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의학 석사 학위 논문

각막윤부줄기세포 결핍 토끼 모델에서 효소감응성 세포시트 기반의 인간 각막윤부상피 세포의 이식

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Cultivated human corneal limbal epithelial transplantation based on enzyme-sensitive cell sheet in a rabbit limbal stem cell deficiency model

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국문 요약

연구 목표: 배양된 사람 각막 윤부상피세포 시트의 이식은 각막 상피와 시력을 회복하는 것에 효과적이라고 증명된 바 있다. 각막 윤부상피세포의 배양은 여러 방법으로 시행되어왔다. 이에 본 저자들은 효소감응성기반의 새로운 배양 기반을 개발하여, 토끼 윤부줄기세포 결핍 모델을 통해 이 방법의 효과를 확인하고자 하였다.

연구 재료 및 방법: 2~2.5kg 나가는 뉴질랜드 토끼 수컷 12 마리가 본 연구에서 사용되었다. 토끼 윤부줄기세포 결핍 모델은 윤부상피절제술과 NaOH 0.5mol/L 를 30 초간 적용한 것을 통해 만들었다. 본 저자들은 토기의 각막 상태를 4 주후에 평가하였고 토끼 윤부줄기세포 결핍 모델을 분류하였다. 그 분류는 각막혼탁 및 각막신생혈관의 발생정도로 4 단계로 나누어 분류하였다. Carboxymethyl cellulose-dopamine (CMC-DOPA) 가 코팅된 Transwell 이 기증자의 각공막 변연부로부터 얻어진 사람 윤부상피세포 배양에 사용되었고, 배양액은 3 일, 5 일, 7 일후에는 매일 교환되었다. 배양된 사람 윤부상피세포 시트 상태는 조직학적 검사와 면역화학적염색에 의해 평가되었으며, 면역화학적염색방법에 사용된 항체로는 anti-CK12, anti-CK3/2p, anti-p63, anti-Zo-1, anti-CK15, Alexa Fluor 647 혹은 555 혹은 488 이 사용되었다. 흉터 조직이 제거된 토끼 각막에 준비된 세포 시트를 이식한 후에, 그 이식 상태를 현미경적 소견, 조직학적 소견, 그리고 면역화학적염색을 통한 소견으로 평가하였으며, 면역화학적염색방법에는 Alexa Fluor 647 가 사용되었다. 이러한 평가는 그룹 1 에서는 2 주후에, 그룹 2 에서는 4 주후에 시행되었다.

연구 결과: 총 12 마리의 수컷의 12 개의 우안이 포함되었으며, 그룹 1 에는 3 개체, 그룹 2 에는 9 개체가 포함되었다. 그룹 1 에서, 각막 혼탁의 단계는 이식 전에 3.67±0.58 그리고 이식 후 2 주째에 3.00±0.00 였으며, 각막 혼탁의 단계는 감소하였으나 통계적인 차이는 없었다 (P = 0.184). 각막 신생 혈관의 단계는 이식 전에 3.33±0.58 그리고 이식 후 2 주째에 3.00±0.00 였으며, 각막 신생 혈관의 단계는 감소하였으나 통계적인 차이는 없었다 (P=0.423). 그룹 2 에서, 각막 혼탁의 단계는 이식 전에 3.78±0.44 그리고 이식 후 4 주째에 3.11±0.33 였으며, 각막 혼탁의 단계는 감소하였고 통계적인 차이가 있었다 (P = 0.004). 각막 신생 혈관의 단계는 이식 전에 3.11±0.60 그리고 이식 후 4 주째에 3.11±0.60 였으며, 각막 신생 혈관의 단계는 차이가 없었다

(P=1.000). 5 개체가 조직학적 검사와 면역화학적염색을 통한 검사가 이루어졌으며, 이중 4 개체에서 사람 윤부상피세포 시트가 4 주간 생존하였다.

결론: 토끼 윤부줄기세포 결핍 모델에서 효소감응성세포시트 기반의 배양된 사람 윤부상피세포 이식은 4 주간 각막 혼탁 감소에 효과가 있었다. 또한, 이식된 세포 시트 중 80%에서 4 주간 생존해 있었다. 이를 통해 볼 때, 배양된 윤부상피세포 시트의 이식은 향후 윤부줄기세포결핍 치료에 중요한 역할을 할 것으로 생각된다.

Keywords: Limbal stem cell(LSC), Limbal stem cell deficiency(LSCD), Enzyme-sensitive cell sheet, Cultivated human corneal limbal epithelial cell, rabbit LSCD model

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Introduction

Limbal stem cells (LSCs) are essential to maintain corneal epithelium and endowed with a capacity for self-renewal and extended proliferative potential[1, 2]. Dysfunction or loss of these cells by chemical or thermal injury, radiation, repeated surgical interventions, immunological disorders, neoplasia, and anirida, results in limbal stem cell deficiency (LSCD). This is characterized by loss of corneal transparency, scarring in the form of conjunctivalization, and visual loss[3].

Management of patients with LSCD depends on the extent of involvement of the limbus (sectoral vs total) and on the unilaterality or bilaterality of the disease. For partial LSCD, mechanical debridement of the conjunctival epithelium from the surface of the cornea can be enough to restore a stable ocular surface as stem cells from the healthy limbal sectors divide and migrate to cover the defect. Scraping of the conjunctival epithelium can be coupled with amniotic membrane transplantation, which may allow for faster healing of the ocular surface[4, 5].

Management of patients with total LSCD has always been challenging because corneal clarity cannot be restored merely by a traditional corneal graft. Penetrating keratoplasty (PKP) is contraindicated in the setting of LSCD. For many years, treatment involved autologous (in cases of unilateral LSCD) or allogeneic keratolimbal grafts[6-12]. However, donor tissues are often insufficient, resulting in difficulty of treatment when needed.

Recent advances in understanding limbal physiology and manipulating limbal stem cells ex vivo allow for the possibility of restoration of a healthy ocular surface with tissue-sparing surgery. This decreases the need for large limbal resection that may jeopardize the homeostasis of the "healthy" eye. These new tissue-sparing techniques also decrease the need for allogeneic tissue in some cases, thus eliminating the need for chronic immunosuppression[8, 13-17].

Transplantation of cultivated limbal epithelial sheets has proven to be effective to restore healthy corneal epithelium and vision[18-23]. Several substrates have been utilized to culture limbal epithelial cells such as human amniotic membranes[18], fibrin gels[24], plasma polymer-coated surfaces[25], biodegradable matrices[26], temperature-responsive culture dishes[27], or feeder layers of mouse NIH 3T3 cells[21].

As shown in previous studies, cultivation of limbal epithelial cell was performed by various methods. We have developed a new culture substrate called enzyme-sensitive substrate by own method, and tried to demonstrate the effectiveness of this method through rabbit LSCD model.

Materials & Methods

1. Preparation of rabbit LSCD model and classification of LSCD grading

Previous studies have described how to make a rabbit LSCD model. Alkali burn of the ocular surface[28-33] in which NaOH is used for ocular surface burning, and the concentration used are 1mol/L [28, 31-35], and 0.5mol/L [29]. The application times are 20s [30], 25s [28, 29], 30s [31-33], and 45s [29]. Limbal mechanical excision, in which the limbal epithelium is mainly removed by limbectomy [36-41], n-heptanol [35, 37, 38], and 75% ethanol [37], or using microsurgical instruments [40, 41].

