Viability of keratocytes in epikeratophakia lenticules

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Abstract

Aim—To study the influence of cryoprotectant, cooling rate, and warming rate on recovery and viability of keratocytes from corneas for cryolathing.

Methods—Corneas were frozen at -50°C for 2 minutes either after exposure to 10% dimethyl sulphoxide in Eagle's MEM for 15 minutes at room temperature (about 22°C), or without earlier exposure to the cryoprotectant. Corneas were cooled either rapidly ($20^{\circ}\text{C}/\text{min}$) or slowly ($1^{\circ}\text{C}/\text{min}$), and they were warmed either rapidly ($>50^{\circ}\text{C}/\text{min}$) by direct transfer into medium at 22°C or slowly ($<20^{\circ}\text{C}/\text{min}$) in air at 22°C . The cryoprotectant was removed by dilution in medium containing 0.5 mol/l sucrose. Recovery of keratocytes was determined by using collagenase digestion to release the cells from the stroma and trypan blue staining. Viability was assessed by the outgrowth of cells from stromal explants in primary tissue culture.

Results—The use of a cryoprotectant before freezing was beneficial, irrespective of the different cooling and warming regimens. Both collagenase digestion and tissue culture revealed that keratocyte survival was improved when corneas were warmed rapidly rather than slowly. The collagenase digestion assay showed an apparently higher recovery of keratocytes after slow cooling (54.3%) than after rapid cooling (34.1%), but no differences in cell viability could be demonstrated by primary tissue culture.

Conclusion—Although in these experiments slow cooling apparently provided the best recovery of keratocyte numbers (though not viability), previous work had revealed some disruption of the epithelial basement membrane after slow cooling. If viable keratocytes and good preservation of epithelial basement membrane are considered to be prerequisites for epikeratophakia lenticules then it is suggested that corneas should be prepared for cryolathing by freezing rapidly after exposure to 10% dimethyl sulphoxide and, following cryolathing, they should be warmed rapidly.

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properties of the cornea. The tissue lens is composed of Bowman's layer and anterior stroma of a donor cornea which has been frozen and lathed.

Epikeratophakia was developed originally for the correction of aphakia by Kaufman in 1980.¹ The procedure was first performed in aphakic adult patients who were spectacle and contact lens intolerant, and who were not good candidates for secondary intraocular lens implantation.² Over the past 15 years, the procedure has undergone many refinements and considerable testing, both experimentally and clinically. The tissue lenses have been adapted for the correction of aphakia in paediatric patients with congenital or traumatic cataracts,³⁻⁵ for the correction of myopia,⁶ and for the treatment of keratoconus.^{7,8}

Among the advantages of epikeratophakia are the simplicity and the reversibility of the procedure, as well as the wide range of available correction. Epikeratophakia, since its advent, has traditionally been considered a safe and effective form of refractive surgery. Although large published series (mostly from the United States) have generally concluded that epikeratophakia is a worthwhile procedure,⁹⁻¹¹ there have been some case reports of failed epikeratophakia.^{12,13} Some of the main complications in these grafts are delayed epithelial healing,¹⁴⁻¹⁶ hazy graft, graft melt,¹⁷ infection, graft dehiscence, refractive error, and interface cyst/opacities.^{18,19} Among these, failure to re-epithelialise the graft surface, which leads either to scarring or melting of the tissue lens, is the most common cause of removal of the tissue lens in epikeratophakia.

Stromal keratocytes have been shown to be irreversibly damaged by freezing during the processing of corneas for lamellar refractive surgery.²⁰⁻²² Since repopulation by host keratocytes can take several months, it is thought that delays in achieving best corrected visual acuity, which may in some cases be up to 1 year,²³ could be linked to a paucity of keratocytes in the donor tissue.

Successful cryopreservation of living cells requires a balance between several interrelated variables, principally cooling rate, warming rate, and the type and concentration of cryoprotectant used.²⁴ Current methods for the production of epikeratophakia lenticules by cryolathing involve rapid cooling of corneas, in the absence of cryoprotectants, to the lathing temperature (around -50°C) followed by slow warming. Such conditions would be expected to be deleterious for mammalian cells. In an attempt to improve keratocyte preservation in epikeratophakia lenticules, a collagenase digestion assay and primary tissue culture were

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Epikeratophakia, or onlay lamellar keratoplasty, is a surgical procedure in which a lenticule made of donor tissue is sutured on to the anterior surface of the cornea to alter the surface topography and, hence, refractive

used in this present study to evaluate, respectively, keratocyte recovery and viability in rabbit corneal tissue following freezing under different conditions of cooling and warming, and in the presence or absence of a cryoprotectant.

