Donor cornea transfer from Optisol GS to organ culture storage: a two-step procedure to increase donor tissue lifespan

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ABSTRACT.

Purpose: Storage time for donor corneas in Optisol GS is limited compared to Eye Bank Organ Culture (EBOC). We here examine the epithelium on donor corneoscleral rims after primary storage in Optisol GS and subsequent incubation in EBOC.

Methods: Morphology was monitored by light and electron microscopy, expression of phenotypic and genotypic markers by immunohistochemistry and RT-PCR and changes in oxidative lipid and DNA damage by ELISA and COMET assay.

Results: A prominent loss of cells was observed after storage in Optisol GS. After maintenance in EBOC, spreading apical cells were Occludin+©, while the staining for E-cadherin and Connexin-43 was less intense. There were an upregulation of Occludin and a downregulation of E-cadherin and Connexin-43. Eye Bank Organ Culture was associated with an ongoing proliferative activity and a downregulation of putative progenitor/stem cell marker ABCG2 and p63. Staining for 8-OHdG and Caspase-3 did not increase, while levels of malondialdehyde and number of DNA strand breaks and oxidized bases increased.

Conclusions: This dual procedure should be pursued as an option to increase the storage time and the pool of available donor corneas. The observed downregulation of markers associated with stemness during EBOC is relevant considering the potential use of donor epithelium in the treatment of ocular surface disorders.

Key words: cell damage – cornea – differentiation – epithelium – limbus – Optisol GS – organ culture – oxidative damage – stem cells

Introduction

‘Cold’ storage at 4°C and organ culture at 32°C are two methods commonly used in order to prolong donor corneal survival, both with similar results as for long-term postoperative outcome (Rijneveld et al. 2008). Prevaling method in the United States is cold storage (CS), and the upper limit for the use of the donor tissue is 14 days. Changes in the epithelium include progressive detachment of epithelial layers, cell death and formation of defects with exposure of the basement membrane (Greenbaum et al. 2004).

In European countries, the commonly used procedure is Eye Bank Organ Culture (EBOC) (Kolstad 1979; Armitage & Easty 1997; Hjortdal et al. 1997; Borderie et al. 2006; Rijneveld et al. 2008; Ehlers et al. 2009). This system permits the maintenance of tissue for up to 4 weeks, and epithelial defects present prior to preservation may to some extent heal during the first 3 days in an organ culture system (Slettedal et al. 2007).

During storage and also during transport and international exchange of donor corneas, the preservation time for tissue in Optisol GS may
approximate the upper recommended limit, and transfer to EBOC has been suggested as an option to increase the lifespan of donor corneas (Nelson et al. 1984; Camposampiero et al. 2003; Rijneveld et al. 2011). Such a dual approach may increase the pool of available donor tissue (Rijneveld et al. 2011) and may, in addition, permit some regeneration of epithelial defects prior to release of tissue for surgery.

In the present study, we used human corneoscleral rims retrieved after transplant procedures and where the donor corneas had been maintained in Optisol GS prior to surgery. Several previous studies have provided a considerable amount of information about the behaviour of the epithelium, the stroma and the endothelium during Eye Bank storage in Optisol GS and in organ culture. For transfer and prolonged storage of donor tissue, each of these storage methods may serve as a second procedure. We here examined the epithelium for changes in architecture, ultrastructure, proliferation and expression of selected phenotypic and genotypic markers associated with a secondary incubation in a commonly used EBOC system. Considering previous reports on cellular stress during Eye Bank storage (Komuro et al. 1999; Jeng et al. 2002), we also monitored epithelial staining for 8-OHdG, Caspase-3 and TUNEL and the levels of lipid peroxides and DNA damage profiles.

