Recent Advance in the Cryopreservation of Corneal Limbal Stem Cells

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ABSTRACT

PURPOSE: This article provides a summary of our research, an overview of the recent progress, and current challenges of cryopreservation and eye banking of corneal limbal stem cell tissue.

METHODS: We reviewed the literature, summarized our research, and presented current challenges involved with cryopreservation and eye banking of corneal limbal stem cell tissue.

RESULTS: Corneal limbal stem cell transplantation is the primary ocular surface treatment for severe chemical burns, radiation injuries, severe infection, ocular pemphigoid, Stevens-Johnson syndrome, and other corneal disorders that result in corneal-limbal stem cell deficiency (LSCD). Numerous techniques have been described to replace corneal limbal stem cells, which are required to produce a normal, new corneal epithelium. Obstacles to overcoming some of the limitations of providing human eye tissue for corneal limbal stem cell transplantation include storage of tissue, tissue culture of the stem cells and the formulation of a tissue culture medium that does not contain cholera toxin and animal products, which are problematic from a safety and U.S. Food and Drug Administration (FDA) regulatory standpoint.

CONCLUSIONS: Biopreservation of ocular limbal stem tissue using cryogenic techniques ("cryopreservation") can extend the viable storage life of transplantable tissues, provide for storage of allograft and autologous graft tissue and perhaps be used to cryopreserve stem cell cultures for certain patients ("eye banking").

KEYWORDS: Cryopreservation, limbal stem cell, cornea, eye bank

The absence of corneal stem cells, or limbal stem cell deficiency (LSCD), may lead to recurrent and persistent epithelial defects, melting, conjunctivalization of the cornea, corneal scarring, ulceration, perforation, and vision loss. LSCD can be the result of congenital disorders, such as the family of autoimmune epitheliopathies, aniridia, and severe congenital disorders that result in lacrimal insufficiency. Secondary disorders that result in corneal limbal stem cell deficiency include: neurotrophic corneal conditions, keratitis, trauma, limbal surgery, chemical toxicity, chemical burns, thermal burns, ocular cicatricial pemphigoid, Stevens-Johnson disease, contact lens wear, ocular rosacea, corneal intraepithelial neoplasia, and limbal cryotherapy. Lamellar or penetrating keratoplasty only provides a temporary replacement of the corneal epithelium. Patients with an abnormal corneal epithelium, which is the result of LSCD, require some type of corneal limbal stem cell transplantation to regenerate the absent or damaged corneal epithelium. Numerous techniques have been described to replace corneal limbal stem cells, which are required to produce a normal new corneal epithelium. Thoft described keratoepitheliokeratoplasty as a means to reconstruct the ocular surface in patients with corneal stem cell deficiency, which he later modified to include limbal stem cells.[1] Kenyon and Tseng performed human trials of limbal stem cell transplants in 1989.[2] In 1997, Pellegrini described the successful transplantation of limbal stem cells by recovering a small allograft and expanding the stem cells on a sheet of fibroblasts in tissue culture.[3] Other corneal limbal stem cell transplant techniques have been subsequently described.[4-12]

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Corneal limbal stem cells are obtained from many sources including eye bank corneolimbal tissue, whole globes, and living related relatives, which represent allogenic transplants. If the patient has viable corneal limbal stem cells which can be recovered and transplanted to the damaged region of the ocular surface, termed an autogenic graft, the risk of immune rejection of the transplanted tissue is eliminated.[13,14] Autologous human limbal stem cell expansion on amniotic membrane (AMN), *in vitro,* has become one of the most potentially effective methods to restore useful vision in patients suffering from various corneal injuries and diseases.[15] *In vitro* expansion of corneal limbal stem cells also reduces the amount of excised tissue required for transplantation. This factor is especially important in the case of recovering autologous corneal stem cell tissue, which can only be obtained from a patient's viable eye, with an intact ocular surface.

In this article, we discuss the cryopreservation research of limbal stem cells by our group and other researchers using different sources of limbal tissues and cells. Secondly, we focus on the current challenges of cryopreserving limbal stem cells and outline future directions for optimizing the cryopreservation parameters for the eye banking of limbal stem cells.

