SCIENTIFIC REPORT

# A novel method for preserving cultured limbal epithelial cells

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**Aim:** To investigate organ culture preservation of cultured limbal epithelial cells in order to enhance the availability of tissue-engineered epithelia that are used to treat patients with limbal stem cell deficiency.

**Methods:** Limbal epithelial cells were cultured for 3 weeks on intact amniotic membrane fastened to a polyester membrane carrier. The cultured epithelia were stored for 1 week at 23˚C in organ culture medium. The preserved epithelia were then examined using a colorimetric cell viability assay, light microscopy and immunohistochemistry.

**Results:** The viability of the preserved epithelia was 84% (20%), and no statistically significant difference was found compared with non-preserved epithelia. In general, the cell borders were maintained, the nuclei showed no sign of degeneration, and the original layered structure was preserved. Mild intercellular oedema was occasionally observed. Expression of p63, K19 and vimentin was maintained.

**Conclusions:** Cultured limbal epithelial cells can be preserved in organ culture medium for 1 week at room temperature, while maintaining the original layered structure and undifferentiated phenotype.

Limbal stem cell deficiency may be treated by transplanting ex vivo expanded limbal epithelial cells.[1] However, the availability of cultured tissue is currently limited owing to logistical and methodological challenges. Firstly, there is a shortage of human donors, which limits the supply of limbal epithelial cells available for tissue engineering. Secondly, the method is expensive and is reserved for eye departments with cell culture facilities. Thirdly, it takes several weeks of cell culture to engineer multilayered epithelial sheets, which makes it difficult to schedule operations. Finally, no efficient method of transporting cultured epithelia has been described to date. The present study aimed to design a preservation method based on organ culture that is technically practical and allows cultured epithelia to be transported between eye departments.

## MATERIALS AND METHODS

### Cell culture and organ culture preservation of limbal epithelial cells

The research was conducted in accordance with the Declaration of Helsinki, and consent was obtained for the use of donor tissue for research purposes. Human amniotic membranes, preserved as reported previously,[2] were attached to the polyester membrane of Netwell culture plate inserts (Costar, Corning, New York, New York, USA) using 6-0 non-absorbable sutures. Eyes were enucleated from cadavers, and explant cultures (n = 32) were prepared as described previously by Meller *et al*.[3]

Limbal explants exposed to dispase (Roche Diagnostics, Basel, Switzerland) were incubated with the stromal side facing the amniotic membrane for 21 days at 37℃ in a medium consisting of N-2-hydroxyethylpiperazine-N'-ethane-sulphonic acid-buffered Dulbecco's modified Eagle's medium containing sodium bicarbonate and Ham's F12 (Sigma-Aldrich, St Louis, Missouri, USA) supplemented with 5% fetal bovine serum, 0.5% dimethyl sulphoxide, 2 ng/ml human epidermal growth factor, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 3 ng/ml hydrocortisone, 30 ng/ml cholera toxin (Biomol, Exeter, UK), 50 μg/ml gentamicin and 1.25 μg/ml amphotericin B. The polyester mesh bottom with the cultured epithelium attached was released using a steel blade and suspended in a sterilised 50 ml glass infusion bottle using an Ethicon Ethilon 6-0 monofilament suture, which was tied to the edge of the polyester membrane (fig 1). The epithelia (n = 16) were incubated for 1 week at 23℃ in an organ culture medium containing N-2-hydroxyethylpiperazine-N'-ethane-sulphonic acid-buffered Dulbecco's modified Eagle's medium with 7.5% sodium bicarbonate, 8% fetal bovine serum, 50 μg/ml gentamicin (Garamycin), 100 μg/ml vancomycin (Abbott Laboratories, Abbott Park, Illinois, USA) and 2.5 μg/ml amphotericin B.