Methods

Forty eight rabbit eyes were obtained from an abattoir immediately post mortem. The eyes were washed three times in sterile 0.9% NaCl, then immersed in two washes of 1% polyvinylpyrrolidone-iodine (2×1 min). The iodine was neutralised with 0.1% sodium thiosulphate, and the eyes were again rinsed three times in sterile 0.9% NaCl. A No 10 scalpel blade was used to make a small incision along the limbus. Rabbit corneal buttons were excised using a pair of corneal excision scissors. Each rabbit cornea was placed on a Teflon block and an 8.0 mm button was cut using a trephine.

CRYOPROTECTANT ADDITION AND REMOVAL

When corneas were to be frozen in the presence of cryoprotectant, they were immersed in 20 ml 10% (v/v) dimethyl sulphoxide (DMSO) in Eagle's minimum essential medium (MEM) for 15 minutes at 22°C. They were then removed from the cryoprotectant medium and blotted dry before being frozen. The cryoprotectant was removed after thawing by transferring corneal buttons into 20 ml 0.5 mol/l sucrose in MEM at 22°C. The sucrose acted as an osmotic buffer to limit the cell swelling that would have been induced by the reduction in DMSO concentration. After 15 minutes, the samples were placed into MEM alone.

COOLING AND WARMING RATES

Corneas were placed into 30 ml glass vials without any medium, and cooled to -50°C in a programmable controlled rate freezer (Planar Kryo 10-16) at either 1°C/min (slow cooling) or approximately 20°C/min (rapid cooling). The corneas were held at -50°C for 2 minutes and then warmed either slowly (<20°C/min) by holding at room temperature (about 22°C), or rapidly (>50°C/min) by directly immersing the corneas into 20 ml medium at room temperature. When cryoprotected corneas were warmed rapidly, the warming medium contained 0.5 mol/l sucrose.

EXPERIMENTAL PROCEDURE

Corneas were allocated to six groups according to experimental treatment: group 1 – unfrozen (control); group 2 – rapid cool, slow warm without cryoprotectant (equivalent to routine processing for epikeratophakia); group 3 – rapid cool, rapid warm without cryoprotectant; group 4 – rapid cool, slow warm with cryoprotectant; group 5 – rapid cool, rapid warm with cryoprotectant; and group 6 – slow cool, rapid warm with cryoprotectant.

ASSAYS

We investigated the effects of different cooling and warming rates on keratocyte survival in cornea buttons using a collagenase digestion assay to estimate numbers of intact cells, and primary tissue culture to determine cell viability.

Collagenase digestion assay^{25 26}

Three corneas from each group were included in this assay. The corneal buttons were placed in petri dishes containing a small amount of MEM and the epithelium of each cornea was removed by gentle scraping with a scalpel blade. The endothelium was removed with a cotton swab, and the corneal button was placed in a 5 ml plastic conical tube containing 2 ml of 0.1% type IV collagenase (Sigma) in MEM buffered with 25 mM HEPES and containing penicillin, streptomycin, amphotericin B, and 2 mmol/l L-glutamine. Each cornea was incubated in this solution in a humidified 5% carbon dioxide atmosphere at 37°C for an optimised time of 4 hours (see below). The specimens were then gently vortexed for 10 seconds, and centrifuged at 1500 rpm for 3 minutes in a swinging bucket rotor. The supernatant was decanted and the pellet was resuspended in 1.0 ml of the culture medium, to which 100 µl trypan blue (0.4%) was added. After 5 minutes, a haemocytometer was filled with the suspension and the cells counted.

The incubation time in collagenase had to be optimised because the previously recommended incubation time of 18 hours^{25 26} left no intact cells. We therefore prepared a series of 20 corneas and incubated them in the collagenase medium. At hourly intervals, two corneas were removed and cell counts performed after trypan blue staining. The optimum time that allowed adequate digestion of the stromal tissue and maximal release of intact cells was found to be 4 hours.

Trypan blue is commonly used in dye exclusion assays for distinguishing between intact cells and cells that have lost plasma membrane integrity. Although not strictly true, it is often assumed that unstained cells are viable and that stained cells are dead. Since non-viable cells could degenerate completely during incubation in the collagenase medium and would not be visible on staining, the count of stained cells would underestimate the total number of damaged cells. Therefore, only unstained cells were counted and the cell concentration, and thus the total number of intact cells in a corneal button, was determined. The percentage recovery of cells was defined as the number of unstained cells in the experimental cornea relative to the control cornea.