A total of 12 (12 males) healthy New Zealand rabbits weighting 2-2.5Kg were used in this study. After general anesthesia, the anterior segment was fully exposed with the eye speculum. The limbal epithelium was removed surgically. A 13mm paper disk embedded in 0.5mol/L NaOH was applied over the cornea and limbal zones of each right eye for 30s, and the eyes were thoroughly washed with normal saline. The debridement of loosing corneal epithelium was done. After application of NaOH, the eye speculum was removed and the rabbits were treated with eye ointment (Forus : Neomycin Sulfate 3.5 mg/g, Dexamethasone 1 mg/g, Polymyxin B Sulfate 6000 IU/g; Samil. Co., Ltd, Seoul, Korea) for 10 days.

After 2 weeks later, we evaluated the state of rabbit's cornea and classified the rabbit LSCD model in group 1. After 4 weeks later, we evaluated the state of rabbit's cornea and classified the rabbit LSCD model in group 2. The classification of rabbit LSCD model was divided into 4 grades in corneal opacity and neovascularization [42].

This study was approved by the Laboratory Animal Ethics Committee of Asan Medical Center of Ulsan University.

2. Method of carboxymethyl cellulose-dopamine (CMC-DOPA) coating

The 1 mg/ml CMC-DA was dissolved in 10 mM Tris buffer solution (Sigma Aldrich) (pH 8.5). The Transwell membranes were coated with the prepared CMC-DA solution for 2 hours at RT. After the coating step, the Transwell plate membranes were washed with distilled water twice and dried in a heating oven for 6 h at 40 °C. Transwell plate membrane sterilization was conducted under an ultraviolet (UV) lamp (λ = 254 nm, 15 W) for 30 minutes.

3. Preparation of cultivated human limbal epithelial sheets

Human donor corneoscleral rims obtained during surgery after 8 mm or less trephination of the graft (surgical corneas) were used. Iris, ciliary body, Decemet's membrane with corneal endothelium, conjunctiva, and excess sclera were surgically removed from the corneosclera. Limbal epithelium was isolated by the treatment with 0.2% Dispase Ⅱ(Roche) in 35mm dish) at 4℃ for overnight. Subsequently , epithelium were treated with 0.25% Trypsin/0.01% EDTA (Gibco. USA) at 37℃ for 1h to generate isolated cells, followed with the dissociation by pipetting to obtain the mixture of single cells and cell aggregates. Dissociated cells were seeded in plastic cell culture inserts (3450, Corning). Cells were grown in 96% DMEM/F12 (Gibco), FBS (4%)(Gibco), recombinant human FGF7 (KGF, 10 ng/mL peprotech 100-19), Y27632 (10 μ M, Enzo), insulin (10 μ g/mL sigma I0516), hydrocortisone (0.5 μ g/mL sigma H0888), tri-iodo-thyronine (2 nM sigma T6397), isoproterenol (250 ng/mL sigma I6379), and 1% antibiotics. Medium was changed at day 3, day 5, and every day after day 7.

Cell culture inserts were coated with 1mg/ml CMC-DA and seeded human limbal epithelial cell on the 6well at 3~5x10^5cells per well. After the epithelial cells reached confluence, human limbal epithelial cells were continued for two weeks with underlying medium replacement daily. To obtain cell sheets were treated medium containing 100U/ml cellulose (Cellulase from *Trichoderma* sp. Sigmaaldrich) the bottom of the insert at 37℃ for 1hr. After detaching cell sheets from the insert, they were aspirated using a pipette and washed using PBS and transferred to a fresh cell culture dish. Human epithelial cell sheets were used for transplantation and imunohistochemistry.

4. Evaluation of cultivated human limbal epithelial sheet status by histology and immunohistochemistry

Cultivated epithelial cell sheets or rabbit tissue were cut in half. One half was fixed with formalin and embedded in paraffin, and the other half was embedded in optimum cutting temperature compound and frozen in liquid nitrogen. Paraffin sections or formalin-fixed cryosections were stained with hematoxylin and eosin (HE) for histologic examination. For immunohistochemistry, cryosections were fixed with 4% paraformaldehyde for 5 minutes and permeablilized with 0.1% triton x-100, and incubated with 10% goat serum for 1hr and were reacted with anti–human mitochondria (1:30, 1hr, RT milipore MAb1273). Paraffin sections were deparaffinized in xylene and rehydrated. We were microwaved in citrate buffer (genemed)or EDTA buffer(trilogy cell marquee) for 20mins. Slides were then washed with phosphate-buffered saline tween (PBST, twice for 10 min) and incubated for 1 h in a 5% BSA, 5% normal goat serum and 5%normal donkey serum. Slides

were then incubated with the anti-CK12 (1:100, overnight, 4℃, abcam ab185627), anti-CK3/2p (1:100, overnight, 4℃ santacruz sc-80000), anti-p63(1:100, overnight, 4℃ abcam ab124762), anti-Zo-1(1:100, overnight, 4℃ thermofisher 61-7300), and anti-CK15 (1:100, overnight, 4℃ thermofisher MA5-11344) antibodies, and incubated with Alexa Fluor 647 or 555 or 488-conjugated anti rabbit, goat, and mouse IgG antibodies(invitrogen). Cell nucleus was counterstained with DAPI (invitrogen). The stain was captured by confocal microscopy (LSM 780).

5. Method of cell sheet transplantation

After observing the rabbit LSCD models, the scar tissue composed of conjunctiva and neovascularization was removed. The prepared limbal epithelial cell sheet was placed on the cornea with scar tissue removed and a 4 point suture at peripheral cornea was performed. For protection, the ocular surface was covered with a contact lens, and a tarsorrhaphy was performed. After cell sheet transplantation, the rabbits were treated with eye ointment (Neomycin Sulfate 3.5 mg/g, Dexamethasone 1 mg/g, Polymyxin B Sulfate 6000 IU/g everyday), and triamcinolone acetonide (0.1 ml injected subconjunctivally every 1 week). An intramuscular injection of cyclosporine was administered daily for 7 days. A week later, the sutures of cornea and eyelid were removed and the contact lens was also removed. In group 1, two weeks after transplantation the rabbits were sacrificed, and in group 2, Four weeks after transplantation the rabbits were sacrificed. After sacrifice, the engrafted corneas were divided into portions for histology, and immunohistochemistry. The primary antibody used for anti-human mitochondria (Alexa Fluor 647).

6. Evaluation of transplantation status

In group 1, clinical outcome was observed in the first, and second week after surgery. In group 2, clinical outcome was observed in the first, second, and fourth week after surgery. Clinical outcome included grade of corneal opacity and corneal neovascularization, and status of fluorescein stained corneal epithelium. According to the ocular surface standard grading table [42], two indexes of corneal opacity and neovascularization grading were compared before transplantation and after second or fourth week. SPSS software was used for statistical analysis. The variables are demonstrated as mean ± standard deviation, and P-value < 0.05 was considered statistically significant. In addition to clinical outcome, results of histology and immunohistochemistry were used to evaluate the status of transplanted cell sheet in 5 eyes.

