Chapter 2

Cornea

Effects of cryopreservation against cultured corneal epithelial cell sheets

The recent development of tissue engineering [1, 2] has enabled the clinical application of cultured corneal epithelial cell sheets. In particular, the cultured corneal epithelial cell sheet is beneficial for the reconstruction of the ocular surface with limbal stem cell deficiencies [3], such as those with chemical and thermal injuries, ultraviolet and ionizing radiation injuries, Stevens-Johnson syndrome, and ocular cicatricial pemphigoid. Because the sheets can be generated from a small piece of limbal tissue that contains corneal epithelial stem cells [2], cultured corneal epithelial sheet transplantation is less invasive than autolimbal transplantation. However, the shortage of human donors is one of the major problems for the timely supply of cultured corneal epithelial cell sheets. Furthermore, the cell culture usually requires several weeks to generate stratified sheets [4] because the corneal epithelial cells possess a lower growth potential than other types of cells such as fibroblasts [1, 2]. The corneal epithelial cells tend to differentiate and lose their viability when the culture period is longer. To overcome these problems, cryopreservation should become an important option to preserve tissue-engineered corneal epithelium.

To date, various methods for the cryopreservation have been reported to maintain the viability of the cells for a long time without affecting the phenotype. The ability of glycerol to protect cells from freezing injury was discovered accidentally. Polge and coworkers found that glycerol can increase the survival rate of spermatozoa dramatically when added to a freezing medium [5]. Since then, cryopreservation techniques have been developed in various fields including blood cell storage, plant biology, and horticulture. Simultaneously, cryoprotective ability has been found in various chemicals. Among those chemicals that have been tested as cryoprotectants, glycerol and DMSO are most commonly used at present. Cryopreservation has also become an important technique in the field of tissue engineering because effective preservation procedures are required for a stable supply and the efficient transportation of the products. The cells are usually the key component of tissue-engineered tissue, providing the tissue specificity and bioactivity required to achieve a therapeutic effect. For the cryopreservation of cultured epithelial and mucosal cell sheets, glycerol has been reported to be superior to DMSO [6]. On the other hand, DMSO is commonly used as a cryoprotectant for corneal grafts, which have been successfully applied in many clinical cases [7]. However, there has been no previous report concerning the effects of cryopreservation on cultured corneal epithelial cells. In the present study, we focused on the influence of these routinely used cryoprotectants and the protocols of cryopreservation for tissue-engineered corneal epithelial cell sheets.

We examined 3 variables: cryoprotectant (DMSO or glycerol), storage temperature (-80°C or -196°C), and the preservation period (4 weeks or 12 weeks of storage). After freezing storage, the effects of cryopreservation on histology and cell viability were analyzed. In what follows the cryopreservation method is presented in detail.

Cell Preparation and Culture

Male Japanese white rabbits were used in this study. Normal corneal limbal tissue was obtained from the unilateral corneal limbus under topical anesthesia. The biopsy sample was rinsed twice in a PBS solution containing antibiotics for 30 minutes at 37°C. It was then treated with 0.25% trypsin solution for 30 minutes at room temperature for cell dissociation. The enzyme activity was neutralized by washing with DMEM containing 10% FCS. The specimen was stirred in DMEM containing 5% FCS for 30 minutes. The cell suspension was filtered through nylon gauze (50 mm), and a suspension of purified corneal cells was obtained. The cell suspension was centrifuged for 5 minutes at 1500 rpm, and the cell pellet was resuspended in culture medium. 3T3-J2 cells were kindly provided by Dr Howard Green, and the cells were treated with 4 mg/ml of mitomycin C (MMC) in DMEM without FCS for 2 hours before epithelial cell inoculation. The 3T3-J2 cells were rinsed with PBS twice to remove MMC. A 3: 1 mixture of DMEM and Ham F12 medium was supplemented with the following: FCS (5%), insulin (5 mg/ml), transferrin (5 mg/ml), hydrocortisone (0.4 mg/ml), cholera toxin (10 ng/ml), triiodothyronine (2 nmol), penicillin (100 U/ml), kanamycin (0.1 mg/ml), and amphotericin (0.25 mg/ml). Human recombinant epidermal growth factor was added at 10 ng/ml when cell adhesion was complete. The 3T3-J2 cells treated with MMC were inoculated into a cell culture dish (10 ml) at a density of 2.0×10^4 cells/cm^2. A collagen membrane filter 33 mm in diameter was placed on the feeder layer, and the epithelial cells were inoculated on the collagen membrane at a density of 1.0×10^4 cells/cm^2. The culture dish was kept in an atmosphere of 10% carbon dioxide. The medium was changed every 2 days. Stratified corneal epithelial cell sheets were obtained after 20 days (Fig. 7).

