·Basic Research·

Effects of preservation time on proliferative potential of human limbal stem/progenitor cells

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Abstract

• AIM: To determine the proliferative potential and the maintenance of stem cell activity in stored human limbal tissues, and correlate this with the preservation time, cell viability and the expression of stem cell markers.

• METHODS: Thirty limbal rims were split into 4 parts and stored in corneal preservation medium at 4℃ for 0, 1, 4, or 7 days. The limbal stem cell and mitotic markers P63, CK19, proliferating cell nuclear antigen (PCNA), and Ki67 were determined by immunohistochemical staining. The proliferative potential of limbal epithelial cells was assessed by cell viability, the ability of generating stratified epithelium, and colony forming assay.

• RESULTS: The stored tissues maintained limbal stratified structure to 7 days and exhibited comparable expression level of stem cell and mitotic markers. The proportion of viable cells decreased with the prolonged preservation time, while colony forming efficiency decreased from the 1st day and disappeared at the 4th day. When inoculated on amniotic membrane, the cells preserved for 1 day formed a stratified epithelium, while the cells from 4 days' preservation formed a discontinuous layer.

• CONCLUSION: The colony forming efficiency of limbal epithelial stem/progenitor cells decreased rapidly with the increasing preservation time, while the expression level of markers and capacity of forming epithelial monolayer on amniotic membrane decreased gradually. The limbal epithelial stem cells lost their function earlier than the lost expression level of stem cell markers. This may help us to better choose the appropriate preservation grafts for future limbal stem cell transplantation.

• KEYWORDS: limbal epithelial cells; proliferative potential; colony forming efficiency; preservation time
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INTRODUCTION

Overwhelming evidence confirmed corneal epithelial stem cells located in the basal region of the limbus and maintained the homeostasis of entire corneal surface[1,2]. Patients with limbal stem cell deficiency were unable to maintain a stable cornea, resulting in conjunctival invasion, vascularization and persistent epithelial defect [3,4]. Therapeutic methods were currently used to repair the defective limbal tissue, including conjunctival limbal autografts from healthy contralateral cornea, keratolimbal allografts from either fresh living related donors or eye bank cadaveric tissue, cultured limbal epithelial transplantation or oral mucosal epithelial transplantation[5].

However, donor corneas are still unable to meet the demand in many Asia countries, especially with the application of cultured limbal epithelial transplantation for the ocular surface reconstruction of patients with limbal stem cell deficiency. So the maintenance of the regeneration capacity of human limbal epithelium, especially the activity of limbal stem/progenitor cells in preservation was paid more attention in the recent years. The preservation media, commercial available as Optisol GS and Dexsol, have been compared by clinical applications and a variety of laboratory techniques. Their main concerns is to maintain the number, morphology, viability and function of corneal endothelial cells for penetrating keratoplasty, and seldom reported the effect on the epithelium stem cells[6,7].

Limbal stem/progenitor cell activity, especially the percent of p63-bright holoclone-forming stem cells in culture was critical for the successful therapy for the patients with limbal stem cell deficiency. However, there are still no specific markers for the identification of limbal stem cells and seldom report describing the functional assessment of limbal stem/progenitor cells after preservation. In the present study, we evaluated the proliferative potential and the maintenance of stem cell activity in stored human limbal tissues, and correlate this with the preservation time, cell viability and the expression level of stem cell markers. The results showed that the colony forming efficiency of limbal epithelial stem/progenitor cells decreased rapidly with the increasing preservation time, while the limbal structure integrity, expression level of limbal stem/progenitor cell markers and the capacity of forming confluent monolayer on amniotic membrane decreased gradually.