Results

1. Clinical outcome

Table 1 shows demographics of rabbit subjects. Total subjects were 12 males and 12 right eyes. Three subjects belong to group 1, and nine subjects belong to group 2.

Table 2 shows clinical outcome in group 1. In group 1, the grading of corneal opacity before and after surgery was 3.67±0.58 and 3.00±0.00, respectively. The grading of corneal opacity decreased, but there was no statistical difference (P = 0.184). In group 1, the grading of corneal neovascularization before and after surgery was 3.33±0.58 and 3.00±0.00, respectively. The grading of corneal neovascularization decreased, but there was no statistical difference (P = 0.423).

Table 3 shows clinical outcome in group 2. In group 2, the grading of corneal opacity before and after surgery was 3.78±0.44 and 3.11±0.33, respectively. The grading of corneal opacity decreased, and there was a statistically significant difference (P = 0.004). In group 2, the grading of corneal neovascularization before and after surgery was 3.11±0.60 and 3.11±0.60, respectively. The grading of corneal neovascularization was no difference (P = 1.000).

Figure 1 shows photographs of pre-operative, post-operative, after 1 week, and 2 weeks in group 1 subject number 4 of 04/16/2018. In pre-operative state, corneal opacity was grade 3 and corneal neovascularization was grade 3. In post-operative state, corneal opacity decreased and neovascularization was not seen. After 1 week, inflammation and neovascularization was observed at peripheral cornea. Corneal opacity decreased and corneal epithelial erosions were observed. After 2 weeks, neovascularization was observed at peripheral cornea and the extent of neovascularization was less than pre-operative state. Corneal opacity was better than pre-operative state and corneal epithelial erosions were observed. Overall, corneal opacity and neovascularization were better than pre-operative state.

Figure 2 shows photographs of pre-operative, post-operative, after 2 weeks, and 4 weeks in group 2 subject number 4 of 03/26/2018. In pre-operative state, corneal opacity was grade 4 and corneal neovascularization was grade 2. In post-operative state, corneal opacity decreased and neovascularization was not seen. After 2 weeks, inflammation and neovascularization was observed at peripheral cornea. Corneal opacity decreased and small corneal epithelial defect was observed in central cornea. After 4 weeks, neovascularization was observed at peripheral cornea and the extent of neovascularization was similar to pre-operative state. Corneal opacity was better than pre-operative state and small corneal epithelial defect disappeared. Overall, corneal opacity was better than pre-operative state, and neovascularization was similar to pre-operative state.

2. Histology and immunohistochemistry

In group 2 subjects, histology and immunohistochemistry were performed in five subjects (shown in Table 1).

Figure 3 shows photographs of pre-operative, post-operative, after 2 weeks, and 4 weeks in group 2 subject number 3 of 03/26/2018. In pre-operative state, corneal opacity was grade 4 and corneal neovascularization was grade 3. In post-operative state, corneal opacity decreased and neovascularization was not seen. After 2 weeks, inflammation and neovascularization was observed at peripheral cornea. Corneal opacity decreased and corneal epithelial defect was observed in central cornea. After 4 weeks, neovascularization was observed at total cornea. Corneal opacity was similar to pre-operative state, but slight better than pre-operative state at paracentral cornea. Also, cornea was covered with conjunctival tissue. Overall, corneal opacity was better than pre-operative state, but neovascularization was worse than pre-operative state. In immunohistochemistry (anti-human mitochondria; Alexa Fluor 647) after 4 weeks, only a small amount of human corneal epithelium was observed in the peripheral cornea. In histology after 4 weeks, rabbit cornea was epithelialized totally and goblet cells were observed in the epithelium.

Figure 4 shows photographs of pre-operative, post-operative, after 1 week, 2 weeks, and 4 weeks in group 2 subject number 4 of 04/02/2018. In pre-operative state, corneal opacity was grade 4 and corneal neovascularization was grade 4. In post-operative state, corneal opacity decreased and neovascularization was not seen. After 1 week, inflammation and neovascularization was observed at peripheral cornea. Corneal opacity decreased and corneal epithelial defect was observed in superior cornea. After 2 weeks, inflammation and neovascularization was aggravated at peripheral cornea. Also, corneal epithelial defect was aggravated in superior cornea. After 4 weeks, neovascularization was observed at total cornea. Corneal epithelial defect was observed at central cornea. Corneal opacity was similar to pre-operative state, but slight better than pre-operative state at central cornea. Overall, corneal opacity was slight better than pre-operative state, but neovascularization was similar to pre-operative state. In immunohistochemistry (anti-human mitochondria; Alexa Fluor 647) after 4 weeks, human corneal epithelium was observed in the total cornea, but the immunostaining was weak at central cornea. In histology after 4 weeks, rabbit cornea was covered with human corneal epithelium and goblet cells were not observed. However, many inflammatory cells deposited under human corneal epithelium.

Figure 5 shows photographs of pre-operative, post-operative, after 1 week, 2 weeks, and 4 weeks in group 2 subject number 2 of 04/09/2018. In pre-operative state, corneal opacity was grade 4 and corneal neovascularization was grade 4. In post-operative state, corneal opacity decreased and

neovascularization was not seen. After 1 week, inflammation and neovascularization was observed at peripheral cornea. Corneal opacity decreased and corneal epithelial defect was not observed. After 2 weeks, inflammation and neovascularization was aggravated at peripheral cornea. Also, corneal epithelial defect was observed in central cornea. After 4 weeks, neovascularization was observed at peripheral cornea, but was stabilized more than 2 weeks ago. Corneal epithelial defect was observed at central cornea. Corneal opacity was better than pre-operative state. Also, cornea was covered with conjunctival tissue. Overall, corneal opacity and neovascularization were better than pre-operative state. In immunohistochemistry (anti-human mitochondria; Alexa Fluor 647) after 4 weeks, human corneal epithelium was observed in the total cornea. In histology after 4 weeks, rabbit cornea was covered with human corneal epithelium and goblet cells were not observed.

Figure 6 shows photographs of pre-operative, post-operative, after 1 week, 2 weeks, and 4 weeks in group 2 subject number 2 of 04/16/2018. In pre-operative state, corneal opacity was grade 4 and corneal neovascularization was grade 3. In post-operative state, corneal opacity decreased and neovascularization was not seen. After 1 week, inflammation and neovascularization was observed at peripheral cornea. Corneal opacity decreased and corneal epithelial erosions were observed at central cornea. After 2 weeks, inflammation and neovascularization was slight aggravated at peripheral cornea. Also, corneal epithelial erosions were observed in central cornea. After 4 weeks, neovascularization was observed at peripheral cornea, but inflammation was observed. Corneal epithelial defect was observed at central cornea. Corneal opacity was better than pre-operative state. Overall, corneal opacity and neovascularization were better than pre-operative state. In immunohistochemistry (anti-human mitochondria; Alexa Fluor 647) after 4 weeks, human corneal epithelium was observed in the total cornea, but the surface was irregular. In histology after 4 weeks, rabbit cornea was covered with human corneal epithelium and goblet cells were not observed.

