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Chapter

Cryopreservation in Ophthalmology

Yuting Shao, Chao Chen, Qi Zhou, Jun Yang, Xiao Lv, Mingyue Lin and Yanlong Bi

Abstract

Amniotic membranes (AMs) and corneas are critical materials in ocular surface reconstruction. AM has specific structures (e.g., basement and two types of cells with stemness characteristics: amniotic epithelial cells and amniotic mesenchymal cells), which contribute to its attractive physical and biological properties that make it fundamental to clinical application. The corneal endothelial cell is a vital part of the cornea, which can influence postoperative vision directly. However, widespread use of fresh AM and cornea has been limited due to their short use span and safety concerns. To overcome these concerns, different preservation methods have been introduced. Cryopreservation is distinguished from many preservation methods for its attractive advantages of prolonged use span, optimally retained tissue structure, and minimized infection risk. This review will focus on recent advances of cryopreserved AM and cornea, including different cryopreservation methods and their indications in ophthalmology.

Keywords: amnion, cornea, cryopreservation, indication, ophthalmology

1. Cryopreservation of amniotic membranes

1.1 Introduction

Ever since Davis [1] first used human amniotic membrane (**Figure 1**) (AM) for skin transplantation, people have been exploring this remarkable biomaterial. AM is located in the innermost layer of the fetal membranes [2]. It is 0.02–0.05 mm thick, lightweight, elastic, almost transparent, and avascular membrane classically composed of three layers: the epithelium, the basement membrane and the stroma layer [2]. There are two types of cells with stemness properties in AM: amniotic epithelial cells (AECs) and amniotic mesenchymal cells (AMSCs) [3], which are responsible for its unique biological properties including anti-inflammatory, anti-scarring, anti-microbial, angio-modulating, immunomodulatory, and anti-cancer effects [4–10]. Due to these properties, AM has become an ideal material for ocular reconstruction including the treatment of persistent epithelial defects and non-healing corneal ulcers, corneal perforations and descemetoceles, bullous keratopathy, as well as corneal disorders with associated limbal stem cell deficiency, pterygium, conjunctival reconstruction, corneoscleral melts and perforations, and glaucoma surgeries. However, its use span is short and many viruses (such as HIV-1/2, hepatitis B, hepatitis C, human T-cell lymphotropic virus, syphilis, and cytomegalovirus)

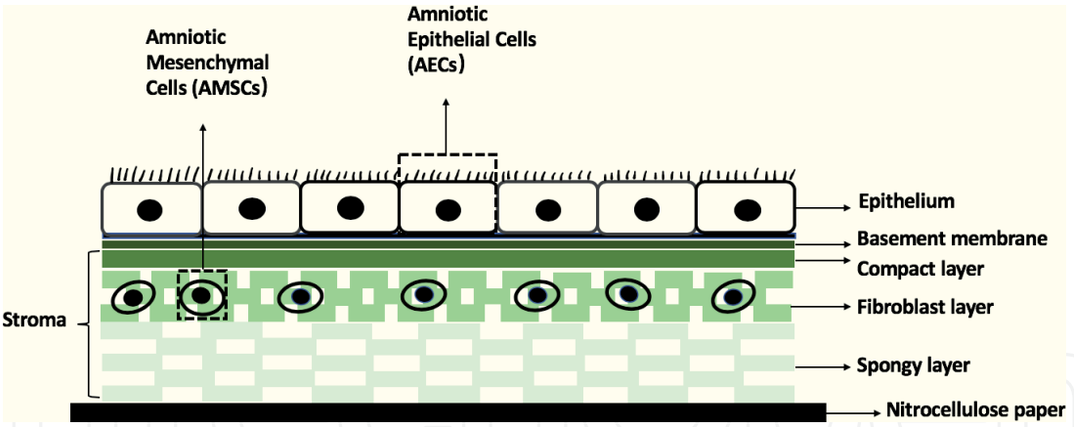


Figure 1.
Amniotic membrane (AM).

can be in their "window period" and escape detection, further limiting the use of fresh AM. To overcome these concerns, different preservation methods have emerged, such as freezing, lyophilization, and cryopreservation. However, most methods result in the destruction of endogenous cells and cause varying degrees of extracellular matrix (ECM) damage, which can affect the functionality of AM and its clinical benefits for wound treatment [11, 12]. Cryopreservation was first introduced by Lee and Tseng and has been proven to achieve high success rate in AM transplantation, which has been distinguished from many methods for its attractive advantages of prolonging use span, optimally retaining tissue structure, and minimizing infection risk [13, 14].

