·Basic Research·

# Investigation of immunogenicity of cryopreserved limbal stem cells

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## Abstract

• AIM: To investigate changes in immunogenicity of cryopreserved limbal stem cells.

• METHODS: Cryopreserved limbal stem cells, fresh primary limbal stem cells and blank controls were inoculated subcutaneously in C57BL-6 mice and the percentage of CD25 cells in limbal explants was determined by flow cytometry at day 21 post inoculation. Morphological studies were performed by light and electron microscopy of limbal explant sections.

• RESULTS: The number of regional and systemic lymphocytes derived from cryopreserved limbal stem cells was lower than that from fresh primary limbal stem cells.

• CONCLUSION: Lymphocytes derived from cryopreserved limbal stem cells showed changes in immunogenicity, but the significance is unknown. The cryopreservation and thawing methods await further study.

• KEYWORDS: rabbit limbal stem cells; cryopreservation; immunogenicity; explant

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## INTRODUCTION

Limbal stem cells are a source of corneal epithelial cells and are located in the matrix of the corneal epithelia within a circular area 1.5mm from the limbal rims. The palisades of Vogt in the limbus are thought to provide limbal epithelial stem cells, which maintain the corneal epithelium[1-3]. Limbal stem cells participate in the renewal and repair of corneal epithelial cells by migration and proliferation, and play a critical role in maintaining the integrity of the ocular surface structure and corneal transparency [4,5]. Therefore, clinically, fresh autologous or homologous limbal stem cells are mostly used for ocular surface reconstruction and satisfactory outcome has been obtained. However, immune rejection remains the most critical factor in transplant failure. How to reduce immunogenicity of limbal stem cells in order to reduce transplant rejection is a very important issue clinically. It has been reported that cryopreservation is an effective method for cornea preservation and cell preservation [6]. Cryopreservation can also selectively kill hypothermia-sensitive cells in organs, therefore, lessening transplant rejection. Changes in immunogenicity of cryopreserved limbal stem cells have not been clearly defined. The current paper investigated the effect of cryopreservation on the immunogenicity of limbal cells and elucidated the possible underlying mechanisms so as to provide an experimental basis for the clinical application of limbal stem cells.

## MATERIALS AND METHODS

**Animals** Thirty 6-week old C57BL-6 mice ($n$=30) and New Zealand white rabbits were purchased from the Animal Center, School of Medicine, Peking University, Beijing, China, and housed at the Experimental Animals Center, China Medical University, Shenyang, Liaoning, China, in environmentally controlled conditions (22℃, a 12 hour light/dark cycle with the light cycle from 6:00 to 18:00 and the dark cycle from 18:00 to 6:00) with adlibitum access to standard laboratory chow and water. The study protocol was approved by the authors' affiliated institution review board and animal experiments were conducted in accordance with the guideline of the local Institutional Animal Care and Use Committees.

### Methods

**Preparation of rabbit limbal tissues** New Zealand white rabbits were anaesthetized by intramuscular urethane and sacrificed by air embolism. Full-thickness limbal explants were prepared under a laminar flow. Descemet's membrane

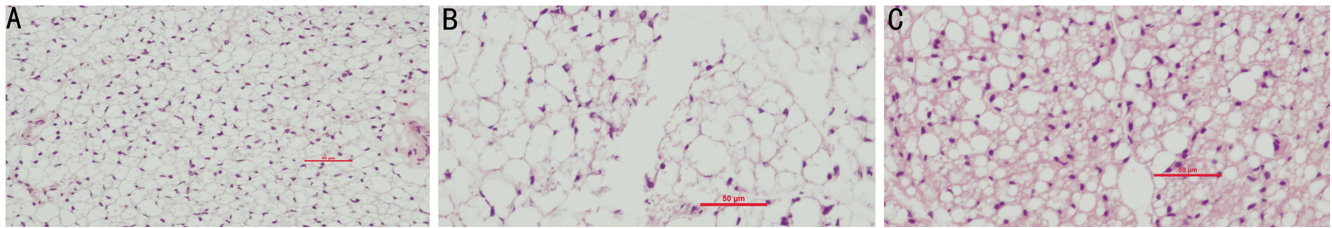

**Figure 1 Light microscopy of mice subcutaneous derived from** A: Blank control; B: Cryopreserved rabbit limbal explants; C: Fresh primary rabbit limbal explants

was removed using a scalpel and full-thickness limbal rims were rinsed twice for 15 minutes each in normal saline containing 20% tobramycin sulfate (v/v) and isolated by removing the sclera with scissors. Superficial limbal explants were processed in the operating room under a stereo microscope. A stromal dissection between the anterior and the mid stroma was performed using a 15° blade and the sclera was carefully removed with scissors resulting in circular superficial limbal rims (2mm). Limbal explants were cultured in cholera toxin-free Green medium. The medium was composed of a 1:1 mixture of calcium-free Dulbecco's Modified Eagle's Medium (DMEM) and Ham F12 medium with 5% fetal bovine serum (FBS) (all from Invitrogen),1mmol/mL HEPES buffer(pH 7.2-7.4), 0.4μg/mL hydrocortisone (Pfizer), 100IU/mL penicillin, 100μg/mL streptomycin, 0.25μg/mL amphotericin B (Invitrogen), and 10ng/mL human recombinant epithelial growth factor (EGF) (Sigma).