Figure 7 shows photographs of pre-operative, post-operative, after 1 week, 2 weeks, and 4 weeks in group 2 subject number 3 of 04/16/2018. In pre-operative state, corneal opacity was grade 3 and corneal neovascularization was grade 3. In post-operative state, corneal opacity decreased and neovascularization was not seen. After 1 week, inflammation and neovascularization was observed at peripheral cornea. Corneal opacity decreased and corneal epithelial defect was not observed. After 2 weeks, inflammation and neovascularization was slight aggravated at peripheral cornea. Also, corneal epithelial erosions were observed in central cornea. After 4 weeks, neovascularization was observed at peripheral cornea, but inflammation was observed. Corneal epithelial erosions and bulla were observed at central cornea. Corneal opacity was similar to pre-operative state. Overall, corneal opacity was similar to pre-operative state, and neovascularization was slight better than pre-operative state. In immunohistochemistry (anti-human mitochondria; Alexa Fluor 647) after 4 weeks,

human corneal epithelium was observed in the total cornea. In histology after 4 weeks, rabbit cornea was covered with human corneal epithelium and goblet cells were not observed. However, inflammatory cells deposited under human corneal epithelium.

Discussion

Transplantation cultivated limbal epithelial sheet has been considered as one of the standard treatments in LSCD [20, 21, 43-46]. However, the protocol for cultivation of limbal epithelial cells differs significantly. Protocols include different culture technique such as isolated epithelial cell cultures or limbal epithelial sheet generation from limbal explants, the application of the mouse 3T3 feeder cell layer, and different substrates [20, 21, 44, 45, 47-49].

In this study, we developed the new method called enzyme-responsive cell sheet and there were several advantages. First, cell culture was effective from isolated donor cell line. Second, cell sheet production and separation without cell degradation from transwells was effective. Third, at least 6 cell sheets from a small amount of cells.

In group 1, corneal opacity improved in two cases (2/3, 66.7%), and there was no change in one case (1/3, 33.6%). No cases were exacerbated. Corneal neovascularization improved in one case (1/3, 33.3%), and there was no change in two cases (2/3, 66.7%). No cases were exacerbated. In group 2, corneal opacity improved in six cases (6/9, 66.7%), and there was no change in three cases (3/9, 33.6%). No cases were exacerbated. Corneal neovascularization improved in one case (1/9, 11.1%), and there was no change in seven cases (7/9, 77.8%). But, there was a deterioration in one case (1/9, 11.1%).

The grade of corneal opacity was 3.67±0.58 before transplantation and 3.00±0.00 after 2 weeks in group 1, which showed improvement, but no statistical difference (P=0.184). However, in group 2, the grade of corneal opacity was 3.78±0.44 before transplantation and 3.11±0.33 after 4 weeks, which showed a statistically significant difference (P=0.004). The grade of corneal neovascularization was 3.33±0.58 before transplantation and 3.00±0.00 after 2 weeks in group 1, which showed improvement, but no statistical difference (P=0.423). However, in group 2, the grade of corneal neovascularization was 3.11±0.60 before transplantation and 3.11±0.60 after 4 weeks, which showed no difference (P=1.000).

As shown above, clinical outcome in this study showed only improvement of corneal opacity in group 2. But, there was no change of corneal neovascularization in group 2. In group 1, there was

improvement of corneal opacity and neovascularization, but no statistically significant difference. Results showed that only corneal opacity of group 2 improved. However, in the all cases of no change in group 1 and 2, the grade of corneal opacity and neovascularization was the same, but microscopic findings showed a decrease in the extent and severity of corneal opacity and neovascularization. This confirms that there is a partial improvement.

The outcome of histology and immunohistochemistry in this study showed that in only 1 case, human limbal epithelial cell sheet did not survive, but in the remaining 4 cases, human limbal epithelial cell sheet survived for 4 weeks. This confirms that enzyme-responsive based cell sheets survived relatively well in immunosuppressed rabbit LSCD eyes for 4 weeks.

There are several reasons for this outcome. First, heterologous transplantation(xenotransplantation) is one of the main reasons for this outcome. Despite the immunosuppressive treatment, xenogenic reaction occurs. We used the immunosuppressive treatment for only 1 week to improve the survival rate of rabbits. Because of immunosuppressive treatment for 1 week, corneal opacity and neovascularization at 2 weeks seem to be worse than at 1 week. If homologous or especially autologous transplantation is performed, better outcome is expected.

Second, the rabbit LSCD model in this study was a severe case of grade 3 or more in corneal opacity and neovascularization, except 1 case in group 2 (shown in table 1). It is already known that the response of treatment in severe cases is low [50]. If transplantation of human limbal epithelial cell sheet is performed, better outcome is expected. In the future, it will be necessary to experiment with low-severity LSCD models.

Third, we did not use the amniotic membrane transplantation(AMT) because we designed the experiment to confirm the sole effect of human limbal epithelial transplantation. AMT is excellent option for the treatment of ocular surface disease by especially chemical burn [50]. If AMT is performed with human limbal epithelial transplantation, better outcome is expected. In the future, it will be necessary to experiment with combination of AMT and human limbal epithelial transplantation.

In conclusion, transplantation of cultivated limbal epithelial cell sheet will play a significant role for LSCD treatment in the future. Tissue-sparing surgery by cultivated limbal epithelial cell sheet is advantage to preserve a healthy eye and decrease or eliminate the need for systemic immunosuppression, especially in the case of homologous transplantation. It is important to note that even with this potential treatment, presurgical planning will still play a catalytic role for the success of such procedures. A wet ocular surface, adequate eyelid function, and control of the ocular

surface inflammatory status needs to be restored before attempting any type of surgical rehabilitation for patients with LSCD.

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Abstract

Purpose: Transplantation of cultivated limbal epithelial sheets has proven to be effective to restore healthy corneal epithelium and vision. Cultivation of limbal epithelial cell was performed by various methods. We have developed a new culture substrate called enzyme-sensitive substrate by own method, and tried to demonstrate the effectiveness of this method through rabbit LSCD model.

Methods: A total of 12 (12 males) healthy New Zealand rabbits weighting 2-2.5Kg were used in this study. Rabbit LSCD model was made by combination of 0.5mol/L NaOH application for 30s and limbectomy. We evaluated the state of rabbit's cornea after 4 weeks and classified the rabbit LSCD model. The classification of rabbit LSCD model was divided into 4 grades in corneal opacity and neovascularization. Carboxymethyl cellulose-dopamine (CMC-DOPA) coated Transwell were used for human limbal epithelial cell culture from human donor corneoscleral rims with medium changed at day 3, day 5, and every day after day 7. Cultivated human limbal epithelial sheet status was evaluated by histology and immunohistochemistry including anti-CK12, anti-CK3/2p, anti-p63, anti-Zo-1, anti-CK15 antibodies and Alexa Fluor 647 or 555 or 488-conjugated anti rabbit, goat, and mouse IgG antibodies. After prepared cell sheet transplantation on the rabbit cornea with scar tissue removed, transplantation status was evaluated by microscopic findings, histology, and immunohistochemistry using anti-human mitochondria (Alexa Fluor 647). The status was evaluated after 2 weeks in group 1, and after 4 weeks in group 2.