METHODS

Limbal Stem Cell Cryopreservation

Current limbal stem cell therapies primarily employ fresh tissues or limbal stem cell expansions on AMN immediately after culture. In the United States, the cold storage method, Optisol GS (Bausch + Lomb, Rochester, NY) at 4ºC to 8ºC is used, which provides a potential storage shelf-life, limited to 2 weeks by the FDA labeling. The main concerns involve maintenance of viable corneal endothelial cells for transplantation (including penetrating keratoplasty and endothelial keraptoplasty). However, research has revealed a reduced proliferation of limbal stem cells after only 4 days of storage in Optisol GS, a fact that guides the preference for fresh tissue use.[16] Extended culture periods of limbal expansions on AMN are also not preferred because of the occurrence of stem cell differentiation. Recently, cryopreservation techniques have been applied to limbal stem cells for their intermediate and long-term storage.[17-23] By preserving limbal stem cells cultivated on AMN from a patient's own ocular tissue, a clinician would be able to perform multiple autologous transplants, if necessary, without subjecting the patient to multiple biopsies. The autologous tissue could be obtained and cryopreserved before the limbal stem cells degenerated (for example, in young aniridia patients), and thus could provide multiple limbal stem cell transplants over decades. The successful application of cryopreservation techniques to preserve limbal stem cells could change the current practice of limbal stem cell therapy and provide new and better treatment options to patients who have corneal surface pathology from many etiologies. Because of the potential benefits for clinical applications, research on the cryopreservation of limbal stem cells has received great attention in recent years. Some of the most significant progress is summarized in the following sections.

Cryopreservation

This technique enables tissue preservation and presents tremendous clinical benefits for patients in need of transplantation and tissue repair.[24] However, cells cannot survive the freezing process without an additive: as cells freeze, water is removed from the solution, in the form of ice, causing an increased concentration of intracellular solutes in the residual unfrozen region. Damage caused by such chemical and osmotic aberration is termed "solution effect" injury. Freezing can also lead to the formation of ice crystals that mechanically disrupt or damage cell organelles and membranes. A cryoprotectant is employed to protect cells from freezing-induced damage by interacting with water molecules to interfere with the ice-forming process. In addition, cryoprotectants can also permeate the cell membrane and prevent the dehydration of cells during the slow cooling process ("penetrating cryoprotectants"). Currently, approximately 100 compounds (including sucrose, DMSO, trehalose, glycerol, and propanediol) have been used for such applications. Dimethyl sulfoxide (DMSO), a common cryoprotectant that was also used in the previous reports of limbal cryopreservation,[17-23] is known to cause epigenetic modifications in cryopreserved cells[25] and has been excluded for usage in fertility-related cryopreservation.

Cryopreservation of isolated limbal stem cells

Using a flow cytometry-based technique, Mi and colleagues[17] have isolated ABCG2-positive/CD34-negative limbal epithelial cells from goat ocular limbal tissues and cultivated them in vitro with DMEF/F-12 plus fetal bovine serum (FBS). After 2 to 4 passages, the cells were recovered and cryopreserved in liquid nitrogen with 10% DMSO plus 25% FBS. The viability of cells was determined to be more than 85% after 1 year of storage. Expression of stem cell markers in these cryopreserved limbal stem cells was also confirmed. When seeded onto denuded AMN and transplanted to recipient goats with experimental LSCD,

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these cryopreserved goat limbal stem cells were able to reconstruct the damaged corneal epithelia and maintain corneal transparency up to 6 months. Interestingly, loss of the donor limbal cells was noted after 2 months, while the transparency of the corneas continued to improve beyond 2 months.

Cryopreservation of limbal stem cell expansions

Limbal explant cultures expanded from human and rabbit limbal biopsies have been used for cryopreservation studies.[18-20] The limbal expansions were maintained either in media containing DMEM/F-12 plus FBS or co-cultured with 3T3-feeder cells and subsequently cryopreserved in FBS, DMSO, or glycerol. After 1 week of cryopreservation in liquid nitrogen, the cell viability of the human stem cell expansion on AMN remained high (87%) after thawing.[18] Rabbit stem cell cultures demonstrated a 66% to 70% cell viability after 4 to 12 weeks of storage in one study[19]and more than 85% after 1 year in another report.[20] The efficacy of using cryopreserved limbal expansions for ocular surface reconstruction was further tested in an experimental LSCD model in rabbits (allograft transplant) and confirmed significant epithelial recovery up to 2 months after thawing the tissue.[20]

Cryopreservation of limbal tissues

Bratanov and colleagues[21] previously showed that limbal tissues obtained from donor corneas and cryopreserved in a solution containing 10% DMSO, 20% human serum albumin, and 70% Optisol GS resumed robust outgrowth after 1 year of storage in liquid nitrogen. However, the authors noted that a loss of stem cell markers, such as p63 or vimentin, was likely due to the prolonged cultivation in vitro (28 days).