MATERIALS AND METHODS

Materials

Human tissue preparation and preservation medium This study was approved by the Ethics Committee of Shandong Eye institute and all experiments were carried out in accordance with the Declaration of Helsinki and Chinese National Guidelines on Stem Cell Research and Therapy. Human limbal samples were obtained from the Eye Bank of Shandong Eye Institute (Qingdao, China) after the central corneal button removed for penetrating keratoplasty. The donor age ranged from 22 to 45 years, mean age of 31.6±5.5 years. The death-to-preservation, preservation-to-surgery, and surgery-to-experiment time intervals of donor corneas were 2.8±0.9 h, 5.5±1.9 h and 1.1±0.3 h, respectively. Thirty corneoscleral rims were divided into 4 parts, 3 corneoscleral rims (12 pieces) for histology and immunohistochemical staining, 9 corneoscleral rims (36 pieces) for cell viability analysis, 9 corneoscleral rims (36 pieces) for colony forming assay, 9 corneoscleral rims (36 pieces) for cell culture on denuded amniotic membrane. Corneoscleral limbal rims were stored in corneal preservation media for 1-7 days at 4℃. The preservation media contains Minimum Essential Medium (9.4g/L, Gibco, Grand Island, NY), Chondroitin sulfate (25.0g/L, Sigma, St. Louis, MO), low-molecular Dextran (10.0g/L, Sigma), HEPES (9.5g/L, Sigma), Tobramycin(100mg/L, Shanghai XinYi Pharmaceutical Co., China), Dexamethone (20.0mg/L, Shandong XinHua Pharmaceutical Co., China) and L-Glutamine (0.375mg/L, Gibco). The media were modified from Dr. Xie's Chinese patent (No. CN1262866) named as "Cornea activity preservation fluid" and could maintain the cornea for 7 days without the detectable death of corneal endothelial cells.

Methods

Histology and immunohistochemical staining Corneoscleral limbal rims were fixed in 4% formaldehyde and paraffin-embedded serial sections were routinely stained with hematoxylin and eosin (HE staining). For the detection of limbal stem/progenitor cell and proliferative activity, sections were blocked with 1% normal goat serum, and incubated overnight at 4℃ with following primary antibodies containing anti-P63 (1:100, Invitrogen), anti-Cytokeratin 19 (1:100, Invitrogen), anti-PCNA (1:100, Santa Cruz), and anti-Ki67 (1:100, Santa Cruz). After washed with PBS, staining was visualized by using HRP- conjugated goat immunoglobulin G secondary antibody (1:100, Santa Cruz) with DAB (3, 3'-diaminobenzidine) as the substrate.

Cell viability analysis Limbal tissues were rinsed 3 times, incubated in 2.4U/mL Dispase II (Roche, Indianapolis, IN) in DMEM for 2 hours at 37℃. The limbal epithelium was removed under a dissecting microscope and treated with 0.25% trypsin+0.02% EDTA for 15 minutes at 37℃. Single cell suspensions were stained with 0.4% trypan blue for the quantification of cell viability with the Countess ® Automated Cell Counter (Invitrogen).

Colony forming assay Clonal efficiency of isolated limbal epithelial cells was evaluated by inoculating single-cell suspensions at a density of 2×10^3 cells/well in 6-well plate on mitomycin C-treated 3T3 feeder layer. Cells were incubated in DMEM/F-12 (1:3) medium supplemented with 10% fetal bovine serum (Gibco), ITS (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1nmol/L cholera toxin (Sigma), 2nmol/L 3,3'5-Triiodo-L-thyronine Sodium salt (Sigma), 0.4ng/mL hydrocortisone succinate (Wako, Osaka, Japan), 2mmol/L L-Glutamine (Invitrogen), penicillin-streptomycin (Hyclone, Logan, UT) and 10ng/mL recombinant human EGF (R&D Systems, Minneapolis, MN). Colony formation was monitored by phase contrast microscopy and analyzed on day 12 after removal of 3T3 feeder layer with 0.25% trypsin+0.02% EDTA, fixation in methanol for 5 minutes and staining with 10% Giemsa staining (Solarbio, Beijing). Colony-forming efficiency (CFE) was calculated as the number of clones/total number of cells seeded per well.

Limbal epithelial cell culture on denuded amniotic membrane Human amniotic membranes were treated with 0.02% EDTA at 37℃ for 2 hours and scraped gently with a cell scraper (Nunc International, Rochester, NY) to remove their amniotic epithelial cells. Single-cell suspensions were seeded on the denuded amniotic membrane at a density of 6×10^4 cells/well in 24-well plate on mitomycin C-treated 3T3 feeder layer. The cells were cultured until confluence (7-10 days) and air-lifted for 4 days, fixed and sectioned for H.E. staining.