Results: Total subjects were 12 males and 12 right eyes. 3 subjects belong to group 1, and 9 subjects belong to group 2. In group 1, the grade of corneal opacity before and after surgery was 3.67±0.58 and 3.00±0.00, respectively. The grade of corneal opacity decreased, but there was no statistical difference (P = 0.184). The grade of corneal neovascularization before and after surgery was 3.33±0.58 and 3.00±0.00, respectively. The grade of corneal neovascularization decreased, but there was no statistical difference (P = 0.423). In group 2, the grade of corneal opacity before and after surgery was 3.78±0.44 and 3.11±0.33, respectively. The grade of corneal opacity decreased, and there was a statistically significant difference (P = 0.004). The grade of corneal neovascularization before and after surgery was 3.11±0.60 and 3.11±0.60, respectively. The grade of corneal neovascularization was no difference (P = 1.000). 5 subjects were tested for histology and immunohistochemistry, and human limbal epithelial cell sheets survived for 4 weeks in 4 out of 5 subjects.

Conclusions: Cultivated human limbal epithelial transplantation based on enzyme-sensitive cell sheet in a rabbit LSCD model was effective in decreasing corneal opacity for 4 weeks. In addition, 80% of transplanted cell sheets were alive for 4 weeks. As a results, transplantation of cultivated limbal epithelial cell sheet will play a significant role for LSCD treatment in the future.

Table 1. Demographics of rabbit subjects.

Operation date (Month/Day/Year)	Rabbit number	Group	Pre-operation corneal opacity grade	Pre-operation corneal neovascularization grade	Pre-operation corneal opacity grade	Post-operation corneal neovascularization grade	Histology and immunochemistry
03/26/2018	3	2	4	3	4	4	O
04/02/2018	4	2	4	4	3	4	O
04/09/2018	2	2	4	4	3	3	O
04/16/2018	2	2	4	3	3	3	O
04/16/2018	3	2	3	3	3	3	O
03/26/2018	4	2	4	2	3	2	X
03/26/2018	5	2	4	3	3	3	X
03/26/2018	6	1	4	3	3	3	X
04/02/2018	2	2	4	3	3	3	X
04/02/2018	6	2	3	3	3	3	X
04/09/2018	1	1	4	4	3	3	X
04/16/2018	4	1	3	3	3	3	X

Table 2. Clinical outcome in group 1(N=3).

	Pre-operation grade	Post-operation grade	P-value
Corneal opacity	3.67±0.58	3.00±0.00	0.184
Corneal neovascularization	3.33±0.58	3.00±0.00	0.423

Table 3. Clinical outcome in group 2(N=9).

	Pre-operation grade	Post-operation grade	P-value
Corneal opacity	3.78±0.44	3.11±0.33	0.004
Corneal neovascularization	3.11±0.60	3.11±0.60	1.000

Figure Legends

Figure 1. Photographs of pre-operative, post-operative, after 1 week, and 2 weeks in group 1 subject number 4 of 04/16/2018. (A) Pre-operative state. (B) Post-operative state. (C) 1 week after transplantation. (D) 2 weeks after transplantation.

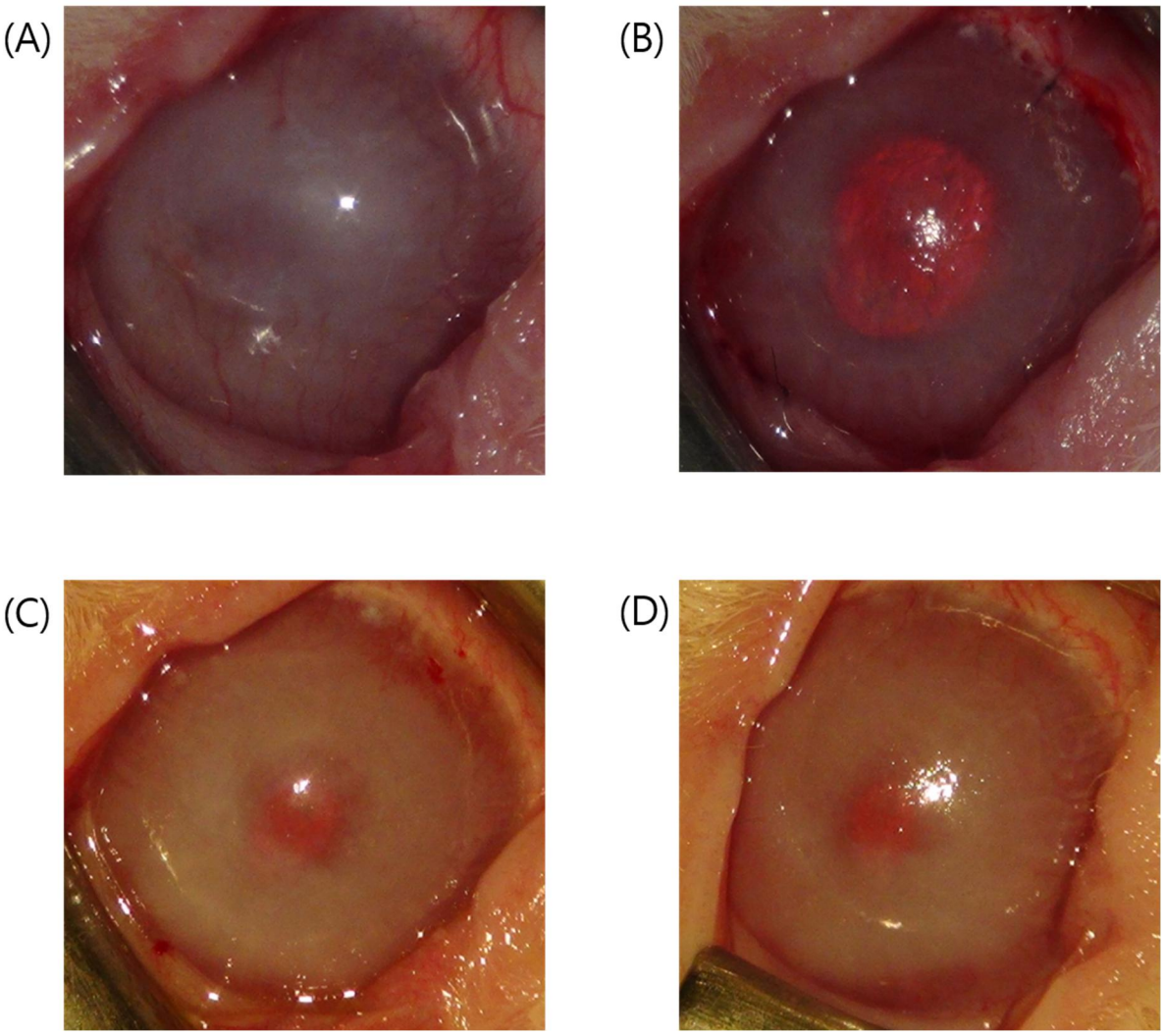

Figure 2. Photographs of pre-operative, post-operative, after 2 weeks, and 4 weeks in group 2 subject number 4 of 03/26/2018. (A) Pre-operative state. (B) Post-operative state. (C) 2 weeks after transplantation. (D) 4 weeks after transplantation.