In this part, we classify the cryopreservation methods applied to amnion by commonly used cryoprotectant and analyze the influence of cryopreservation on AM combined with specific clinical trials.

1.2 General cryopreservation techniques

The AM is normally washed using balanced saline solution containing antibiotics such as streptomycin, penicillin, neomycin, and amphotericin prior to storage. Pieces of AM resting on a carrier are placed in a vial containing cryoprotectant solution at a controlled cooling rate. Storage temperatures of −80°C are often utilized, with the maximum storage times ranging between 1 and 2 years [1, 11, 12].

The main disadvantage of cryopreservation is the requirement of a deep-freezing facility, which is expensive, cumbersome, and frequently unavailable, especially in underdeveloped countries. In addition, maintaining stable storage temperatures during transportation is also relatively difficult.

1.3 Cryopreservation methods on AM

1.3.1 Glycerol-cryopreservation

Glycerol storage was first introduced in the Netherlands in 1984 to preserve donor skin for transplantation [13]. Positive results over subsequent decades have led to its clinical acceptance, including in the preservation of AM. Glycerol has led to higher cell viability and higher bFGF secretion for up to three months of AM storage [14]. After strict preservation and sterilization processes, pieces of AM resting on a carrier are placed in a vial containing storage solution. Tseng's laboratory introduced a methodology of glycerol (86%) in Dulbecco's Modified Eagle Medium at a ratio of 1:1 [15, 16]. The most common

cryopreservation protocol reported in the literature involves the use of 50% glycerol and storage at −80°C [17–21]. Undiluted and 98% glycerol have also been reported to be clinically effective [15].

In 2011, Thomasen et al. [21] showed that long-term storage of 50% glycerol cryopreserved AM for durations up to 24 months at −80°C did not significantly impair the histology of AM. Wagner et al. [14] used 85% glycerol for cryopreserved AM, and their histological examinations had no significant alterations following cryopreservation, either for straight cryopreservation or with glycerol. They also demonstrated that neither tensile strength nor Young's modulus was significantly influenced by the storage method. In addition, they also detected a significant increase in tensile strength over storage time, independent of the storage method.

Some groups have found that storage of AM in 50% glycerol at −80°C decellularizes the AM and results in low viability [17–20]. Interestingly, the results from Wagner et al. [14] research showed that epithelial cells were not significantly reduced during freezing in comparison to stromal cells, possibly indicating a higher sensitivity of stromal AM cells to freezing damage than epithelial cells (**Figure 2**). Through repeated measurement analysis, storage time showed a significant effect on cell viability. Prabhasawat et al. [22, 23] reported that the use of a highly concentrated glycerol solution abolishes AM cell viability. The possible toxic effect of glycerol is responsible for that.

To summarize, glycerol-cryopreserved AM retains the histological characteristics of fresh AM independent of an increase in glycerol concentration. Tensile strength and elasticity can also be better preserved, with tensile strength increasing with storage time. However, the cell viability of cryopreserved AM was significantly affected by storage time and glycerol concentration. In particular, the stromal cells were more sensitive. A previous study [24] showed that this method had little effect on the growth factors of AM. More research is needed to confirm the effect of glycerol cryopreservation on AMs.