Primary tissue culture

Five corneas from each group were prepared for culture. After thawing, the corneal buttons were placed in a petri dish containing a small amount of MEM. The endothelium and Descemet's membrane were scraped off from each cornea with a scalpel blade. The edge of

Figure 1 Numbers of keratocytes isolated from central 8 mm corneal buttons following different treatment regimens. Each bar represents the mean ($\pm$ SD) of three corneas. Control=unfrozen tissue (group 1); RC/SW (-CP)=rapid cool, slow warm, no cryoprotectant (group 2); RC/RW (-CP)=rapid cool, rapid warm, no cryoprotectant (group 3); RC/SW (+CP)=rapid cool, slow warm with cryoprotectant (group 4); RC/RW (+CP)=rapid cool, rapid warm with cryoprotectant (group 5); and SC/RW (+CP)=slow cool, rapid warm with cryoprotectant (group 6).

the stroma was roughly teased with forceps until a separation could be seen between two layers of stroma. The epithelial side was pulled away from the rest of the stroma using forceps. The stromal buttons were cut into eight equal segments with a scalpel blade, giving a total of 40 explants in each group. Each segment was placed in the centre of a separate well of a 24 well culture plate. A drop of fetal bovine serum (FBS) was added to each well to aid attachment of the stromal explants. After several hours, 1 ml culture medium was added to each well. The medium consisted of TC 199 with 2.2 g/l NaHCO_3 , 2 mmol/l L-glutamine, 10% FBS, antibiotics (100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 g/ml amphotericin B), and 1% non-essential amino acids. The cultures were incubated in 5% carbon dioxide in air in a humidified incubator at 37°C. The culture medium was changed every 3 days.

The times when keratocytes started to migrate from the individual pieces of tissue and the times to confluence were recorded. The tissue fragments were left on the growth sur-

face for as long as possible, and removed only when they had either shrivelled up or had started floating. If no growth was detected after 30 days of incubation, the explant was discarded.

STATISTICAL METHODS

For group comparisons of cell growth, we used analysis of variance and Tukey's method for multiple unplanned pairwise comparisons. The level of significance was set at 5%.

Results

GROUP 1 – UNTREATED CONTROL

Collagenase digestion released a mean of 31.1×10^4 (SD 3.7×10^4) unstained keratocytes from each unfrozen cornea (Fig 1). Primary tissue culture demonstrated cell growth from 22 of the 40 stromal explants obtained from five unfrozen corneas (Table 1). Cells appeared from the explants after 7.7 (SD 2.6) days (Fig 2) and reached confluence 19.9 (1.7) days later (Fig 3).

GROUP 2 – RAPID COOL, SLOW WARM, NO CRYOPROTECTANT

These corneas showed the lowest cell counts in the collagenase digestion assay (Fig 1) with a mean of 0.3×10^4 cells (0.1×10^4). This was equivalent to a recovery of only 1% compared with the controls (group 1). None of the explants of corneas frozen under these conditions demonstrated any capacity for cell growth.

GROUP 3 – RAPID COOL, RAPID WARM, NO CRYOPROTECTANT

In the absence of cryoprotectant, rapid warming resulted in the recovery of 4.7×10^4 (1.3×10^4) cells per cornea (15.1% compared with control) (Fig 1). Despite this improvement in recovery when compared with slow warming (group 2), there was no evidence of cell viability since none of the tissue explants initiated any cell growth.

Figure 2 Effects of different treatment regimens on keratocyte growth. Each bar represents the mean ($\pm$ SD) of time to appearance of cells from explants. Control=unfrozen tissue (group 1); RC/RW (+CP)=rapid cool, rapid warm with cryoprotectant (group 5); and SC/RW (+CP)=slow cool, rapid warm with cryoprotectant (group 6).

Figure 3 Effects of different treatment regimens on keratocyte growth. Each bar represents the mean ($\pm$ SD) of time to confluence. Control=unfrozen tissue (group 1); RC/RW (+CP)=rapid cool, rapid warm with cryoprotectant (group 5); and SC/RW (+CP)=slow cool, rapid warm with cryoprotectant (group 6).