### Cell viability analysis

Mitochondrial function, an indicator of cell viability, was measured using a colorimetric assay, as reported previously.[4–6] This technique is based on mitochondrial enzyme reduction of the water-soluble tetrazolium salt-8-(2(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt) and spectrophotometric quantification of the water-soluble formazan dye generated. Initially, a calibration curve was created to investigate the relationship between the optical density and the number of viable cells in samples from non-preserved cultured epithelial cells. Disks (n = 12) of cultured epithelium were trephinated using biopsy punches (Kai Industries, Gifu, Japan) of different diameters (2, 3, 4, 5 and 6 mm). They were then incubated in 20 μl CCK-8 solution (Alexis Corporation, Lausen, Switzerland) and 200 μl organ culture medium for 2 h. The solution was analysed colorimetrically at 450 nm in an automated microplate reader (Kinetic-QCL, Bio-Whittaker, Walkersville, Maryland, USA). The discs were subsequently trypsinised, and cell numbers were counted directly using the trypan blue dye exclusion technique. Based on measurements of 3 mm epithelial discs, the optical density after preservation (n = 8) was calculated as the percentage of that before preservation (n = 8).

### Light microscopy and immunohistochemistry

Preserved epithelia (n = 8) and non-preserved epithelia (n = 8) were fixed in neutral buffered 4% formaldehyde and embedded in paraffin. Serial sections of 5 μm thickness were routinely stained with haematoxylin and eosin. Immunohistochemistry was performed with a panel of antibodies (table 1). To visualise the immunoreactions, we used a standard peroxidase technique (DAB detection kit) in a Ventana ES Immunohistochemistry Instrument (Tucson, Arizona, USA). Optimal antibody dilutions were determined by titration using the positive controls

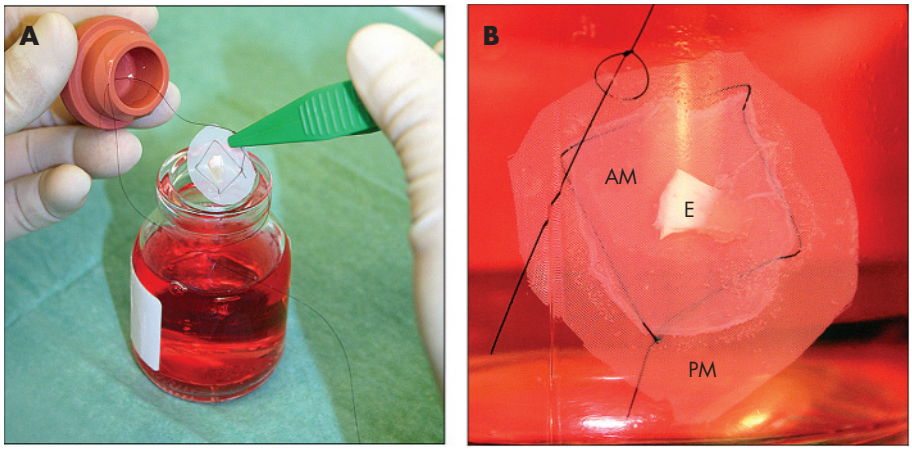


**Figure 1** Organ culture preservation of cultured epithelium. (A) Experimental design showing the graft attached to the polyester membrane carrier, the rubber stopper and the glass infusion bottle containing the storage medium. (B) Cultured epithelium fully immersed in organ culture medium. The amniotic membrane was fastened to the polyester membrane carrier at four corners using a 6-0 monofilament suture. AM, amniotic membrane; E, limbal explant; PM, polyester membrane.

recommended by the manufacturers. The expression pattern was evaluated by two independent investigators.

### Statistical analysis

Data are presented as mean (SD). SPSS V.14.0 was used to assess the cell viability (correlation analysis and *t* tests for two independent groups). A p value of $<0.05$ was considered significant.

## RESULTS

### Viability

A linear relationship was observed between the optical density and the viable cell number in samples from non-preserved cultured epithelial cells (correlation $r = 0.97$). The optical density of the non-preserved epithelia was 0.27 (0.03), whereas that of the preserved epithelia was 0.23 (0.05), giving a viability percentage of 84% (20%). No significant difference was found between the two groups ($p = 0.07$).