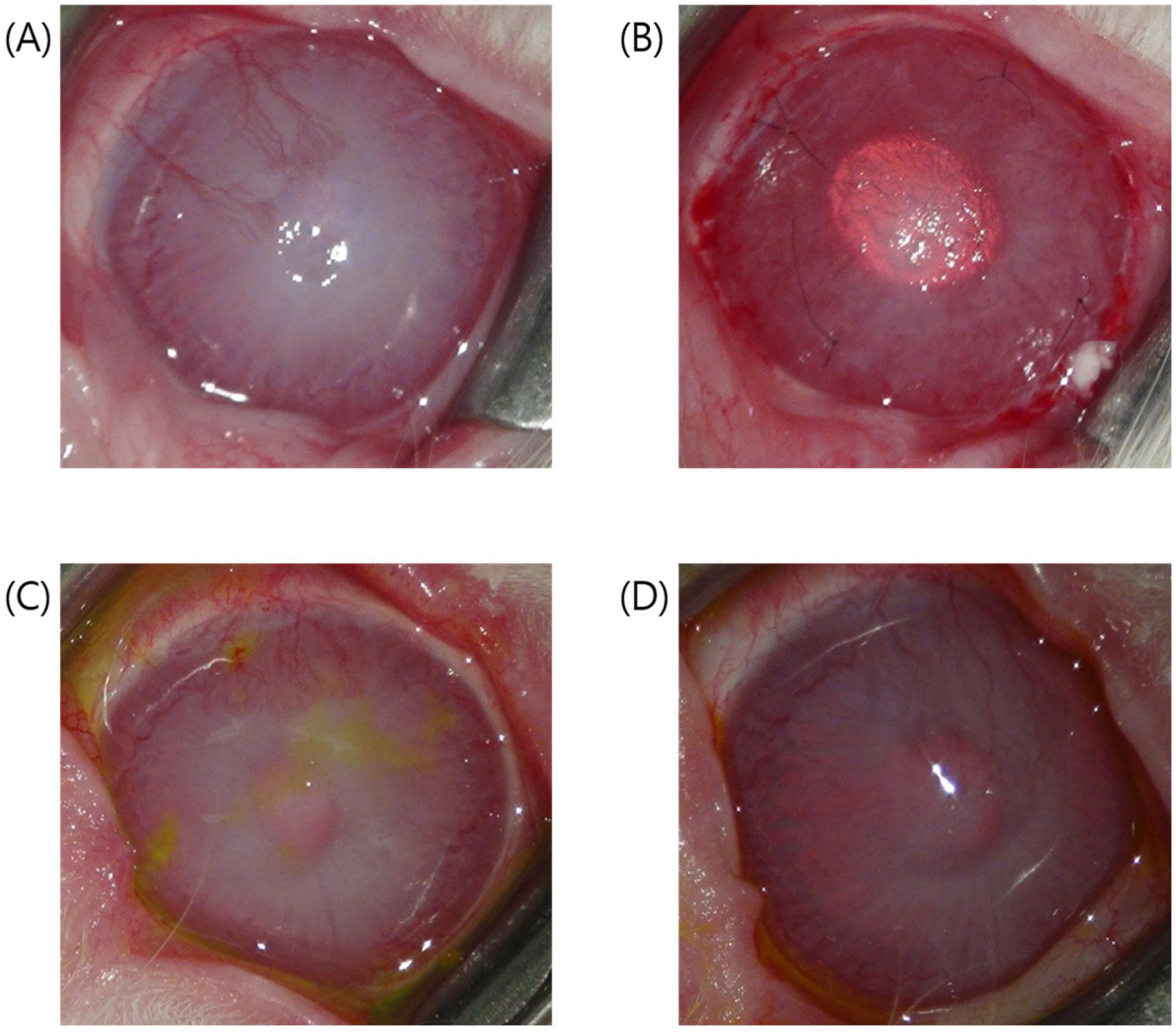

Figure 3. Photographs of pre-operative, post-operative, after 2 weeks, and 4 weeks in group 2 subject number 3 of 03/26/2018. (A) Pre-operative state. (B) Post-operative state. (C) 2 weeks after transplantation. (D) 4 weeks after transplantation. (E) Stained with hematoxylin and eosin for histologic examination. (F) Stained with anti-human mitochondria (Alexa Fluor 647) for immunochemistry examination.

