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# Expansion and cryopreservation of porcine and human corneal endothelial cells

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ABSTRACT

Impairment of the corneal endothelium causes blindness that afflicts millions worldwide and constitutes the most often cited indication for corneal transplants. The scarcity of donor corneas has prompted the alternative use of tissue-engineered grafts which requires the *ex vivo* expansion and cryopreservation of corneal endothelial cells. The aims of this study are to culture and identify the conditions that will yield viable and functional corneal endothelial cells after cryopreservation. Previously, using human umbilical vein endothelial cells (HUVECs), we employed a systematic approach to optimize the post-thaw recovery of cells with high membrane integrity and functionality. Here, we investigated whether improved protocols for HUVECs translate to the cryopreservation of corneal endothelial cells, despite the differences in function and embryonic origin of these cell types. First, we isolated endothelial cells from pig corneas and then applied an interrupted slow cooling protocol in the presence of dimethyl sulfoxide ($Me_2SO$), with or without hydroxyethyl starch (HES). Next, we isolated and expanded endothelial cells from human corneas and applied the best protocol verified using porcine cells. We found that slow cooling at 1 °C/min in the presence of 5% $Me_2SO$ and 6% HES, followed by rapid thawing after liquid nitrogen storage, yields membrane-intact cells that could form monolayers expressing the tight junction marker ZO-1 and cytoskeleton F-actin, and could form tubes in reconstituted basement membrane matrix. Thus, we show that a cryopreservation protocol optimized for HUVECs can be applied successfully to corneal endothelial cells, and this could provide a means to address the need for off-the-shelf cryopreserved cells for corneal tissue engineering and regenerative medicine.



## 1. Introduction

The cornea is the transparent tissue at the front of the eye that serves as the major refractive component and protects the eye from physical damage and infection. It is about 550 μm thick and comprises three cellular layers namely the epithelium, the stroma, and the endothelium, a single layer of hexagonal cells that acts as a barrier and pump to restrict movement of water into the stroma [12]. When excess water is imbibed by the stroma the cornea swells, resulting in blurred vision and eventual blindness. Corneal blindness afflicts over 10 million people worldwide and current treatment often involves replacement of the entire cornea or the defective endothelial layer; however, there is a global shortage of donor corneas [18].

The density of the human corneal endothelial cell layer (3000 to 4000 cells per $mm^2$ at childbirth) naturally decreases with age at the rate of 0.5–0.6% per year [6]. These cells are not known to regenerate *in vivo* as they are arrested in the G1 phase of the cell cycle [63]. Instead the cells undergo an increase in the range and distribution of cell sizes (polymegethism) and a departure from their typical hexagonal shape to various polygonal structures (pleomorphism) [59]. Age- and disease-related loss of these cells constitutes the most often cited reason for about 150,000 corneal transplants performed annually worldwide [39]. In the US alone in 2015, 48,792 corneal transplants were performed, with endothelial keratoplasty (27,208) being the most common, according to the annual report of the Eye Bank Association of America [65]. The availability and quality of donor corneas are primary concerns and yet a majority of donors fall in the age range of 50–80 years old [65]. Due to the age-dependent deterioration of the cornea about

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half of donated corneas are deemed unsuitable for transplant for failure to meet the minimum endothelial cell density of 2000–2200 cells/mm$^2$ [2].

Human corneas for transplant are commonly stored at 2–8 °C for up to 14 days [1] or by organ culture, which allows up to 4 weeks of storage [10,11]. Cryopreservation can offer an unlimited storage time and eliminate the time-dependent deterioration of donor tissue. The first cryopreservation technique developed for rabbit, cat and human corneas used 7.5% dimethyl sulfoxide ($Me_2SO$) and 10% sucrose [9]. Rabbit and human corneas have also been vitrified using 6.8 M propane-1,2-diol and shown to retain endothelial function [3]. Nonetheless, cryopreserved human corneas have not been adopted by eye banks for routine use due to their significantly lower performance compared with fresh corneas, attributed in part to polymegethism and pleomorphism and a reduction in viability of the endothelial cells after cryopreservation [7,8].

In order to address the global shortage of transplantable donor corneas, recent efforts have been expended towards the development of protocols for the expansion of human corneal endothelial cells for injection into the anterior cornea [35] or for seeding onto natural or synthetic scaffolds for transplantation [20,23,32,56]. These approaches require a readily accessible source of a sufficient number of viable and functional corneal endothelial cells. Typically, human corneal endothelial cells are cryopreserved in suspension in the presence of 10% $Me_2SO$ and 90% fetal bovine serum (FBS), slowly cooled to −80 °C in an isopropanol bath freezing container and then transferred to liquid nitrogen [54]. Porcine corneal endothelial cells in suspension have also been cryopreserved in 20% $Me_2SO$ with a post-thaw recovery of 72 ± 4% [58]. Vitrification procedures have also been employed [16], but they use high concentrations of toxic cryoprotectants and are more susceptible to changes in protocol parameters such as cooling and warming rates than are slow cooling protocols. Slow cooling allows super-cooled water to leave the cells osmotically before the onset of harmful intracellular ice formation. We have developed an interrupted slow cooling protocol (graded freezing) that not only identifies the extent and occurrence of cryoinjury, but also provides a convenient method for investigating the degree of protection conferred by permeating and non-permeating cryoprotective agents [27,49]. As a permeating cryoprotectant, $Me_2SO$ colligatively reduces the amount of ice formed at a given temperature and mitigates solute effects injury [26]. The mechanisms of cryoprotection by hydroxyethyl starch (HES), a non-permeating cryoprotectant, have been reviewed, which include cell dehydration that mitigates damage due to intracellular ice formation, stabilization of the cell membrane, and ability to bind water molecules and keep them in a glassy state [29,53]. We have previously applied the combination of $Me_2SO$ and HES in the optimization of cryopreservation of human umbilical vein endothelial cells (HUVECs) using interrupted cooling protocols [55]. Here, we investigated whether the optimized protocol will be applicable to corneal endothelial cells.

