardinal 10-0 nylon sutures at 3, 6, 9, and 12 clock-hour positions (Fig. 14h and i). Additionally, there was a single running suture with 16 to 18 bites using same suture materials (Fig. 14j). After surgery, gentamicin and triamcinolone were injected subconjunctivally.

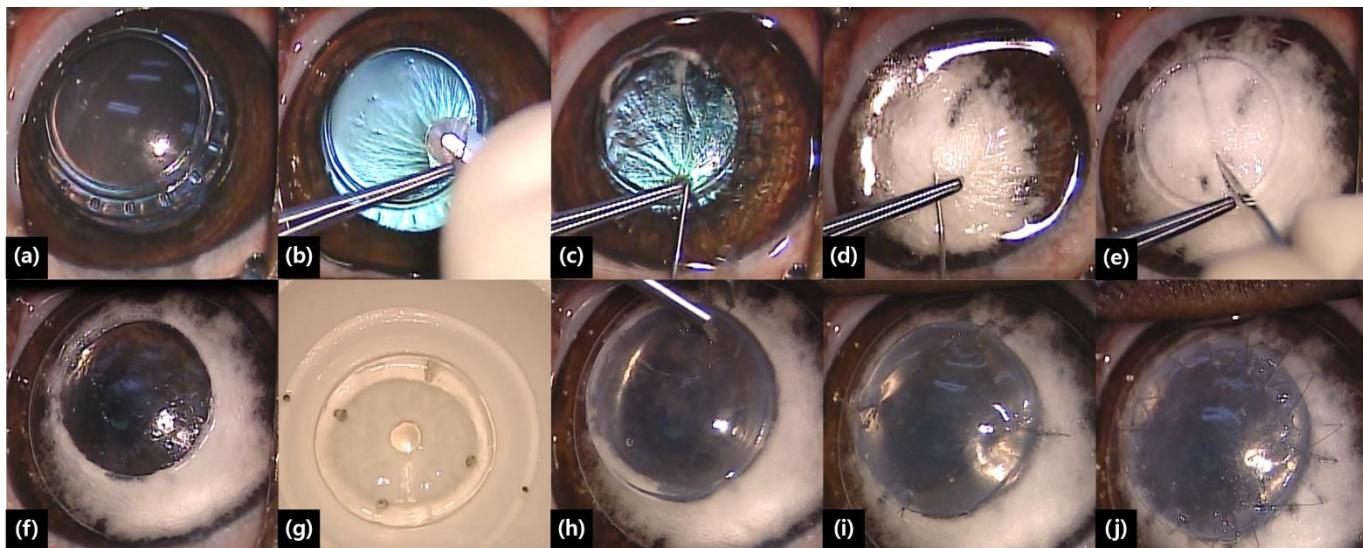


Fig. 14. Procedures of DALK using the BBT in dogs. (a) Center of the cornea was trephined 750 μm using an 8-mm vacuum trephine. (b) A partial-thickness superficial anterior keratectomy was performed (c) A 30-gauge needle attached to a 5-cc syringe was inserted at the base of the trephination gutter into the corneal stroma. (d) 4 mL of air were gently injected causing DM to separate from the deep stroma. Intrastromal blanching was observed during this procedure. (e) A blanched stroma was incised with a 15° slit-knife to allow air to escape and to collapse the bubble for stroma removal. (f) DM was exposed after excising the remaining stroma using corneal scissors. (g) After DM and endothelium were stripped off, the donor cornea was punched from the endothelial side using an 8.5-mm-diameter punch. (h) This prepared donor corneal button was fitted onto the exposed Descemet's plane of the recipient cornea. (i) 4 cardinal sutures were used with 10-0 nylon at the 3, 6, 9, and 12 clock-hour positions. (j) A single running suture was performed with 16 to 18 bites using the same suture materials.

3. Post-operative care and evaluation

After surgery, one drop of atropine (1%, Isopto Atropine[®]) was applied two times a day for 3 days and levofloxacin (0.5%, Cravit[®]) was administered every 6 hours for 7 days. Cyclosporine ointment (2mg/g, Optimimmune[®], Schering-Plough, Segre, France) was administered every 12 hours until 30 days post operatively. The all corneal sutures were removed under the topical anesthesia with proparacaine hydrochloride (0.5%, Alcaine, Alcon, Forest, New Zealand) at the day-21 post-surgery.

The eyes were examined by slit-lamp biomicroscopy (SL-D7[®]), tonometry (Tonovet[®]), and indirect ophthalmoscopy (Vantage plus[®]) for the evaluation of corneal condition and graft rejection at 7, 14, 21, 28 and 150 days post-surgery. In this periods, menace response, dazzle reflex, and pupillary light reflex (PLR) were evaluated, and IOP and CCT were measured. Also, blepharospasm, corneal edema, corneal vascularization, and haze developments at the central cornea and suture line were examined clinically. The level of corneal haze was evaluated using the previously reported clinical grading system as follows (Fantes *et al.*, 1990). Grade 0: a completely clear cornea; Grade 0.5: a trace amount of haze observed with careful oblique illumination; Grade 1: a mild obscuration of the iris details; Grade 2: a more prominent haze not interfering with the visibility of fine iris details; Grade 3: a moderate obscuration of the iris and lens; Grade 4: a complete opacification

At the same time, the CCT was measured using the ultrasonic pachymeter (PACHMETE DGH 55[®]).

The results of CCT and IOP are expressed at mean \pm SD. Statistical analyses were performed using SPSS V20 for windows (SPSS Inc.). One way ANOVA with Bonferroni's post-hoc assessment was used to test for significance when comparing the CCT. P-values < 0.05 were considered significant.

4. Histopathological evaluation

The dogs were euthanized with T61 under general anesthesia through tiletamine and zolazepam (Zoletil[®]; 5 mg/kg IV) at day 150 after surgery, and the eyes enucleated and fixed in 10% formalin and embedded in paraffin. Six tissue sections of 4 um thickness were obtained at the central corneal part for each eyes. Three sections were stained with H&E stain according to the standard procedure. A light microsocope (BX51[®]) equipped with a digital camera (DP71[®]) was used for photomicrography to evaluate the stromal structures at the transplanted junction.

5. Immunofluorescence detection of α -SMA

Immunofluorescent staining for α -SMA was performed using the three paraffin embedded sections for each eyes. These tissue sections were treated with the monoclonal antibody for α -SMA (A5228; Sigma-Aldrich St. Louis, MO, USA) at a 1:100 dilution in 1xPBS for 2 hours and with a secondary antibody (Alexa Fluor 488 goat anti-mouse IgG, A-11017; Life technologies, Gaithersburg, MD, USA) and Propidium iodide (P3566; Life technologies) at a dilution of 1:100 for 1 hour. Sections were viewed and photographed with FluoViewTM 300 fluorescence microscope (Olympus, Tokyo, Japan).

Results

The mean CCT was $817 \pm 23 \mu\text{m}$ for the 3 eyes before surgery. The central cornea was trephined a depth of $750 \mu\text{m}$ for approximately 80% of the corneal thickness. DALK was performed for the 3 eyes successfully without any DM tearing. After the surgical procedure, no rejection of the corneal implants was observed in the eyes for 5 months postoperatively (Table 3). The menace response, dazzle reflex, and PLR were normal in all experimented eyes during this period. IOP was in the normal range and did not show significant changes. Fluorescein dye staining test was positive in two cases at the 7 days after surgery, and the blepharospasm was examined in the two cases at the same examination time. Because of the corneal ulcer was located near the suture line, the ulcer and blepharospasm were related with spur of the suture materials. The all abnormal responses were not showed after suture removal.

A corneal haze was developed from 7 days after surgery at the central transplanted cornea and around the suture line (Table 3). The corneal haze of central corneal part was reduced from 21 days after surgery. The corneal haze around the junction of the donor and recipient cornea and suture lines increased at 21 days after surgery and stayed until all experimental period. The central portion of the transplanted cornea stayed transparent while a corneal haze developed around the transplanted margin (Fig. 15). Stromal edema of the donor cornea was present from just after operation until 3 weeks after

Table 3. Clinical evaluations after corneal transplantation in three dogs

Pre-OP	Post-OP				
	Day 7	Day 14	Day 21	Day 28	Day 150
Menace response	N	N	N	N	N
Dazzle reflex	N	N	N	N	N
PLR	N	N	N	N	N
IOP	16.3 ± 0.6	16.7 ± 1.5	16.3 ± 0.6	16.0 ± 1.0	16.0 ± 1.0
Fluorescein dye	-(3)	-(1) / +(2)	-(3)	-(3)	-(3)
Blepharospasm	-(3)	-(1) / +(2)	-(2) / +(1)	-(3)	-(3)
Neovascularization	-(3)	-(3)	-(3)	-(3)	-(3)
CCT	817 ± 23	$1024.1 \pm 28.5^*$	$1002.5 \pm 37.0^*$	$958.6 \pm 27.3^*$	$940.7 \pm 35.8^*$
Haze (central cornea)	0	1.7 ± 0.6	1.7 ± 1.2	0.5 ± 0.4	0.2 ± 0.3
Haze (suture line)	0	2.0 ± 1.0	2.0 ± 1.0	2.3 ± 0.6	2.0 ± 0.8

The data was presented with mean \pm SD for IOP, CCT and haze. N, normal; -, negative; +, positive; (n), n for numbers. * P < 0.05, significantly different compared with CCT of the Pre-OP.

surgery in all 3 beagles (Fig. 16) and then began to decrease from 4 weeks after the surgery. The corneal thickness was significantly increased at the Day 7, 14, 21, and 28 after surgery compared to the CCT of Pre-operative examined values (Table 3). The menace response was normal even though the transplanted corneas were edematous.

After the sutures were removed, the corneal haze around the implant margin decreased, and the corneal haze near the suture line disappeared. The marginal haze at the junction between the donor and recipient corneas decreased to almost nothing at 150 days after the operation (Fig. 15). At 150 days after the surgery, a new spotted haze observed in the central part of the cornea which looks like a deposited mineral spot. The spotted haze was found in the deep stroma near DM on the slit image (Fig. 16f).

In the histopathological examination, the stroma and epithelium of the donor cornea had normal structures (Fig. 17). A modified arrangement of corneal stroma was confirmed in the junction portion between the donor and recipient corneas. In addition, keratocytes and epithelial proliferation and a distortion in the stromal structure were detected at the junction of the transplant. There were more severe pathologic changes in the recipient cornea than in the donor parts. Some particles of the donor's DM in the cornea were observed in the stroma layer (Fig. 17b).

The SMA-positive cells were detected in the junction of the donor cornea and recipient cornea (Fig. 18). More of the SMA-positive cells were present in the

hyperplastic epithelial parts and anterior stroma beneath the epithelium. The green fluorescence was not detected in the deep stroma near the recipient's DM.

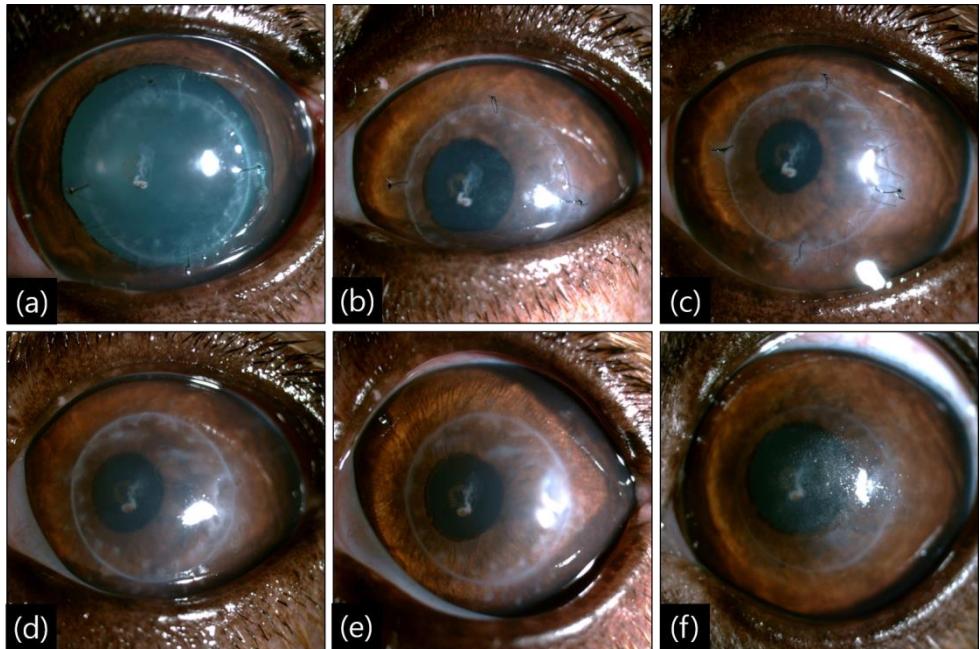


Fig. 15. Slit-lamp biomicroscopy after DALK. (a) 12 hours after surgery, (b) 7 days, (c) 14 days, (d) 21 days, (e) 28 days and (f) 150 days after surgery with direct diffuse illumination.

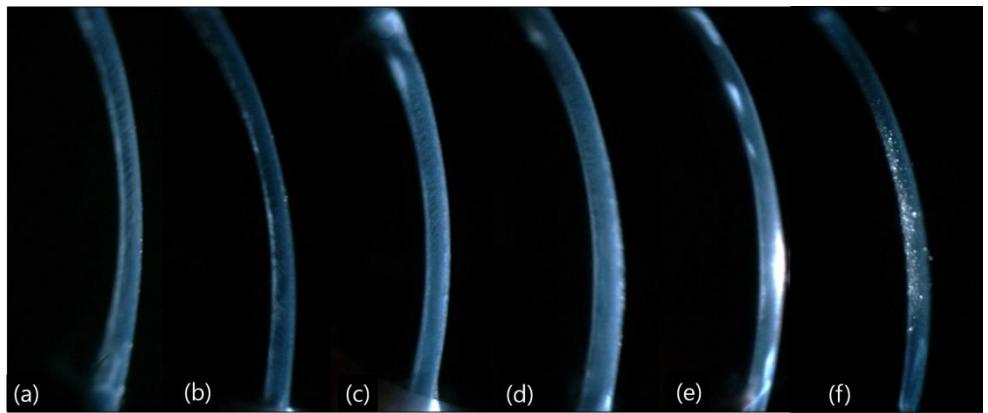


Fig. 16. Slit-lamp biomicroscopy with direct narrow slit (1 mm) for the stroma. (a) 12 hours after surgery, (b) 7 days , (c) 14 days, (d) 21 days, (e) 28 days and (f) 150 days after surgery.

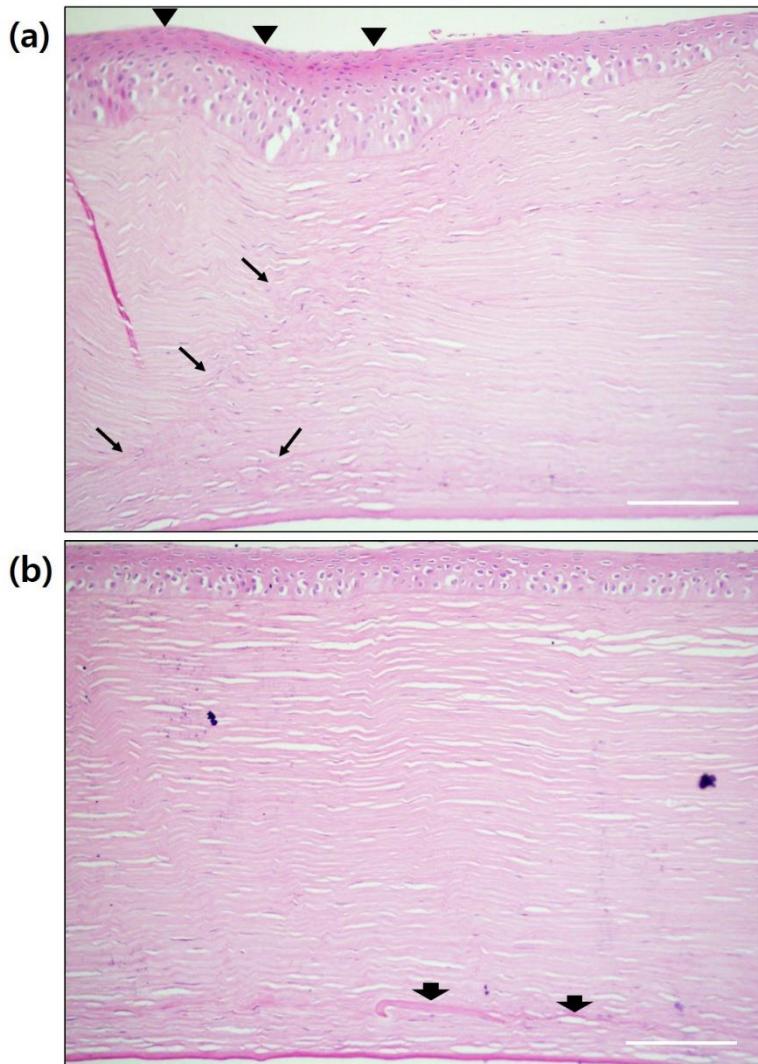


Fig. 17. Histopathological evaluation with H&E staining at 150 days after surgery. (a) The epithelium was overgrown (arrow head) at the junction of the donor and recipient corneas. In addition, deformed stromal structures were detected at the transplant junction (arrow). (b) The central corneal part had a normal corneal structure. A small piece of Descemet's membrane was left in the stroma (thick arrow). 200 \times magnification. Scale bar = 50 μ m.

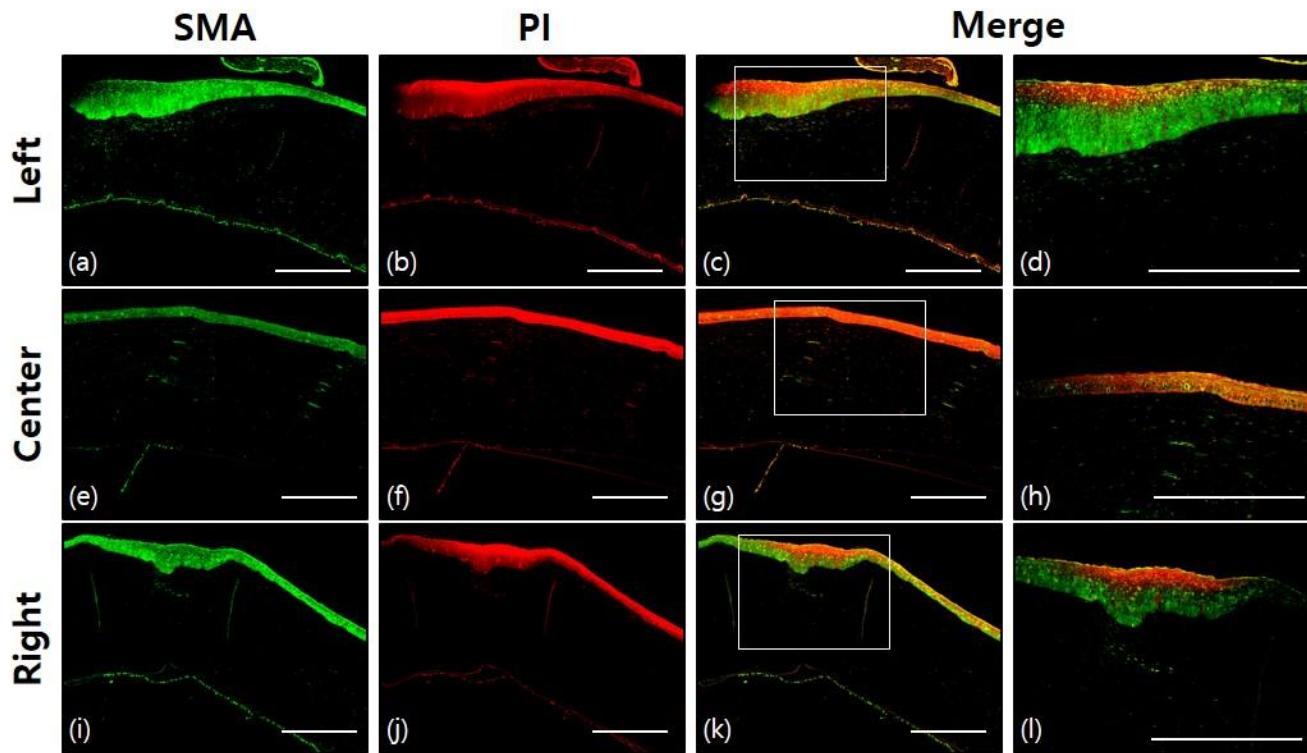


Fig. 18. Alpha-SMA staining 150 days after DALK. Cell nuclei were stained red with propidium iodide (PI) and SMA was stained green fluorescence. Representative image of the SMA-positive cells detected in the left and right transplanted junction. The scale bar = 200 um. Magnification 100X (a-c, e-g, i-k), 200X (d, h, l).