### Light microscopy and immunohistochemistry

On the whole, the cell borders were maintained, and the nuclei showed no sign of degeneration (fig 2B). The epithelia attached well to the amniotic membrane. Mild intercellular oedema was occasionally observed. No change in staining pattern was revealed for K19, vimentin, K3, K5 and K14. Minimal changes were disclosed for Ki67, p63, Cx43, E-cadherin and integrin β-1 (table 1, fig 2).

## DISCUSSION

Past studies have examined the epithelial proliferative potential of organ cultured corneoscleral rims as a source of limbal epithelial cells.[7–9] However, no previous reports have examined organ culture preservation of ex vivo expanded limbal epithelial cells. This study shows that cultured limbal epithelial cells can be preserved in organ culture medium at room temperature for 1 week, while maintaining the original layered structure and undifferentiated phenotype.

The initial challenge was to find a suitable carrier for the amniotic membrane. Ultimately, only polyester membrane culture plate inserts met all our requirements. The membranes (1) were able to withstand the tension of the sutures and keep the amniotic membrane distended; (2) were easily released from the culture plate insert; (3) fitted into the glass infusion bottle; and (4) were easy to detach from the amniotic membrane.

Organ culture preservation of donor corneas is currently the most widely used corneal storage method in Europe,[10] and the medium supplies the nutrients needed to maintain cellular metabolism in the tissue.[11] The present study was conducted at room temperature (23°C), which eliminated the need for

**Table 1** Semiquantitative immunohistochemical characteristics of non-preserved cultured epithelium and cultured epithelium preserved for 1 week at 23°C (room temperature)

| | | | Non-preserved epithelium | | | 1-Week storage at 23°C | | |
|---|---|---|---|---|---|---|---|---|
| **Specificity** | **Clone** | **Source, dilution** | **B** | **SB** | **S** | **B** | **SB** | **S** |
| p63 | 4A4 | DAKO,* 1/25 | 3 | 2 | 0-1 | 3 | 3 | 0-1 |
| Cytokeratin 19 | RCK108 | DAKO,* 1/200 | 2 | 2 | 2 | 2 | 2 | 2 |
| Vimentin | VIM 3B4 | Ventana Medical Systems† | 3 | 2 | 1 | 3 | 2 | 1 |
| Ki67 | MIB-1 | DAKO,* 1/75 | 1 | 0-1 | 0 | 0-1 | 0 | 0 |
| Cytokeratin 3 | AE5 | ImmuQuest,‡ 1/500 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cytokeratin 5 | XM26 | Novocastra Lab.,§ 1/600 | 3 | 3 | 3 | 3 | 3 | 3 |
| Cytokeratin 14 | LL02 | Novocastra Lab.,§ 1/80 | 3 | 3 | 3 | 3 | 3 | 3 |
| Connexin 43 | Polyclonal | Sigma-Aldrich,¶ 1/500 | 2 | 2 | 1 | 1 | 1 | 0 |
| E-cadherin | NCH-38 | Novocastra Lab.,§ 1/25 | 1 | 2 | 0-1 | 1 | 2 | 0 |
| Integrin β-1 | 7F10 | Novocastra Lab.,§ 1/10 | 2 | 1 | 0-1 | 2 | 0 | 0 |

B, basal layer; S, superficial layer; SB, suprabasal layer.
0 = undetectable; 1 = weak positivity; 2 = moderate positivity; 3 = strong positivity.
*Glostrup, Denmark.
†Tucson, Arizona, USA.
‡Cleveland, UK.
§Newcastle, UK.
¶St Louis, Missouri, USA.