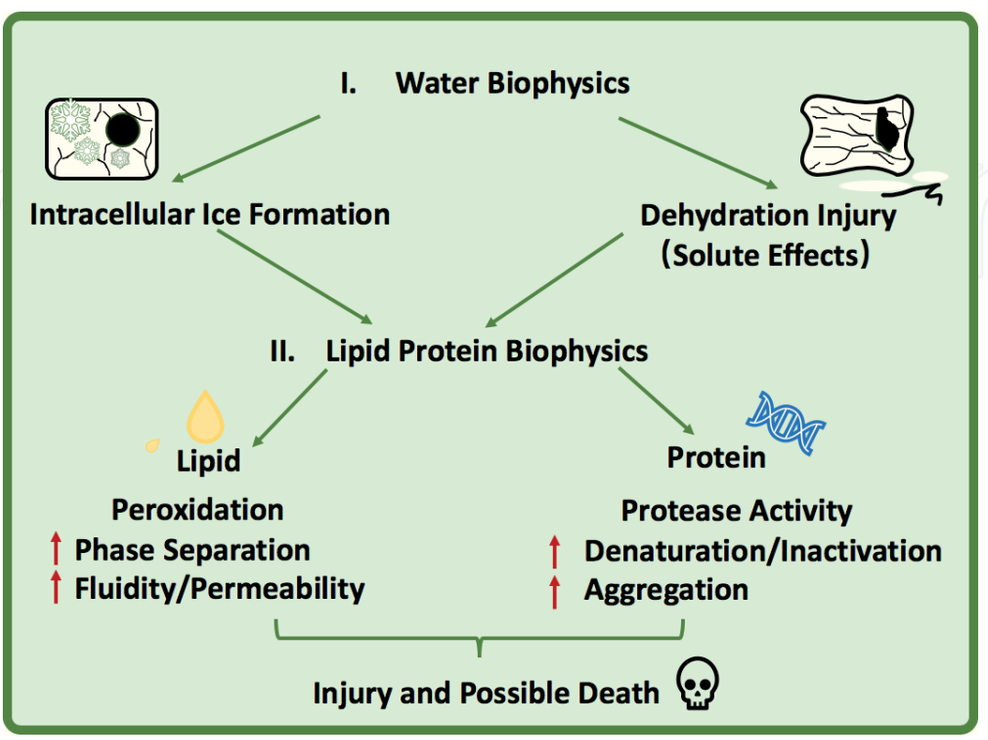


Figure 2.
Pathways of cellular injury during freeing.

1.3.2 DMSO-cryopreservation

DMSO has been used as an alternative for AM in glycerol-cryopreservation. An increasing concentration of DMSO is used instead of washing the AM with an antibiotic-saline solution after placenta collection [12]. Azuara-Blanco et al. [25] used 4%, 8%, and 10% DMSO, while Kubo et al. [26] used 0.5 M, 0.1 M, and 0.15 M DMSO for washing. AMs can be stored in 10% or 0.15 M DMSO at −80°C for several months without significant damage. In general, solutions containing DMSO are used less often for AM cryopreservation compared to glycerol, due to high toxicity [12]. However, AM storage solutions containing DMSO have been studied a lot regarding its ability to increase cell viability in AM under experimental conditions [2].

A cryopreservation method with DMSO from Duan-Arnold's group [24] showed a retained cell viability of over 80%. Cryopreserved AM tested after three months of storage showed no changes in the tissue architecture and collagen IV, which exists in the basement membrane, compared with fresh AM. However, in 2015, Yazdanpanah et al. [8] showed that the viability of epithelial cells in fresh AMs was estimated at 97% after staining with trypan blue, decreasing to about 50% in DMSO cryopreserved tissues after six months. They evaluated the effects of cryopreservation on AM angiogenesis modulation activity compared to fresh tissue in an animal model, showing that cryopreserved AM has the same effect on angiogenesis as fresh AM. The epithelial surface of cryopreserved AM inhibited angiogenesis, and the mesenchymal surface augmented vessel sprouting and length. In 2013, Tehrani et al. [27] used 10% DMSO as a cryoprotectant to evaluate the antibacterial properties of AM after preservation in vitro. The results of this study showed that the antibacterial property of AM was maintained after cryopreservation, but was dependent on bacterial genus and strain.