Discussion

The BBT for DALK has been proven to be a valuable method as an alternative to PK with the purpose of reshaping the cornea profile in human patients with keratoconus by removing and replacing most of the corneal ectasia (Fontana *et al.*, 2007). Thus, DALK is performed without substituting the host's healthy endothelium, and it could reduce the risk of immunological rejection (Fontana *et al.*, 2007). In addition, DALK has been performed on rabbits (Tham *et al.*, 2002) and horses (Martins *et al.*, 2013) experimentally. In this study, BBT with DALK was performed on the canine cornea to establish the feasibility of this technique in dogs. The shape and transparency of the transplanted cornea were successfully maintained, and 5 months after the surgery, there still was no rejection in any of the experimental eyes.

Corneal grafts show a higher success rate than other organ transplants. To the best of the authors' knowledge, so far there have been no reports on BBT in dogs. BBT, which is normally done for human ophthalmology, has not been a feasible procedure in canine eyes (Leiva *et al.*, 2014). However, according to our results, BBT is not necessarily impossible although it seems to require a skilled surgeon who knows the technique. A small portion of the stromal layer of the recipient cornea after BBT remained in this experiments similar to the report by Leiva et al. (2014). When the thickness of the remaining stroma in this experiment was estimated by histopathological examination, however, its thickness was relatively less in this study compared to the thickness in the other previous reports. Similar to this results, very small parts of the stroma remain which

cause the development of corneal haze in reports on human ophthalmology (McKee, 2013).

In addition, part of DM was left in the stromal layer which determines the junction between the recipient cornea and the donor cornea. Part of the membrane may remain during the process of removing DM from the donor cornea. DM incarceration was reported on human ophthalmology under microscopic examination (Lang *et al.*, 1986). The stromal structures of the human cornea after air injection look similar to those of the dog cornea (Arenas *et al.*, 2012). Therefore, the results of human ophthalmology support this speculation about the incarcerated part of donor DM.

In this results, the swelling of the stroma of the transplanted cornea was observed during the first 3 weeks after surgery. The edema did not progress and normalized three weeks after the surgery. In clinical examinations, an eye that has rejection after corneal transplantation shows conjunctival hyperemia, anterior chamber reaction, keratic precipitates, and graft edema (Panda *et al.*, 2007). Corneal transplant rejection is a process in which a corneal graft that has been clear for 5-7 days in horses (Brooks *et al.*, 2008) or 2 weeks in humans suddenly develops graft edema in conjunction with anterior segment inflammatory signs (Panda *et al.*, 2007). The corneal edema could be produced as part of a sudden rejection to the transplant. However, in this study, corneal edema was present in all the transplanted corneal parts from the first examination just after the surgery. Thus, the corneal edema might be developed during the surgical procedure especially in the procedure for donor cornea preparation. When another examination was done 3 weeks after the operation, the corneal edema had resolved itself in all the cases.

A corneal haze appeared near the junction of the transplant and the suture lines; furthermore, the epithelial overgrowth was observed in these areas. In particular, these reactions were identified on the recipient side of the cornea, but the epithelial cells of the central part of the donor cornea maintained their normal morphology. Alpha-SMA positive cells were detected in these transplanted junction. Especially, the majority of green fluorescein positive cells were revealed at the bottom of the overgrown and deformed epithelium. Therefore, these deformed hyperproliferative epithelium were demonstrated to be the main cause of the haze.

Overgrown epithelial cells were detected more on the inside of the recipient cornea. Corneal epithelial breach can be caused by exposed suture knots and a loose suture, which are some of the predictors for the occurrence of corneal haze during the post-operative period (Tham *et al.*, 2002). These factors give rise to irritation, leading to a subsequent corneal ulcer and corneal vascularization. Therefore, these reactions could be predisposing factors to graft rejection (Tham *et al.*, 2002). In these cases, the haze may invade the adjacent host cornea which is nearer to the vascularization (Fontana *et al.*, 2007).

The spotted haze in the deep stroma near DM was observed at 150 days postoperatively in the clinical evaluation. But, the green fluorescein positive cells were not detected in these areas. This means that there was an absence of myofibroblast which was the main type of cells for the corneal haze generation. Whereas, some of the donor DM particles and pre-DM fibers were observed at the same area in histological examinations. Accordingly, the spotted haze was suggested to be the result of light

scattering by these ununiformed DM particles. Since corneal edema had been accompanied with these ununiformed DM particles until the day 28 postoperatively, the spotted haze could only be revealed after the edema had disappeared on the day 150 postoperatively.

For human ophthalmology, much equipment has been developed for delicate surgical techniques such as an excimer laser or femtosecond laser (Albarez *et al.*, 2014; Levinger *et al.*, 2014). This equipment has been used to reduce corneal haze as well as other post-surgical complications. In the veterinary field, BBT can be used to maintain the vision of patients that have a wide range of corneal diseases while preserving the healthy endothelium. Furthermore, considering the quality of life of the animal, BBT is a better surgical method for restoring vision.

Conclusions

Corneal transplantation using DALK with the BBT could be performed in a dog that has a large corneal defect and vision loss with a healthy endothelium.

GENERAL CONCLUSIONS

This study was designed to develop the AK technique for the standardization of corneal haze model and DALK in dogs.

AK, which was performed 375 μm depths and 8 mm diameter, was used to achieve the desired corneal thickness after resection and produced a more sufficient corneal haze than CK technique. Therefore, this technique would contribute to improving standardization of the corneal haze model for the evaluation of therapeutic agents' effects.

The effect of onion extract ointment on corneal haze suppression was evaluated to test the utility of AK for the experimental model. The corneal haze was significantly suppressed in the onion extract treated group compared with the control group. Also, the expression of α -SMA, a specific marker for myofibroblasts, was significantly decreased in the onion extract ointment treated group compared with the control group. Accordingly, the onion extract ointment could be useful as one of therapeutic agents in preventing corneal haze development through the down-regulation of fibroblast transdifferentiation into myofibroblast.

DALK using a BBT was applied in three dogs to establish the feasibility of corneal transplantation. The central portion of the transplanted cornea remained transparent while corneal haze developed around the transplanted margin. The menace response was normal, and the transplant rejection was not observed in all eyes until the end of experiments. Consequently, corneal transplantation using DALK with the BBT could be performed in a dog that has a large corneal defect with a healthy endothelium.

Through these studies, AK could contribute to improving standardization of the corneal haze model. This is the useful experimental technique for the evaluation of corneal haze suppression, and the onion extract ointment could have potential as a therapeutic agent in preventing corneal haze development. Also, this study demonstrated the feasibility of corneal transplantation in dog eyes by DALK using a BBT.

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국문초록

개에서 각막흐림모델과 심부표층각막이식을 위한 공기주입 각막절제술의 개발

지도교수 서 강 문

김 수 현

서울대학교 대학원
수의학과 임상수의학 전공

본 연구는 개에서 표준화된 각막흐림모델과 심부표층각막이식을 위한 공기주입 각막절제술의 개발을 목적으로 실시되었다.

균일한 깊이와 크기로 각막 부분절제를 실시하고 효과적인 각막흐림을 발생시키기 위해 공기주입 각막절제술을 개발했다. 도축 후 적출된 돼지 눈을 네 군으로 나누어, 각각 250, 375, 500, 750 μm 의 깊이로 각막 실질을 절개했다. 절개된 단면에서 절개면 위쪽의 실질로 4 mL의 공기를 주입한 후 불투명한 흰색으로 변한 부분을 제거했다. 375 μm 이상의 깊이로 절개한 군들에서 절개한 각막 깊이와 공기 주입 후 절제된 각막의 깊이 사이에

유의적인 차이가 나타나지 않았다. 각막흐림의 발생 정도를 평가하기 위해 여섯 마리의 개에 공기주입 각막절제술과 기존의 각막절제술을 실시했다. 기존의 각막절제술에 비해 공기주입 각막절제술을 실시했을 때, 각막흐림의 발생과 평활근 액틴의 발현이 유의적으로 많았다. 그러므로, 공기주입 각막절제술은 원하는 두께로 각막절제가 가능하고 충분한 각막흐림을 발생 시킨다.

공기주입 각막절제술의 각막흐림 평가에 대한 실험적 유용성을 확인하기 위하여, 본 기법의 적용 후 양파 추출물 안연고의 각막흐림 발생 억제효과를 평가했다. 공기주입 각막절제술을 실시 한 후, 네 군으로 나누어 인공눈물, 프레드니솔론 아세트산염, 양파 추출물 안연고, 전환성장인자- β 1을 각각 적용 했다. 인공눈물 점안 군과 비교 했을 때, 술 후 14일부터 프레드니솔론 아세트산염과 양파 추출물 안연고를 점안한 군에서 각막흐림의 발생이 유의적으로 억제 되었다. 또한, 면역형광염색 결과 프레드니솔론 아세트산염과 양파 추출물 안연고를 점안한 군에서 평활근 액틴의 발현을 의미하는 녹색 형광강도가 유의적으로 억제되었다. 결과적으로, 양파 추출물 안연고의 각막흐림 억제 효과가 입증되었으며, 공기주입 각막절제술은 이러한 효과를 평가하기에 유용한 방법이었다.

개에서 각막이식의 실현 가능성 평가하기 위해, 공기주입 각막절제술의 기반이 된 공기주입기법을 이용한 심부표층각막이식술을 세 마리의 실험 견에서 실시했다. 임상적인 안검사 결과 이식된 각막의 주변부와 뒤흥계판

근처의 이식 경계면 일부에서 국소적인 각막흐림이 발생되었으며, 면역형광염색 실험에서 각막흐림의 주요 원인인 근섬유모세포의 표시인 평활근 액틴이 이식 경계면에서 확인되었다. 이 평활근 액틴은 뒤경계판 부분에서는 확인되지 않았으며, 조직 사진에서 이 부위에 공여 각막의 뒤경계판 조각의 일부가 관찰되었다. 국소적인 각막흐림을 제외하면, 술 후 150일까지 이식 거부반응 없이 각막의 구조 및 시력이 유지되었다.

본 연구의 결과 공기주입 각막절제술은 표준화된 각막흐림모델로서 각막흐림의 실험적 평가에 유용한 방법이며, 공기주입기법을 이용한 심부표층각막이식술은 개에서 이식 거부반응을 유발하지 않고 적용이 가능했다.

주요어: 각막흐림, 공기주입 각막절제술, 공기주입기법, 개,

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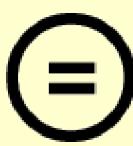
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A DISSERTATION
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

**Development of Air Assisted Lamellar Keratectomy
for Corneal Haze Model and Deep Anterior
Lamellar Keratoplasty in Dogs**

개에서 각막흐림모델과 심부표층각막이식을 위한
공기주입 각막절제술의 개발

by

Soohyun Kim

MAJOR IN VETERINARY CLINICAL SCIENCES
DEPARTMENT OF VETERINARY MEDICINE
GRADUATE SCHOOL
SEOUL NATIONAL UNIVERSITY

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**Development of Air Assisted Lamellar Keratectomy
for Corneal Haze Model and Deep Anterior
Lamellar Keratoplasty in Dogs**

**by
Soohyun Kim**

**Supervised by
Professor Kangmoon Seo**

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Development of Air Assisted Lamellar Keratectomy for Corneal Haze Model and Deep Anterior Lamellar Keratoplasty in Dogs

Supervised by

Professor Kangmoon Seo

Soohyun Kim

ABSTRACT

The purpose of the present study was development of air assisted lamellar keratectomy (AK) technique for the standardized corneal haze model and deep anterior lamellar keratoplasty in dogs.

The AK was performed to achieve a constant ablation depth and size and to generate the efficient corneal haze. The *ex vivo* porcine corneas were categorized into four groups depending on the following trephined depths: 250, 375, 500 and 750 μm . After 4 ml air was injected to the stroma at the base of the trephination groove, the fuzzy region of the white opaque cornea was removed. No significant differences were observed between the trephined corneal depths for resection and the ablated corneal thickness at depths deeper

than 375 μm . AK and conventional keratectomy (CK) were applied to six beagles and development of corneal haze was evaluated weekly until postoperative day 28. The occurrence of corneal haze and α -smooth muscle actin (SMA) expression following AK were significantly higher than those following CK. Therefore, AK was used to achieve the desired corneal thickness after resection and produce a sufficient corneal haze.

The effect of onion extract ointment on corneal haze suppression was evaluated to verify the experimental utility of AK technique for the evaluation of haze development. After the AK was performed, an artificial tear (group C), prednisolone acetate (group P), onion extract ointment (group O), and transforming growth factor (TGF)- β 1 (group T) were applied to each group. The haze was significantly suppressed in the group P and O compared with the group C from day-14 after the surgery. Also, the total green intensity for α -SMA was significantly less expressed in the group P and O than in the group C. Thus, the onion extract ointment was demonstrated to have the suppression effects for corneal haze development and AK technique was sufficient to evaluate these effects.

The feasibility of deep anterior lamellar keratoplasty (DALK) using big-bubble technique (BBT), upon which the AK technique was based, was evaluated in three dogs. The eyes were examined until 150 days after the operation of DALK. The central portion of the transplanted cornea remained transparent while corneal haze developed around the transplanted margin. The marginal haze was rarely observed between the donor and recipient corneas at 150 days after the operation. A spotted haze developed in the central part of the deep stroma near the Descemet's membrane (DM). Alpha-SMA positive cells were detected at the transplanted margin in which the corneal haze appeared clinically.

Based on the results of the present studies, AK technique could be useful for the evaluation of corneal haze and contribute to improving the standardized corneal haze model.

Keywords: air assisted lamellar keratectomy, big-bubble technique, corneal haze, deep anterior lamellar keratoplasty, dog, onion extract ointment

Student number: 2011-21670

List of Abbreviations

AK	Air assisted lamellar keratectomy
ANOVA	Analysis of variance
BBT	Big-bubble technique
CCT	Central corneal thickness
CK	Conventional keratectomy
DALK	Deep anterior lamellar keratoplasty
DAPI	4',6-diamidino-2-phenylindole
DM	Descemet's membrane
DMEM	Dulbecco's modified Eagle's medium
H&E	Hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IOP	Intraocular pressure
MMC	Mitomycin C
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline
PK	Penetrating keratoplasty
PLR	pupillary light reflex
PRK	Photorefractive keratectomy
ROS	Reactive oxygen species
SD	Standard deviation
SMA	Smooth muscle actin
TGF-β1	Transforming growth factor - β 1

CONTENTS

GENERAL INTRODUCTION	1
CHAPTER I.	
Air assisted lamellar keratectomy for the corneal haze model	
Abstract	6
Introduction	7
Materials and Methods	
1. Experimental design	9
2. Animals	10
3. <i>Ex vivo</i> experiments on porcine eyes	11
4. <i>In vivo</i> experiments on canine eyes	14
5. Clinical grading of corneal haze	15
6. Quantitative corneal haze grading	16
7. Histopathological and immunofluorescence analyses	17
8. Quantification of α -SMA positive cells	18
9. Statistical analyses	19
Result	20
Discussion	30
Conclusions	34

CHAPTER II.

Effect of onion extract on corneal haze suppression after air assisted lamellar keratectomy

Abstract	36
Introduction	37
Materials and Methods	
1. Corneal fibroblast culture and cell viability test for onion extract	40
2. A process of manufacture for the onion extract ointment	42
3. Animals	43
4. Corneal haze generation by the air assisted lamellar keratectomy	44
5. Corneal haze grading	47
6. Immunofluorescence analyses	48
7. Quantification of α -SMA positive cells	49
8. Statistical analyses	50
Results	51
Discussion	59
Conclusions	63

CHAPTER III.

Deep anterior lamellar keratoplasty of dog eyes using the big-bubble technique

Abstract.....	65
Introduction.....	66
Materials and Methods	
1. Animals.....	68
2. Surgical technique.....	69
3. Post-operative care and evaluation.....	72
4. Histopathological evaluation.....	74
5. Immunofluorescence detection of α -SMA.....	75
Results.....	76
Discussion.....	84
Conclusions.....	88
GENERAL CONCLUSIONS.....	89
REFERENCES.....	91
ABSTRACT IN KOREAN.....	103

GENERAL INTRODUCTION

Cornea is a unique transparent structure in the body. Also, the cornea has no blood supply to maintenance the transparency. This transparent structure of the cornea is essential to maintain clear vision. Various diseases or corneal surgeries destroy this transparency by developing the corneal haze resulting in the vision loss (Sakimoto *et al.*, 2006). Because the precise mechanisms of the corneal haze formation are unclear, therapies specifically targeting its prevention are limited. Therefore, experimental corneal haze models are needed for the research of the haze developing mechanisms and for the development of newer pharmacological agents.

Several experimental models have been introduced for the experiments to the research of corneal epithelial diseases, stromal fibrosis, and haze.

Experimental methods	References
Mechanical debridement	de Medeiros <i>et al.</i> , 2008
Irregular-phototherapeutic keratectomy (PTK)	Mohan <i>et al.</i> , 2008
Chemical burning	Yang <i>et al.</i> , 2010
Corneal alkali burn	Luo <i>et al.</i> , 2013
<i>ex vivo</i> bovine corneal irritation model	Bhasker <i>et al.</i> , 2015
Photorefractive keratectomy (PRK)	Alcalde <i>et al.</i> , 2015
Novel <i>in vivo</i> corneal model of fibrosis	Gronkiewicz <i>et al.</i> , 2016

However, the resection depth by mechanical debridement (de Medeiros *et al.*, 2008) is not deep enough to develop sufficient corneal haze for the experimental evaluation. For

this reason, this method has been limited to the evaluation of the epithelial healing. The chemical burning methods (Yang *et al.*, 2010; Luo *et al.*, 2013; Bhasker *et al.*, 2015) are difficult to assess an affected depth owing to loss of transparency and induced severe pain postoperatively. Recently, the novel *in vivo* corneal model of fibrosis (Gronkiewicz *et al.*, 2016) was reported using alkali burning. However, fibrosis was accompanied not only by haze but also by severe neovascularization and edema. Thus, this would not be an appropriate method for the evaluation of corneal haze. The delicate stromal ablation could be achieved with the desired depth and size in the PRK (Mohan *et al.*, 2008; Alcalde *et al.*, 2015). But, these methods require expensive specialized laser equipment and the result haze is insufficient for evaluation of treatment effects.

From these aspects, this study was designed to establish AK technique for standardization of corneal haze model by modification of BBT. The stromal structure is deformed to become loose by injecting air when the BBT was applied. In this study, the BBT was modified so that the air injected to the partial stromal layer. This method permitted visualization of the superficial stroma and the loosely deformed stromal structure. Additionally, the occurrence of corneal haze resulting from the AK technique was evaluated by comparing it with the occurrence of corneal haze resulting from the CK technique.

To verify experimental utility of the AK technique for the corneal haze development, the onion extract ointment was used to evaluate the capability of maintaining corneal transparency by suppressing the corneal haze development. Onion extract contains antioxidant phytochemicals, sulfur, and other numerous phenolic compounds (Benkeblia,

2005). These compounds have been reported to possess many biological activities which are effective in various diseases. Especially, onion extract was shown to have fibroblast-inhibiting properties for the reduction of proliferative activity (Ho et al., 2006). Because the key factors of the corneal haze expression are the differentiation of keratocytes to myofibroblasts (Gupta *et al.*, 2011), the fibroblast-inhibiting effect of onion extract could be a good therapeutic candidate as a new medicine for corneal haze suppression.