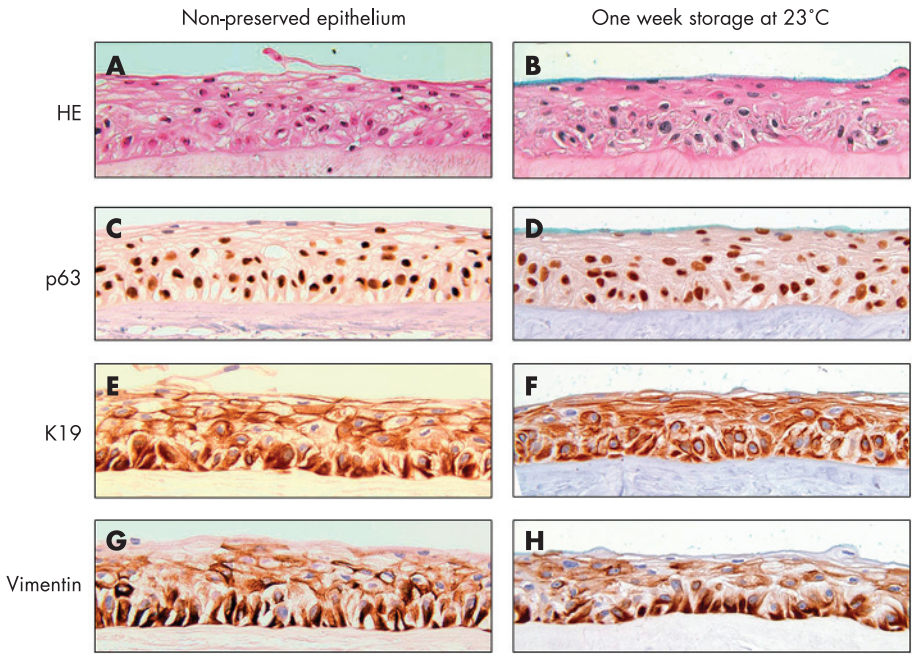


**Figure 2** Sections stained with haematoxylin and eosin (A, B), and immunostaining of p63 (C, D), K19 (E, F) and vimentin (G, H) in non-preserved cultured epithelial cells (A, C, E and G) and cultured epithelial cells preserved for 1 week at 23°C (B, D, F and H). Original magnification: ×100.

heating cabinets and made it easier to distribute the transplants between eye departments. A few reports have been published that consider the influence of room temperature (23–25°C) on corneas stored in culture media such as McCarey–Kaufman medium,[12] K-Sol medium,[13] TC 199 medium[14] and RPMI 1640 organ culture medium.[15] However, in these studies, the corneal endothelium has been the main focus of attention.

The linear relationship observed between the optical density and the cell number is consistent with the results of Kito *et al*,[6] who reported a high correlation ($R^2 = 0.976$). The results of the cell viability assay and the light microscopy examination indicated that the majority of the cultured epithelial cells were viable after preservation. Mild intercellular oedema occurred occasionally, which has previously been reported after organ culture storage.[16 17]

As there were no data available for direct comparison with our immunohistochemical findings, we compared our data with the results of a study by Joseph *et al*,[8] which investigated limbal explants stored in organ culture medium for 3–4 weeks. The expression of p63, vimentin, Ki67 and Cx43 was close to their results. However, in their study, only a few cells were positive for K19, and K3 was expressed in the superficial layer.

There is no standardised method of culturing limbal epithelial cells. In the present study, we used intact amniotic membrane without 3T3 fibroblast feeder layers or air-lifting, because previous studies have suggested that this method preserves the characteristics of limbal epithelial stem cells.[3 18 19] However, the use of air-lifting has been reported to provide the epithelial sheet with increased mechanical strength,[20] which may be beneficial prior to preservation in organ culture. Further studies are warranted to increase the knowledge of the effects of storage conditions on epithelial biology and morphology. In addition, more work needs to be done to investigate whether limbal epithelial cells can be stored for longer periods, in other tissue culture media, using other cell culture protocols and at other temperatures.

In conclusion, our study demonstrates that organ culture may preserve cultured epithelia for transplantation.

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