To sum up, the literature we collected on DMSO-cryopreserved AM showed no significant differences in tissue integrity and biological properties (antibacterial and angiogenesis modulation) compared with fresh AM. However, although many research groups use DMSO as a cryoprotectant, the data related to cell viability vary. These conflicting results can be attributed to several factors, including differing cryopreservation procedures and storage times.

1.4 Controversy on cryopreserved AM

1.4.1 Variable cell viabilities

In 2000, Kruse et al. [18] believed that devitalized AM exhibited therapeutic effects, and their data showed that the preservation of viable cells in AM provided no additional benefits. This conclusion led to the development of cryopreservation methods including AM devitalization steps. One of them, known as the CRYOTEK® process, includes a freezing step before cryopreservation, resulting in devitalized tissue [28]. However, Yan et al. [29] demonstrated that the combination of exogenous cells and acellular AM resulted in faster wound closure compared with acellular AM alone. Duan-Arnold et al. [24] demonstrated that endogenous viable cells allow cryopreserved AM with higher angiogenic, anti-inflammatory, antioxidant, fibroblast, and keratinocyte chemo-attractive activities when compared with AM in devitalization. Before 2001, most studies reported that cell viability of 50% or less at cryopreserved post-thaw with cells failing to survive after 18 months of storage at −80°C [26, 30]. Since then, scientists have been attempting to improve the cryopreservation method, for improved cell viability retention. For example, the cryopreservation protocol invented by the group of Duan Arnold et al. [24]

can maintain 70% or greater cell viability after 24 months of storage at −80°C. AM storage solutions containing dimethyl sulfoxide (DMSO) have been studied, mostly under experimental conditions, and shown the ability to increase AM cell viability [2]. Although the survival of amniotic cells is related to storage time, different cryopreservation steps can also affect cell viability, thus exerting different clinical effects.

1.4.2 Storage temperature

The best storage temperature (−196°C or − 80°C) is also a controversial issue for cryopreservation. AMs stored at −196°C have showed morphology similar to fresh AM in both preservation media, and AM stored at −80°C showed disruption of the stromal matrix [2, 31]. However, −80°C is still widely used by international scientists.

To sum up, cryopreservation protocols are not standardized. Preparation and sterilization before cryopreservation, as well as the selection of cryoprotectant during cryopreservation, will lead to high variability in cell viability [2, 32]. Different storage temperature and storage time also affects the structure and function of amniotic membrane. It is important to establish adaptable protocols for the clinical banking of AM that include verification of graft quality and viability before its release for transplantation, whether in the trial or clinical stage.

1.5 Commercially available cryopreserved AM

1.5.1 PROKERA®Slim (PKS)/PROKERA®(PK)

PROKERA®Slim (PKS) (Bio-Tissue, Inc., Miami, FL, USA) is a Class II medical device approved by the Food and Drug Administration in 2003 to be used as a temporary AM patch for delivering the biological actions of AM to a corneal surface without using sutures. It contains a piece of cryopreserved AM clipped into a concave poly-carbonate dual-ring system, like a symblepharon ring, that conforms to the corneal and limbal surface like a contact lens. The ring system has an inner diameter of 15 or 16 mm.

It has become the most common commercially available cryopreserved AM product in ophthalmology and is applied to various ocular surface and orbital disorders. It is a safe and effective method that makes AM transplantation sutureless and adhesiveless, contributing to healing and reconstruction of the ocular surface and orbit with minimal side effects [33]. However, PROKERA is not recommended for eyes with functioning blebs or glaucoma drainage implants because of the oppositional positioning of the retaining ring [34].

2. Cryopreservation of cornea

2.1 Introduction

Corneal disease is one of the world's leading causes of blindness. Corneal scarring and haze due to various factors can affect vision, making corneal transplantation an important means of treatment for corneal diseases [35–37]. Advances in corneal preservation techniques have improved the survival rate of corneal grafts [38] and have largely contributed to the development of modern corneal transplant surgery [39]. With the flourishing of corneal preservation technology, breakthroughs have been made in preservation times and corneal activity. Nevertheless,

cryopreservation is the only current method that can virtually preserve tissue structure for a long time.