Statistical Analysis The differences between control and experimental conditions were tested with Student's t tests. A P value of less than 0.05 was considered to be statistically significant.

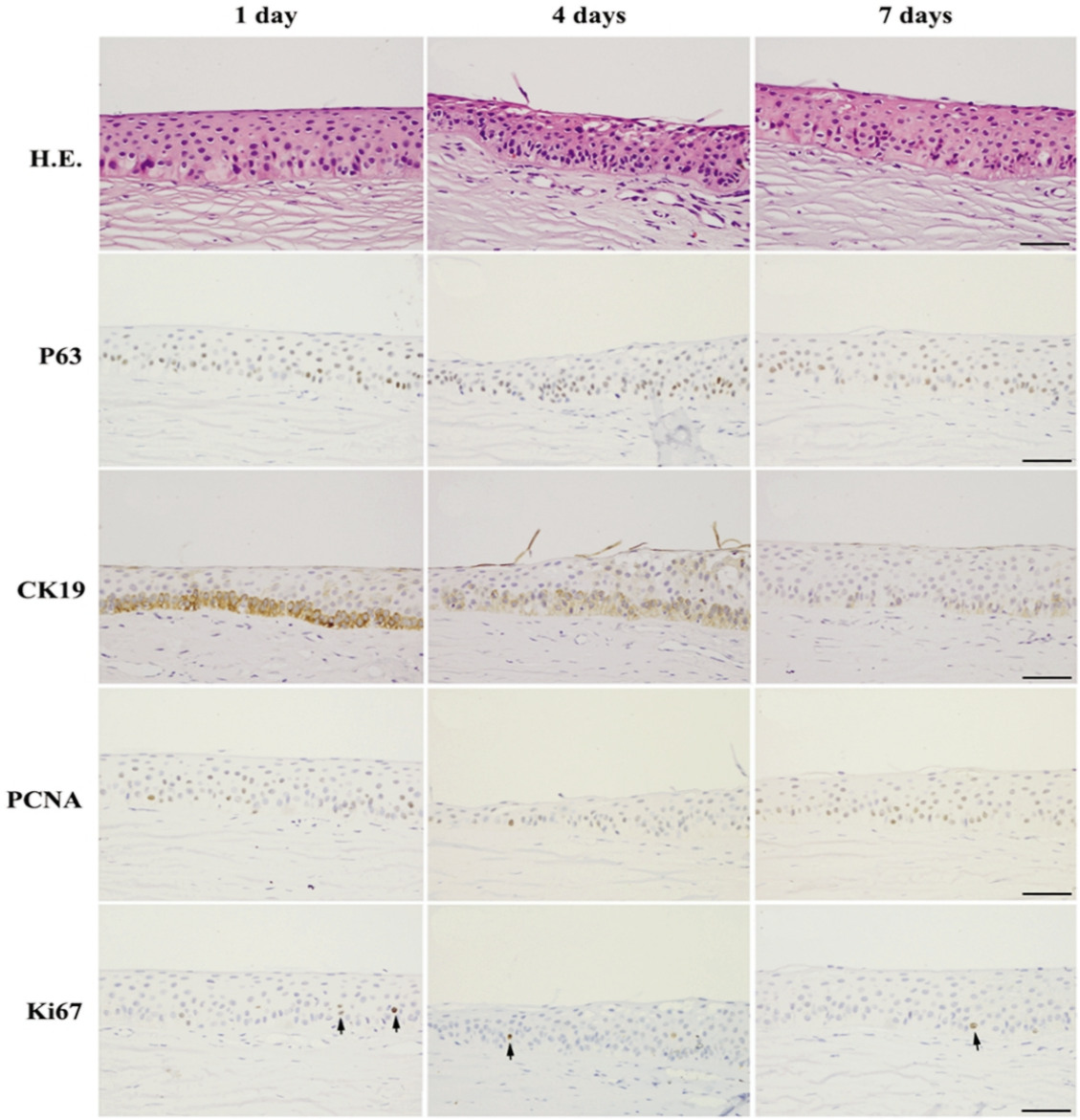

Figure 1 Section staining with HE and immunohistochemistry in limbal tissues after preservation. The limbal epithelium maintained typical stratified epithelial structure during the preservation of 7 days, but showed visible vacuolar degeneration in the superficial layer (4 days' preservation) or even in the basal layer (7 days' preservation). The expression level of P63, PCNA and Ki67 in the basal epithelium remained stable with the prolonged preservation time, while the expression density of CK19 decreased significantly after the preservation for 4 and 7 days. Arrows: positive cells with Ki67 staining. Bar: 100μm.