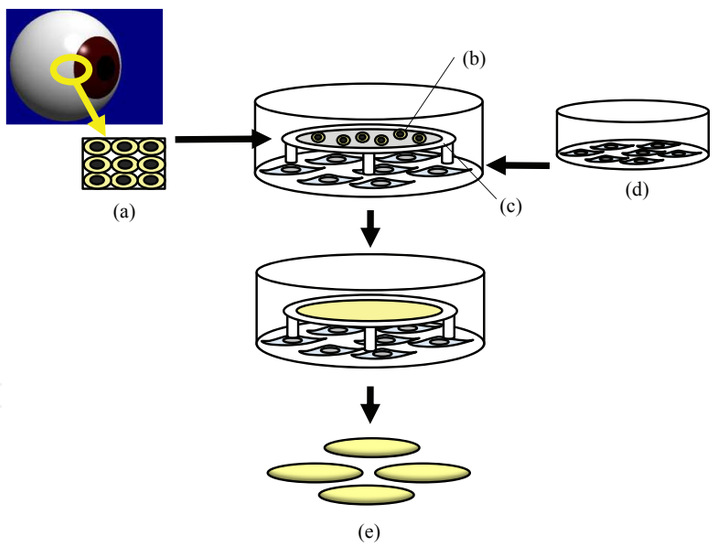

Fig. 7. Preparation for the cultured corneal epithelial cell sheets on the collagen filter membrane. (a) Limbal tissue from corneal limbus. (b) Isolated corneal epithelial cells. (c) Collagen filter membrane. (d) 3T3 cells treated with mitomycin C. (e) Culture corneal epithelial sheets (From Kito et al. 2005. Reprinted with permission).

Preparation of samples for cryopreservation

The cell sheets were punched out with a biopsy punch (8 mm in diameter) and mechanically removed from the sheets on the collagen membrane to avoid the effect of enzymes. In total, 68 samples were used for the viability analysis, and 9 samples were used for the histologic analysis.

Freezing protocols for corneal epithelial cell sheets

The freezing medium was prepared as 10% cryoprotectant and 15% FCS added to DMEM. Glycerol and DMSO were used as cryoprotectants. Cultured corneal epithelial cell sheets were frozen under a slow freezing schedule in a 1.8-mL cryotube with 1.5 ml of a freezing medium. The sheets with glycerol as a cryoprotectant were equilibrated for 40 minutes at 4°C, whereas those with DMSO were equilibrated for 20 minutes at 4°C as indicated in a previous report [8]. The incubation period was provided to allow for equilibration of cryoprotectants within tissues. The samples were incubated longer in glycerol because it penetrates the cell membrane more slowly than DMSO [9]. The 4°C incubation temperature for DMSO was selected to minimize the potential toxic effect [10]. The cultured corneal epithelial cell sheets were divided into 8 groups by storage temperature, cryoprotectant used, and storage period, i.e., samples cyropreserved with glycerol at -80°C for 4 weeks (n = 7) or 12 weeks (n = 9), samples cryopreserved with glycerol at -196°C for 4 weeks (n = 7) or 12 weeks (n = 7), samples cryopreserved with DMSO at -80°C for either 4 weeks (n = 7) or 12 weeks (n = 11), and samples cryopreserved with DMSO at -196°C for 4 weeks (n = 10) or 12 weeks (n = 10). The samples were cooled in a controlled rate freezing chamber placed in a -80°C freezer at a rate of 21°C/min from +4 to -80°C after equilibration with the cryoprotectants. Then, the samples stored at -196°C were transferred directly to the liquid nitrogen tank after overnight incubation at 280°C (Fig. 8).