Meanwhile, the development of modern eye banks have been accompanied by the advancement of corneal preservation technology. The establishment of an eye bank provides favorable conditions for corneal transplantation [40, 41].

2.2 Corneal transplantation and preservation

The idea of replacing the turbid cornea with transparent tissue was first proposed by Pellier de Quengsy in 1789 [42, 43]. In 1824, Reisinger exploited animal corneas in surgery [44], which was named keratoplasty. Later in the nineteenth century, a large number of animal experiments helped doctors realize that inter-species transplantation was a necessary condition to avoid corneal opacity after transplantation [45–47]. In view of this, researchers began to experiment with human corneal transplantation. Early corneal transplantation relied on living donor tissues due to fears relating to transplanting dead tissue. The first successful full-thickness corneal transplantation (including all corneal layers) was completed in 1905 [48]. It was not until the 1930s that the cornea of the deceased donor was used and the entire eye was kept in a glass jug (wet room) for several days [49].

In 1912, Magitot reported that excised corneal grafts could be preserved in red blood cells at 5°C for eight days [50–52]. The grafts were successfully used for corneal lamellar transplantation [53]. At first, the freshness of the cornea was considered key to corneal transplantation [54, 55]. However, Ukrainian doctor Filatov systematically reported the application of corpus corneal tissue to clinical practice [56], which possessed an inter-generational meaning. It opened a new era of corneal preservation and transplantation [57–59]. These developments led to the establishment of the world's first ophthalmology bank in New York in the 1940s. The preservation technique of the original eye bank was very simple [60, 61]: eyeballs were kept in a small glass bottle in a humid and cool environment [62]. Immediate removal of the eyeball after donor death was the only way to ensure the quality of the corneal grafts [63–65]. Eye banks were established in major cities, such as London, to guarantee that eyeballs were promptly forwarded [66, 67]. In the early 1950s, the activity of CECs was first considered as an important factor affecting transplantation [68–70]. The emphasis on preservation techniques was transferred to maintain the activity and integrity of CECs [71, 72]. Since then, corneal preservation techniques have been increasingly successful, resulting in approximately 40,000 corneal transplants per year in the United States, 20,000 per year in Europe, and thousands per year in other countries, such as India.

2.3 Corneal preservation methods

Corneal preservation is divided into two categories according to the survival of CECs: inactive and active preservation [73–75]. The former method includes dry preservation and cryopreservation [76–78] and operates under the principle of removing corneal moisture while inhibiting enzyme activity and autolysis in cells for long-term preservation [79, 80]. Common preservatives are glycerin, molecular sieves, and silica gel [81–83], which can preserve intact lamellar collagen structure [84]. Active preservation comprises short-term (hours to two days), medium-long term (7 to 30 days) and long-term (months to years) preservation. In terms of storage conditions, it utilizes normal (34~37°C), low (usually 4°C) and deep low (subzero) temperatures [85–88].

Short-term corneal storage mainly refers to the preservation of wet rooms, the simplest and most convenient of all corneal storage technologies. For this reason,

it is still the basic technology for preserving cornea in the eye banks of developing countries. As for medium-term corneal preservation, corneal preservation solution is stored at 4°C for 4 to 14 days [89].

The prolongation of corneal preservation allows more preparation for patients and flexible adjusting of operation times, while also satisfying blood test and corneal transportation times. With the improvement of preservation techniques, the composition of the corneal preservation solution has been constantly changing. A certain concentration of chondroitin sulfate is added to modify M-K solution, which can alleviate the swelling state during preservation. Optisol corneal medium preservation solution was proposed by Lindstrom and has become the most common preservation solution in US eye banks, which is mainly a mixture of K-liquid and Dexsol solution [90]. Long-term corneal preservation refers to organ culture storage and cryopreservation. Organ culture is to simulate the presence of a normal human cornea environment with medium at 30–37°C [91].

At present, there are several corneal preservation methods applied in global eye banks, but none of those is perfect. Each preservation method has its own advantages and disadvantages, which differ from the preservation temperature, the composition of the preservation solution, and the penetrant preventing matrix edema.