RESULTS

Limbal Epithelial Structure and Phenotypic Maintenance During the preservation time up to 7 days, limbal epithelium maintained typical stratified epithelial structure and did not assumed significant morphological changes from the results of HE staining. However, a few cells showed visible intracellular vacuoles in the superficial layer (4 days' preservation) or even in the basal layer (7 days' preservation) (Figure 1). Moreover, the basal epithelium remained the positive staining with limbal stem/progenitor cell markers (P63 and CK19) and mitotic markers (PCNA and Ki67) (Figure 1). It should be mentioned that the density of CK19 staining became weaker with the prolonged preservation time, and the other 3 markers did not show visible change in these sections.

Cell Viability Analysis To exclude the variation by manual counting, an automated cell counting method was adapted by using trypan blue staining. As shown in Figure 2, the proportion of viable cells was stable around 75% during the first days' preservation, while from the 4th day, the percent of viable cells in the total cells decreased significantly, and the variation in the same group also became different significantly.

Limbal Epithelial Cell Culture on Denuded Amniotic Membrane The capacity of forming epithelial monolayer or multilayer is important for the cultured limbal epithelium transplantation for the reconstruction of damaged ocular surface. The limbal epithelial cells from tissues preserved for different times were inoculated on the denuded amniotic membranes and co-cultured with the mitomycin C-treated

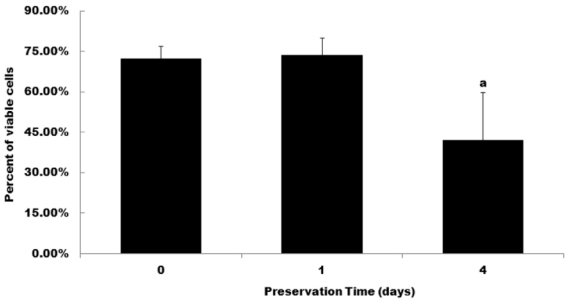

Figure 2 Cell viability analyses during the preservation of limbal tissues The proportion of viable cells was stable during the 0 to 1 day' preservation, while decreased significantly from the 4th day of preservation. [a] $P<0.05$ *vs* 0 day and 1 day group, respectively. Three experiments were repeated and representative results were shown (N=3).

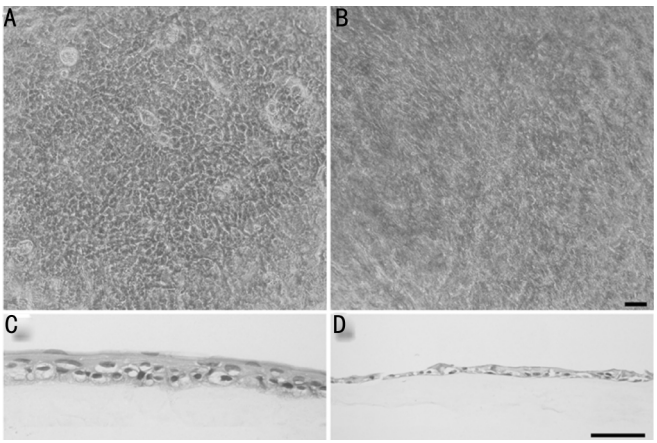

Figure 3 Limbal epithelium cultured on denuded amniotic membrane The limbal epithelial cells from tissues preserved for 1 day (A, C) or 4 days (B, D) were inoculated on the denuded amniotic membranes and co-cultured with the mitomycin C-treated 3T3 feeder layer for 11-14 days. The cells from 1 day's preservation became stratification with air-liquid culture (A) and formed multilayer epithelial structure (C), while the cells from 4 days' preservation could hardly stratified into multilayer structure on amniotic membrane, and cell morphology was not as regular as the cells from the tissues preserved for 1 day (B, D). Bar: 50μm.