Since the anatomy and physiology of the eye of the pig is similar to that of the human in many aspects [21,30,44], and due to their availability and accessibility, our first aim was to isolate corneal endothelial cells from pig eyes and to culture and expand the cells to a number sufficient to apply the cryopreservation protocol that had been optimized for HUVECs. While animal-derived primary cells are suitable for these experiments, the use of human corneal endothelial cells is desirable for investigations directed towards clinical applications. Our next aim was to obtain cadaveric human corneas that have been deemed unsuitable for transplant, isolate corneal endothelial cells, culture them in supplemented media conducive to cell proliferation, and subject them to the protocol verified using porcine corneal endothelial cells. As cells can undergo biological alterations during the freeze/thaw process, our last aim was to demonstrate that viable and structurally intact corneal endothelial cells are recovered post-thaw. Corneal endothelial cell monolayers express typical markers such as zonula occludens (ZO)-1, a tight junction protein that links other junctional components with the actin cytoskeleton. ZO-1 and filamentous (F)-actin are both important for the barrier function of the endothelium [5,36]. As a further confirmation of the recovery of living cells after cryopreservation, we also examined the ability of fresh and thawed corneal endothelial cells to form tubes in the reconstituted basement membrane Matrigel [4].

## 2. Materials and methods

### 2.1. Cell isolation and cultures

The method for isolating porcine corneal endothelial cells was adapted from previously published procedures for corneal endothelial cells from bovine and porcine sources [24,48] with some modifications. Eyes were obtained through a local abattoir from six-month old pigs (cross-breed of Duroc and Landrace) slaughtered for meat processing and human consumption. The eyes were placed in a leak-resistant specimen container and refrigerated until pick-up. Within 5 h of collection, the eyes were treated with chlortetracycline antibiotic (50 μg/mL, Sigma-Aldrich, Oakville, ON, Canada) for 15 min, and then transferred to a designated biosafety cabinet. The cornea was excised with a scalpel along with 1–2 mm of surrounding scleral tissue. The cornea was placed, endothelial side up, in a sterile 12-well tissue culture plate and rinsed with $Ca^{2+}$-/$Mg^{2+}$-free phosphate-buffered saline solution (PBS, Gibco, Life Technologies, Burlington, ON, Canada). The inner side of the cornea was treated with trypsin/EDTA (0.05%/0.02%, Gibco) and incubated for 10 min at 37 °C. The enzymatically-dislodged cells were collected with a sterile pipette tip, centrifuged, then re-suspended in DMEM supplemented with 4.5 mg/mL glucose, 4 mM L-glutamine, 10% FBS and 1% antibiotic-antimycotic agent (10,000 units/mL of penicillin, 10,000 μg/mL of streptomycin, and 25 μg/mL of amphotericin B) (all from Gibco). The cells pooled from at least three corneas were seeded in T25 tissue culture flasks and incubated at 37 °C/5% $CO_2$ until confluent. Media changes were carried out every 2–3 days. Once confluent (~10 days), the primary cells were trypsinized and counted using a Coulter® Z2™ particle count and size analyzer (Beckman Coulter, Mississauga, ON, Canada). Cells were sub-cultured up to 10 population doublings.

The method for isolation and culture of human corneal endothelial cells was based on modified published procedures in which proteolytic digestion, seeding density and matrix for cell growth have been optimized [40–42]. This part of the study was approved by the Human Research Ethics Board of the University of Alberta. Donor eyes from deceased donors (n = 5) were procured through the Lions Eye Bank (Rockyview General Hospital, Calgary, AB, Canada) after they were deemed unsuitable for transplant due to poor tissue quality (n = 3 donors) or medical conditions (n = 2 donors). Informed consent for use in research was obtained from the next of kin and donor confidentiality was maintained. The average age of donors (1 male, 4 female) was 70.2 ± 5.8 years (range: 63–76 years). The time from death to refrigeration at 2–8 °C in balanced salt solution at the eye bank was an average of 6.1 ± 2.0 h (range: 4–8.5 h). All eyes were received wrapped in gauze moistened with saline in containers cooled using gel packs. Primary cultures were initiated within an average of 5.2 ± 2.9 days of death (range: 3–10 days). Upon receipt, the eyes were washed three times with $Ca^{2+}$-/$Mg^{2+}$-free PBS containing 1% antibiotic-antimycotic for 15 min each time. Using sterile scalpel and dissecting scissors, the cornea was excised along with 1–2 mm of surrounding scleral tissue and then washed with 1 mL of PBS/