For the repair of deep corneal defects, various surgical managements using an autograft or preserved biological membranes have been used in veterinary ophthalmology. However, most of these surgical methods for large corneal defects would not be able to recover corneal transparency. Transplantation of various corneal parts is an essential technique for the treatment of severe corneal damage to maintain the corneal transparency and clear vision in human medicine (Bahar *et al.*, 2008). On the contrary, corneal transplantation is very limited in dogs due to insufficient resources of donor cornea and frequent graft rejections. DALK is one of the corneal transplantation methods to remove and replace the pathologic corneal stroma while preserving the host endothelium, which eliminates the risk of endothelial graft rejection (Karimian *et al.*, 2010). Thus, this method could suggest the possibility of successfully performing corneal transplantation to the dog without graft rejection.

The AK is an easy and simple surgical technique for the standardized corneal haze model. The AK could achieve the standardized stromal ablation in the desired resection size and depth without a specialized laser equipment. Furthermore, the degree of stromal haze generation was enough for the experimental evaluation of haze. In this study, the AK

was demonstrated to be a useful experimental model to evaluate the suppressive effect of onion extract on corneal haze. DALK, one of the corneal transplantation techniques injecting an air into the stroma of AK, was performed to confirm the availability of this technique in dogs.

CHAPTER I.

**Air assisted lamellar keratectomy for the corneal haze
model**

Abstract

To standardize the corneal haze model in the resection depth and size for efficient corneal haze development, AK was performed. The *ex vivo* porcine corneas were categorized into four groups depending on the trephined depth: 250 (G1), 375 (G2), 500 (G3) and 750- μm (G4). The stroma was equally ablated at the five measurement sites in all groups. Significant differences were observed between the trephined corneal depths for resection and ablated corneal thickness in G1 ($p < 0.001$). No significant differences were observed between the trephined corneal depth for resection and the ablated corneal thickness in G2, G3, and G4. The resection percentage was similar in all groups after microscopic imaging of corneal sections. AK and CK method were applied to six beagles, after which development of corneal haze was evaluated weekly until postoperative day 28. The occurrence of corneal haze in the AK-group was significantly higher than that in the CK-group beginning 14 days after surgery. Alpha-SMA expression was significantly higher in the AK-group ($p < 0.001$) than the CK-group. AK was considered as a useful method to achieve the desired corneal thickness after resection and produce sufficient corneal haze.

Introduction

Corneal transparency, which is one of the most important factors influencing vision, is a functional translation of the detailed ultrastructure of the stroma that is primarily attributed to the narrow, uniform diameter of collagen fibrils (Meek *et al.*, 2001). Corneal haze is associated with disruption of the collagen fiber array (Møller-Pedersen, 2004) and proliferation of newly formed myofibroblasts during the fibrotic response (Fini, 1999). The development of corneal haze resulting from refractory corneal diseases and PRK has been reported (Sakimoto *et al.*, 2006). Although fibrotic response is an essential component of the normal corneal healing process (Hu *et al.*, 2009), significant corneal opacity can be induced during this process and mediate a decline in visual acuity. Therefore, a crucial aspect of corneal wound healing is minimizing corneal haze.

Several experimental models have been introduced in the effort to develop treatments to prevent or reduce corneal haze, including mechanical debridement, chemical burning, and PRK (de Medeiros *et al.*, 2008; Soong *et al.*, 2008; Yang *et al.*, 2010). Corneal wound healing is a complex process controlled by various factors (Stepp *et al.*, 2014), and the size and depth of experimental corneal defects are important factors that must be considered for objective experimental modeling. The desired resection depth is not easily attained using experimental methods that include mechanical debridement and chemical burns. Conversely, PRK is better able to achieve the desired ablation thickness, but requires specialized expensive laser equipment. Thus, there is a need for a standardized and easily applied method.

Anwar and Teichmann introduced the BBT to expose DM by injecting air into the deep stroma (Anwar *et al.*, 2002). In this method, the stromal structure is deformed to become loose, and a large bubble is made between the deep stroma and the Descemet's membrane by injecting air. A very small stromal layer resided after applying this method (McKee, 2013). When this technique was applied, the injected air blanched the stroma, resulting in loss of transparency. In this study, the air injection method was applied to the partial stromal layer with slight modification for blanching to acquire the desired depth. This method permitted visualization of the superficial stroma and the loosely deformed stromal structure.

The purpose of this study was to establish a standardized corneal haze model for wound size and depth by using AK, which is a modification of the BBT. Additionally, the occurrence of corneal haze resulting from the AK was evaluated by comparing it with the occurrence of corneal haze resulting from the conventional method.

Materials and Methods

1. Experimental design

Fifty porcine eyes obtained from a slaughterhouse and 12 canine eyes were used in this study. Ten porcine eyes were included in each group according to the trephined corneal depth for resection of 250 (G1), 375 (G2), 500 (G3), and 750 μm (G4). Ten eyes were used to make histopathological sections of normal corneas ($n = 5$) and air-injected corneas ($n = 5$).

Six female beagles were used. One eye of each dog was selected at random for AK group (three right and three left eyes, $n = 6$), while the contralateral eye received CK group (three right and three left eyes, $n = 6$). Basal corneal haze and corneal haze was evaluated *in vivo* at 7, 14, 21, and 28 days after surgery. The dogs were sacrificed 4 weeks postoperatively for Periodic acid Schiff (PAS) and immunofluorescent (anti α -SMA antibody) staining to study the formation of myofibroblasts.

2. Animals

Fifty porcine eyes obtained from a slaughterhouse and six healthy female beagles were used. Prior to the experiment, all dogs underwent an ophthalmic examination including slit-lamp biomicroscopy (SL-D7[®]; Topcon, Tokyo, Japan), indirect ophthalmoscopy (Vantage plus[®]; Keeler, Windsor, UK), rebound tonometry (Tonovet[®], Tiolat, Helsinki, Finland), Schirmer's tear test (Schirmer tear test[®], Intervet, Summit, NJ, USA) and fluorescein staining (Fluorescein paper[®]; Haag Streit AG, Koeniz, Switzerland), and dogs with ocular or systemic diseases were excluded. The animal use and experimental protocols were approved by the Institutional Animal Care and Use committee (SNU-121108-4 and 121123-10; Seoul National University, Korea).

3. *Ex vivo* experiments on porcine eyes (air assisted lamellar keratectomy)

Porcine eyes were placed on a specially designed frame (Fig. 1a). The Intraocular pressure (IOP) was 10–20 mmHg as measured by an applanation tonometer (TonoPen XL[®]; Mentor, FL, USA). The center of the cornea was trephined to 250 (G1), 375 (G2), 500 (G3), and 750 μm (G4) using an 8 mm diameter trephine (Barron radial vacuum trephine[®]; Katena products, Inc., Denville, NJ, USA). The surgical field was kept dry after trephination to minimize stromal edema. A 30 gauge needle was attached to a 4 mL air-filled syringe. The needle was bent 5 mm from its tip so that the terminal segment angled upwards at approximately 60°, while the bevel faced up. The tip was introduced parallel to the corneal surface into the central stroma at the base of the trephination groove (Fig. 1b and f). The plunger of the air-filled syringe was pressed until intrastromal blanching was observed. The fuzzy region of the white opaque cornea was removed using a corneal dissector and blunt-tipped corneal scissors.

Corneal thickness was measured at five places (the central, superior, inferior, nasal, and temporal surface) within the central 8 mm diameter area of the cornea using an ultrasonic pachymeter (PACHMETE DGH 55[®]; DGH Technology Inc., Pennsylvania, USA) before and after applying the AK (Fig. 1g). The pachymetry values are expressed as the average \pm standard deviation (SD) of 25 successive readings. If the SD of a measurement was $> 10 \mu\text{m}$, the value was discarded.

Ablated corneal depth was calculated using the corneal thickness pre- and post-operation, and the calculated thickness and trephined depth were compared.

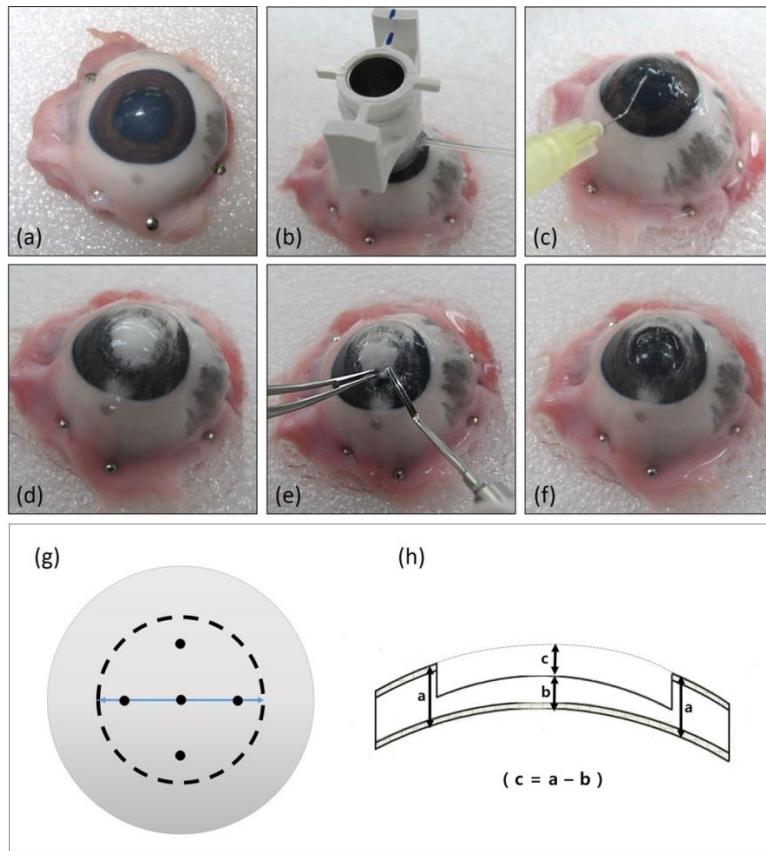


Fig. 1. Procedures of air assisted lamellar keratectomy. (a) The enucleated porcine eye was placed on a specially designed frame. (b) The center of the cornea was trephined using a vacuum trephine. (c) Four mL of air was injected at the base of the trephination gutter into the corneal stroma using a 30-gauge needle attached to a syringe. (d) Intrastromal blanching was observed. (e) The blanched cornea was removed using a corneal dissector and blunt-tipped corneal scissors. (f) Appearance after keratectomy using AK. (g) Schematic diagrams for measuring corneal thickness. The dotted line indicates the ablated corneal area and the blue arrows show an 8 mm diameter. The measurement sites of corneal thickness by ultrasonic pachymeter are marked by black dots. (h) Diagram of the corneal cross section. The thickness of the ablated area “b” was subtracted from the normal corneal thickness “a” to calculate the ablated corneal thickness “c”.

4. *In vivo* experiments on canine eyes (anesthesia and surgical procedures)

Each dog was positioned in dorsal recumbency while under general anesthesia with isoflurane after intubation and induction with tiletamine and zolazepam (Zoletil[®], Virbac, Carros, France; 2.5 mg/kg IV). The head was stabilized with a vacuum pillow, and the ocular surface was disinfected with 0.5% povidone iodine solution. Upper and lower eyelids were braced using an eyelid speculum. Keratectomy was performed in the two groups using either AK or CK after administration of atracurium (ATRA[®], Hanapharm, Seoul, Korea; 0.01 mg/kg IV) for central positioning of the cornea. The center of the cornea was trephined for 375 µm using an 8 mm diameter trephine in both groups. A conventional superficial keratectomy was performed with a #66 lamellar blade. Following surgery, one drop of atropine (1%, Isopto Atropine[®], Alcon, Antwerp, Belgium) was applied only once. Additionally, one drop of levofloxacin (0.5%, Cravit[®], Santen, Osaka, Japan) was applied three times daily until day 7 after surgery in all groups

5. Clinical grading of corneal haze

The level of haze in the cornea was measured by slit lamp biomicroscopy (SL-D7[®]) at 7, 14, 21, and 28 days after surgery and graded as follows (Fantes *et al.*, 1990): Grade 0: completely clear cornea; Grade 0.5: trace haze seen with careful oblique illumination; Grade 1: mild obscuration of iris details; Grade 2: a more prominent haze not interfering with visibility of fine iris details; Grade 3: moderate obscuration of the iris and lens; Grade 4: complete opacification of the stroma in the area of ablation.

Haze grading was performed in a blinded manner by three independent veterinarians.

6. Quantitative corneal haze grading

The slit images were taken under standardized conditions (1 mm wide, 14 mm long slit beam and a 45° angle from the temporal aspect of the cornea without background illumination to evaluate corneal haze preoperatively) at 7, 14, 21, and 28 days post-surgery. Each photograph was converted to an 8 bit gray-scale image using digital image analysis software (ImageJ ver. 1.46r; <http://rsbweb.nih.gov/ij/>). The selected area of the corneal section (100×3 pixels) was isolated, and an intensity of 0–255 was determined by averaging the gray-scale (intensity) indices of individual pixels within the area. Total intensity levels within the selected area were measured.

7. Histopathological and immunofluorescence analyses

Beagle eyes were enucleated by the conventional trans-scleral method after euthanasia with T61 after general anesthesia through administration of tiletamine and zolazepam (Zoletil®; 2.5 mg/kg IV). Porcine and canine corneas were excised from the globe by cutting with a blade and tenotomy scissors 2–3 mm from the limbus. Samples were fixed in 10% buffered formalin and embedded in paraffin. Six tissue sections were obtained at the central corneal part for each eyes. Three sections were stained with PAS according to the standard procedure. A light microscope (BX51®; Olympus, Tokyo, Japan) equipped with a digital camera (DP71®; Olympus) was used for photomicrography. The thickness of the non-ablated and ablated corneas on the histopathological section was determined by digital image analysis and the percentage of the ablated corneal thickness was calculated (Fig. 1h).

Immunofluorescent staining for α-SMA, a marker for myofibroblasts, was performed in three sections for the each eyes using mouse monoclonal antibody for α-SMA (M085101; DAKO, Carpinteria, CA, USA) with Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR, USA). The immunohistochemistry slides were mounted with SlowFade Gold antifade reagent with DAPI (Molecular Probes) and imaged using a fluorescence microscope (BX51®) equipped with a digital camera (DP71®).

8. Quantification of α -SMA positive cells

The green fluorescence of α -SMA positive cells in six randomly selected, non-overlapping, full-thickness central corneal columns, extending from the anterior stromal surface to the posterior stromal surface, were detected from the central ablated cornea of each specimen as previously described (Mohan *et al.*, 2003). The intensity of green fluorescence in each column was evaluated by digital image analysis. The mean green fluorescence intensity for the six column in each samples was used for the final results.

9. Statistical analyses

All measurements were performed in triplicate, and the results are expressed as the mean \pm SD. Statistical analyses were performed using SPSS V20 for Windows (SPSS Inc., Chicago, IL, USA). A student's *t*-test was used to test for significance between the two groups. One-way analysis of variance (ANOVA) with Bonferroni's post-hoc assessment was employed to test for significance when comparing three or more groups. *P* values < 0.05 were considered significant.

Results

The mean IOP before trephination was 13.7 ± 0.2 mmHg (range, 11.3–16.0 mmHg) after fitting the eyes on the specially designed frame. No significant differences were observed between the mean IOP of each group based on one-way ANOVA with Bonferroni's post-hoc test ($p = 0.957$). Mean corneal thickness pre- and post-operation and the ablated cornea for each of the five measurement sites are shown in Table 1. No significant differences were observed among the five measurement sites within each group. The ablated corneal thickness in G1 ($p < 0.001$) was significantly different from the trephined corneal depth (Table 2). No significant differences were detected between ablated corneal thickness and the trephined corneal depth for resection in G2 ($p = 0.214$), G3 ($p = 0.381$) or G4 ($p = 0.439$).

No significant differences were observed between the ablated percentages measured by ultrasonic pachymetry and digital image analyses of the histopathological sections in any of the groups (Fig. 2). The calculated percentage of corneal resection on the photomicrograph in G1 was significantly different ($p = 0.013$) from the desired percentage of resection. (Fig. 2). The stroma of the air-injected cornea above the needle insertion layer was severely deformed by the small air bubbles relative to the normal cornea (Fig. 3a and b). The most deformed stromal fiber due to air bubbles was removed after resection; hence, the surface of the wounded cornea was smooth following application of AK (Fig. 3c). Several small bubbles inserted into the stroma at the levels of the trephination gutter paralleled the stromal fibril layer on the outside margin of the trephined area.

Table 1. Corneal thickness at each measurement site

Corneal thickness pre-operation (μm) [*]					Mean \pm SD	P value [†]
	Central	Inferior	Superior	Nasal	Temporal	
G1	984.1 \pm 3.1	974.3 \pm 17.7	979.0 \pm 13.0	969.7 \pm 14.2	976.1 \pm 16.4	976.6 \pm 5.4
G2	995.7 \pm 5.8	987.3 \pm 7.7	985.7 \pm 9.0	991.5 \pm 5.8	988.2 \pm 8.3	989.7 \pm 4.0
G3	1021.2 \pm 41.8	1011.4 \pm 47.3	995.1 \pm 14.2	993.4 \pm 14.4	993.7 \pm 15.5	1003.0 \pm 12.7
G4	972.0 \pm 19.9	968.5 \pm 27.1	969.7 \pm 26.6	967.2 \pm 26.4	969.6 \pm 23.9	969.4 \pm 1.8
Corneal thickness post-operation (μm) [*]					Mean \pm SD	P value [†]
	Central	Inferior	Superior	Nasal	Temporal	
G1	616.9 \pm 34.2	612.5 \pm 31.3	622.0 \pm 53.9	616.7 \pm 56.9	617.1 \pm 55.0	617.0 \pm 3.4
G2	621.5 \pm 2.8	613.3 \pm 2.2	610.0 \pm 5.4	617.9 \pm 2.8	613.4 \pm 2.2	615.2 \pm 4.5
G3	503.1 \pm 51.7	500.5 \pm 56.9	504.1 \pm 55.3	500.4 \pm 64.2	501.2 \pm 61.5	501.9 \pm 1.6
G4	250.8 \pm 19.2	251.3 \pm 27.1	257.8 \pm 28.6	253.9 \pm 24.6	250.4 \pm 25.5	252.8 \pm 3.1
Resected corneal thickness (μm) [*]					Mean \pm SD	P value [†]
	Central	Inferior	Superior	Nasal	Temporal	
G1	367.3 \pm 33.8	361.9 \pm 46.5	357 \pm 42.3	352.9 \pm 53.2	359 \pm 42.1	359.6 \pm 5.4
G2	374.2 \pm 5.0	374.0 \pm 9.3	375.7 \pm 13.6	373.6 \pm 3.8	374.8 \pm 8.9	374.5 \pm 0.8
G3	518.1 \pm 57.7	510.9 \pm 69.0	491.1 \pm 58.4	493 \pm 65.2	492.5 \pm 53.3	501.1 \pm 12.5
G4	721.2 \pm 21.4	717.2 \pm 32.7	711.9 \pm 35.5	713.3 \pm 37.8	719.2 \pm 35.2	716.6 \pm 3.9

G1, 250 μm trephined group (n = 10); G2, 375 μm trephined group (n = 10); G3, 500 μm trephined group (n = 10); G4, 750 μm trephined group (n = 10) for resection using air assisted lamellar keratectomy. *The values represent the mean \pm SD. †One-way ANOVA was used to investigate differences between measurement sites in each group followed by a Bonferroni's post-hoc test.