3T3 feeder layer for 11-14 days. The results showed the cells from 1 day's preservation formed monolayer in 7 days, and became stratification when air-liquid culture for 7 days (Figure 3A), while the cells from limbal tissues preserved for 4 days could also proliferate into confluence in 10 days (Figure 3B), but hardly stratified into multilayer structure on amniotic membrane. Moreover, the morphology of limbal epithelial cells on amniotic membrane from the tissues preserved for 4 days was not as regular as the cells from the tissues preserved for 1 day (Figure 3B). The results of section staining also indicated the culture from 1 day's preservation formed 3-4 layers stratified epithelium structure (Figure 3C), while the cells from the tissues preserved for 4 days formed only 1-2 un-continuous cell layer on the denuded amniotic membrane (Figure 3D).

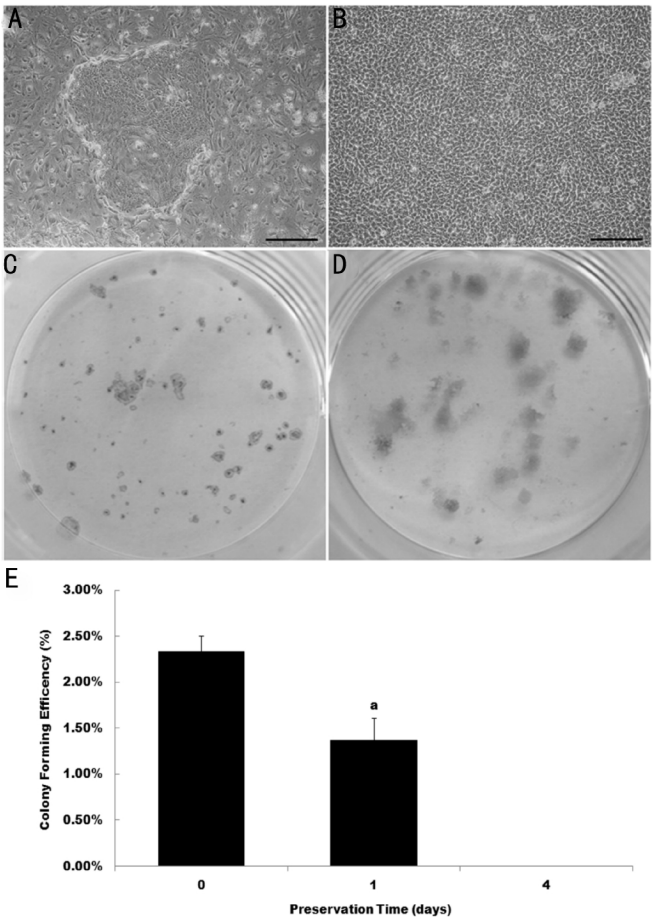

Figure 4 Colony forming capacity of limbal epithelial cells from preserved tissues. Limbal epithelial cells were inoculated on fibroblast feeder cells and incubated for 12 days Cell colonies were easily formed in the epithelial cell culture from the limbal tissues preserved for 1 day (A), but the cells from preserved tissues for 4 days formed intact epithelial monolayer without visible colony (B). Giemsa staining showed intact and dense-staining colonies formed by the cells from the tissues preserved for 1 day (C), while the cells preserved for 4 days only formed "smear cluster" with proliferating epithelial cells (D). Quantitative analysis revealed that colony forming efficiency decreased from about 2.5% at 1 day to 0 at 4 days' preservation of donor limbal tissue (E). [a] $P<0.05$ *vs* 0 day and 4 days group, respectively. Bar: 500μm.

Colony Forming Capacity of Limbal Stem/Progenitor Cells Colony forming capacity of limbal stem/progenitor cells represents the repopulation function to repair the defect of damaged corneal epithelium. Limbal epithelial cells were inoculated on fibroblast feeder cells and incubated for 12 days. Cell colonies were easily observed in the epithelial cell culture from the limbal tissues preserved for 1 day (Figure 4A). However, almost no colony formed by the limbal epithelial cells preserved for 4 days, but the cells actually formed intact epithelial monolayer on feeder cells (Figure 4B). Giemsa staining showed the cells from the tissues preserved for 1 day formed intact and dense-staining colonies (Figure 4C), while the cells preserved for 4 days

did not form visible colony, but formed "smear cluster" with proliferating epithelial cells (Figure 4D). Quantitative analysis revealed that colony forming efficiency decreased from about 2.5% at 1 day to 0 at 4 days' preservation of donor limbal tissue (Figure 4E).