Table 1 Growth of keratocytes from stromal explants. Each group contained a total of 40 explants from five corneas: n is the number of explants from which cells grew. The mean (SD) times in days to the first appearance of cells and from then to confluence are also shown

	n	Appearance (days)	Confluence (days)
Group 1 – control	22	7.7 (2.6)	19.9 (1.7)
Group 2 – RC/SW (no CP)	0	–	–
Group 3 – RC/RW (no CP)	0	–	–
Group 4 – RC/SW (+CP)	1	19	20
Group 5 – RC/RW (+CP)	12	8.4 (3.3)	19.7 (2.0)
Group 6 – SC/RW (+CP)	8	9.0 (2.4)	18.5 (1.8)

RC=rapid cool, SC=slow cool, RW=rapid warm, SW=slow warm, CP=cryoprotectant.

GROUP 4 – RAPID COOL, SLOW WARM WITH CRYOPROTECTANT

Only one of the 40 explants from corneas frozen under these conditions showed any cell growth, with cells appearing only after 19 days of culture: confluence was reached after a further 20 days (Table 1). These corneas contained on average 5.4×10^4 (0.6×10^4) keratocytes, which was 17.4% of controls.

GROUP 5 – RAPID COOL, RAPID WARM WITH CRYOPROTECTANT

Again, rapid warming gave higher recovery of cells than slow warming (group 4), with 10.6×10^4 (1.8×10^4) cells per cornea (34.1% compared with controls) (Fig 1). More important, however, was the improvement in viability with a total of 12 of the 40 explants initiating cell growth (Table 1); but this was less than the controls (group 1) (χ^2 test: $p < 0.01$). Keratocytes began to migrate from the explants after 8.4 (3.3) days, and the cultures became confluent after a further 19.7 (2.0) days. These times were no different from the controls (ANOVA: $p > 0.05$) (Figs 2 and 3).

GROUP 6 – SLOW COOL, RAPID WARM WITH CRYOPROTECTANT

Finally, slow cooling gave the highest cell recovery of 16.9×10^4 (3.7×10^4) cells per cornea (54.3% compared with control) (Fig 1). Cells migrated from eight of the 40 explants after 9 (2.4) days of culture, and confluence was reached after a further 18.5 (1.8) days (Table 1). Although fewer explants in this group displayed cell growth than in the unfrozen control group (χ^2 test: $p < 0.01$), the times to first appearance of cells and to confluence were no different from the controls ($p > 0.05$) (Figs 2 and 3).

Discussion

Processes in the preparation of lenticules for epikeratophakia, which may include freezing, lathing, lyophilisation, and the use of the corneal press, have all been implicated in the cause of structural damage to the tissue lens. The potential adverse effects of cryolathing on the cornea are loss of epithelial cells, epithelial basement membrane damage, Bowman's layer fractures, keratocyte death, and stromal collagen disruption.^{22 27 28} Studies of human specimens obtained on removal of failed

epikeratophakia lenticules or during penetrating keratoplasty have described similar complications.^{14–16} Previous studies have demonstrated that the conditions of freezing during preparation of epikeratophakia lenticules can affect keratocyte survival,^{21 25 26 29} but most of these have focused on the use of cryoprotectants or on cooling rate, with little attention being paid to the rate of warming of the frozen tissue.

The solution, KM-26, originally developed by Barraquer for cryolathing corneal tissue contains 8% glycerol and 4% DMSO. Evaluations of this solution have demonstrated that keratocyte survival is better in the presence of cryoprotectants,²¹ although one study suggested that the presence of the DMSO was deleterious.²⁹ On the other hand, recovery of keratocytes has been reported to be markedly better in a solution denoted CPTES, which does not contain any cryoprotectant, than in KM-26.³⁰ This CPTES solution is an electrolyte solution containing a high K^+ concentration, a low Na^+ concentration and the organic buffer TES: it was originally developed as a carrier solution for cryoprotectants.³¹

In the present study, the presence of 10% DMSO markedly improved both the recovery and viability of keratocytes. Not only is this a somewhat higher concentration than in KM-26, but the tissue was exposed to the cryoprotectant solution for 15 minutes before freezing, which is far longer than the 2 minute exposure typically used for KM-26.²¹ This would have allowed more time for permeation of the cryoprotectant into the stromal tissue. The extent of permeation of KM-26 is uncertain: glycerol tends to be far less permeable than DMSO and, coupled with a short exposure time, the main effect of KM-26 may simply be partial dehydration of the tissue with little permeation of cryoprotectant. This could still be protective because the prior dehydration of the tissue would alter both the amount and distribution of ice within the stroma in ways that may favour keratocyte survival.