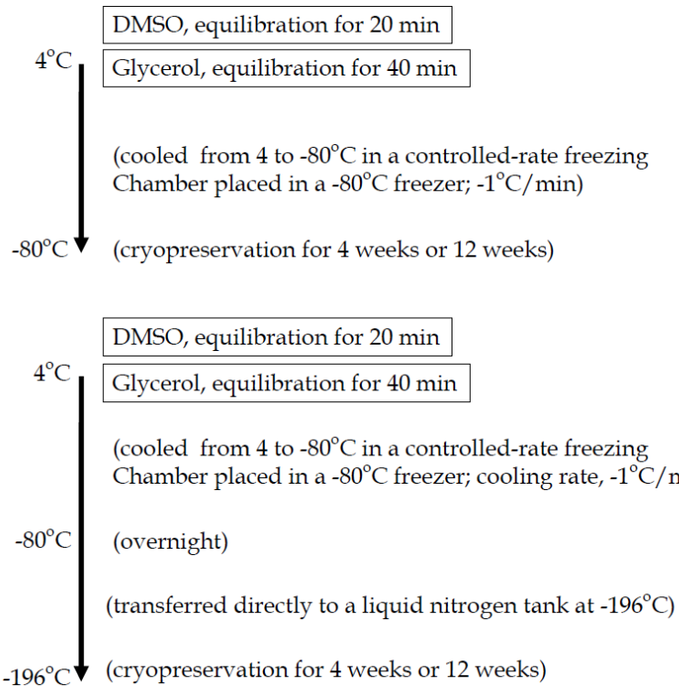

Fig. 8. Freezing protocols. Cultured corneal epithelial cell sheets were frozen under a slow freezing schedule. The sheets with glycerol as a cryoprotectant were equilibrated for 40 minutes at 4°C, whereas those with DMSO were equilibrated for 20 minutes at 4°C. An incubation period was provided to allow for equilibration of cryoprotectants within tissues. The samples were incubated longer in glycerol because it penetrates the cell membrane more slowly than DMSO. The 4°C incubation temperature for DMSO was selected to minimize potential toxic effects (From Kito et al. 2005. Reprinted with permission).

Thawing protocols

After cryopreservation, the samples were placed in a water bath at 37°C and thawed rapidly under continuous gentle stirring. The warming rate could not be measured in this study; however, the average rate under a similar condition was reported at approximately 150°C/min during the entire thawing process [8]. When the frozen cryoprotectant solution had nearly all melted, the cryotubes were removed from the water bath, and the samples were transferred into culture dishes. To avoid cell damage from osmotic stress, 6 ml of chilled DMEM containing 10% FCS was gradually added, and the melted cryoprotectant solution was diluted to less than 2% of DMSO or glycerol concentration. After equilibration for several minutes, the culture dishes were emptied of all but about 1.5 ml of diluted cryoprotectant solution. Then a second gradual dilution was performed to achieve a cryoprotectant concentration of less than 0.5%. All of the diluted cryoprotectant solution was then decanted and replaced with new culture medium. The cell sheets were allowed to equilibrate for several more minutes and washed again with the same medium.

Structural damage such as vacuolar degeneration was more clearly observed in the corneal epithelial cell sheets cryopreserved with DMSO than those with glycerol, especially at -80°C, whereas only minor morphologic changes were observed in the corneal epithelial cell sheets cryopreserved in glycerol at both temperatures. Colorimetric cell viability assay revealed that the storage conditions at the lower temperature (-196°C) showed higher cell survival than those at the higher storage temperature (-80°C). The difference between the two cryoprotectants, however, was not significant. Among the conditions used in this study, the samples cryopreserved with glycerol at -196°C showed the highest cell survival rate (70.3 ± 8.3% and 66.4 ± 14.7% for 4 and 12 weeks, respectively). The difference between this group and those stored at -80°C was significant for both 4 and 12 weeks of storage using either glycerol or DMSO. Although the cryopreserved cell sheets could not maintain their original layered structure after thawing, viable cell sheets could be regenerated.

The cell survival rates obtained after freezing storage were reasonable; however, the cryopreserved sheets could not maintain their original layered structure. More works needs to be done to better preserve the corneal epithelial cell sheets.

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Tissue engineering, which aims at regenerating new tissues, as well as substituting lost organs by making use of autogenic or allogenic cells in combination with biomaterials, is an emerging biomedical engineering field. There are several driving forces that presently make tissue engineering very challenging and important: 1) the limitations in biological functions of current artificial tissues and organs made from man-made materials alone, 2) the shortage of donor tissue and organs for organs transplantation, 3) recent remarkable advances in regeneration mechanisms made by molecular biologists, as well as 4) achievements in modern biotechnology for large-scale tissue culture and growth factor production.

This book was edited by collecting all the achievement performed in the laboratory of oral and maxillofacial surgery and it brings together the specific experiences of the scientific community in these experiences of our scientific community in this field as well as the clinical experiences of the most renowned experts in the fields from all over Nagoya University. The editors are especially proud of bringing together the leading biologists and material scientists together with dentist, plastic surgeons, cardiovascular surgery and doctors of all specialties from all department of the medical school of Nagoya University. Taken together, this unique collection of world-wide expert achievement and experiences represents the current spectrum of possibilities in tissue engineered substitution.

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