DISCUSSION

Limbal transplantation and cultured limbal epithelial transplantation are the promising treatment modality for limbal stem cell deficiency with the overall success rate of 76%. For cultured limbal epithelial transplantation, cultures in which holoclone-forming cells constituted more than 3% of the total number of clonogenic cells were associated with successful transplantation in 78% of patients, however, only 1% of patients obtained stable ocular surface when transplanted with the cultures less than 3% stem cells, which suggested the success rate of cultured limbal epithelial transplantation was associated with the quality of donor tissues or the percentage of holoclone-forming limbal stem cells in cultures[8]. In the present study, we investigated the changes of colony-forming capacity of limbal epithelial cells preserved in corneal storage medium. The results showed that the colony forming efficiency of limbal epithelial stem/progenitor cells decreased rapidly with the increasing preservation time, but the limbal structure integrity, expression level of limbal stem/progenitor cell markers and the capacity of forming confluent monolayer on amniotic membrane decreased gradually.

Most corneal preservation media were designed for the maintenance of corneal endothelium function, the preservation of limbal epithelium wasn't investigated until the recent decade. Previous results suggested that long-term preservation of limbal explants always caused severe disturbances of epithelial integrity, progressive viability loss of epithelial cells, thus impaired the proliferation and migration when cultured *in vitro*. According to the use of corneal storage media, previous articles could be divided into two groups on the evaluation of limbal epithelial preservations. The first group used Optisol GS or Dexsol, the two commercial storage media available in North America, to preserve human cornea at 4℃. The results showed that donor epithelium lost gradually with the length of storage, and nearly all the cells were lost after 4 days of storage, while the basal cell layer was maintained for a longer time [9]. Our results also confirmed the culture of limbal epithelial cells after 4 days of storage could not form typical stratified epithelial structure, although the limbal tissue assumed comparable expression level of limbal stem/progenitor cell markers, intact epithelium structure and the capacity of reaching confluence on amniotic membrane.

The second group used organ culture storage to preserve at ambient temperature (23℃). The assessment of cultured limbal epithelial cells suggested a slight decrease in viability following 2 weeks' storage, and the capacity of forming multilayer structure of limbal epithelial cells was preserved following 2 weeks of storage but lost after 3 weeks [10]. Shanmuganathan *et al* [11] provided information that limbal tissue can be held in organ culture for up to 30 days without affecting the proliferative potential of limbal epithelial cells. These above results suggested that both short preservation time and ambient temperature organ culture preservation were preferable for the success of limbal epithelial culture *in vitro*.

Previous reports always measure epithelial outgrowth area as the indications to judge the quality of cultured limbal epithelial cells [12-14]. However, the proliferation of limbal epithelial cells was dependent on the expansion of limbal stem/progenitor cells, including colony-forming stem cells, transit-amplifying cells and even terminal differentiated cells [15,16], which suggested the rate of epithelial outgrowth cannot reflect the percentage and activity of limbal stem cells in explants. In the present study, we compared the stem cell colony-forming capacity and the growth into confluence on amniotic membrane. The results showed colony-forming capacity of limbal epithelial stem/progenitor cells decreased more rapidly than the loss of growth on amniotic membrane and the expression level of stem cell markers. To further prove our findings, more intensive time interval maybe needed for determining growth capacity of limbal stem cells decreased *in vitro*.

In this experiment, our original medium also can preserve the integrity of the corneal epithelium for 4 days, and further comparison with other conventional medium such as Optisol GS and Dexsol may provide more information about the preservation ability of our medium. It should be mentioned that more factors would influence the stem cell activity and epithelial cell growth of stored limbal tissues, such as storage temperature, storage medium, death to retrieval time, donor age, except the duration of corneal storage. Further research should be to check the stem cell activity preservation of commercially available storage medium Optisol GS, Dexsol or the organ-cultured storage medium in Europe, and design new media specific for the storage of limbal epithelium. These fundamental researches may help us to better choose the appropriate preservation grafts for future limbal stem cell transplantation.

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