**Culture of rabbit limbal stem cells** The excised circular rabbit limbal tissues were washed three times in phosphate buffered saline (PBS) and digested with dispase II (Sigma) with the epithelial side up at 4℃ for 18 hours. The limbal tissues were then washed three times with PBS and put in a new Petri dish and the limbal epithelia were obtained by scraping the limbal tissues using an iris repositor under an anatomic microscope. The epithelial cells were digested with 0.25% trypsin and 0.02% EDTA at 37℃ for 10 minutes and subsequently rendered into single cell suspensions using a 19 and 23 gauge needle. The suspension was centrifuged at 1 000rpm for 8 minutes and the pellets were dissolved in limbal tissue media. The cells were inoculated in a 10mL Petri dish at a density of $10^5$cells/mL at 37℃ with 5% $CO_2$ and the media were replenished every three days and the cells were observed under an inverted microscope. No feeders were used to grow cells.

**Cryopreservation of rabbit limbal stem cells** When fresh primary rabbit limbal stem cells became 90% to 100% confluent, they were digested with 0.25% trypsin and 0.02% EDTA at 37℃ until they became detached from the dish surface. The cells were rendered into single cell suspensions using a 19 and 23 gauge needle. The cellular pellets were collected after centrifugation at 1 000rpm for 8 minutes and were dissolved in the cryopreservation solution containing DMEM/F12, FBS and DMSO at a ratio of 5:4:1. The cells were stored at -80℃ until being thawed one month later.

**Flow cytometry** The flow cytometry analysis was performed on a BD FACSCalibur system (Becton Dickinson). The staining of each population, combined with the forward scatter channel (FSC) and the side scatter channel (SSC) data, identifies cells present in a sample and allows the counting of the relative proportions of each. Cell suspensions ($10^5$ cells/mL) in PBS/BSA buffer were fixed with 4% paraformaldehyde(Fix Buffer I;BD Bioscience)for 10 minutes at 37℃ . After washing with PBS/BSA, cells were permeabilized and incubated for 30 minutes at 4℃. Aliquots were distributed into different test tubes for primary antibody binding (CD25-FITC, and CD3-PE) and incubated at room temperature for 30 minutes. An anti-mouse IgG1 FITC secondary antibody was added, and cells were incubated for 30 minutes at room temperature. Relative size, granularity or internal complexity, and relative fluorescence intensity were registered with the Cell Quest Pro software (BD Bioscience).

**Statistical Analysis** The mice were divided into three groups (10 mice/group) to receive a subcutaneous inoculation of cryopreserved rabbit limbal stem cells ($5\times10^6$ cells/0.2mL), fresh primary rabbit limbal stem cells ($5\times10^6$ cells/0.2mL), or 0.2mL cell-free culture media. The C57BL/6 mice were sacrificed at day 21 post inoculation and the subcutaneous tissue was removed and stored in OCT gel in liquid nitrogen. After thawing, the issue was paraffin embedded, cryosectioned and H&E stained. The immune reaction was studied under light microscope and immune cells in the tissue section were observed under an electron microscope.

## RESULTS

We studied the mice subcutaneous morphological features. Light microscopy revealed that the density of nucleated cells was significantly higher in cryopreserved rabbit limbal explants and fresh primary rabbit limbal explants than that of controls (Figure 1). The nucleus appeared round, concave on one side, with scant cytoplasm, and the chromatin was dense and clumped, resembling lymphocytes. The number of lymphocyte-like cells was significantly higher in