Table 2. Measurement of the mean resected corneal thickness in each group

	Mean corneal thickness (μm) [*]			Trehined corneal depth (μm)	P value [†]
	Pre	Post	Resected		
G1	976.6 ± 5.4	617.0 ± 3.4	359.6 ± 5.4	250	< 0.001 [‡]
G2	989.7 ± 4.0	615.2 ± 4.5	374.5 ± 0.8	375	0.214
G3	1003.0 ± 12.7	501.9 ± 1.6	501.1 ± 12.5	500	0.381
G4	969.4 ± 1.8	252.8 ± 3.1	716.6 ± 3.9	750	0.439

G1, 250 μm trephined group (n = 10); G2, 375 μm trephined group (n = 10); G3, 500 μm trephined group (n = 10); G4, 750 μm trephined group (n = 10) for resection using air assisted lamellar keratectomy. ^{*}Values represent the mean \pm SD. [†]Significant differences within the same groups between the mean ablated corneal thickness and trephined corneal depth for resection were identified by the Student's *t*-test. [‡]Statistically significant.

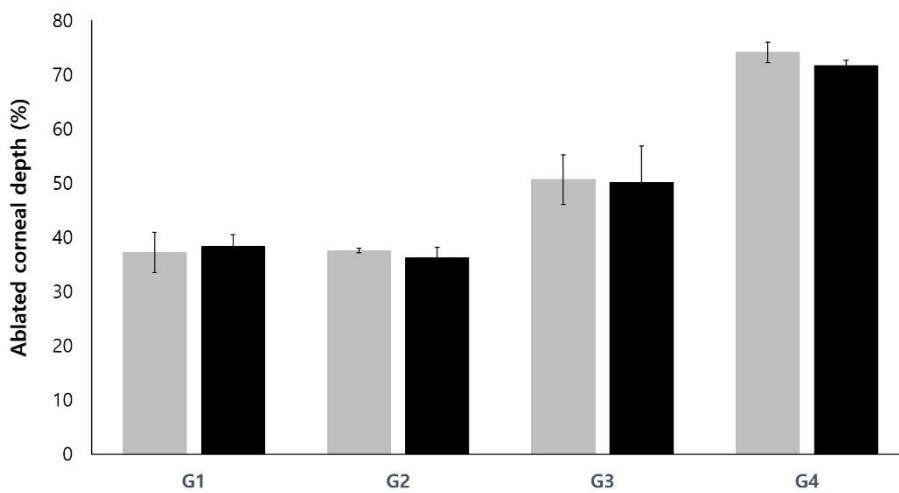


Fig. 2. Ablated corneal thickness (%) calculated based on the pachymetry and photomicrographic image analyses. G1, 250 μm trephined group; G2, 375 μm trephined group; G3, 500 μm trephined group; G4, 750 μm trephined group, ■ ; Pachymetry, and □ ; Digital image-analysis.

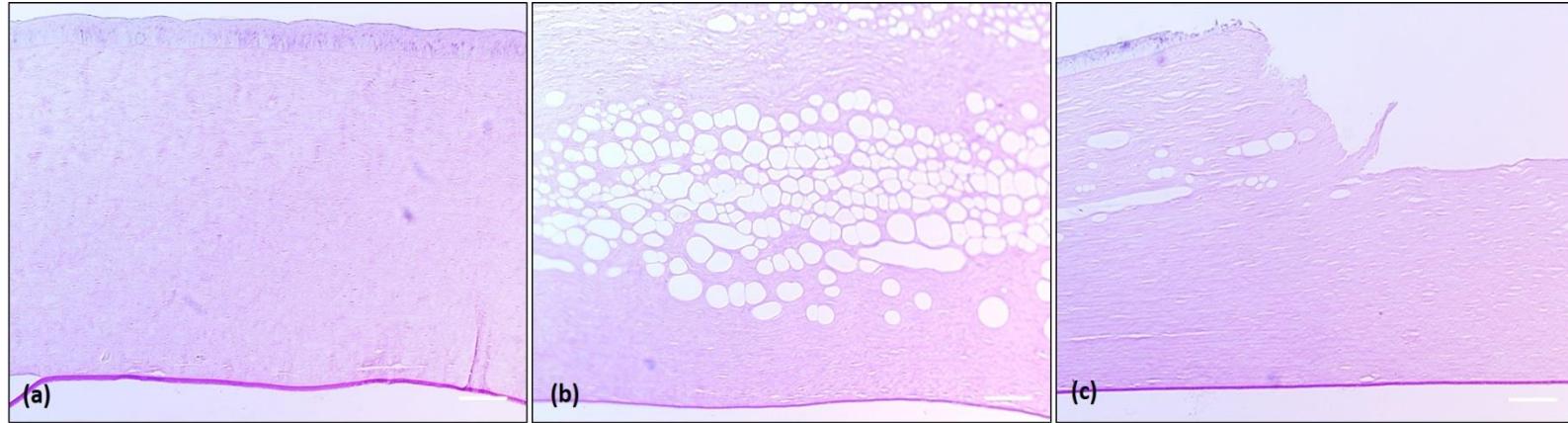


Fig. 3. Photomicrograph of porcine corneas. (a) Normal porcine cornea showing aligned stromal layers. (b) Stroma of the air-injected cornea was deformed by small air bubbles above the layer for needle insertion. (c) After resection by the air assisted lamellar keratectomy showing smooth ablated surface. 200 \times magnification. Scale bar = 50 μm .

Corneal haze was noted at 7 days after surgery and appeared to peak about 21 days after surgery in both groups (Fig. 4). Clinical corneal haze in the AK group was significantly greater than that in the CK group beginning 14 days after surgery (day 14; $p = 0.036$, day 21; $p = 0.044$, day 28; $p = 0.009$) (Fig. 5a). Furthermore, the quantity of corneal haze was more developed in the AK group than in the CK group from 14 days after surgery ($p < 0.001$) (Fig. 5b).

The corneal sections of the canine eyes showed a distinct stromal remodeling pattern in the AK group relative to the normal cornea and the CK group upon PAS staining (Fig. 6a and c). Alpha-SMA-positive cells were selectively detected in the anterior stroma immediately beneath the epithelial basement membrane in the CK and AK groups upon immunohistochemistry (Fig. 6d and f). Total green intensity of the entire stroma was significantly enhanced in the CK ($p < 0.001$) and AK groups ($p < 0.001$) relative to that in the normal cornea. Moreover, total green intensity in the AK group was significantly higher than that in the CK group ($p < 0.001$, Fig. 7).

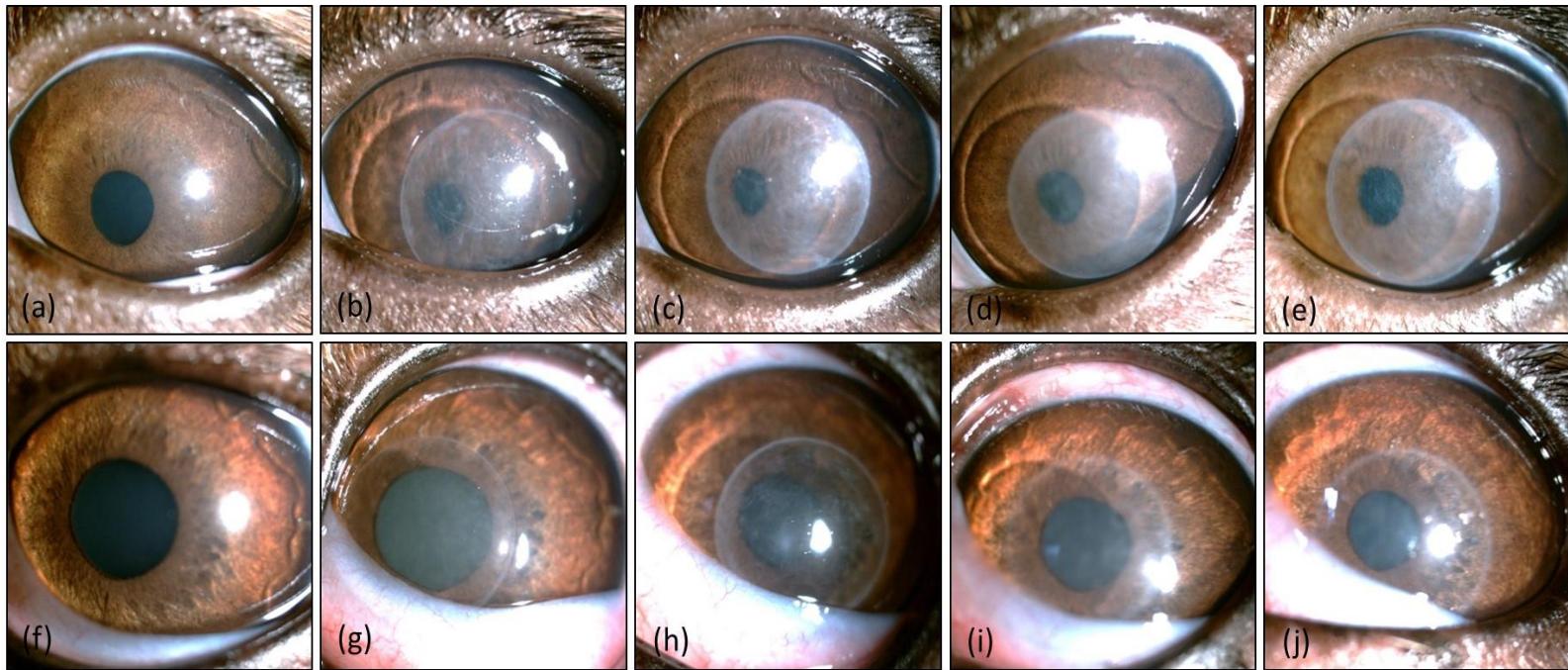


Fig. 4. Slit-lamp biomicroscopy of the corneas with subepithelial haze. Greater corneal haze developed following AK (a-e) than CK (f-j). a and f; pre-operative, b and g; day 7, c and h; day 14, d and I; day 21, e and j; day 28 after the surgery. The size of the captured corneal section was fixed by controlling the magnification of the slit lamp (10 \times magnification) under diffuse illumination (45°).

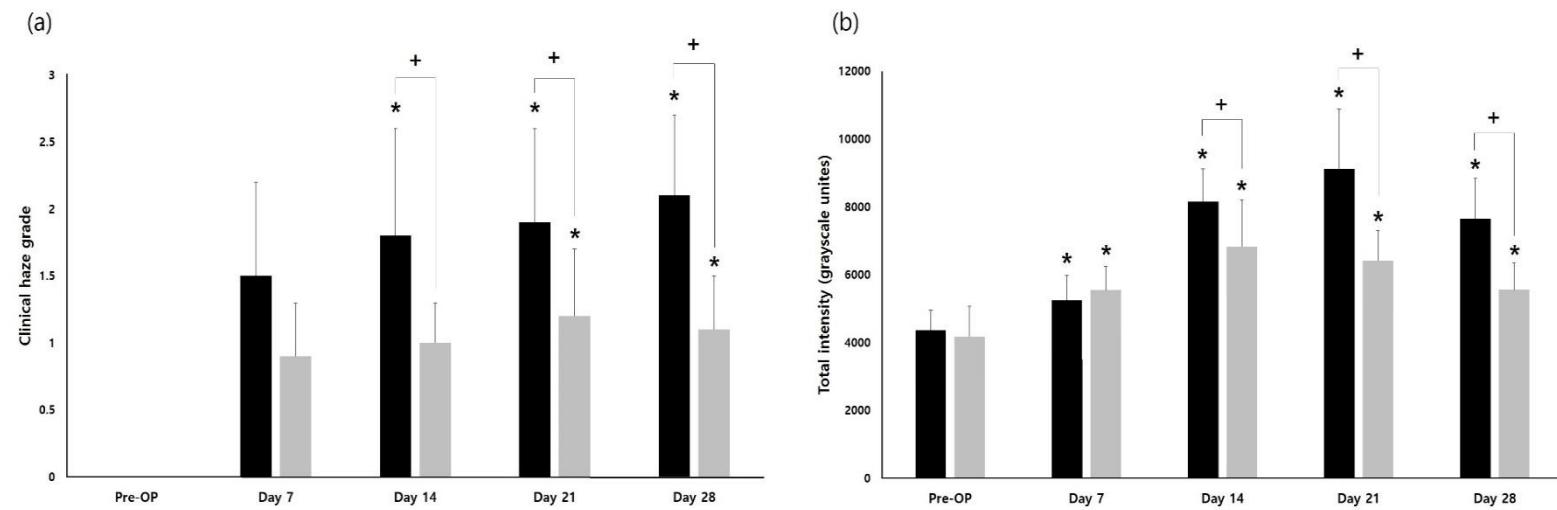


Fig. 5. Corneal haze measurement by clinical haze grading and quantitative method. (a) Clinical corneal haze grading. (b) Quantification of corneal haze. ■ = AK group and □ = CK group. Significant differences are indicated by + Student's *t*-test, $p < 0.05$ and *one-way ANOVA with Bonferroni's post-hoc test, $p < 0.05$.

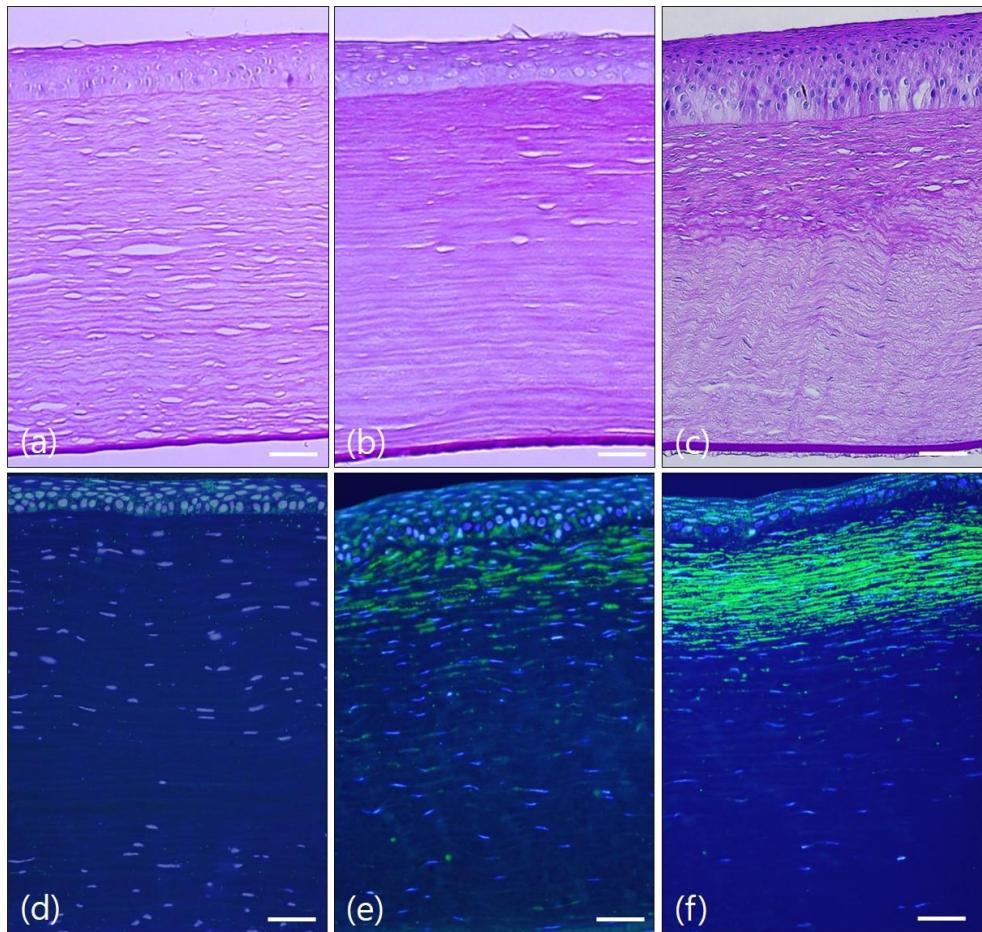


Fig. 6. Histopathology with PAS stain (a–c) and immunohistochemistry for α -SMA (d–f) for the evaluation of corneal haze. DAPI-stained nuclei are shown in blue, SMA-stained cells are shown in green. a and d; normal cornea, b and e; CK group, c and f; AK group. 200 \times magnification. Scale bar = 50 μ m.

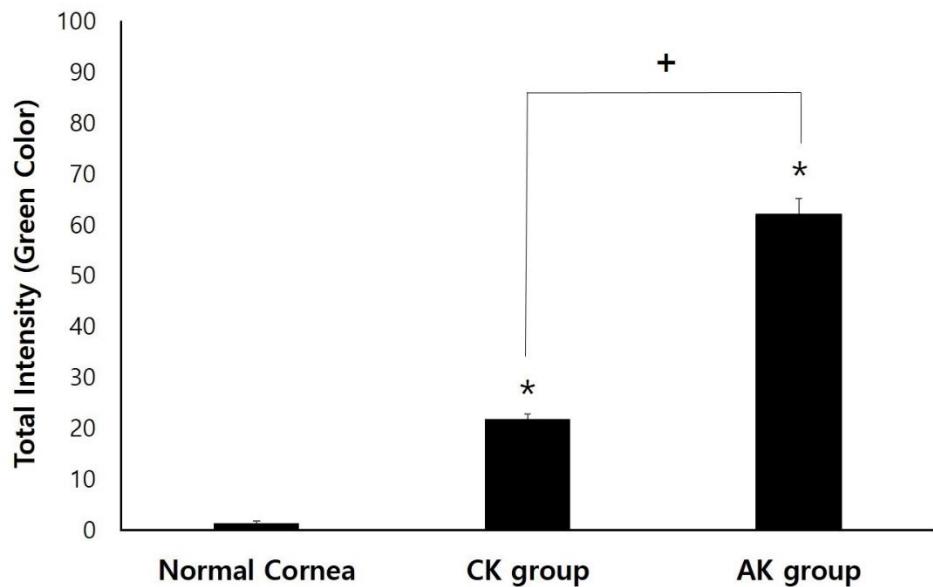


Fig. 7. The quantification of SMA-positive cells by green fluorescence detection.
Significant differences are indicated by + Student's *t*-test, *one-way ANOVA with Bonferroni's post-hoc test, $p < 0.05$.

Discussion

Corneal haze is a common complication following corneal surgery that results in diminished corneal transparency (Sakimoto *et al.*, 2006). Because the precise mechanisms of the formation of corneal haze are unclear, therapies specifically targeting its prevention are limited. Mitomycin C (MMC) has been widely used to prevent corneal haze following surface ablation for myopia (Teus *et al.*, 2009). However, multiple complications of MMC treatment have been reported, including limbal and scleral necrosis, abnormal wound healing, and loss of keratocytes (Safianik *et al.*, 2002). These adverse effects have encouraged the development of newer pharmacological agents that can effectively inhibit the formation of corneal haze without causing serious side effects.

Several experimental methods have been used to induce a corneal wound and haze, including mechanical debridement, chemical burns, and superficial keratectomy (Rieck *et al.*, 1992). Mechanical debridement is primarily used as a model to check the treatment effectiveness of epithelial healing, so the resection is not deep enough to develop sufficient corneal haze. Chemical burning tends to cause loss of keratocytes, further decreasing their density during slow healing injuries (Lin *et al.*, 2004), and resection depth after applying these methods is difficult to assess owing to loss of transparency. PRK is a superficial keratectomy that employs an excimer laser and has been conducted to achieve accurate ablation (Soong *et al.*, 2008). Expensive specialized equipment is needed for this technique, and the haze that develops is grade 1 or less on a scale of four total grades (Mohan *et al.*, 2008), which is insufficient for evaluation of treatment effects. For these reasons, standardized experimental models are needed in this research field.

The first goal of this study was to improve the method of superficial lamellar keratectomy by modifying the BBT to achieve standardized resection size and depth. This study demonstrated that AK resulted in uniform resection depth at each of the five measurement sites without expensive laser equipment. Additionally, ablated corneal thickness using this technique was the same as the trephined corneal depth in G2, G3, and G4. The percentage of ablated corneal thickness on the histopathological section calculated with a digital imaging program was not significantly different from the results obtained using ultrasonic pachymetry. In contrast, the resection thickness of G1 was significantly thicker than the desired depth. The trephined depth was not deep enough to insert a needle into in this group and excess air infiltrated under the stroma at the level of needle insertion. For these reasons, more stroma was ablated than desired. Accordingly, the AK achieved the standardized resection depth at a trephined corneal depth greater than 375 μm .