Materials and Methods

Tissue

Corneas were stored in Optisol GS (Bausch & Lomb Incorporated, Rochester, NY, USA) at 4°C until transplantation, and the remaining corneoscleral rim acquired for our study. Half of the rims ($n = 20$) were immediately processed for analysis (Optisol GS, group 1), while the other half ($n = 20$) was transferred to Eye Bank OC for 1 week prior to analysis (Optisol GS + OC, group 2). This experimental design was selected in order to examine the effect of OC on tissue previously stored in Optisol GS. In studies on DNA damage, additional 10 rims were used. Mean donor age in group 1 was 61.5 (SD 10.1) years and in group 2, 64.8 (SD 8.8) years; post-mortem time to preservation in the two groups was 9.9 (SD 4.0)/10.5 (SD 3.9) hr, time from preservation to transplantation was 9.8 (SD 1.3) days, and time in OC was 6.0 (SD 0.7) days. Female/male ratio was 11:29. In the group for DNA damage, age was 57 (SD 13.4) years, post-mortem time was 7 (SD 5.9) h, storage in Optisol GS was 8.7 (SD 1.5) days, and time in organ culture was 6.7 (SD 0.8) days. The study was approved by the Regional Committee for Medical Research Ethics of Eastern Norway, all tissue was consented for research, and the Helsinki Declaration was adhered to throughout the study.

Eye Bank storage systems

Cold storage medium at 4°C: Optisol GS (Bausch & Lomb Incorporated, Rochester, NY, USA) was used in CS medium. Eye Bank Organ Culture medium at 32°C: Eagle’s MEM with Earle salts and t-glutamate (Gibco, Invitrogen, Paisley, UK), sodium bicarbonate (2.20 μg/ml), HEPES buffer (2.98 μg/ml), 8% heat-inactivated foetal calf serum, amphotericin B (5 μg/ml), gentamicin (50 μg/ml) (Sigma Aldrich, Saint Louis, MO, USA) and Vancomycin (100 μg/ml) (Alpharma ApS, Copenhagen, Denmark), pH 7.1–7.2, was used in EBOC medium.

Light microscopy, immunostaining and ultrastructure

From each rim, duplicate or triplicate samples were removed, fixed in 4% formaldehyde and embedded in paraffin; 3-μm sections were stained with haematoxylin and eosin (H&E), and nuclei of the epithelium were counted and their appearance was recorded. Sections were also stained for Ki-67 (SP6, 1:200; Thermo Scientific, Freeport, CA, USA), PCNA (PC10, 1:1500; DakoCytomation, Glostrup, Denmark), p63 (AA4 + Y4A3, 1:1600; Thermo Scientific), p63z (C-12, 1:1500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), ABCG2 (BXP-21, 1:80; Sigma Aldrich, Saint Louis, MO, USA), E-cadherin (NCH-38, 1:50; DakoCytomation), Vimentin (SP20, 1:200; Thermo Scientific, Freeport, CA, USA), Occludin (ab64482, 1:70; AbCam, Cambridge, UK), 8-OHdG (15A3, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Connexin-43 (C6219, 1:500; Sigma Aldrich, Saint Louis, MO, USA) and Caspase 3 (ASP175 SA1, 1:200; Cell Signaling, Beverly, MA, USA). Cyto-keratin 3 (AE3, 1:500; ImmunoQuest, Cleveland, UK) was used to confirm a corneal epithelial phenotype. An Autostainer360 (Lab Vision Corporation, Fremont, CA, USA) was used, and positive immunoreactions were detected by a secondary antibody conjugated with peroxidase-labelled polymer with diaminobenzidine. Positive and negative controls were used as recommended by distributor. Total cell number, Ki-67+, PCNA+ and p63+ cells were counted at 40x magnification by three independent observers in the peripheral corneal epithelium. Student’s t-test and Statistical Package for the Social Sciences (spp version 18; IBM Corporation, Armonk, NY, USA) were used. In the limbal area, the number of Ki-67+ and ABCG2+ cells and their distribution in basal versus suprabasal layers were recorded (triplicate counts at 40x magnification, see Results). For 8-OHdG and Caspase-3, the reaction was evaluated in sections from six random samples maintained in Optisol GS or in Optisol GS + organ culture.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end DNA labelling (TUNEL) was used to detect apoptotic cells (DeadEnd™ Colori-metric TUNEL System; Promega, Madison, WI, USA).

For transmission electron microscopy (TEM), samples were fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer, washed, postfixed in osmium tetroxide, dehydrated and embedded in Epon. Ultrathin sections (60–70 nm) were cut on a Leica Ultracut UCT (Leica, Wetzlar, Germany), contrasted with uranyl acetate and lead citrate and examined in a transmission electron microscope (Teerna G2 Spirit BioTWIN 120 kV, LaB6; FEI Company, Eindhoven, the Netherlands).