Cryopreservation of limbal expansions on AMN

Success of cryopreserving human limbal expansions on AMN was first demonstrated by Yeh and coworkers.[22] Limbal tissue blocks from donor corneas were cultivated on denuded AMN using an air-lifting method in DMEM/F-12 supplemented with FBS and cholera toxin. After 2 to 3 weeks of culture, the stratified epithelial sheets/AMN were cryopreserved in various combinations of DMSO/FBS. The authors further determined the after-thaw cell viability to be ~50% between 1 to 8 weeks of storage in the liquid nitrogen. The somewhat lower post-thaw cell viability may be related to the cultivation method (air-lifting) and/or the particular cell viability assay adopted in the particular report. Revived cryopreserved limbal expansions resumed robust proliferation and were confirmed to express a stem cell marker, ABCG2, in the post-thaw outgrowths.

Cryopreservation of limbal tissues and limbal expansion on AMN (Combined approach)

We recently conducted a proof-of-principle study using a combined approach to generate two types of transplantable materials from a single corneal tissue source.[23] Fig. 1 outlines the procedure: Limbal tissue blocks dissected from donor corneas were either used

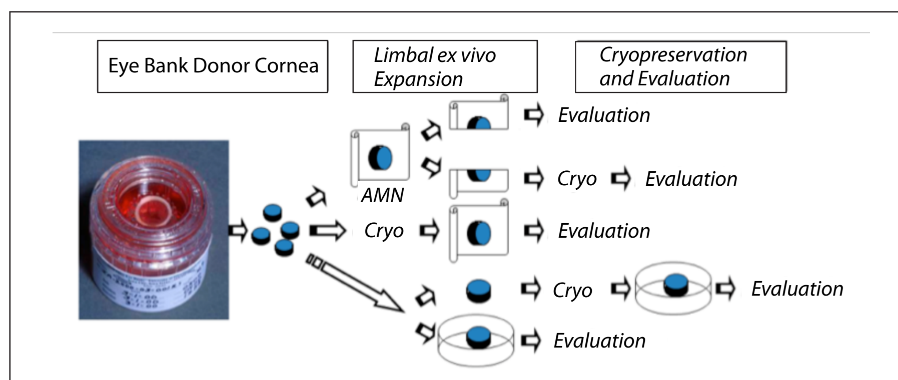

Fig. 1. Summary of the limbal explant cultures procedures in Ewel et al.[23]
Comparison study was performed to evaluate the impacts of cryopreservation on the limbal stem cells. Donor cornea buttons were obtained from Minnesota Lions Eye Bank, St. Paul, MN. Two-millimeter circular corneolimbal discs were dissected from donor corneal tissues. The corneolimbal discs were bisected; half of the tissue blocks from each donor were cultured immediately as a control, and the other half were immediately cryopreserved. The limbal tissue blocks were handled with great care and the explant cultures of limbal stem cells from each donor were performed on either glass chamber slides for immunostaining analysis, or on 1 cm x 3 cm sections of denuded AMN for cell viability analysis. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and the medium was changed every 2 days. The limbal expansions were maintained in monolayer cultures (without air-lifting) for 2 weeks before recovering tissues or cryopreservation.
Abbreviations: AMN, amniotic membrane; **Cryo,** cryopreservation.

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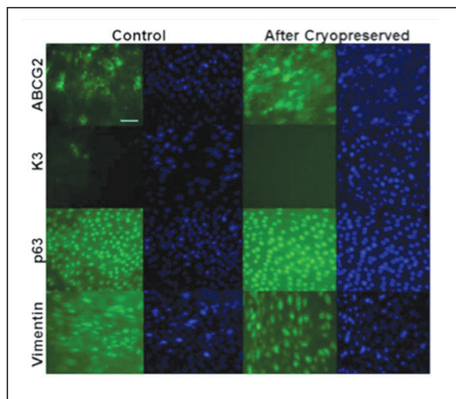

Fig. 2. Characterization of limbal explant cultures by immunofluorescence staining

Explant cultures grown on glass chamber slides were analyzed for limbal stem cell markers by immunohistochemistry. Cells were stained with antibodies specific for ABCG2, K3, p63, and vimentin (FITC). Results of K12 stainings are not shown. Cell nuclei were counterstained with Hoechst 33342 (DAPI). Slides were mounted with cover slips and analyzed for signal detection by a Zeiss Axiovert 200M epi-fluorescence microscope.