The corneal stroma is a precisely formed collagen fibril layer that has a unique parallel arrangement in a mucoid matrix scattered with keratocytes (Freund *et al.*, 1995). Because of these stromal structures in the normal cornea, dissection between stromal fibers requires specialized skill. Unlike the normal corneal stroma, the histopathological structure of the air-injected cornea was severely deformed by air bubbles and easily detached using a corneal dissector in this study. Additionally, the injected air bubbles were distributed uniformly over the entire internal area of trephination. These findings indicate that this modified method could be applied more easily and quickly to generate a standardized stromal defect compared to previous models.

Corneal haze developed in all eyes included in this experiment, beginning at day 7 after surgery and appearing to peak about 21 days after surgery. Surface ablation triggers a cascade of physiological events that culminate in mild to severe corneal fibrosis (de Medeiros *et al.*, 2008). Modern technologies that involve mechanical removal of the corneal epithelium have demonstrated upregulation of various pro-inflammatory interleukins that indirectly contributes to corneal fibrosis (Chang *et al.*, 2008). Haze following PRK may result from corneal wound healing, which is likely to be initiated by keratocyte apoptosis and subsequent over-proliferation of cells (Mohan *et al.*, 2003). According to our histopathological results, stromal reorganization was observed after healing from the surface resection represented by differentiation of fibroblasts into myofibroblasts with epithelial cell hyperplasia.

This results show that greater corneal haze developed after applying the AK than after CK. Additionally, α -SMA-positive cells were significantly more numerous in the AK group than those in the CK group upon immunohistochemical evaluation. Expression of α -SMA in fibroblasts is a specific marker of myofibroblast differentiation (Jester *et al.*, 1995). The morphological and immunohistochemical findings suggest that more keratocytes were replaced by smooth muscle-like myofibroblasts during wound healing after the AK. Mechanical tension is an important underlying factor in the molecular mechanisms of tissue repair and fibrosis. Mechanical stress induces myofibroblast differentiation in human corneal fibroblasts (Eckes *et al.*, 2006; Garrett *et al.*, 2004). Thus, it seems reasonable to conclude that the AK could induce more severe corneal haze due to myofibroblasts after the mechanical tension induced by injecting air bubbles. The stromal

structure was severely deformed by the injected air, which is an indication of air bubbles generating mechanical tension on stromal fibers.

Conclusions

In summary, these results suggest that AK can be useful to achieve the desired resection depth of the corneal stroma. The AK induced more corneal haze than the conventional method of superficial keratectomy. Therefore, this technique would contribute to improving standardization of the corneal haze model.

CHAPTER II.

**Effect of onion extract on corneal haze suppression after
air assisted lamellar keratectomy**

Abstract

This study evaluated the effect of onion extract on corneal haze suppression after applying the AK. The AK was performed on 24 canine eyes. They were treated with an artificial tear (group C), prednisolone acetate (group P), onion extract (group O), and TGF- β 1 (group T) three times per day from 7 to 28 days after the surgery. Corneal haze occurred on all eyes and was observed beginning at 7 days after the surgery. The haze was significantly decreased in groups P and O from day 14 compared with the group C using the clinical (group P; $P=0.021$, group O; $P=0.037$) and objective evaluation method (group P; $P=0.021$, group O; $P=0.039$). In contrast, it was significantly increased in group T from day 14 compared with group C based on the clinical ($P=0.002$) and objective evaluation method ($P<0.001$). Subsequently, these eyes were enucleated after euthanasia, and immunohistochemistry with α -SMA antibodies was done. The total green intensity for α -SMA was significantly more expressed in group T and significantly less expressed in groups P and O than in group C. Onion extract could have potential as a therapeutic in preventing corneal haze development by suppressing the differentiation of fibroblasts into myofibroblasts.

Introduction

Many corneal diseases are associated with the development of opacities in the stroma. Corneal haze presents as a superficial opacification of the anterior corneal stroma leading to a transient decrease in corneal transparency after lamellar keratectomy for dermoid, corneal inclusion cyst, and corneal tumor, corneoconjunctival transposition or autologous lamellar keratectomy for deep corneal ulcer in veterinary ophthalmology. Also, it is one of the most important complications of PRK, and its incidence and intensity increase in eyes treated for higher degrees of refractive error in human medicine (Heitzmann *et al.*, 1993). In most transparency disorders, corneal haze may be induced by a combination of two or more predominant factors like corneal edema, scarring, accumulated macromolecules, and reflective keratocytes (Møller-Pedersen *et al.*, 2004). Moreover, the formation of corneal haze involves the apoptosis of keratocytes and the proliferation and transformation of fibroblasts into myofibroblasts (Wilson *et al.*, 2001). Therefore, one of the most crucial aspects of corneal healing from refractive surgery is the minimization of corneal haze.

The efficacy of MMC in reducing the incidence of corneal haze has led to its widespread use in most refractive surgery practices (Teus *et al.*, 2009). However, multiple complications such as limbal/scleral necrosis, abnormal wound healing, and loss of keratocytes are reported with the topical use of MMC (Safianik *et al.*, 2002). These results encourage the development of newer pharmacologic agents that can effectively inhibit the formation of corneal haze without causing serious side effects. Recent research on trichostatin A (TSA), a histone deacetylase inhibitor, reported that it inhibits TGF- β 1-

induced accumulation of the extracellular matrix and myofibroblast formation *in vitro* and markedly decreases haze *in vivo* (Sharma *et al.*, 2009). However, there are no commercially available products for clinical use.

Allium cepa (onion) and onion extract have been reported to be effective in cardiovascular disease, because of their hypolipidemic, anti-hypertensive, anti-diabetic, and antithrombotic effects, and to possess many other biological activities including antimicrobial, antioxidant, anticarcinogenic and immunomodulatory activities (Corzo-Martínez *et al.*, 2007). Especially, flavonoids in onion extract reduce scar formation by inhibiting fibroblast activities (Cho *et al.*, 2010). Recently, commercial products composed of onion extract have been used to reduce hypertrophic scar formation (Ho *et al.*, 2006). Myofibroblasts are an important cell in connective tissue remodeling that differentiates during wound healing and fibrosis development in the pathogenesis of such diseases as hypertrophic scars, liver or pulmonary fibrosis (Desmouliere *et al.*, 2005), and corneal haze formation (Milani *et al.*, 2013). The myofibroblasts could represent an important target for corneal haze treatment like in the treatment for hypertrophic scar formation. Thus, onion extract could be useful as a therapeutic in preventing the development of corneal haze by suppressing the differentiation of fibroblasts into myofibroblasts.

AK is one of the experimental models for the development of corneal haze (Kim *et al.*, 2015). In this method, the wound size and depth were standardized by modification of the bubble technique for corneal transplantation. Also, it could induce more corneal haze than the conventional superficial keratectomy.

The aim of this study was to evaluate the efficacy of onion extract ointment in corneal haze development after applying to the haze model with the AK for canine eyes. In addition, the effect of onion extract ointment in the down-regulation of myofibroblast expression was examined with immunohistochemistry using the α -SMA antibody.

Materials and Methods

1. Corneal fibroblast culture and cell viability test for onion extract

Corneal fibroblasts were cultured from porcine eyes, which obtained from a local slaughterhouse, for the cell viability test of the onion extract. The corneal button which removed by an 8 mm diameter trephine (Barron radial vacuum trephine[®], Katena) was obtained. After then, the epithelial cells of the corneal buttons were scrapped off using a # 10 scalpel blade, DMs were peeled off, and the corneal stromas were washed with PBS (pH 7.4) (10010-023; GIBCOTM, Grand Island, NY, USA). The corneal buttons were cut into four small pieces and incubated overnight in a humidified CO₂ incubator at 37°C in DMEM (11995-065; GIBCOTM) containing 20 mM HEPES (15630-080; GIBCOTM) and 1.25 mg/mL collagenase type I (17100-017; GIBCOTM). The digested tissues were mixed with media by pipetting and filtered a 100 µm cell strainer (08-771-19; FalconTM, New Jersey, USA). Then, they were centrifuged at 800 g for 5 minutes and resuspended in 2 mL of DMEM containing 20 mM HEPES, 50 µg/mL gentamicin (15750-078; GIBCOTM), 1.25 µg/mL amphotericin B (A20678; GIBCOTM) and 10% fetal bovine serum (10437-028; GIBCOTM). This keratocyte-containing cell suspension was then seeded on 6-well plastic dishes and incubated in a humidified CO₂ incubator at 37°C. Eighty percent confluent cultures of cornea fibroblasts (passage 1-3) were used for experiments.

Trypan blue dye exclusion test was used to evaluate cell viability of corneal fibroblasts after treating onion extract. Onion extract (W281719; Sigma-Aldrich, St. Louis, MO, USA) was treated to the each well at 0, 0.01, 0.1, 1, 10, 50, and 100 μ L/mL concentrations diluted with Dimethyl sulfoxide (DMSO) (AMR-0231-1; Amresco, Solon, OH, USA) for 24 hr. Then, they were resuspended using 0.05% trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA) (25300-054; GIBCOTM) and trypan blue solution (0.4% wt/vol, 15250-061; GIBCOTM) was mixed with the resuspension cells. The suspensions were loaded into a hemocytometer and scored with a light microscope. Cells that stained blue were scored as nonviable.

2. A process of manufacture for the onion extract ointment

A 1% onion extract ointment was made with 10 g white petrolatum (white petrolatum 1 g/g, Sungkwang Pharm., Cheonan, Korea) and 0.1 mL onion extract (W281719, Sigma-Aldrich) mixture in a water bath. The concentration of onion extract ointment depended on the *in vitro* viability test. Before use main study, the onion extract ointment was applied every 12 hours for 2 weeks at the normal cornea of six healthy beagle dogs to test the abnormal allergic reactions, blepharospasm, conjunctival hyperemia, corneal epithelial disorders and other ocular abnormality, by ophthalmic examinations every the other days.

3. Animals

Twenty-four eyes from 12 healthy beagles were used in this study. Before the experiment, all dogs underwent an ophthalmic examination including slit-lamp biomicroscopy (SL-D7[®]), indirect ophthalmoscopy (Vantage plus[®]), rebound tonometry (Tonovet[®]), Schirmer's tear test (Schirmer tear test[®]) and fluorescein staining (Fluorescein paper[®]). Dogs with ocular or systemic diseases were excluded. The animal use and experimental protocols were approved by the Institutional Animal Care and Use committee (SNU-121123-10; Seoul National University, Korea). All dogs were divided into 4 groups consisting of 6 eyes in each group; control (Group C; n = 6), Prednisolone acetate treatment (Group P; n = 6), Onion extract treatment (Group O; n = 6), and TGF-β1 treatment (Group T; n = 6).

4. Corneal haze generation by the air assisted lamellar keratectomy

AK was performed following a method reported previously (Kim *et al.*, 2015). General anesthesia was performed by intravenous injection of tiletamine and zolazepam (Zoletil[®]; 2.5 mg/kg) for induction, and maintained with isoflurane (Ifiran[®], Hana Pharm, Seoul, Korea; MAC 0.5 – 1.5%). Atracurium (ATRA[®]; 0.01 mg/kg IV) was administrated for central positioning of the cornea during the surgery. The AK was performed for all eyes. Briefly, the center of the cornea was trephined 375 µm using an 8 mm diameter trephine (Barron radial vacuum trephine[®], Katena) (Fig. 8a). The surgical field was kept dry after the trephination to minimize stromal edema. Four mL of air were injected at the base of the trephination gutter into the corneal stroma using a 30-gauge needle attached to a syringe. The needle was bent 5 mm from its tip so that the terminal segment angled upwards approximately 60°, while the bevel faces up (Fig. 8b). The tip was introduced parallel to the corneal surface into the central stroma at the base of the trephination groove. The plunger of the air-filled syringe was pressed until intrastromal blanching was observed (Fig. 8c). The fuzzy region of the white opaque cornea was removed using a corneal dissector and blunt-tipped corneal scissors (Fig. 8d).

After surgery, one drop of atropine (1%, Isopto Atropine[®]) was applied for 3 days for the purpose of cycloplegic effect. Levofloxacin (0.5%, Cravit[®]) eye drops were administered three times daily until 7 days after the surgery. After the seventh day, artificial tear eye drops (0.1% sodium hyaluronate; Lacure[®], Samil

Pharm., Seoul, Korea) for group C, prednisolone acetate 1% (Pred-Forte[®], Allergan, Irvine, California, USA) for group P, an onion extract (W281719, Sigma-Aldrich, St. Louis, MO, USA) ointment 10 mL/mg diluted with a white petrolatum (Vaseline[®], SungKwang Pharm, Cheonan, Korea) for group O, and TGF-β1 (T7039, Sigma-Aldrich) 1 ng/mL diluted with artificial tear eye drops for group T were administered three times daily each for 3 weeks.

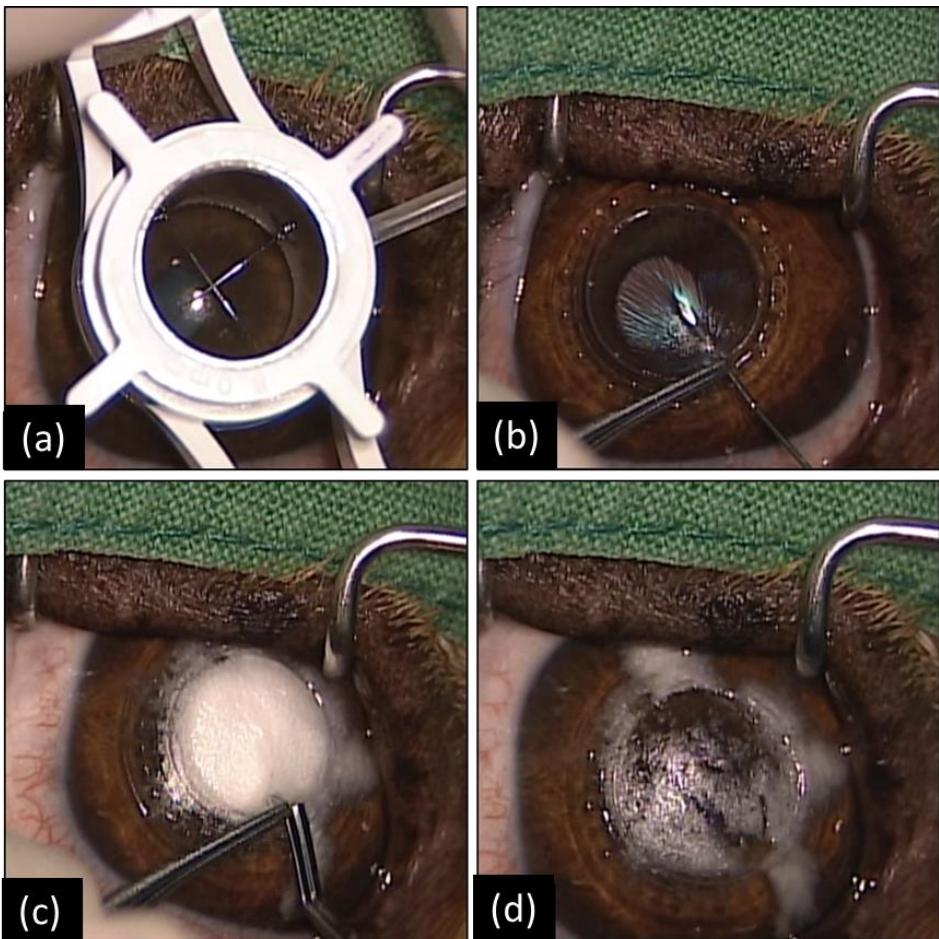


Fig. 8. The procedures of air assisted lamellar keratectomy in dogs. (a) The center of the cornea was trephined $375\text{ }\mu\text{m}$ using an 8 mm vacuum trephine. (b) Four mL of air was injected at the base of the trephination gutter into the corneal stroma using a 30-gauge needle attached to a syringe. (c) Intrastromal blanching was observed, and the blanched cornea was removed using a corneal dissector and blunt-tipped corneal scissors. (d) Appearance after keratectomy using the air assisted lamellar keratectomy.

5. Corneal haze grading

The level of haze in the cornea was evaluated by slit lamp biomicroscopy (SL-D7[®]) at 7, 14, 21, and 28 days after the surgery using two kinds of method: the previously reported clinical grading system (Fantes *et al.*, 1990) and a quantitative method. With the clinical grading, grade 0 was a completely clear cornea; grade 0.5 had a trace amount of haze observed with careful oblique illumination; grade 1 was a mild obscuration of the iris details; grade 2 was a more prominent haze not interfering with the visibility of fine iris details; grade 3 was a moderate obscuration of the iris and lens; and grade 4 was complete opacification of the stroma in the area of the ablation. Haze grading was performed in a blinded manner by three independent veterinarians.

For quantitative haze grading, slit images were taken under standardized conditions: a 1 mm wide, 14 mm long slit beam and a 45° angle from the temporal aspect of the cornea without background illumination. Then, each photograph was converted into an 8 bit gray-scale image using digital image analysis (ImageJ ver. 1.46r). The selected area of the corneal section (100 x 3 pixel) was isolated, and an intensity of 0 to 255 was determined by averaging the gray-scale (intensity) indices of the individual pixels within the area. Total intensity levels within the selected area were measured.

6. Immunofluorescence Analyses

The beagle eyes were enucleated with the conventional trans-scleral method after euthanasia with T61 after general anesthesia through tiletamine and zolazepam (Zoletil[®]; 5.0 mg/kg IV). Corneas were excised 2 – 3 mm from the limbus with forceps and tenotomy scissors. The samples were fixed in 10% buffered formalin and embedded in paraffin. Three tissue sections (4 µm thickness) were obtained at the central corneal part for each eyes. Immunofluorescence staining for α-SMA, a marker for myofibroblasts, was performed. These tissue sections were incubated at room temperature with the monoclonal antibody for α-SMA (M085101, DAKO) at a 1:200 dilution in 1× PBS for 90 minutes and with a secondary antibody (Alexa Fluor 488 goat anti-mouse IgG; Molecular Probes) at a dilution of 1:500 for 1 hour. Tissues were mounted with mounting medium and DAPI (SlowFade[®] Gold antifade reagent, Molecular Probes) to visualize the nuclei in the tissue sections. Sections were viewed and photographed with a fluorescence microscope (BX51[®]) equipped with a digital camera (DP71[®]).

7. Quantification of α -SMA-positive cells

Fluorescence intensity of green color for the α -SMA positive cells was detected using the digital image analyzer (Image J) by comparing size-matched areas from 6 randomly chosen fields of view in the experimental areas. These 6 fields were non-overlapping, full-thickness central corneal columns, extending from the anterior stromal surface to the posterior stromal surface following a method previously reported (Mohan *et al.*, 2003). The mean green fluorescence intensity for the six column in each samples was used for the final results.

8. Statistical analyses

Statistical analysis was performed with SPSS V20 for Windows (SPSS Inc.). Data were expressed as the mean \pm SD, and the level of significance was $P < 0.05$. One-way ANOVA with Bonferroni's post-hoc assessment was used to test for the significance of the objective haze grading and the total green color intensity between the groups. In addition, in one-way repeated measures ANOVA following pairwise comparison, Bonferroni's adjustment was performed to evaluate the objective haze grades against time in the same group. For the clinical corneal haze grading, the values between groups were compared using the Kruskal-Wallis analysis or Friedman test with the Wilcoxon signed rank test.

Results

Cell viability was over 95% at the concentration of 0, 0.01, 0.1, 1, and 10 uL/mL onion extract in vitro evaluation (data not shown). The 1% onion extract ointment were showed no adverse effects and allergic reaction, like blepharospasm, conjunctival hyperemia, corneal epithelial disorders and other ocular abnormalities, when applied every 12 hours to the normal beagle cornea for 2 weeks.

