Transplantation of autologous corneal epithelial cell sheet cultured from human limbus

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ABSTRACT

Transplantation of autologous corneal epithelial cell sheet cultured from human limbus

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With patients with complete limbal stem cell deficiency due to chemical and thermal burns, corneal conjunctivalization with the invasion of goblet cells occurs, leading to corneal opacification and visual loss. In this study using limbal biopsies of patients, we developed a culture system in which limbal keratinocytes are cultured on lethally-irradiated 3T3 cells. The kertinocytes were cultured to confluency to form sheets for transplantation. The resulting autologous corneal epithelial cell (HCEC) sheets (Holoeye®) were used to treat damaged limbus of cornea due to chemical burn. We sought to evaluate the effectiveness of corneal epithelial sheets on corneal wound repair.

Holoeye® was prepared with HCECs at passage 2. p63, keratin 19 and connexin 43 were expressed throughout the sheet whereas sp1 was not expressed indicating that differentiated cells exist.

Three eyes of 3 patients underwent Holoeye® transplantation. In all 3 eyes, the entire corneal surface was free from epithelial defects after surgery, showing complete survival of the transplanted corneal epithelium. Therefore, cultivated corneal epithelial cell sheet (Holoeye®) transplantation can be used for severe stem cell deficiencies

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Key words: limbal stem cell deficiency, corneal epithelial sheet (Holoeye®)

Transplantation of autologous corneal epithelial cell sheet cultured from human limbus

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(Directed by Assistant Professor Hyung Keun Lee)

I. INTRODUCTION

The anterior ocular surface is covered with corneal, limbal, and conjunctival epithelial cells. The cornea epithelium is a stratified squamous epithelium devoid of goblet cells, with a cuboidal basal layer lying on the avascular cornea stroma by the Bowman′s layer. The conjunctival epithelium consists of loosely organized cell layers populated by mucin−secreting goblet cells, which maintains the tear film on the ocular surface.[1, 2] The epithelium of the limbus serves as a junctional zone between

the cornea and conjunctival epithelium and provides the source of corneal epithelial regeneration.[3-7]

In patients with complete limbal stem cell deficiency due to chemical and thermal burns, corneal conjunctivalization with the invasion of goblet cells occurs, leading to corneal opacification and visual loss.[8] Autologous lamellar kerato-limbal grafts along with conventional allogenic penetrating keratoplasty can be used to treat these patients.[9-11] However, a large limbal withdrawal from the uninjured eye is required for transplantation of autologous limbal grafts, which cannot be performed on patients with bilateral limbal deficiency. In addition, the donor eye is subjected to a potential risk of limbal deficiency. To circumvent these limitations, corneal cells from the limbus were serially cultured and applied to the damaged cornea.[12] It was reported that autologous corneal cells cultured on fibrin or amniotic membrane restored bilateral or partial limbal deficiency, respectively.[13]

In this study using limbal biopsies of patients, we developed a culture system in which limbal keratinocytes are cultured on lethally-irradiated 3T3 cells according to

the method described for epidermal keratinocytes.[14,15] The kertinocytes were cultured to confluency to form sheets for transplantation. The resulting corneal epithelial cell sheets (Holoeye®) were used to treat damaged limbus of cornea due to alkali burn. We sought to evaluate the effectiveness of corneal epithelial cell sheets on corneal wound repair.

II. MATERIALS AND METHODS

1. Subjects

The study consisted of 3 eyes of 3 patients who underwent cultured autologous human corneal epithelial cell (HCEC) transplantation at Yonsei University medical center from December 2003 through September 2004. They consisted of two males and one female clinically diagnosed as total stem cell deficiency due to chemical burns. In accordance with the Helsinki Agreement, informed consents were obtained from all three patients.