Real-time RT-PCR

RNA was extracted using Qiazol reagent (Qiagen, Hilden, Germany). Following DNase treatment (Ambion, Austin, TX, USA), RNA was quantified by spectrophotometer (Nanodrop,
Wilmington, DE, USA). Reverse transcription (RT) was performed using the High Capacity cDNA Archive Kit (Applied Biosystems, Abingdon, UK) with 200 ng of total RNA per 20 μl RT reaction. Comparative relative quantification was performed using the StepOnePlus Real-Time RT PCR system (Applied Biosystems) and Taqman® Gene Expression assays following protocols from the manufacturer (Applied Biosystems). All samples were run in triplicate. Data were analysed by the 2^ΔΔCt method for genes as the fold-change in expression, normalized to GAPDH as endogenous reference and expressed relative to Optisol GS, which was arbitrarily chosen as calibrator (Table 1).

**Table 1. Primes used for real-time RT-PCR.**

| Gene name                         | Gene symbol | Alias       | Taqman assay ID       |
|-----------------------------------|-------------|-------------|-----------------------|
| ATP-binding cassette subfamily G2 | ABCG2       | BCRP        | HS01053790_ml         |
| Gap junction protein alpha 1, 43 kDa | GJA1       | CX43        | HS00748445_ml         |
| Occludin                          | OCLN        | –           | HS00170162_ml         |
| Glyceraldehyde-3-phosphate dehydrogenase | GAPDH     | GAPD        | HS99999905_ml         |
| Tumour protein p63                | TP63        | p63         | HS00978340_ml         |
| Antigen identified by monoclonal antibody K67 | KI-67     | MKI67       | HS01032443_ml         |
| E-cadherin (epithelial)           | E-cadherin  | ECAD        | HS01023894_ml         |
| Tumour protein p53                | TP53        | p53         | HS00153349_ml         |

**Lipid peroxidation by-products**

**ELISA**

Lipid peroxidation was measured using a commercial kit (Lipid Peroxidation Microplate Assay Kit; Oxford Biomedical Research, Rochester Hills, MI, USA) following the manufacturer’s instructions. Reactive oxygen species degrade polyunsaturated lipids, forming malondialdehyde (MDA). The assay is based on the reaction of two molecules of a chromogenic reagent, N-methyl-2-phenylindole, with one molecule of MDA at 45°C to yield a stable chromophore with a maximal absorbance at 586 nm. The amount of MDA can be monitored by reading the absorbance at 586 nm, which is proportional to its concentration.

**DNA damage**

Epithelium was removed by scraping in medium (Optisol GS or EBOC) on ice. A single-cell suspension is obtained by gentle pipetting and by centrifuging at 200 g for 5 min at 4°C, and cells were resuspended in 30 μl of phosphate-buffered saline (PBS). The comet assay (single-cell gel electrophoresis) (Azqueta et al. 2009) was used to measure strand breaks, oxidized pyrimidines (by digestion with endonuclease III; endo III), oxidized purines [with formamidopyrimidine-DNA glycosylase (FPG)] and cyclobutane pyrimidine dimers (with T4 endonuclease V; T4EndoV). Under fluorescence microscopy, after electrophoresis, comet-like images are seen, where the comet tail represents broken DNA that is able to move in the direction of the anode. The percentage of DNA in the tail indicates the frequency of breaks. For each sample, 25 comets per gel, two gels per treatment, were scored using a Nikon Eclipse TS-100 fluorescence microscope with semiautomated image analysis system (Comet Assay IV; Perceptive Instruments, Suffolk, UK), and the median was calculated as an index of the frequency of DNA lesions. To calculate net enzyme-sensitive sites, comet scores from buffer-incubated gels were subtracted from the scores of gels incubated with the different enzymes.

Untreated lymphocytes were used as a negative control, and as positive control, lymphocytes from healthy volunteers treated on ice with 2 μm photosensitizer Ro 19-8022 plus visible light (500-W tungsten-halogen source at 33 cm) to induce 8-oxoGua. They were treated as the corneal epithelium cells but incubated only with enzyme buffer or FPG, respectively.

Statistical Package for the Social Sciences (spss) was used for statistical analysis. A p-value <0.05 was considered statistically significant.