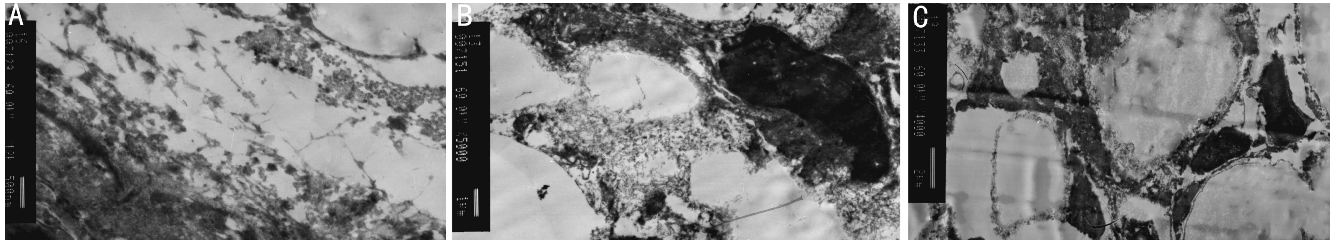


**Figure 2 Electron microscopy of mice subcutaneous derived from** A:Blank control; B: Cryopreserved rabbit limbal explants; C: Fresh primary rabbit limbal explants

A

File: 20100427.002
Sample ID: F4-T
Acquisition Date: 27-Apr-1(
Gate: G1
Gated Events: 16827

| Quad | Events | % Gated |
|---|---|---|
| UL | 11649 | 69.23 |
| UR | 527 | 3.13 |
| LL | 4619 | 27.46 |
| LR | 32 | 0.19 |

B

File: 20100423.004
Sample ID: F2-T
Acquisition Date: 23-Apr-1(
Gate: G1
Gated Events: 18297

| Quad | Events | % Gated |
|---|---|---|
| UL | 5780 | 31.59 |
| UR | 854 | 4.67 |
| LL | 11204 | 61.23 |
| LR | 459 | 2.51 |

C

File: 20100423.001
Sample ID: F1-CON
Acquisition Date: 23-Apr-1(
Gate: G1
Gated Events: 23625

| Quad | Events | % Gated |
|---|---|---|
| UL | 39 | 0.17 |
| UR | 3 | 0.01 |
| LL | 23560 | 99.72 |
| LR | 23 | 0.10 |

**Figure 3 Analysis of mice blood cells from cryopreserved rabbit limbal explants, fresh primary rabbit limbal explants or blank controls cultured for three weeks by flow cytometry for the percentage of CD3⁺/CD25⁺ cells. Single cell suspensions were analyzed according to their size (FSC) and granularity (SSC) profiles** A: Cryopreserved rabbit limbal explants; B: Fresh primary rabbit limbal explants; C: Controls

cryopreserved rabbit limbal explants and fresh primary rabbit limbal explants than that of controls. Electron microscopy showed that cells derived from cryopreserved rabbit limbal explants and fresh primary rabbit limbal explants confirmed that these cells were lymphocytes and contained abundant free ribosomes and lysosomes (Figure 2). We further analyzed the percentage of $CD3^+/CD25^+$ in mice blood by flow cytometry. Figure 3 shows the dot plots

corresponding to the analysis of cell suspensions from limbal explants cultured for three weeks by flow cytometry. The percentage of $CD3^{+}/CD25^{+}$ cells was 3.13% and 4.67% and 0.01% in cryopreserved rabbit limbal explants, fresh primary rabbit limbal explants, and controls. The difference between cryopreserved rabbit limbal explants and fresh primary rabbit limbal explant rabbit limbal explant is small, this difference is not statistically significant.

## DISCUSSION

Cryopreservation can arrest cellular metabolism by deep low temperature and cells can restore their metabolic activity after thawing under appropriate conditions, exerting their biological function [7]. Following cornea transplantation, the predominant corneal immune reaction is mediated by T lymphocytes and the subsets of Th and Tc cells play predominant roles in the process [7,8]. Our results are consistent with those reported in the literature that thawed cryopreserved limbal stem cells resulted in immune reaction in the inoculated mice. A significant difference was noted in the number and density of lymphocytes between blank control, cryopreserved rabbit limbal explants, and fresh primary rabbit limbal explants. The possible mechanisms include hypothermic injury to the cryopreserved cells [9], i.e. the injury of cells by ice crystals and the toxicity of cryoprotectants [10-12].

There are still many aspects of the current study that require further improvement. The cryopreservation of limbal stem cells that did not use programmable freeze control system may cause injury by ice crystals against cells [9] and the thawing method also needs further improvement. The subcutaneous tissue should be fixed immediately and paraffin embedded after the rabbits were sacrificed and should not be placed directly in liquid nitrogen in OCT gel. Immune rejection is a series of cascading events with interaction among lymphocytes. Th cells, upon activation, release IL-2 and IFN-γ. HLA (H-2 in mouse), which plays role in transplant rejection, was not determined. The cryopreservation time was relatively long and affected biological activities of cells.

Until now, there are still many controversies in the studies of the biological properties of cryopreserved limbal cells. The current study investigated changes in the immunogenicity of cryopreserved limbal cells and could provide an experimental basis for clinical application of limbal cells and further expand the use of limbal stem cells as donor cells in the clinic.

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