2. Human corneal epithelial cell (HCEC) culture

Human corneal epithelial cell (HCEC) was isolated from the limbal area (1-2 mm^2) of normal eyes of 14 donors of different ages. HCECs were cultured on a lethally

irradiated feeder layer of 3T3 cells.[14,15] In brief, samples were treated with trypsin (0.05% trypsin and 0.01% EDTA) at 37°C for ~80 min. Cells were collected every 20 minutes. Cells were plated ($1.5x10^4$ /cm^2) on lethally irradiated 3T3 cells($2.4x10^4$ /cm^2) and cultured in 5% CO^2 and humidified atmosphere in DME and Ham′s F12 media (2:1 mixture) containing FCS (10%), insulin (5 mg/ml), adenine (0.18 mM), hydrocortisone (0.4 mg/ml), cholera toxin(0.1 nM), triiodothyronine (2 nM), glutamine (4 mM), and penicillinstreptomycin (50 IU/ml). Epidermal growth factor (10 ng/ml) was added at 10 ng/ml beginning at the first feeding, 3 days after plating. Cultures were then fed every other day. Subconfluent primary cultures were passaged at a density of $6x10^3$ cells/cm^2 and cultured as above. For serial propagation, cells were passaged as above, always at the stage of subconfluence, until they reached senescence.

These cells were cultured up to 9 passages (referred to as P1 for primary culture and P10 for passage 9). At every passage, cells were fixed for immunostaining and lysed for immunoblotting for corneal markers described below.

For determination of the Colony-forming efficiency, cells (300–2,000) from each biopsy and from each cell passage of serially cultivated mass and clonal cultures were plated onto 3T3 feeder layers and cultivated as above. Colonies were fixed 12 days later, stained with rhodamine B and scored under a dissecting microscope. Values are expressed as the ratio of the number colonies on the number of inoculated cells. All colonies were scored whether progressively growing or aborted.[15] The number of cell generations was calculated using the following formula: $x = 3.322 \log N/No$, where N equals the total number of cells obtained at each passage and No equals the number of clonogenic cells. Clonogenic cells were calculated from the colony-forming efficiency data (see above), which were determined separately in parallel dishes at the time of cell passage.

For three patients enrolled in the study, a limbal biopsy of contralateral eye from each patient was obtained to isolate HCECs. These cells were cultured to confluency for 11days, after which autologous corneal epithelial cell sheets were detached from the culture flask and grafted onto damaged cornea along with amniotic membrane.

3. Immunoblotting and Immunostaining

Confluent sheets of epithelium generated by either mass or clonal cultures were detached from the vessels with Dispase II.[16] Specimens were fixed in paraformaldehyde (4% in PBS) overnight at 4 °C and embedded in paraffin. Sections were immunostained with antibodies raised against keratin 3 (anti-human mouse IgG, Biodesign, Saco, Maine, USA), p63(anti-human mouse IgG, Pharmigen, San Diego, CA, USA), sp1(anti-human mouse IgG, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and connexin 43(anti-human mouse IgG, Chemicon , Temecula, CA, USA).

Five μm frozen sections of Holoeye® were prepared on which imunofluorescent staining was performed according to the standard procedure, using primary antibodies against connexin 43, p63, sp1 and keratin19. Mounting solution containing DAPI (Vectashield®, Vector laboratories, Burlingame, CA, USA) was used, and the resulting fluorescence was analyzed with Axioskop 40 (Carl Zeiss Co., Jena,

Germany).

4. Surgical procedure of transplantation of autologous corneal epithelial sheets (Holoeye®)

A 360° peritomy was performed 5 mm peripheral to the limbus in the recipient eye. The resulting conjunctival frill was excised and bleeders cauterized. A #15 blade attached to a Band–Parker handle was used to perform superficial keratectomy, leaving a relatively clear stromal bed. We then treated the residual subconjunctival fibroblasts for 5 minutes with 0.04% mitomycin-C by placing the small tips of microsponges containing mitomycin-C inside the subconjunctival spaces avoiding the bare sclera, together with vigorous saline washing. We secured the cultured corneal epithelial cell sheet (Holoeye®) onto the corneal surface with 10-0 nylon sutures and then covered it with amniotic membrane. Temporary tarsorrhaphy or bandage contact lens was applied on the recipient eye. Topical application of 0.3% ofloxacine

(Ofloxacine®, Samil Pharm., Seoul, Korea) and 1 % predforte (Predforte®, Samil pharm., Seoul, Korea) 4 times a day were prescribed to prevent postoperative inflammation and rejection in the operated eye. Then the amniotic membrane was removed in the second week after operation.