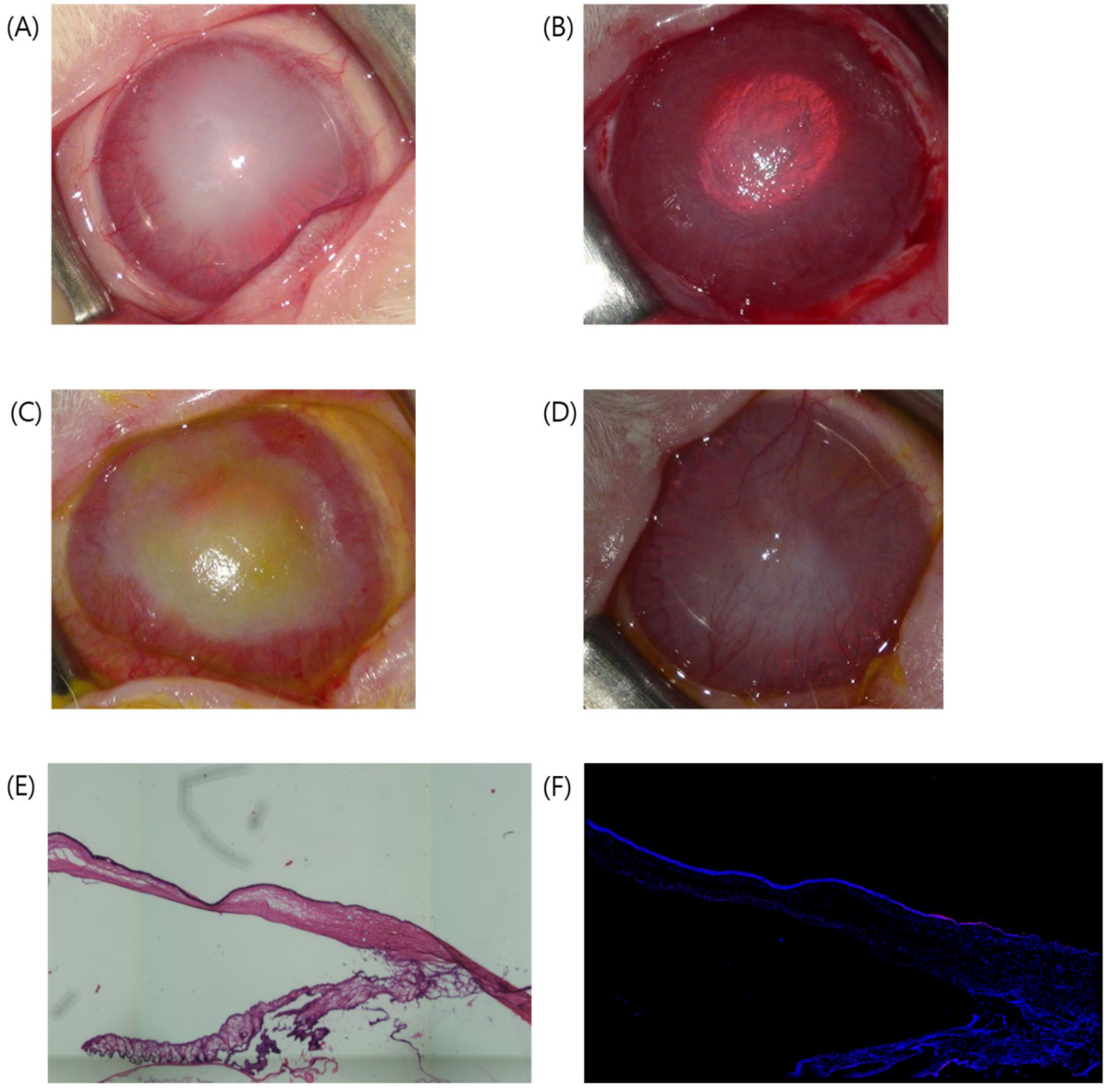

Figure 4. Photographs of pre-operative, post-operative, after 1 week, 2 weeks, and 4 weeks in group 2 subject number 4 of 04/02/2018. (A) Pre-operative state. (B) Post-operative state. (C) 1 week after transplantation. (D) 2 weeks after transplantation. (E) 4 weeks after transplantation. (F) Stained with hematoxylin and eosin for histologic examination. (G) Stained with anti-human mitochondria (Alexa Fluor 647) for immunochemistry examination.

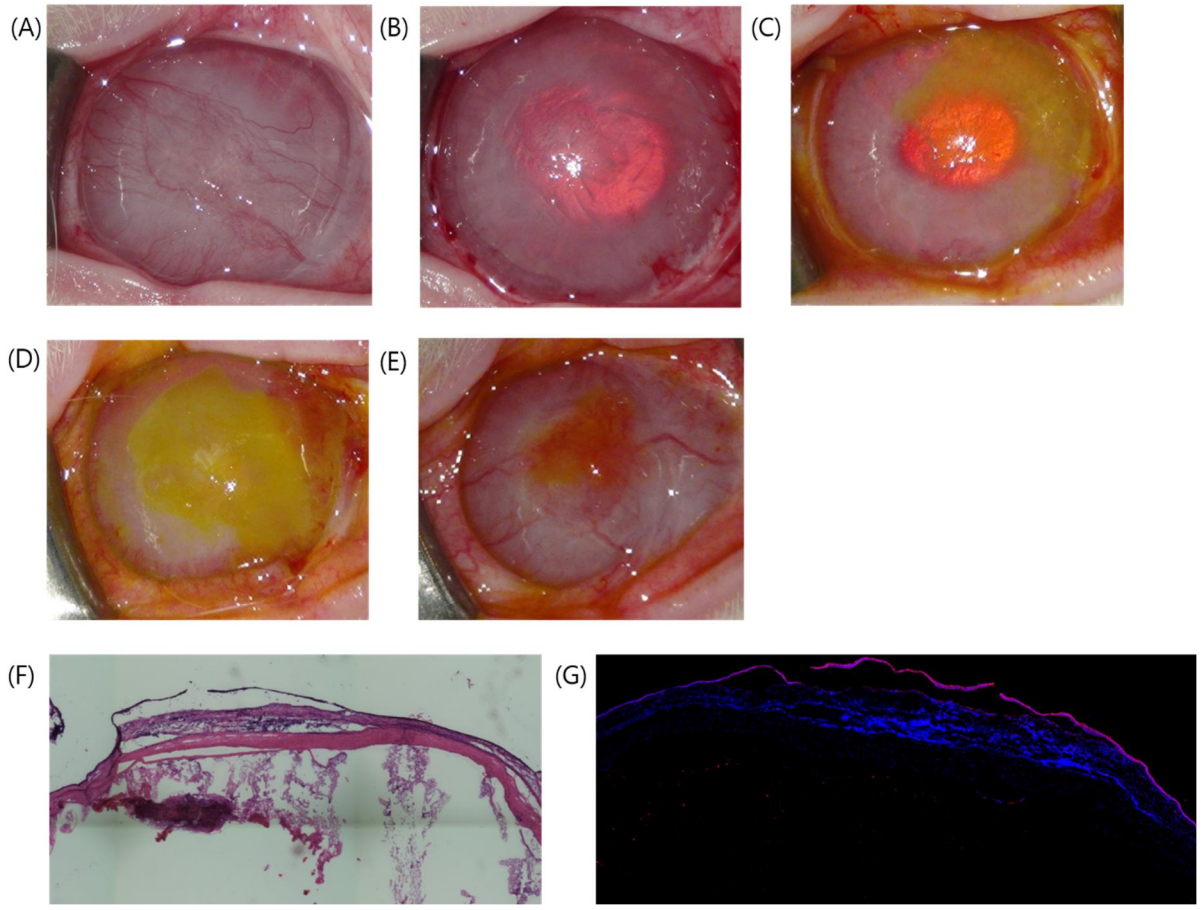

Figure 5. Photographs of pre-operative, post-operative, after 1 week, 2 weeks, and 4 weeks in group 2 subject number 2 of 04/09/2018. (A) Pre-operative state. (B) Post-operative state. (C) 1 week after transplantation. (D) 2 weeks after transplantation. (E) 4 weeks after transplantation. (F) Stained with hematoxylin and eosin for histologic examination. (G) Stained with anti-human mitochondria (Alexa Fluor 647) for immunochemistry examination.