Corneal haze was developed after the AK depending on each treatment group (Fig. 9). Control corneas treated with the artificial tear eye drops (group C) had significant developed corneal haze in clinical grading until 21 days after surgery (day 14; $P = 0.032$, day 21; $P = 0.041$, and day 28; $P = 0.210$) compared with day 7 (Fig. 10). Topical application of 1% prednisolone acetate (group P) and 1% onion extract ointment (group O) caused a statistically significant decrease in corneal haze in group P (day 14; $P = 0.021$, day 21; $P = 0.012$, day 28; $P = 0.001$), and in group O (day 14; $P = 0.037$, day 21; $P = 0.008$, day 28; $P = 0.003$) compared with the same groups for haze grading at day 7. Additionally, corneal haze was significantly increased in the TGF- β 1 treated group (group T) (day 14; $P = 0.002$, day 21; $P < 0.001$, day 28; $P < 0.001$ compared with the haze grading at day 7). For the degree of clinical corneal haze in each group, it decreased in groups P and O from day 14 (day 14; $P = 0.016$ and $P = 0.007$, day 21; $P < 0.001$ and $P = 0.026$, day 28; $P = 0.001$ and $P = 0.011$, respectively) compared with group C. In addition, corneal haze significantly increased in group T at days 21 ($P = 0.022$) and 28 ($P = 0.001$) compared with group C.

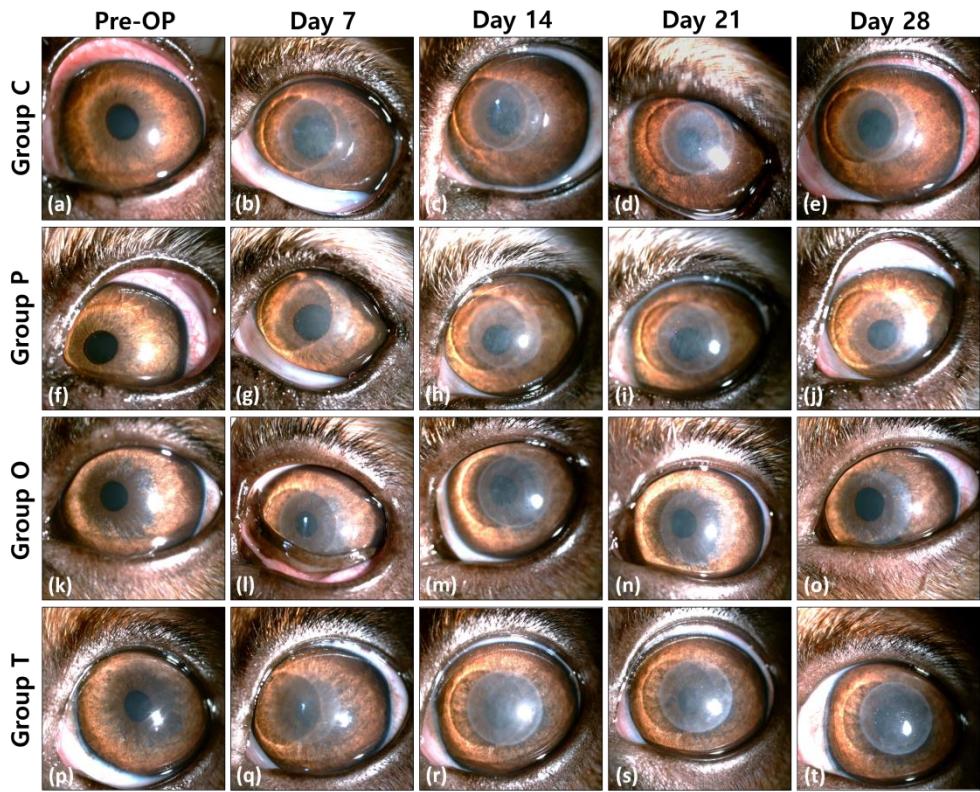


Fig. 9. The evaluation of corneal haze with a slit-lamp biomicroscopy. Group C; artificial tear treatment group (a-e), Group P; prednisolone acetate treatment group (f-j), Group O; onion extract ointment treatment group (k-o), Group T; TGF- β 1 treatment group (p-t). a, f, k and p; pre-operation, b, g, l and q; day 7, c, h, m and r; day 14, d, i, n and s; day 21, e, j, o and t; day 28 after surgery.

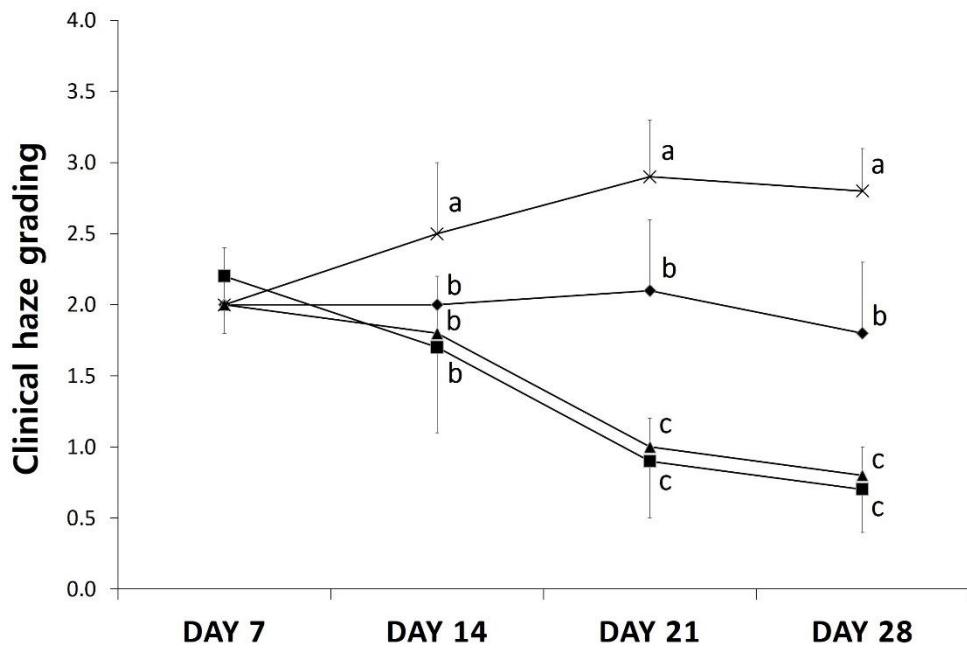


Fig. 10. Clinical corneal haze grading. Mean grades for the clinical evaluation of corneal haze at days 7, 14, 21, and 28 after surgery for each group. ▲ = group C, ■ = group P, ▲ = group O, ✕ = group T. ^{a, b, c} Values with a different superscript were significantly different ($P < 0.05$) between groups in the same evaluation day.

Corneal haze was observed beginning 7 days after surgery in all groups, and appeared to peak about 21 days after surgery in the control group with an objective evaluation method. The total intensity of the grayscale units for the hazed cornea was significantly increased at days 21 ($P < 0.001$) and 28 ($P < 0.001$) compared with day 7 in group C (Fig. 11). The total intensity in groups P (day 14; $P = 0.021$, day 21; $P < 0.001$, day 28; $P < 0.001$) and O (day 14; $P = 0.039$, day 21; $P < 0.001$, day 28; $P < 0.001$) significantly decreased compared with that at day 7. The total intensity of the grayscale units in group T significantly increased at days 14 ($P < 0.001$), 21 ($P < 0.001$), and 28 ($P < 0.001$) compared with that at day 7.

The corneal haze significantly decreased in groups P ($P < 0.001$) and O ($P < 0.001$) at day 21, and in groups P ($P < 0.001$) and O ($P = 0.002$) at day 28 compared with group C. Furthermore, the corneal haze significantly increased in group T at days 14 ($P < 0.001$), 21 ($P < 0.001$) and 28 ($P = 0.003$) compared with group C.

The corneal haze was also evaluated by immunohistochemical staining of myofibroblasts and by quantification of α -SMA-positive cells in tissue sections (Fig. 12). In group C, the corneas exhibited high numbers of α -SMA-positive myofibroblast cells, mostly in the anterior stroma below the epithelium. Topical application of prednisolone acetate (group P) and onion extract (group O) significantly reduced the numbers of α -SMA-positive cells in the stroma. In contrast, TGF- β 1 application (group T) significantly increased the numbers of α -SMA-positive cells compared with that in group C.

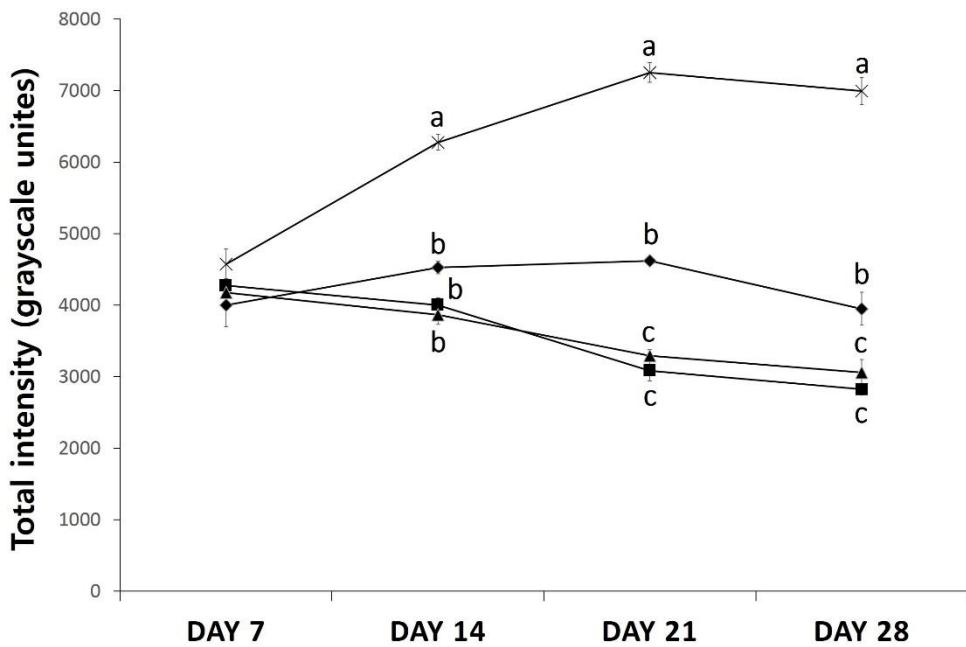


Fig. 11. Total intensity (grayscale units) levels within the corneal section.

◆ = group C, ■ = group P, ▲ = group O, ✖ = group T. ^{a, b, c} Values with a different superscript were significantly different ($P < 0.05$) between groups in the same evaluation day.

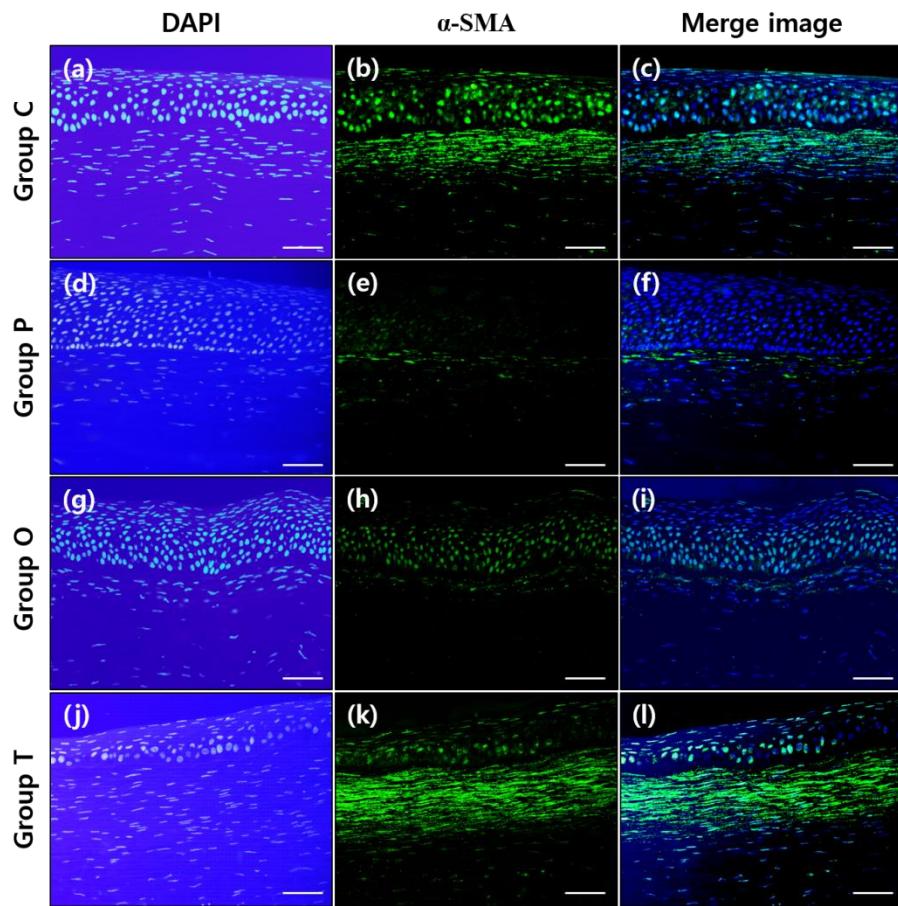


Fig. 12. Immunohistochemistry for α -SMA. (a) stained nucleus with DAPI in control group (group C), (b) α -SMA-positive cells (green color) in the group C, (c) merge image a and b, (d) stained nucleus with DAPI in prednisolone acetate treatment group (group P), (e) α -SMA-positive cells (green color) in the group P, (f) merge image d and e, (g) stained nucleus with DAPI in onion extract treatment group (group O), (h) α -SMA-positive cells (green color) in the group O, (i) merge image g and h, (j) stained nucleus with DAPI in TGF- β 1 treated group group (group T), (k) α -SMA-positive cells (green color) in the group T, (l) merge image j and k. 400 \times magnification, scale bar = 50 μ m.

The total green intensity of the entire stroma was significantly enhanced in group T ($p < 0.001$) compared with that in group C (Fig. 13). The total green intensity in groups P ($p < 0.001$) and O ($p < 0.001$) was significantly lower than that in group C.

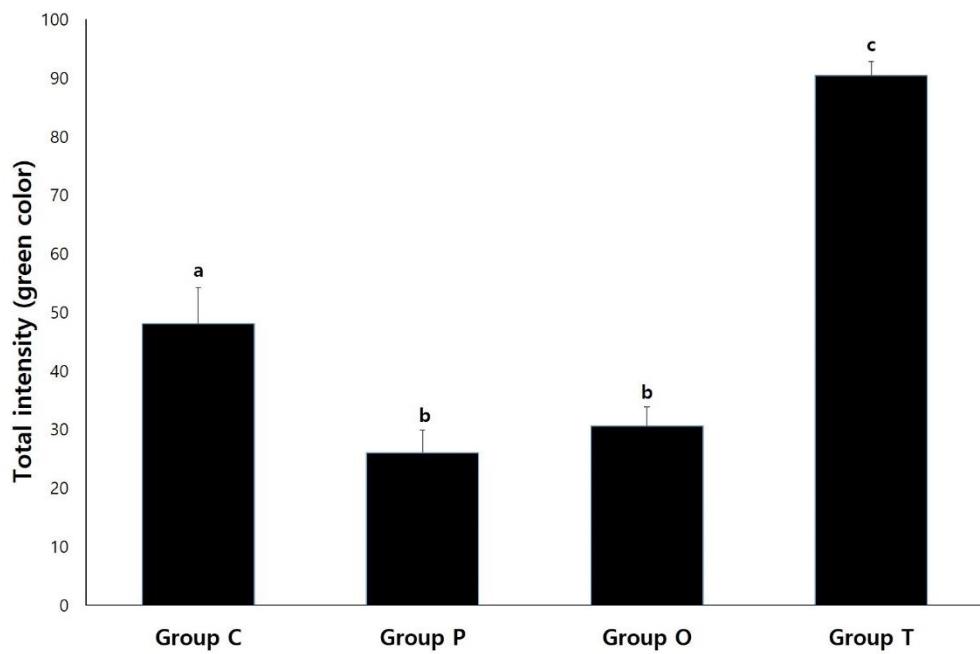


Fig. 13. The total intensity of green fluorescence. Group C; artificial tear treated group, Group P; 1% prednisolone acetate treated group, Group O; onion extract 10 mL/mg treated group, Group T; TGF- β 1 1ng/mL treated group, ^{a, b, c} Values with a different superscript were significantly different ($P < 0.05$).

Discussion

Formation of corneal haze involves the apoptosis of keratocytes (Wilson *et al.*, 2001) and transdifferentiation of keratocytes into myofibroblasts in response to endogenous epithelial derived cytokines (Jester *et al.*, 2003). TGF- β 1 directly activates keratocytes and leads to the formation of myofibroblasts as well as the subsequent reformation of subepithelial stromal tissue (Saika, 2006). Myofibroblasts scatter more light than that of undifferentiated fibroblasts or keratocytes, not only from their nuclei, but also from their cell bodies and dendritic processes (Møller-Pedersen, 2004). In addition, this population of cells participates in extracellular matrix remodeling, resulting in a denser and more disorganized extracellular matrix (Jester *et al.*, 1999). Intracellular microfilament fibers such as F-actin and α -SMA were expressed much higher in myofibroblasts than in keratocytes. These cellular components were enabled myofibroblasts to contract and close wounds, but also rendered the cornea less translucent (Meek *et al.*, 2004). Collectively, these changes lead to a loss of corneal transparency.

For a clear cornea, MMC is widely used intraoperatively by clinicians to prevent PRK-induced corneal haze although there are several complications reported with its topical use (Camellin, 2004). There are no effective medicines to control corneal haze except for MMC treatments. Because the application of steroid eye drop occasionally results in rapid corneal stromal melting, use of these drugs for achieving better corneal transparency is restricted. Thus, this study have shown the effects of onion extract ointment in corneal haze prevention and suppression of myofibroblasts from stromal ablation using the AK for the development of new treatment and prevention strategies.

Corneal fibroblasts were viable in the 10 uL/mL concentration of the onion extract. There were no adverse effects or allergic reactions for the 1% onion extract ointments. Therefore, this concentration of onion extract ointments was used in this study to evaluate efficacy of onion extract. According to these results, corneal haze grading and expression of α -SMA-positive cells significantly decreased in the onion extract treated group compared with the control group. Because α -SMA is a specific marker for myofibroblasts, these results suggest that onion extract ointment have suppressive effects on corneal haze.

Treatment with prednisolone acetate showed significant suppression of corneal haze compared with the artificial tear treatment in this paper. Postoperative use of topical corticosteroids has been controversial after PRK. Topically applied steroids, acting as an anti-inflammatory agent, have effectively suppressed corneal haze formation after excimer laser keratectomy in experimental studies (Kaji *et al.*, 2000; Nien *et al.*, 2011). But, this reduction in haze appears to be due in part to a delay in the wound-healing response (Nien *et al.*, 2011). Also, glucocorticoids increase the lytic action of corneal collagenase, suggesting that this effect might be responsible for the corneal destruction in clinical conditions (Brown *et al.*, 1970). Accordingly, the onion extract ointment could use more safely than steroid eye drops, which could induce corneal melting.

Onion (*Allium cepae*) extract contains a great amount of antioxidant phytochemicals, sulfur and other numerous phenolic compounds (Benkeblia, 2005). These compounds have been reported to be effective in cardiovascular diseases because of their hypolipidemic, anti-hypertensive, anti-diabetic, and antithrombotic effects, and to possess many other biological activities including antimicrobial, antioxidant, anticarcinogenic,

antimutagenic, antiasthmatic, immunomodulatory, and probiotic activities (Corzo-Martínez *et al.*, 2007). Especially, onion extract was shown to have fibroblast-inhibiting properties, to reduce proliferative activity, and to produce substances in the extracellular matrix (Ho *et al.*, 2006). Recently, commercial products composed of onion extract have been used to reduce scar formation on the skin (Ho *et al.*, 2006). According to the results of this study, onion extract suppressed the differentiation of myofibroblasts, and as a result, corneal haze developed significantly less than that of the control. Onion extract would be a good therapeutic candidate as a new medicine for corneal haze suppression.