III. RESULTS

1. Growth potential of HCECs

The growth potential of corneal epithelial cell was determined by measuring the colony forming efficiency (CFE) at passage 2 through 9. The cultured media show that the growth is greatest in passage 3 (Figure 1A). The CFE abruptly decreased to 25 % at passage 4 but gradually from passage 4 to passage 9(2%) (Figure 1B).

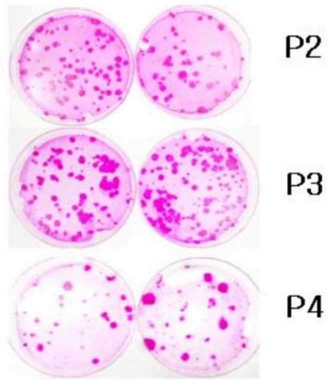


Figure 1A. Determination of the colony-forming efficiency (CFE). Culture of the Human corneal epithelial cells (HCEC) from passage 2 to 4 which were stained with Rhodamine B. HCEC growth is greatest in passage 3.

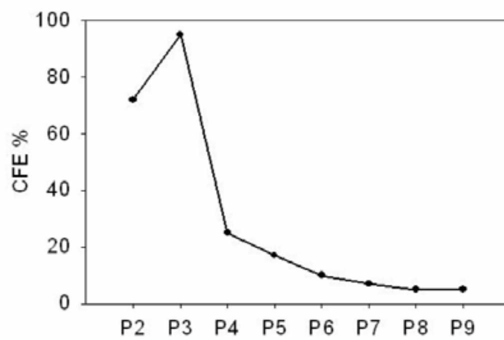
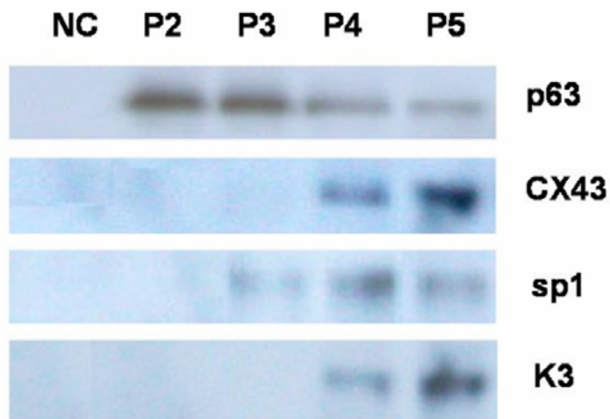


Figure 1B. Growth potential of Human corneal epithelial cells (HCEC) by measuring colony forming efficiency (CFE). CFE is the greatest in passage 3 decreasing to 25% at passage 4.

2. Differentiation program of HCEC markers

In order to investigate the degree of stemness in these cultures, immunoblotting using antibodies against p63 was performed. The level of p63 expression was highest at passage 2 and gradually decreased as the passage number increased. In contrast to p63, the expression of differentiation markers such as connexin 43 and keratin 3 increased upon subculturing with the highest level observed at passage 5. Interestingly, the expression of sp1 preceded that of either connexin 43 or K3 (Figure 2).