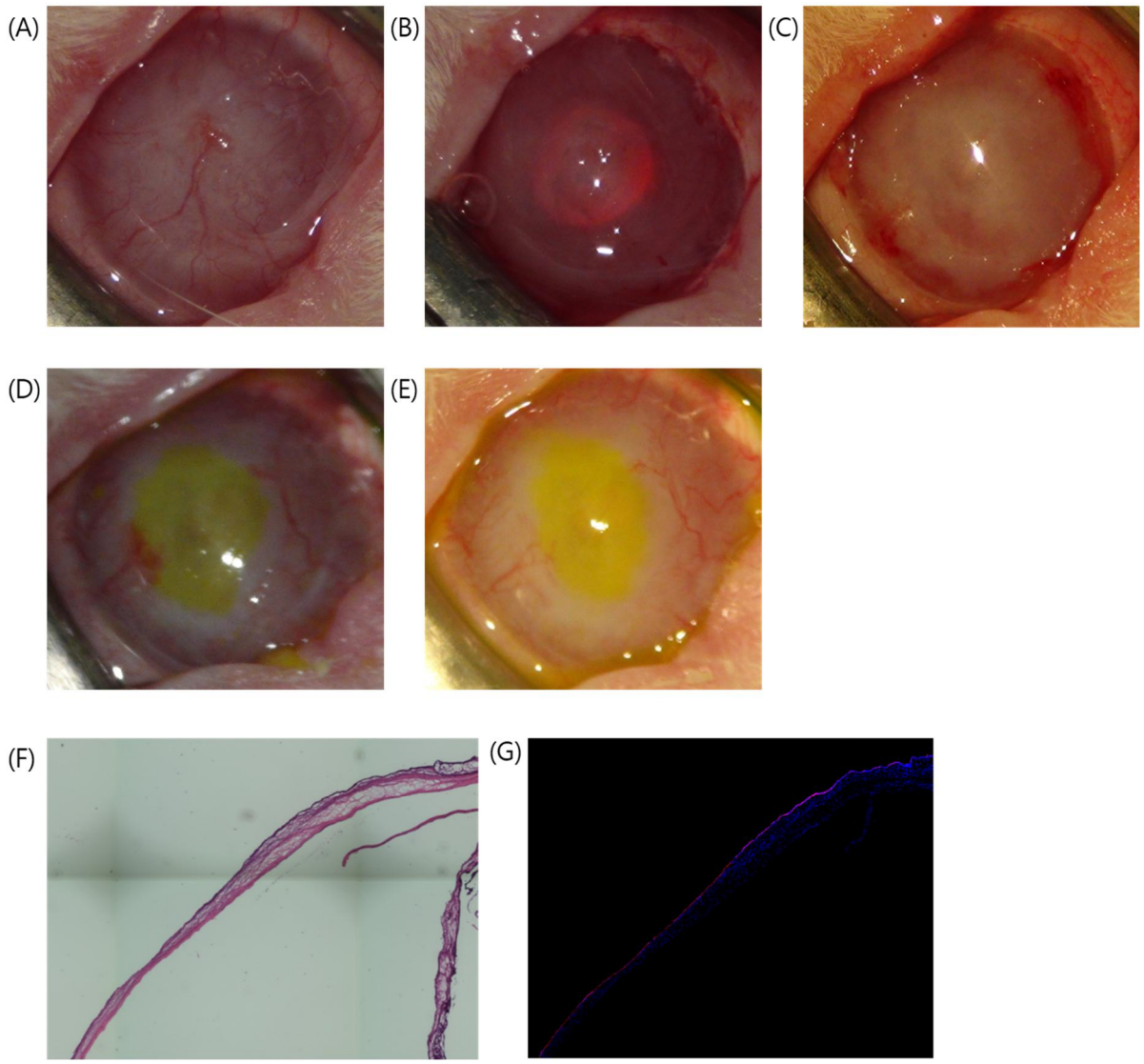

Figure 6. Photographs of pre-operative, post-operative, after 1 week, 2 weeks, and 4 weeks in group 2 subject number 2 of 04/16/2018. (A) Pre-operative state. (B) Post-operative state. (C) 1 week after transplantation. (D) 2 weeks after transplantation. (E) 4 weeks after transplantation. (F) Stained with hematoxylin and eosin for histologic examination. (G) Stained with anti-human mitochondria (Alexa Fluor 647) for immunochemistry examination.

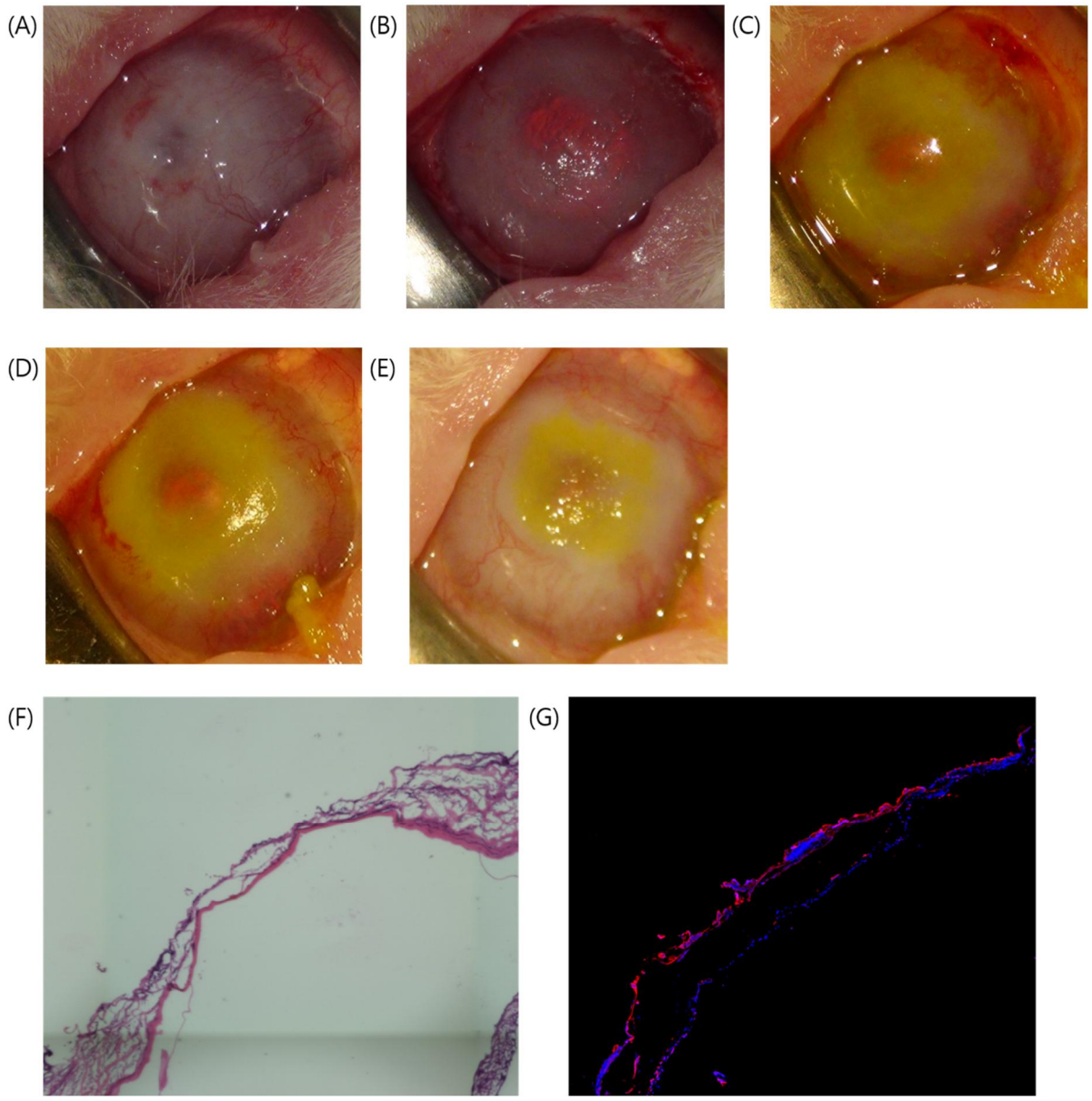

Figure 7. Photographs of pre-operative, post-operative, after 1 week, 2 weeks, and 4 weeks in group 2 subject number 3 of 04/16/2018. (A) Pre-operative state. (B) Post-operative state. (C) 1 week after transplantation. (D) 2 weeks after transplantation. (E) 4 weeks after transplantation. (F) Stained with hematoxylin and eosin for histologic examination. (G) Stained with anti-human mitochondria (Alexa Fluor 647) for immunochemistry examination.