Scale bar = 100μm.

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directly for explant cultures on AMN or subjected to a cryopreservation procedure modified from a previous report[21] that used a cryopreservation solution (10% DMSO, 20% FBS, and 70% Optisol GS). After storage for 4 weeks in liquid nitrogen, these cryopreserved limbal tissues were thawed and cultivated in vitro for 2 weeks. Robust outgrowth was observed on these cryopreserved limbal tissues, similar to the non-cryopreserved ones. Immunostaining results revealed prominent expressions of ABCG2, p63, and vimentin, yet sparse signals of cytokeratin K3 (Fig. 2) or K12 (Unpublished results by SC Kaufman and K Suri). Additionally, Western blots and reverse transcription-polymerase chain reaction (RT-PCR) results further corroborated the immunostaining observations.[23] Interestingly, although not statistically significant, an elevated expression of ABCG2 was noted in the limbal explant cultures derived from cryopreserved tissues. The upregulation of ABCG2, a putative limbal stem cell marker that has been shown to play roles in oxidative stress response in the corneal cells,[24] may serve as a protective mechanism in response to the freeze-thaw process.

Our study also supports the feasibility of cryopreserving the limbal expansions on AMN. Instead of growing the limbal explant cultures to stratified epithelia (air-lifting method),[22] we used a submersion technique to maintain limbal explant cultures as monolayers, a method used for the autologous limbal stem cells transplant in humans.[15] The cryopreserved limbal expansions on AMN (denuded) displayed 87.5% cell viability, post-thawing (Fig. 3), a result that is comparable to or better than previous reports[18,22] on cryopreserved human limbal expansions. A robust outgrowth of corneal limbal stem cells was also observed.[23]

The previously noted studies that involve limbal stem cell expansion on AMN support the feasibility of cryopreserving surplus limbal stem cell tissue for repeat or later use. Successful cryopreservation of ocular limbal tissues also allows corneal surgeons to establish eye banking of corneal autograft tissues and limbal expansions on AMN that would provide a source of stem cells for individuals who have aniridia, for stem cell expansion graft tissue from patients who have unilateral disease, or from living relatives. In addition to the obvious benefits of eye banking, cryopreservation of limbal stem cells may also enhance the capacity for stress response(s) (eg, ABCG2)[26] or reduce the

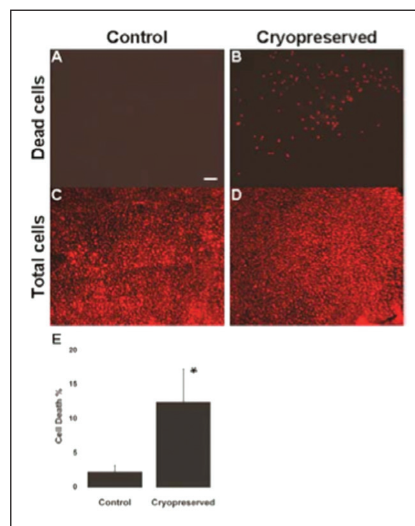

Fig. 3. Cell death of cryopreserved limbal tissues after thawing

Propidium iodide (PI) staining of limbal stem cells expanded on amniotic membranes before (A) and after thawing from cryopreservation (B). After incubation in the stain, cultures were washed extensively, and then AMNs were carefully removed from their nitrocellulose backings to be laid flat on microscope slides. The number of dead cells was determined by counting PI-positive cells. Cells within the same viewing field were then permeated with 1% Triton X-100, washed extensively, and stained again with PI to determine total cell numbers to calculate the percentage of cell death, as shown in (C) and (D). Images were taken at 100x magnification, scale bar = 150μm. Quantification of cell death expressed as percentage of total cells (E). (n=6; bar=Standard Error of the Mean; P=0.007)

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immunogenic nature (HLA-DR)[27] that can enhance graft tissue survival in an allogeneic environment.