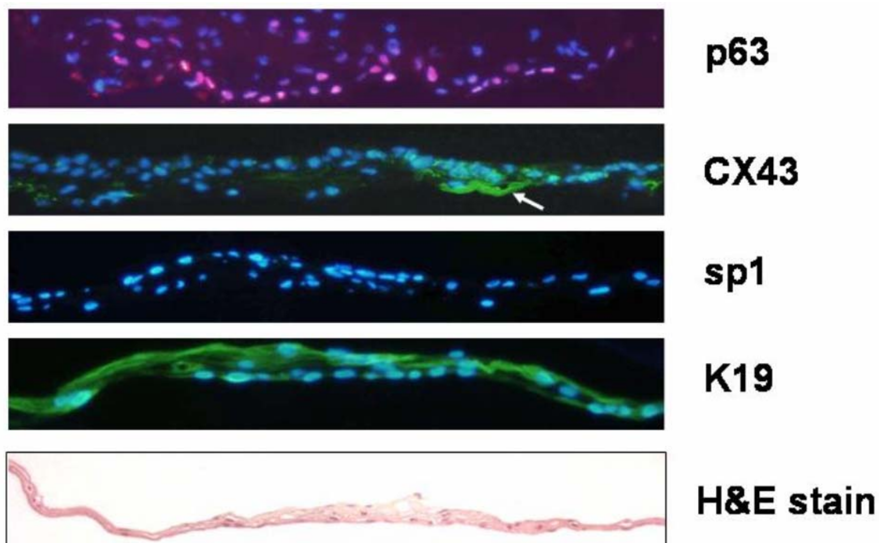
P: passage

CX 43: connexin 43

Figure 2. Western blot analysis of Human corneal epithelial cells (HCEC) using antibodies against p63, sp1, connexin 43, and keratin 3 from passage 2 to passage 5. p63 was detected in all passages, with the highest level at passage 2. In contrast, connexin 43 and keratin 3 showed highest level at passage 5. sp1 was detected prior to detection of connexin 43 and keratin 3. The first lane(NC) is the control with no HCEC loading. Each line was loaded with same amount of total HCEC.

3. Expression of Marker proteins in Holoeye®

Holoeye® was prepared with HCECs at passage 2 and the resulting frozen sections were analyzed for expression of proliferation/differentiation markers. p63 and keratin 19 were expressed throughout the sheet whereas sp1 was not expressed. Different from the data with cells (Figure 2), connexin 43 was expressed sporadically in the sheet indicating that differentiated cells exist to some extent in Holoeye®.



CX 43: connexin 43

K 19: keratin 19

H&E stain: Hematoxylin and eosin stain

Figure 3. Immunohistochemical staining of p63, connexin 43, sp1 and keratin 19 on frozen section of Holoeye®. p 63, Connexin 43 and keratin 19 was expressed in Holoeye® but, sp1 was not expressed. *Hoechst 33342 staining was used as counterstaining (blue).*

4. Transplantation of Holoeye® onto the damaged cornea

Case1: A 30-year old male presented to our emergency center in October 2003 due to acidic burn on both eyes. Visual acuity was counting fingers on the right eye and 20/20 on the left eye. Total subepithelial opacity with large epithelial defect with superior limbic ischemic damage was noted in his right eye and minimal epithelial defect with opacity was noted on the left eye. Emergent treatment with massive irrigation of normal saline was done. Within a few days epithelial defect healed in the left eye. But symblepharon was formed causing shortening of both superior and inferior fornices in the right eye with remaining epithelial defect. Symblepharon lysis with amniotic membrane transplant was performed on the right eye but the epithelial defect still remained after 2 months of treatment. Several limbal cell transplants were done without success resulting in total conjunctivalization of the cornea (Figure 4A). In September 2004, limbal cell harvest was done from the left eye at 3 o′clock position and cultivated limbal cell (Holoeye®) transplantation was carried out. Four

weeks after surgery, the grafts had taken up well, and complete reepithelialization was documented in 3 months (Figure 4D).

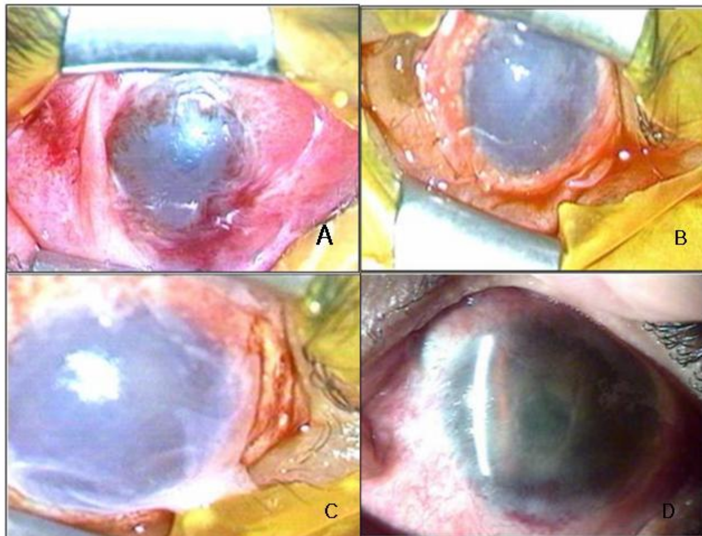


Figure 4. Case1: 30-year old patient with alkali burn on the right eye. Photographs were taken during and after the operation. A. Total conjunctivalization was noted after chemical burn. B. Holoeye® applied in the damaged cornea. C. Amniotic membrane was applied to secure the Holoeye® D. 3 months after the operation; total reepithelialization was noted with normal looking cornea epithelium.