Addition and removal of permeating cryoprotectants, such as DMSO, cause osmotically induced fluctuations in cell water volume.³² Removal of cryoprotectants by dilution causes cell swelling, which could be deleterious. To lessen this osmotic stress during removal of the DMSO, we included sucrose, which does not permeate cells, in the dilution medium. This would have increased the osmolality of the dilution medium to approximately 900 mosmol/kg, and would have partly balanced the osmotic effect of the reduction in DMSO external concentration. It is likely, therefore, that this protocol was less damaging to keratocytes than single step dilution with, for example, balanced salt solution or MEM.

By using a slow, controlled rate freeze on a modified Barraquer cryolathe, Lee *et al*²⁵ demonstrated that keratocyte viability increased when the cooling rate was reduced from the standard 33°C per minute to 1–3°C per minute. Lamellar grafts in rabbits performed with tissue frozen slowly were thinner, clearer, and had less interface irregularity than grafts from rapidly frozen tissue. The slowly

frozen grafts also possessed greater numbers of keratocytes at 3 and 7 days after surgery.

Although we found a higher recovery of keratocytes in the presence of DMSO with slow cooling (54.3%) than with rapid cooling (34.1%), there was little difference between rapid and slow cooling in terms of cell viability, as demonstrated by primary tissue culture. There was, however, a large effect of warming rate on both cell recovery and viability. Slow warming, such as would be achieved by allowing tissue to thaw on the cryolathe chuck, reduced recovery of keratocytes compared with rapid warming in medium, and reduced viability to such an extent that only one out of 40 stromal explants showed any growth of cells. We previously showed a similarly beneficial effect of rapid warming on the structural integrity of epithelial basement membrane.³³ The effect of warming rate is likely to be associated with the recrystallisation and reorganisation of ice within the stroma. Recrystallisation is the growth of large ice crystals at the expense of small, less thermodynamically stable crystals, and this is thought to contribute to cryoinjury in tissues.²⁴ Rapid warming would allow far less time for this phenomenon to occur.

The present study has demonstrated not only that the conditions of freezing and thawing affect keratocytes, but has confirmed that the recovery of cells that can exclude trypan blue does not necessarily indicate viability. The collagenase digestion assay allowed the release of keratocytes from the stromal collagen matrix, and the exclusion of trypan blue demonstrated that they had retained some degree of plasma membrane integrity. But unstained cells were also recovered from tissue that had been frozen under conditions that resulted in little or no outgrowth of cells from explants (groups 2, 3, and 4). Although the outgrowth of cells from stromal explants can be variable (only 22 out of 40 explants from unfrozen corneas showed growth of cells), this assay at least provides a definite demonstration of the presence of viable cells. Accordingly, the results with this stricter assay were different from the collagenase digestion, which gave only an estimate of cell numbers present in stroma but no indication of viability.

Successful epikeratophakia requires not only recovery of an intact and durable epithelial sheet over the newly grafted lenticule, but repopulation of the lenticule by host keratocytes, especially if donor keratocytes are depleted during cryolathing. Keratocyte repopulation and the re-establishment of a normal corneal extracellular matrix play a significant role in the recovery of corneal clarity following frozen lamellar keratoplasty.²⁰ In a study of epikeratophakia in rabbits, the repopulation of keratocytes was almost complete on day 90 and gradually returned to normal within a year.³⁴ Failure of keratocyte repopulation could be explained on the basis of disorganisation of collagen lamellae, which may account for the variable visual acuity results.³⁵ Alternatively, a loss of keratocytes has been shown following de-epithelialisation, suggesting an interaction

between corneal epithelium and stroma in rabbits.³⁶⁻³⁸

Current methods for the production of lenticules for epikeratophakia often involve rapid freezing, cryolathing, and slow warming of corneal tissue in the absence of cryoprotectants. This procedure has been shown to be responsible for extensive keratocyte destruction, considerable distortion of the epithelial basement membrane, and condensation of anchoring fibrils in the tissue lens.³³ In terms of keratocyte viability, the most important factors were the presence of cryoprotectant and use of rapid rather than slow warming. Slow cooling did give greater recovery of keratocytes, but there was no advantage over rapid cooling in terms of cell viability. Indeed, the slow cooling procedure may give rise to greater disruption of stromal lamellae and structural alteration of the epithelial basal lamina, which in turn may compromise the re-epithelialisation of donor lenticule.³³ Thus, rapid cooling with cryoprotection followed by rapid warming would appear to result in the best balance between keratocyte viability and preservation of tissue structure.

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