2.4 Cryopreservation

After donor death, the sudden stop of the aqueous humor causes nutrient and oxygen shortages, leading to final depletion at room temperature, which can, in turn, lead to autolysis of the corneal cells and initial damage to the cornea [92]. During the period from donor death to corneal removal and storage, the donor's corpse is exposed to room temperatures, necessitating minimal time delays to ensure that the initial donor cornea is healthy and intact along with functional endothelial cells.

The acceptable short storage time, as well as organ damage, poses a logistical challenge to organ storage and ultimately affects grafts and patient survival. Prolonged storage times can cause many transplantable organs, further exacerbating the growing imbalance between organ supply and demand. Organ cryopreservation is used to preserve long-lived cells and tissues. Theoretically, the storage of biological materials, including cells, tissues, and organs for transplantation at a low temperature (i.e., in liquid nitrogen at −160°C) is uncertain [93, 94]. Such a technique would have the potential to alter the way in which organs are recovered, distributed, and utilized for transplantation. However, ice is the biggest enemy in the cryopreservation of organs and tissues. Ice crystals, especially intracellular ice, can cause significant cellular damage and destroy the complex macroscopic tissues of intact organs. In this field, current developments are used to avoid the formation of ice, or mitigate it, during cryogenic storage. Any successful organ cryopreservation strategy requires a delicate balance between the relative needs of cryopreservation and toxicity in these situations.

2.5 Corneal cryopreservation technology

In 1954, Eastcott first adopted a cryopreserved human cornea for transplantation successfully [95, 96], pretreating the keratin tissue in glycerol before freezing it in a mixture of alcohol and carbon dioxide for cryopreservation of the full-thickness cornea [97]. This method generally removes the cornea under the protection of a cryogen to −80°C, and stores it in liquid nitrogen at −196°C. Therefore, the CECs are in a dormant state. The state can completely inhibit the metabolism

of cells, eliminate the toxic effects caused by the accumulation of metabolites, and avoid the need to change the liquid during organ culture. In addition, it also restrains microorganisms during cryopreservation, protecting the cornea from microbial invasion.

The components currently contained in corneal cryoprotectants include DMSO, propylene glycol, ChS, and sucrose. DMSO is a relatively stable protective agent to maintain the integrity of corneal cells, while sucrose molecules act as buffers in corneal protection, and ChS improves CEC activity in cryopreservation [98]. DMSO began to be treated as a tissue preservative to preserve cultured rabbit CECs by Smith [99]. Shortly thereafter, Mueller injected a preservation solution containing DMSO into the anterior chamber of an eyeball, placing the eyeball in a preservation solution containing glycerol. The cornea was removed before surgery for full-thickness transplantation [100, 101]. In 1965, Capella [102] used DMSO as an antifreeze to improve a cryopreservation method, ensuring corneal graft activity. According to another report [103], the clinical application of cryopreservation techniques has little differences in techniques. The corneal tissue must be preserved eight hours after death. By increasing the level of DMSO, it eventually reaches a concentration of 7.5%. The classic four-step cooling is to initiate a cooling rate at 1.5~2°C/min, drop the temperature to −30°C, change to 5–7°C/min, and ultimately maintain −80°C [104, 105].

It is still essential to further explore the rate of cooling to keep CEC activity and reduce cell loss [106, 107]. Temperature-controlled thawing before transplantation is a key step in protecting the corneal endothelium. At present, the prevalent view is that rapid rewarming could decrease the contact of cells with high concentrations of electrolytes and reduce cell damage [108]. The thawing process of the cryopreserved cornea must be strictly controlled, as the solute containing DMSO has endotoxicity once the temperature exceeds 37°C [109]. Cryopreservation would impair the morphology and function of the corneal endothelium. During the thawing process, an ascending solute concentration, the formation of crystals, changes in pH, and osmotic pressure will reduce the survival rate of CECs [110]. Glycerol, polyvinylpyrrolidone, and DMSO can all be used as cryopreservatives, but DMSO is currently the most widely used [111, 112].