Case 2: A 56 year-old woman sustained injury to her left eye when NaOH accidentally fell into her eye in May 2002. After initial treatment by a local ophthalmologist, she came to our institute 1 day after the accident. On evaluation, the right eye was unremarkable in all respects. Limbal ischemia was evident with total epithelial defect and stromal opacity in the left eye. The epithelial defect failed to heal with medical management. Amniotic membrane transplant was done two times which failed to reepithelize the persistent epithelial defect. In January 2003, autologous limbal cell transplant was performed without success. Three months after the surgery, conjunctival ingrowth had covered the graft at nasal and temporal portion with superficial vascularization. Stromal opacity with neovascularization was also noted indicating nonfunctioning limbal cells (Figure 5A). Therefore, cultivated limbal stem cell (Holoeye®) transplant was performed in December 2003. Seven days after the operation, the Holoeye® completely covered the cornea without epithelium defect (Figure 5B, C). Two months after the surgery, penetrating keratoplasty was

performed on the left eye. Eight months after surgery, the visual acuity was 20/50 with clear epithelium (Figure 5D).

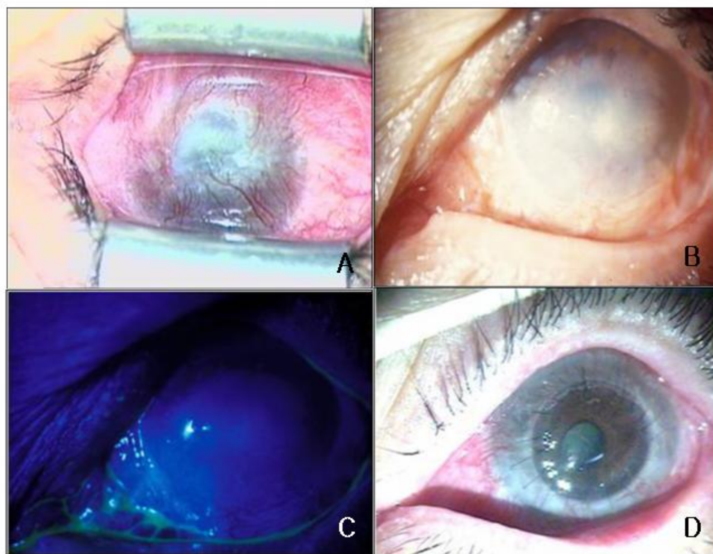


Figure 5. Case 2: 56-year old patient with alkali burn on the left eye. Photographs taken during before and after the operation. A. Total conjunctivalization with neovascularization of the stroma was noted after chemical burn. B. Holoeye® applied in the damaged cornea completely covers the cornea in postoperative day 7. C. Fluorescent staining of the cornea indicating intact epithelium. D. 2 months after the operation, penetrating keratoplasty was performed.

Case 3: A 30-year-old man sustained injury to his left eye when hydrochloric acid accidentally fell into his eye in July 2003. After initial treatment by his local ophthalmologist, he went to another institute where he received amniotic membrane transplant two times. On evaluation in January 2004, visual acuity in the left eye was 10/200. Total limbal ischemia was evident with subepithelial opacity and stromal vascularization. Hence, limbal cell biopsy was performed in January 2004. After cultivation, cultivated limbal stem cell (Holoeye®) transplantation was performed. Complete reepithelialization was attained at postoperative 2 weeks. In November 2005 penetrating keratoplasty was performed on his left eye. Nine months after surgery, the visual acuity was 20/200 with clear corneal epithelium.

IV. DISCUSSION

Recently, therapeutic techniques for reconstruction of the ocular surface have been greatly advanced by the introduction of autologous or allogenic limbal epithelial cell transplantation. Still, limitations lie in using autologous or allogenic limbal epithelial cells, such as the amount and frequency of the limbal cells that could be used in autologous grafts and allograft rejection in allogenic grafts. Therefore, cultivation of autologous limbal epithelial cells could diminish such limitations. But, the effectiveness of this therapeutic trial will be determined by the quality of the cultivated corneal epithelial cells. In this study, we have demonstrated that cultured corneal epithelial cells contain similar differentiation with normal corneal keratocyte resulting in restoring the damaged corneal limbus with normal looking healthy limbus with additional advantages.