RESULTS

Challenges for Future Studies

Although the feasibility of cryopreserving limbal stem cells for therapeutic applications has been confirmed by our group and others,[17-23] in-depth investigations such as characterization of the cryopreservation behavior of limbal stem cells and optimization of the cryopreservation parameters will be necessary before applying this new tool in clinical procedures. Future directions for research of the limbal stem cell cryopreservation should at least include the five following issues:

(1) Optimal cryopreservation conditions for the preservation of limbal stem cells

We performed preliminary cryopreservation experiments on limbal tissue and limbal *ex vivo* expansion on AMN using published cryogenic conditions.[21,22,23] However, these studies, explored only a small number of cryopreservation parameters. A more extensive investigation of optimal cryopreservation conditions (including cooling rate and better alternative cryoprotectants) and an in-depth characterization of limbal stem cells are essential to optimize cryopreservation, eye banking of limbal tissue, and further facilitate limbal stem cell-based therapy.

(2) Evaluation of corneal tissue integrity from long-term storage

Currently, the freezing damage and its potential adverse effects on limbal stem cells are evaluated with assays that measure post-thawing cell viability, colony forming (proliferation potential), and expression of limbal stem cell markers. The potential impact of long-term cryopreservation on the "stemness" of the limbal stem cells needs to be determined for any practical application, including multiple transplantation procedures or eye banking. The freezing and thawing damage can also be evaluated using techniques such as cryoscanning/transmission electron microscopy and low-temperature Raman microspectroscopy to gain a better insight into optimal cryoinduction. Raman/FTIR microspectroscopies have been used to measure the chemical activity of water and other molecules inside the cells at temperatures above freezing and thus allow direct optimization of the intracellular medium (intracellular water, organic materials, and cryoprotectants).

3) Culture media

A safe and effective culture medium with humanized growth supplements is also needed to eliminate safety and regulatory concerns for limbal *ex vivo* expansion on AMN. Most of the cryopreservation studies of limbal stem cells that we reviewed were based on the formula of a widely used, FBS-based cornea medium.[15] This particular limbal cell culture medium includes xenogenic components and biotoxin (cholera toxin), which presents major safety concerns for clinical therapy. The use of FBS as a medium-supplement in limbal stem culture is widely practiced, despite the risk of possible contamination with viruses, prions, bacteria, mycoplasma, yeast, fungi, and endotoxins.[28] There are also commercially available serum-free media with bovine pituitary extract added for corneal epithelial cultures. The safety concerns (for example, bovine spongiform encephalopathy or other prion-related diseases) of media adjuvants remain. These concerns necessitate the development of "humanized" supplements to overcome such problems. Clinically, autologous serum from transplant recipients may present a better alternative to FBS. In addition, epidermal growth factor (EGF), insulin, and transferrin of human or recombinant sources can replace the current supplements. Our previous report[23] also used epinephrine to replace cholera toxin in the culture medium for the cryopreservation study.

(4) Proper storage devices for liquid nitrogen storage

Our preliminary study showed noticeable cell death caused by manipulating limbal expansions on AMN that were fitted into standard cryotubes (unpublished results by SC Kaufman and C Yuan). Therefore, cryopreservation storage vessels designed with appropriate physical specifications for convenient retrieval and minimal manipulation of the limbal expansions on AMN would be beneficial. Appropriate storage devices can also help reduce freezing damage. Currently, various alternative cryopreservation storage vessels, such as KryoVue peel pouch (American Fluoroseal Corp, Gaithersburg, MD), are available commercially and need to be tested in this new limbal stem cell cryopreservation application.

(5) Transplantation of cryopreserved limbal cells in LSCD animal models

So far, only cryopreserved limbal stem cells from rabbits and goats have been tested for treating LSCD. Transplantation of cryopreserved human limbal stem cells is needed to confirm the efficacy of treating specific ocular disorders and to evaluate long-term outcomes.

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CONCLUSIONS

In summary, we have presented evidence supporting the feasibility of cryopreserving corneal limbal stem cells using both donor limbal tissues and limbal explant cultures expanded on AMN. Efforts to develop optimal protocols for the biopreservation of limbal stem cells could extend the shelf life of transplantable donor tissues, increase the availability of quality tissues for eye banks, and allow multiple usages from single explant cultures for transplantation and eye banking purpose. These procedures could, therefore, advance and expand the potential of limbal stem cell therapy.

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