2.6 Effect of corneal cryopreservation

The ultra-low temperature preservation method overcomes the drawbacks of most other corneal preservation methods, significantly prolonging corneal preservation time, reducing pollution, and avoiding the toxic effects of its own metabolic substances. Electron microscopy can observe changes in the subcellular morphology of CECs caused by cryopreservation, some of which are considered irreversible [113]. Studies have shown that, after cryopreservation, the barrier function of endothelial cells is impaired. Compared with wet room preservation and MK solution preservation, cryopreserved corneal grafts have been completely transparent for a long time after surgery. For one-year cryopreservation, 55% of endothelial cells were deactivated, while the rate of CECs preserved by MK solution was only 21–22% [114]. There are barely significant structural differences in microbiological, histological, and ultrastructural features when comparing long-term cryopreservation of tissue (>7 years) and short-term cryopreserved cat corneal sclera (<1 year) [115]. As such, tissues cryopreserved for up to 10 years could be used for tectonic support without structural or microbial barriers.

Under suitable conditions, no crystal solidification occurs during the freezing process, called vitrification [116]. Vitrification requires a high concentration of

cryoprotectant, yet theoretically, tissue could be stored in a very low temperature environment without forming intracellular or extracellular crystals, and corneal endothelium damage could be avoided significantly [117]. Glycerol, 1,2-propanetriol, and 2,3-butanediol are all considered as eligible cryopreservation agents for corneal vitrification [118, 119].

Studies have found that an effective concentration of a single cryopreservative is toxic to CECs, yet the mixture of preservatives or the addition of preservatives at low temperatures seems to reduce toxicity [120]. As a means of corneal preservation, further study is warranted to investigate whether vitrification would achieve good results. In 1981, Sperling used corneal grafts in a corneal preservation solution at the early stage and carried out a cryopreservation operation later. After rewarming, the cornea was transferred to a preservation solution, identifying corneal activity. The following studies indicated that the corneal grafts maintained transparency 71% of the time after 1 year and 58% of the time after 12 years [121].

In our previous study, we performed lamellar keratoplasty combined with keratopigmentation in 22 corneal leukoma eyes using glycerol-cryopreserved corneal tissues, and no graft-rejection occurred during the 3 years of follow-up. Moreover, the outcome of a low graft rejection rate in glycerol-cryopreserved corneal tissues was also confirmed by our preceding study in treating Terrien marginal degeneration. In the subsequent study, for patients with refractive herpes simplex keratitis, 3 eyes of 27 eyes (11.1%) suffered allograft stromal rejection, all eyes reversed after prompt medication. Meanwhile, only 2 eyes (7.41%) exhibited refractive herpes simplex keratitis recurrence and the main site was located at the margin of the graft and the recipient bed. This result is consistent with the theory that grafts survive better when compared with reports clarifying that up to 33% of patients have suffered recurrence using fresh grafts. The recurrence rate in fresh grafts may be partially related to the long-term usage of topical steroid eye drops; however, it may be much more closely correlated with fewer keratocytes in the cryopreserved donor tissue to reactivate immune-inflammatory responses [122–124]. Based on the above information, glycerol-cryopreserved corneal tissues can be effectively and biosafely used with a low rejection and recurrence rate in corneal transplantation, especially in developing countries where good donor corneas are difficult to get.

3. Conclusion

The cryopreservation method can preserve the activity of the AM and cornea for extended periods up to several years, solving the problem of preservation time and activity deterioration. However, equipment complications, expensive technical support, and transport difficulties have become impediments to widespread use. The functional status of AM, endothelial cells, and corneal transparency have been of vital importance in the development of cryopreservation. As researchers become more aware of the function and properties of CECs, attempts to find a more conducive method and media for the preservation of AMs and corneas will continue.

Conflict of interest

We declare that we have no conflicts of interest.

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Author details

Yuting Shao†, Chao Chen†, Qi Zhou, Jun Yang, Xiao Lv, Mingyue Lin and Yanlong Bi*
Department of Ophthalmology, Tongji Hospital, Tongji University School of Medicine, Shanghai, China

*Address all correspondence to: biyanlong@tongji.edu.cn

† Co-first author.

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