Mechanical removal of the corneal epithelium and PRK up-regulate TGF- β 1 (Gupta *et al.*, 2011). TGF- β 1, a potent profibrotic cytokine, is a key regulator for the differentiation of myofibroblasts during corneal wound healing. It directly activates keratocytes and leads to the formation of myofibroblasts as well as the subsequent reformation of the subepithelial stroma (Gupta *et al.*, 2011). Consequently, these mechanisms could promote the clinical expression of corneal haze after corneal surgery. In these results, corneal haze significantly increased by treatments of additional TGF- β 1 compared with the control. Furthermore, one study showed the prevention of PRK-induced haze through the use of anti-TGF- β 1 antibodies (Møller-Pedersen *et al.*, 1998). Thus, the suppression of TGF- β 1 expression is critical in the prevention of corneal haze.

Fibroblasts differentiate into myofibroblasts through a Smad 2/3 signaling pathway and enhance NADPH oxidases (Nox) 4-derived ROS signaling cascades (Cucoranu *et al.*, 2005). Depletion of Nox4, an essential mediator of Smad 2/3 transcription factor activation in response to TGF- β 1, down-regulates α -SMA mRNA, and overexpression of

Nox4 induces α -SMA expression (Clempus *et al.*, 2007). The precise mechanisms of onion extract have not yet been completely elucidated. The corneal haze grade was significantly lower in the onion extract treated group than in the control group, and the expression of α -SMA was also down-regulated by the onion extract treatments shown in the results of this study. Among the many flavonoid compounds, quercetin is a major component of onion extract (Sellappan *et al.*, 2002), and it has been shown to have powerful antioxidative activity with metal ion binding properties and radical scavenging abilities (Erden *et al.*, 2000). In addition, quercetin has a scavenging effect on superoxide anions and hydroxyl radicals, and it prevents lipid peroxidation by blocking the action of xanthine oxidase and chelating iron (Hwang *et al.*, 2009). These effects could have important roles in the suppressive effect of onion extract against corneal haze formation. These results suggest that onion extract could block TGF- β 1 signaling cascades by scavenging ROS to reduce α -SMA expression and subsequently corneal haze development. Further experiments are needed to understand the exact mechanisms of onion extract.

The limitation of this study is that the evaluation time was short and there were not enough to prove exact mechanism of onion extract ointment against corneal haze formation. Therefore, more studies will be needed to understand the mechanisms.

Conclusions

In summary, onion extract ointment could be useful as a therapeutic in the suppression of corneal haze development after apply the AK through the down-regulation of fibroblast transdifferentiation into myofibroblasts. This effect could be from the scavenging ability of the onion extract.

CHAPTER III.

**Deep anterior lamellar keratoplasty of dog eyes using the
big-bubble technique**

Abstract

The aim of this study was to establish the feasibility of corneal transplantation using BBT for performing DALK in three dogs. After the cornea was trephined 750 µm, 4 mL of air was injected, and the blanched stroma was removed to expose DM. The donor corneal button, which was gently stripped off the DM, was sutured onto the bare DM of the recipient cornea. The dogs received topical antibiotics every 6 hours for 7 days and 2% cyclosporine ointment every 12 hours for 1 month. The eyes were examined post-operatively at 7, 14, 21, 28 and 150 days. The central portion of the transplanted cornea stayed transparent while corneal haze developed around the transplanted margin. Menace response was normal even though the transplanted cornea was edematous until 3 weeks after surgery. A marginal haze was rarely observed between the donor and recipient corneas at 150 days after the operation. A spotted haze developed in the central part of the deep stroma near the DM. In the histopathological examination, the stroma and epithelium of the donor cornea had normal structures. Corneal transplantation using DALK with BBT can be performed in dogs preserving the healthy endothelium.

Introduction

Corneal ulceration is a common and clinically important ocular disease in dogs. Especially in the case of large and deep corneal defects, various surgical managements have been tried to promptly and effectively repair the cornea, including conjunctival pedicle graft (Soontornvipart *et al.*, 2003), corneal-scleral transposition (Parshall *et al.*, 1973) and autogenous corneal graft (Brightman *et al.*, 1989). In addition, preserved biological membranes including pericardium (Alio *et al.*, 2013), intestinal submucosa (Bussieres *et al.*, 2004), amniotic membrane (Barros *et al.*, 1998) and renal capsule (Andrade *et al.*, 1999) are used in medicine and in veterinary general surgery for deep corneal defects and perforations. However, these surgical methods for large corneal defects will not be able to recover corneal transparency. Additionally, the indicated cases are restricted to certain types of techniques.

Transplantation of various corneal parts is an essential technique for the treatment of severe corneal damage in humans (Bahar *et al.*, 2008), and it has been performed successfully in horses for therapeutic and tectonic reasons (Brooks *et al.*, 2005). Furthermore, Penetrating keratoplasty (PK), one of the transplantation methods, has been performed in experimental models in the rabbit cornea (Niederer *et al.*, 2007). However, corneal transplantation is very limited in dogs due to insufficient resources of donor cornea and frequent graft rejections characterized by corneal vascularization, graft failure, and subsequent corneal edema. Therefore, there are few reports on canine corneal transplantation (McEntyre *et al.*, 1968).

DALK removes and replaces the pathologic corneal stroma while preserving the host endothelium, which eliminates the risk of endothelial graft rejection and has a reduced effect on the endothelial cell count (Karimian *et al.*, 2010). Thus, DALK is indicated for a patient with a healthy endothelium to achieve a high success rate for corneal transplantation as an alternative procedure to PK (Karimian *et al.*, 2010). Several surgical methods were used to bare the DM during DALK including layer-by-layer manual dissection (Tsubota *et al.*, 1998), hydro-delamination (Sugita *et al.*, 1997), viscoelastic dissection (Melles *et al.*, 1999) and air injection (Anwar *et al.*, 2002). Especially, the BBT introduced by Anwar and Teichmann is a method that injects air which forms a large bubble in the stroma to detach DM during DALK (Anwar *et al.*, 2002).

The purpose of this study was to establish the feasibility of corneal transplantation with BBT when performing DALK in dogs.

Materials and Methods

1. Animals

Three eyes from 3 healthy male beagles with normal corneas were used in this study. The donor corneas were obtained from dogs scarified in other experiments unrelated to this study. The animal use and experimental protocols were approved by the Institutional Animal Care and Use Committee (SNU-140520-1; Seoul National University, Korea).

Complete ophthalmic examinations were performed before the experiment with a rebound tonometry (Tonovet[®]), Schirmer's tear test (Schirmer tear test[®]), slit-lamp biomicroscopy (SL-D7[®]) and indirect ophthalmoscopy (Vantage plus[®]) with a 30-diopter indirect lens (Classic BIO Lens[®], Volk Optical Inc., Mentor, OH, U.S.A.). None of the beagles had any corneal diseases.

2. Surgical technique

The surgical procedure was performed under general anesthesia with isoflurane after induction with tiletamine and zolazepam (Zoletil[®]; 2.5 mg/kg IV) with the BBT as described by Anwar and Teichmann (2002). Before the surgery, the central corneal thickness (CCT) was measured with an ultrasonic pachymeter (PACHMETE DGH 55[®]). The central axial cornea was trephined 750 µm with an 8 mm diameter trephine (Barron radial vacuum trephine[®], Katena) to create an incision of approximately 80% thickness (Fig. 14a). A partial-thickness superficial anterior keratectomy was performed by dissection with a #66 lamellar blade (Katena products) (Fig. 14b). The introduction of a small amount of air into the anterior chamber by intracameral injection at the limbus was done with a 26 G needle. A 30 G needle attached to a 4 mL filled syringe with the tip manually bent to approximately 30 degrees was introduced with its bevel down into the cornea stroma through the trephination groove and advanced to the center of the cornea (Fig. 14c). At this point, air was injected gently, being forced through the posterior stromal lamella along the path of least resistance, causing the DM to separate from the deep stroma (Fig. 14d). A blanched corneal stroma was incised with a 15° slit-knife (Alcon Laboratories, Fort Worth, Texas, USA) to let the air escape and to collapse the bubble (Fig. 14e). A corneal dissector was carefully inserted and advanced into the cleavage plane that was created until its tip approached the opposite trephination groove. Corneal scissors were used to remove the remaining corneal tissue and expose the DM (Fig. 14f). The donor

cornea was gently stripped off the DM and endothelium with a cellulose sponge or forceps. Then, the donor cornea was punched from the endothelial side with an 8.5 mm diameter Barron punch (Katena, Denville, New Jersey, USA) (Fig. 14g). This prepared donor corneal button was initially sutured onto the bare DM with 4 cardinal 10-0 nylon sutures at 3, 6, 9, and 12 clock-hour positions (Fig. 14h and i). Additionally, there was a single running suture with 16 to 18 bites using same suture materials (Fig. 14j). After surgery, gentamicin and triamcinolone were injected subconjunctivally.

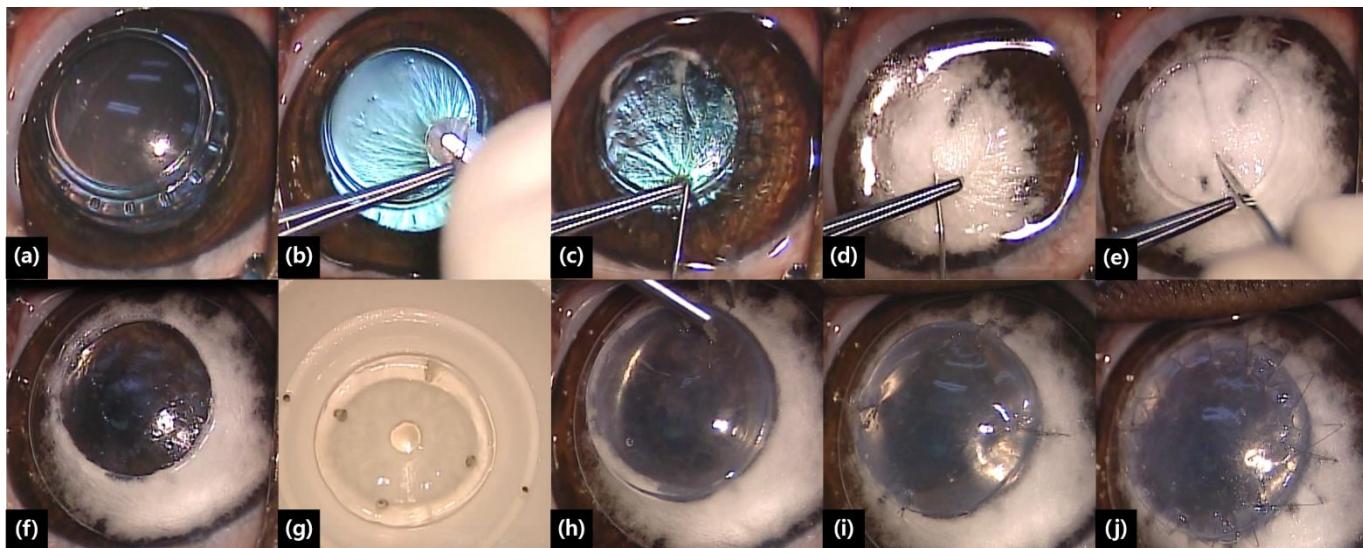


Fig. 14. Procedures of DALK using the BBT in dogs. (a) Center of the cornea was trephined 750 μm using an 8-mm vacuum trephine. (b) A partial-thickness superficial anterior keratectomy was performed (c) A 30-gauge needle attached to a 5-cc syringe was inserted at the base of the trephination gutter into the corneal stroma. (d) 4 mL of air were gently injected causing DM to separate from the deep stroma. Intrastromal blanching was observed during this procedure. (e) A blanched stroma was incised with a 15° slit-knife to allow air to escape and to collapse the bubble for stroma removal. (f) DM was exposed after excising the remaining stroma using corneal scissors. (g) After DM and endothelium were stripped off, the donor cornea was punched from the endothelial side using an 8.5-mm-diameter punch. (h) This prepared donor corneal button was fitted onto the exposed Descemet's plane of the recipient cornea. (i) 4 cardinal sutures were used with 10-0 nylon at the 3, 6, 9, and 12 clock-hour positions. (j) A single running suture was performed with 16 to 18 bites using the same suture materials.

3. Post-operative care and evaluation

After surgery, one drop of atropine (1%, Isopto Atropine[®]) was applied two times a day for 3 days and levofloxacin (0.5%, Cravit[®]) was administered every 6 hours for 7 days. Cyclosporine ointment (2mg/g, Optimimmune[®], Schering-Plough, Segre, France) was administered every 12 hours until 30 days post operatively. The all corneal sutures were removed under the topical anesthesia with proparacaine hydrochloride (0.5%, Alcaine, Alcon, Forest, New Zealand) at the day-21 post-surgery.

The eyes were examined by slit-lamp biomicroscopy (SL-D7[®]), tonometry (Tonovet[®]), and indirect ophthalmoscopy (Vantage plus[®]) for the evaluation of corneal condition and graft rejection at 7, 14, 21, 28 and 150 days post-surgery. In this periods, menace response, dazzle reflex, and pupillary light reflex (PLR) were evaluated, and IOP and CCT were measured. Also, blepharospasm, corneal edema, corneal vascularization, and haze developments at the central cornea and suture line were examined clinically. The level of corneal haze was evaluated using the previously reported clinical grading system as follows (Fantes *et al.*, 1990). Grade 0: a completely clear cornea; Grade 0.5: a trace amount of haze observed with careful oblique illumination; Grade 1: a mild obscuration of the iris details; Grade 2: a more prominent haze not interfering with the visibility of fine iris details; Grade 3: a moderate obscuration of the iris and lens; Grade 4: a complete opacification

At the same time, the CCT was measured using the ultrasonic pachymeter (PACHMETE DGH 55[®]).

The results of CCT and IOP are expressed at mean \pm SD. Statistical analyses were performed using SPSS V20 for windows (SPSS Inc.). One way ANOVA with Bonferroni's post-hoc assessment was used to test for significance when comparing the CCT. P-values < 0.05 were considered significant.

4. Histopathological evaluation

The dogs were euthanized with T61 under general anesthesia through tiletamine and zolazepam (Zoletil[®]; 5 mg/kg IV) at day 150 after surgery, and the eyes enucleated and fixed in 10% formalin and embedded in paraffin. Six tissue sections of 4 um thickness were obtained at the central corneal part for each eyes. Three sections were stained with H&E stain according to the standard procedure. A light microsocope (BX51[®]) equipped with a digital camera (DP71[®]) was used for photomicrography to evaluate the stromal structures at the transplanted junction.

5. Immunofluorescence detection of α -SMA

Immunofluorescent staining for α -SMA was performed using the three paraffin embedded sections for each eyes. These tissue sections were treated with the monoclonal antibody for α -SMA (A5228; Sigma-Aldrich St. Louis, MO, USA) at a 1:100 dilution in 1xPBS for 2 hours and with a secondary antibody (Alexa Fluor 488 goat anti-mouse IgG, A-11017; Life technologies, Gaithersburg, MD, USA) and Propidium iodide (P3566; Life technologies) at a dilution of 1:100 for 1 hour. Sections were viewed and photographed with FluoViewTM 300 fluorescence microscope (Olympus, Tokyo, Japan).

Results

The mean CCT was $817 \pm 23 \mu\text{m}$ for the 3 eyes before surgery. The central cornea was trephined a depth of $750 \mu\text{m}$ for approximately 80% of the corneal thickness. DALK was performed for the 3 eyes successfully without any DM tearing. After the surgical procedure, no rejection of the corneal implants was observed in the eyes for 5 months postoperatively (Table 3). The menace response, dazzle reflex, and PLR were normal in all experimented eyes during this period. IOP was in the normal range and did not show significant changes. Fluorescein dye staining test was positive in two cases at the 7 days after surgery, and the blepharospasm was examined in the two cases at the same examination time. Because of the corneal ulcer was located near the suture line, the ulcer and blepharospasm were related with spur of the suture materials. The all abnormal responses were not showed after suture removal.

A corneal haze was developed from 7 days after surgery at the central transplanted cornea and around the suture line (Table 3). The corneal haze of central corneal part was reduced from 21 days after surgery. The corneal haze around the junction of the donor and recipient cornea and suture lines increased at 21 days after surgery and stayed until all experimental period. The central portion of the transplanted cornea stayed transparent while a corneal haze developed around the transplanted margin (Fig. 15). Stromal edema of the donor cornea was present from just after operation until 3 weeks after

Table 3. Clinical evaluations after corneal transplantation in three dogs

Pre-OP	Post-OP				
	Day 7	Day 14	Day 21	Day 28	Day 150
Menace response	N	N	N	N	N
Dazzle reflex	N	N	N	N	N
PLR	N	N	N	N	N
IOP	16.3 ± 0.6	16.7 ± 1.5	16.3 ± 0.6	16.0 ± 1.0	16.0 ± 1.0
Fluorescein dye	-(3)	-(1) / +(2)	-(3)	-(3)	-(3)
Blepharospasm	-(3)	-(1) / +(2)	-(2) / +(1)	-(3)	-(3)
Neovascularization	-(3)	-(3)	-(3)	-(3)	-(3)
CCT	817 ± 23	$1024.1 \pm 28.5^*$	$1002.5 \pm 37.0^*$	$958.6 \pm 27.3^*$	$940.7 \pm 35.8^*$
Haze (central cornea)	0	1.7 ± 0.6	1.7 ± 1.2	0.5 ± 0.4	0.2 ± 0.3
Haze (suture line)	0	2.0 ± 1.0	2.0 ± 1.0	2.3 ± 0.6	2.0 ± 0.8

The data was presented with mean \pm SD for IOP, CCT and haze. N, normal; -, negative; +, positive; (n), n for numbers. * P < 0.05, significantly different compared with CCT of the Pre-OP.

surgery in all 3 beagles (Fig. 16) and then began to decrease from 4 weeks after the surgery. The corneal thickness was significantly increased at the Day 7, 14, 21, and 28 after surgery compared to the CCT of Pre-operative examined values (Table 3). The menace response was normal even though the transplanted corneas were edematous.

After the sutures were removed, the corneal haze around the implant margin decreased, and the corneal haze near the suture line disappeared. The marginal haze at the junction between the donor and recipient corneas decreased to almost nothing at 150 days after the operation (Fig. 15). At 150 days after the surgery, a new spotted haze observed in the central part of the cornea which looks like a deposited mineral spot. The spotted haze was found in the deep stroma near DM on the slit image (Fig. 16f).

In the histopathological examination, the stroma and epithelium of the donor cornea had normal structures (Fig. 17). A modified arrangement of corneal stroma was confirmed in the junction portion between the donor and recipient corneas. In addition, keratocytes and epithelial proliferation and a distortion in the stromal structure were detected at the junction of the transplant. There were more severe pathologic changes in the recipient cornea than in the donor parts. Some particles of the donor's DM in the cornea were observed in the stroma layer (Fig. 17b).

The SMA-positive cells were detected in the junction of the donor cornea and recipient cornea (Fig. 18). More of the SMA-positive cells were present in the

hyperplastic epithelial parts and anterior stroma beneath the epithelium. The green fluorescence was not detected in the deep stroma near the recipient's DM.