We were able to cultivate corneal epithelial cells from 14 biopsies of donors ranging from 2 to 75 years old and to maintain corneal stem cells from these patients

for more than 4 passages in culture independent of their age (data not shown). As the passage number increased in cultivating the corneal epithelium, the expression of p63, known to be a cell proliferation marker, gradually decreased in HCECs but that of connexin 43(a protein induced early in differentiation) and of keratin 3(a known corneal differentiation marker) increased. This observation indicates that a normal differentiation program takes place in HCECs as in the case of human epidermal keratocytes. A confluent corneal epithelial sheet (Holoeye®) was prepared by culturing HCECs at passage 2 for 11 days. In the sheet, a differentiation marker, keratin 3, along with connexin 43 was detected, which contrasted the result with subconfluent cultures of P2 (Figure 2). However, p63 and keratin 19 were abundantly expressed as in the subconfluent cultures (Figure 3). Therefore, our cultured corneal epithelial sheets contain both corneal stem cells and differentiating cells, closely resembling human corneal epithelium. When these sheets were applied on the damaged cornea, complete coverage was observed in 3 patients and maintained for, at least, 3 months with no damage to the donor eye.

V. CONCLUSION

In the previous methods of limbal cell transplantation, limbal deficiency of the donor eye due to removal of a relatively large piece of limbus for transplantation has been reported in rabbits.[4] Our method could substantially reduce the likelihood of this complication, because only a small piece of limbus is removed. Moreover, this method can also be used when both eyes have limbal deficiency but when the location of the deficiency differs in the two eyes. There are also results of ex vivo expansion of autologous limbal epithelial cells on amniotic membrane which provide sufficient limbal epithelial cells for transplantation.[17] Our method has an advantage that no amniotic membrane is needed in cultivating the corneal epithelial sheet and while two to three weeks is required for cultivation in the previous method, our method requires only 11 days. Also, since there is no barrier such as the amniotic membrane between the basal layer of the corneal epithelial sheet and the damaged cornea, we believe that this may hasten the healing process.

Based on these results, we conclude that the use of cultured corneal epithelial sheet, Holoeye®, could accelerate the reepithelialization of corneal wound with less risk to the donor cornea.

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국 문 요 약(in Korean)

각막 윤부에서 자가 배양한
corneal epithelial cell sheet 의 이식

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연세대학교 대학원 의학과

노미인

화학 또는 열화상에 의한 전체 각막 윤부 결핍증 환자에서는 각막표면의 결막화가 발생하며 각막신생혈관, 만성 염증 및 불량한 상피 지속성, 섬유화 증식으로 인한 각막의 반흔화로 인하여 각막은 투명성과 무혈관성을 잃게 되어 결국 실명에 이르게 된다.

본 연구에서는 환자의 각막 윤부에서 얻은 조직을 lethally-irradiated 3T3 cell 을 이용하여 윤부 상피세포를 배양한 후 융합하여 corneal epithelial sheet (Holoeye®)를 만든 후 화학 화상에 의해 윤부에 손상이 있는 환자에 이식하여 각막 재생에 미치는 효과를 알아보고자 하였다.

화학적 화상으로 각막 혼탁이 있는 3 안을 대상으로 배양된 autologous corneal epithelial cell sheet (Holoeye®)을 이식하였다. 이에 대한 면역학적 검사 결과 p63 과 K19 는 발현되었으나 sp1 은 발현되지 않았다. 또한 Connexin 43 은 sheet 의 전반에 걸쳐서 발현이 되었다. 이는 Holoeye®에 분화중인 세포가 있음을 보여주는 것으로 각막 상피 세포의 특성을 지니고 있음을 알 수 있다.

Holoeye®를 이식한 3 안에서 수술 후에 각막 상피의 결손이 사라졌으며 이는 Holoeye®가 성공적으로 이식이 되었음을 보여주는 것이다. 따라서

cultivated corneal epithelial sheet (Holoeye®)는 각막 윤부 결핍증의 치료에 유용한 방법으로 생각된다.

핵심 되는 말: 각막 윤부 결핍증, corneal epithelial cell sheet(Holoeye®)