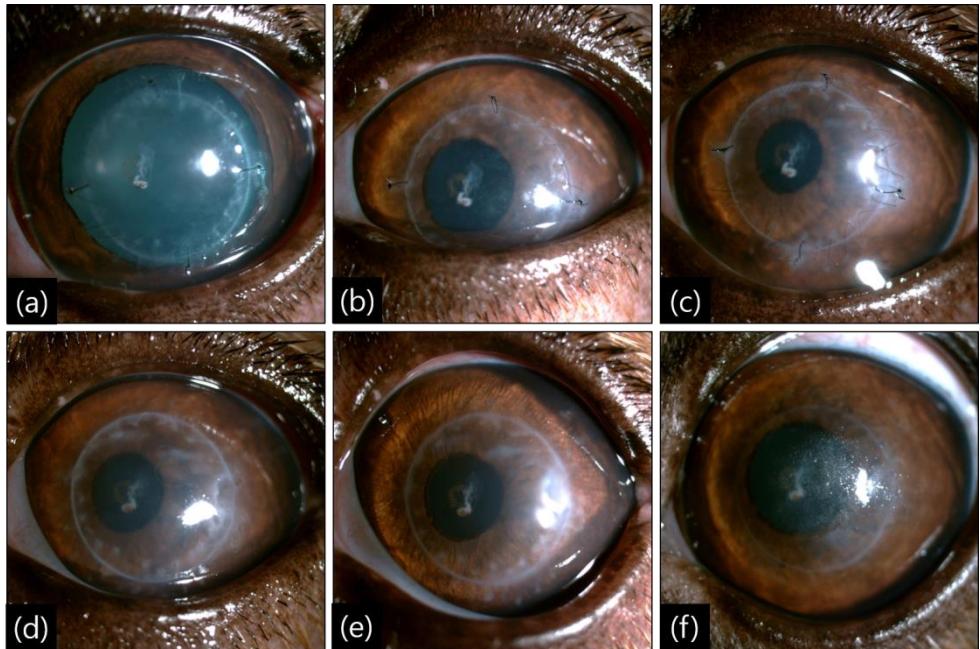


Fig. 15. Slit-lamp biomicroscopy after DALK. (a) 12 hours after surgery, (b) 7 days, (c) 14 days, (d) 21 days, (e) 28 days and (f) 150 days after surgery with direct diffuse illumination.

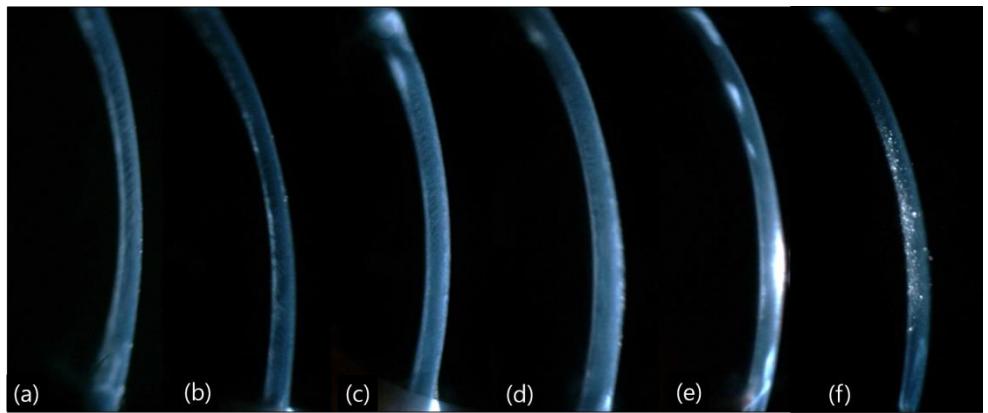


Fig. 16. Slit-lamp biomicroscopy with direct narrow slit (1 mm) for the stroma. (a) 12 hours after surgery, (b) 7 days , (c) 14 days, (d) 21 days, (e) 28 days and (f) 150 days after surgery.

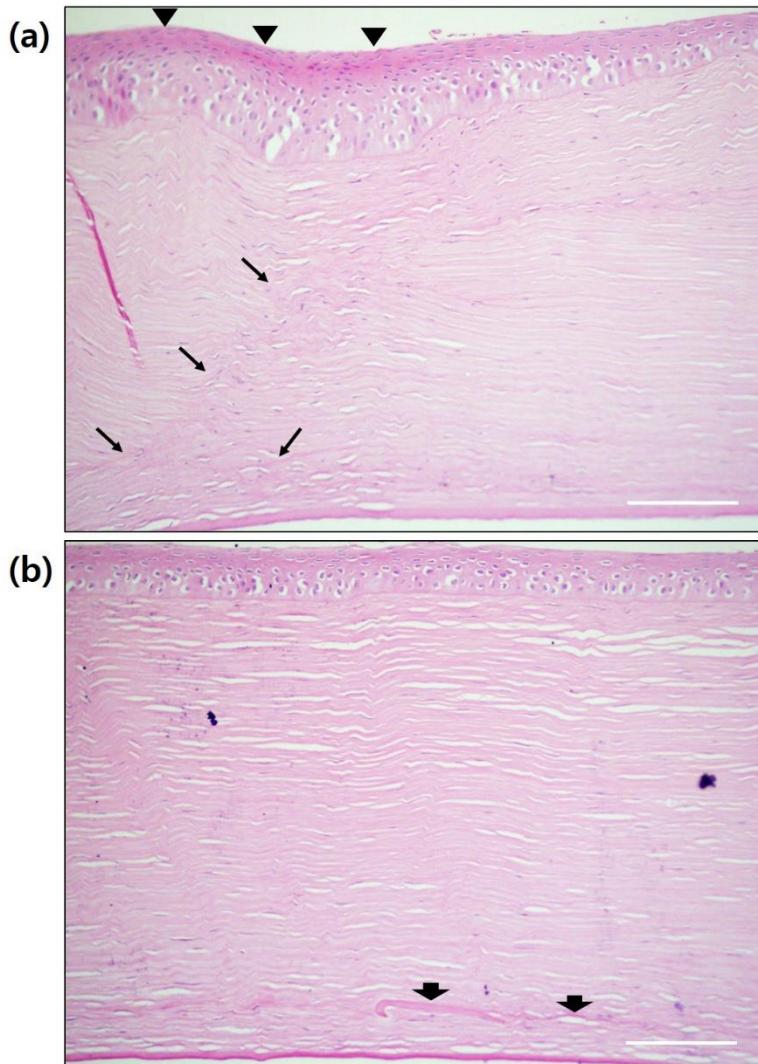


Fig. 17. Histopathological evaluation with H&E staining at 150 days after surgery. (a) The epithelium was overgrown (arrow head) at the junction of the donor and recipient corneas. In addition, deformed stromal structures were detected at the transplant junction (arrow). (b) The central corneal part had a normal corneal structure. A small piece of Descemet's membrane was left in the stroma (thick arrow). 200 \times magnification. Scale bar = 50 μ m.

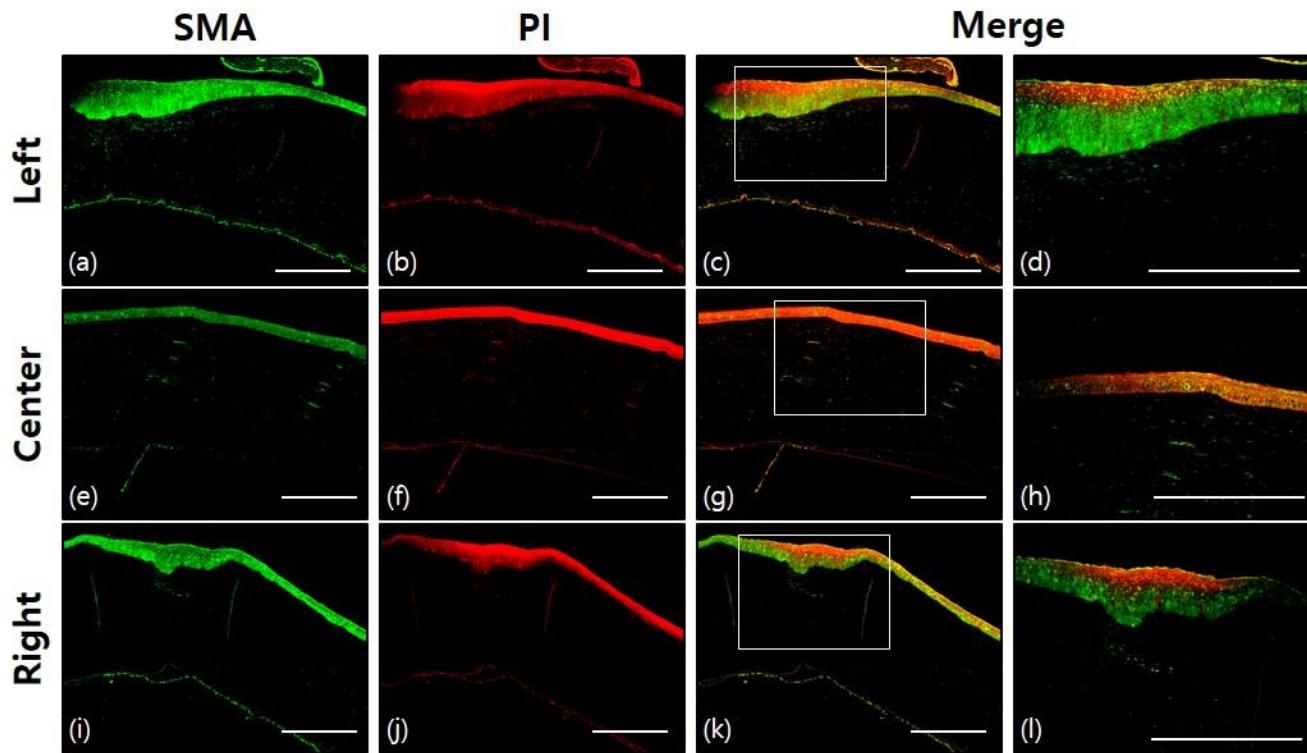


Fig. 18. Alpha-SMA staining 150 days after DALK. Cell nuclei were stained red with propidium iodide (PI) and SMA was stained green fluorescence. Representative image of the SMA-positive cells detected in the left and right transplanted junction. The scale bar = 200 um. Magnification 100X (a-c, e-g, i-k), 200X (d, h, l).

Discussion

The BBT for DALK has been proven to be a valuable method as an alternative to PK with the purpose of reshaping the cornea profile in human patients with keratoconus by removing and replacing most of the corneal ectasia (Fontana *et al.*, 2007). Thus, DALK is performed without substituting the host's healthy endothelium, and it could reduce the risk of immunological rejection (Fontana *et al.*, 2007). In addition, DALK has been performed on rabbits (Tham *et al.*, 2002) and horses (Martins *et al.*, 2013) experimentally. In this study, BBT with DALK was performed on the canine cornea to establish the feasibility of this technique in dogs. The shape and transparency of the transplanted cornea were successfully maintained, and 5 months after the surgery, there still was no rejection in any of the experimental eyes.

Corneal grafts show a higher success rate than other organ transplants. To the best of the authors' knowledge, so far there have been no reports on BBT in dogs. BBT, which is normally done for human ophthalmology, has not been a feasible procedure in canine eyes (Leiva *et al.*, 2014). However, according to our results, BBT is not necessarily impossible although it seems to require a skilled surgeon who knows the technique. A small portion of the stromal layer of the recipient cornea after BBT remained in this experiments similar to the report by Leiva et al. (2014). When the thickness of the remaining stroma in this experiment was estimated by histopathological examination, however, its thickness was relatively less in this study compared to the thickness in the other previous reports. Similar to this results, very small parts of the stroma remain which

cause the development of corneal haze in reports on human ophthalmology (McKee, 2013).

In addition, part of DM was left in the stromal layer which determines the junction between the recipient cornea and the donor cornea. Part of the membrane may remain during the process of removing DM from the donor cornea. DM incarceration was reported on human ophthalmology under microscopic examination (Lang *et al.*, 1986). The stromal structures of the human cornea after air injection look similar to those of the dog cornea (Arenas *et al.*, 2012). Therefore, the results of human ophthalmology support this speculation about the incarcerated part of donor DM.

In this results, the swelling of the stroma of the transplanted cornea was observed during the first 3 weeks after surgery. The edema did not progress and normalized three weeks after the surgery. In clinical examinations, an eye that has rejection after corneal transplantation shows conjunctival hyperemia, anterior chamber reaction, keratic precipitates, and graft edema (Panda *et al.*, 2007). Corneal transplant rejection is a process in which a corneal graft that has been clear for 5-7 days in horses (Brooks *et al.*, 2008) or 2 weeks in humans suddenly develops graft edema in conjunction with anterior segment inflammatory signs (Panda *et al.*, 2007). The corneal edema could be produced as part of a sudden rejection to the transplant. However, in this study, corneal edema was present in all the transplanted corneal parts from the first examination just after the surgery. Thus, the corneal edema might be developed during the surgical procedure especially in the procedure for donor cornea preparation. When another examination was done 3 weeks after the operation, the corneal edema had resolved itself in all the cases.

A corneal haze appeared near the junction of the transplant and the suture lines; furthermore, the epithelial overgrowth was observed in these areas. In particular, these reactions were identified on the recipient side of the cornea, but the epithelial cells of the central part of the donor cornea maintained their normal morphology. Alpha-SMA positive cells were detected in these transplanted junction. Especially, the majority of green fluorescein positive cells were revealed at the bottom of the overgrown and deformed epithelium. Therefore, these deformed hyperproliferative epithelium were demonstrated to be the main cause of the haze.

Overgrown epithelial cells were detected more on the inside of the recipient cornea. Corneal epithelial breach can be caused by exposed suture knots and a loose suture, which are some of the predictors for the occurrence of corneal haze during the post-operative period (Tham *et al.*, 2002). These factors give rise to irritation, leading to a subsequent corneal ulcer and corneal vascularization. Therefore, these reactions could be predisposing factors to graft rejection (Tham *et al.*, 2002). In these cases, the haze may invade the adjacent host cornea which is nearer to the vascularization (Fontana *et al.*, 2007).

The spotted haze in the deep stroma near DM was observed at 150 days postoperatively in the clinical evaluation. But, the green fluorescein positive cells were not detected in these areas. This means that there was an absence of myofibroblast which was the main type of cells for the corneal haze generation. Whereas, some of the donor DM particles and pre-DM fibers were observed at the same area in histological examinations. Accordingly, the spotted haze was suggested to be the result of light

scattering by these ununiformed DM particles. Since corneal edema had been accompanied with these ununiformed DM particles until the day 28 postoperatively, the spotted haze could only be revealed after the edema had disappeared on the day 150 postoperatively.

For human ophthalmology, much equipment has been developed for delicate surgical techniques such as an excimer laser or femtosecond laser (Albarez *et al.*, 2014; Levinger *et al.*, 2014). This equipment has been used to reduce corneal haze as well as other post-surgical complications. In the veterinary field, BBT can be used to maintain the vision of patients that have a wide range of corneal diseases while preserving the healthy endothelium. Furthermore, considering the quality of life of the animal, BBT is a better surgical method for restoring vision.

Conclusions

Corneal transplantation using DALK with the BBT could be performed in a dog that has a large corneal defect and vision loss with a healthy endothelium.

GENERAL CONCLUSIONS

This study was designed to develop the AK technique for the standardization of corneal haze model and DALK in dogs.

AK, which was performed 375 μm depths and 8 mm diameter, was used to achieve the desired corneal thickness after resection and produced a more sufficient corneal haze than CK technique. Therefore, this technique would contribute to improving standardization of the corneal haze model for the evaluation of therapeutic agents' effects.

The effect of onion extract ointment on corneal haze suppression was evaluated to test the utility of AK for the experimental model. The corneal haze was significantly suppressed in the onion extract treated group compared with the control group. Also, the expression of α -SMA, a specific marker for myofibroblasts, was significantly decreased in the onion extract ointment treated group compared with the control group. Accordingly, the onion extract ointment could be useful as one of therapeutic agents in preventing corneal haze development through the down-regulation of fibroblast transdifferentiation into myofibroblast.

DALK using a BBT was applied in three dogs to establish the feasibility of corneal transplantation. The central portion of the transplanted cornea remained transparent while corneal haze developed around the transplanted margin. The menace response was normal, and the transplant rejection was not observed in all eyes until the end of experiments. Consequently, corneal transplantation using DALK with the BBT could be performed in a dog that has a large corneal defect with a healthy endothelium.

Through these studies, AK could contribute to improving standardization of the corneal haze model. This is the useful experimental technique for the evaluation of corneal haze suppression, and the onion extract ointment could have potential as a therapeutic agent in preventing corneal haze development. Also, this study demonstrated the feasibility of corneal transplantation in dog eyes by DALK using a BBT.

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국문초록

개에서 각막흐림모델과 심부표층각막이식을 위한 공기주입 각막절제술의 개발

지도교수 서 강 문

김 수 현

서울대학교 대학원
수의학과 임상수의학 전공

본 연구는 개에서 표준화된 각막흐림모델과 심부표층각막이식을 위한 공기주입 각막절제술의 개발을 목적으로 실시되었다.

균일한 깊이와 크기로 각막 부분절제를 실시하고 효과적인 각막흐림을 발생시키기 위해 공기주입 각막절제술을 개발했다. 도축 후 적출된 돼지 눈을 네 군으로 나누어, 각각 250, 375, 500, 750 μm 의 깊이로 각막 실질을 절개했다. 절개된 단면에서 절개면 위쪽의 실질로 4 mL의 공기를 주입한 후 불투명한 흰색으로 변한 부분을 제거했다. 375 μm 이상의 깊이로 절개한 군들에서 절개한 각막 깊이와 공기 주입 후 절제된 각막의 깊이 사이에

유의적인 차이가 나타나지 않았다. 각막흐림의 발생 정도를 평가하기 위해 여섯 마리의 개에 공기주입 각막절제술과 기존의 각막절제술을 실시했다. 기존의 각막절제술에 비해 공기주입 각막절제술을 실시했을 때, 각막흐림의 발생과 평활근 액틴의 발현이 유의적으로 많았다. 그러므로, 공기주입 각막절제술은 원하는 두께로 각막절제가 가능하고 충분한 각막흐림을 발생 시킨다.

공기주입 각막절제술의 각막흐림 평가에 대한 실험적 유용성을 확인하기 위하여, 본 기법의 적용 후 양파 추출물 안연고의 각막흐림 발생 억제효과를 평가했다. 공기주입 각막절제술을 실시 한 후, 네 군으로 나누어 인공눈물, 프레드니솔론 아세트산염, 양파 추출물 안연고, 전환성장인자- β 1을 각각 적용 했다. 인공눈물 점안 군과 비교 했을 때, 술 후 14일부터 프레드니솔론 아세트산염과 양파 추출물 안연고를 점안한 군에서 각막흐림의 발생이 유의적으로 억제 되었다. 또한, 면역형광염색 결과 프레드니솔론 아세트산염과 양파 추출물 안연고를 점안한 군에서 평활근 액틴의 발현을 의미하는 녹색 형광강도가 유의적으로 억제되었다. 결과적으로, 양파 추출물 안연고의 각막흐림 억제 효과가 입증되었으며, 공기주입 각막절제술은 이러한 효과를 평가하기에 유용한 방법이었다.

개에서 각막이식의 실현 가능성 평가하기 위해, 공기주입 각막절제술의 기반이 된 공기주입기법을 이용한 심부표층각막이식술을 세 마리의 실험 견에서 실시했다. 임상적인 안검사 결과 이식된 각막의 주변부와 뒤흥계판

근처의 이식 경계면 일부에서 국소적인 각막흐림이 발생되었으며, 면역형광염색 실험에서 각막흐림의 주요 원인인 근섬유모세포의 표시인 평활근 액틴이 이식 경계면에서 확인되었다. 이 평활근 액틴은 뒤경계판 부분에서는 확인되지 않았으며, 조직 사진에서 이 부위에 공여 각막의 뒤경계판 조각의 일부가 관찰되었다. 국소적인 각막흐림을 제외하면, 술 후 150일까지 이식 거부반응 없이 각막의 구조 및 시력이 유지되었다.

본 연구의 결과 공기주입 각막절제술은 표준화된 각막흐림모델로서 각막흐림의 실험적 평가에 유용한 방법이며, 공기주입기법을 이용한 심부표층각막이식술은 개에서 이식 거부반응을 유발하지 않고 적용이 가능했다.

주요어: 각막흐림, 공기주입 각막절제술, 공기주입기법, 개,

심부표층각막이식술, 양파 추출물 안연고

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