**Results**

**Optisol GS storage (group 1)**

Loss of superficial layers of the epithelium and additional detachment of scattered basal cells or groups of basal cells were observed on all rims. Most samples stained negative for the tight junction protein Occludin (Fig. 1) normally present between cells in the superficial layers, while most cells stained positive for E-cadherin and Connexin-43 (Fig. 1) markers for adherens and gap junctions, respectively. The proliferation marker Ki67 was positive in cells in

![Fig. 1. Immunostaining of adherens, tight and gap junction-associated markers Occludin (A, B), E-cadherin (C, D) and Connexin-43 (E, F) after storage in Optisol GS and after Optisol GS + EBOC. EBOC, Eye Bank Organ Culture.](image-url)
the basal and in the suprabasal layer in the corneal epithelium (Fig. 2). Low-degree staining was also seen in the limbal area (4%), and the ratio between positive cells in the basal versus suprabasal layer was 0.3. Cells positive for the proliferation/progenitor/stem cell markers p63 and p63α were seen in the basal and suprabasal layer in the corneal as well as in the limbal epithelium. Vimentin stained basal limbal cells densely, while only few isolated suprabasal and some peripheral basal corneal cells were positive (Fig. 2). Similarly, dense staining for progenitor/stem cell marker ABCG2 was confined to cells in the basal limbal area, and staining of isolated suprabasal and corneal epithelial cells was less intense (Fig. 2).

DNA base oxidation and cell proliferation/DNA repair as revealed by positive staining for 8-OHdG and PCNA, respectively, ranged between slight and intense. Staining for Caspase-3, a marker of apoptosis, was particularly evident in some cells with gross nuclear alterations, but a low to moderate activity was also seen in some cells with an apparent normal nuclear morphology (Fig. 3). The TUNEL assay was positive in some cells with deformed nuclei (picture not shown).

Transmission electron microscopy generally showed an organized cytoplasm with detectable mitochondria, endoplasmic reticulum, intermediate filaments, a regular chromatin pattern, intercellular desmosomal complexes and basal hemidesmosomal attachments (Fig. 4B–D).

Group 1 and group 2

RT-PCR

Results from gene expression analysis after Optisol GS and after Optisol GS + OC storage are presented in Fig. 5. In the latter group, there was a significant increase in the expression of the tight junction marker Occludin, while Connexin-43 and E-cadherin, markers for gap and adherens junctions, respectively, were downregulated. As for proliferation, Ki67 was upregulated, while the expression of putative progenitor/stemness-related genes ABCG2 and p63 was downregulated. P53 showed a reduction from 1 (SD 0.15) in Optisol GS to 0.4 (SD 0.03) after EBOC.

Lipid peroxidation and DNA damage

Lipid peroxidation, assayed by measuring MDA, was detected after Optisol GS as well as after Optisol GS + OC
and the concentration increased from 4.6 ± 2.1 mM/mg protein to 7.3 ± 0.9 mM/mg protein. In addition, the level of strand breaks was very low with a mean value of 5.7 ± 3.6% tail DNA in cold-stored tissue. Enzyme-sensitive sites were generally not much increased by organ culture, except for some samples that showed a substantial increase in EndoIII-sensitive sites (oxidized pyrimidines) that increased markedly in three of the 10 samples, while the levels of FPG-sensitive sites were similar in the two groups. The levels of T4endoV-sites increased in all but one sample (Fig. 6).

**Discussion**

The morphological alterations observed in the epithelium after storage in Optisol GS were similar to those described in previous studies, including a loss of superficial cell layers and occasional exposure of the basement membrane (Greenbaum et al. 2004). This deterioration explains the lack of staining for the tight junction protein Occludin in the samples. However, a primary storage in Optisol GS did not inhibit an increase in the gene expression and translation during subsequent organ culture.

We observed an intense immunostaining for E-cadherin and Connexin-43, markers detecting adherens and gap junctions, after Optisol GS. Translation, as well as the expression of these proteins, in particular that of Connexin-43, was reduced after organ culture. Varying expression of both E-cadherin and Connexin-43 has previously been described during wound healing and regeneration (Matic et al. 1997; Chang et al. 2008). Further, the expression of Connexin-43 also depends on the phenotype, a decreased level being observed in the basal limbal population (Hernandez Galindo et al. 2003). Our findings may therefore indicate an ongoing epithelial regeneration after 1 week in EBOC.

In epithelium on corneas stored in Optisol GS, subsequent organ culture was associated with an upregulation of proliferation marker Ki67, and the counting of cells with intact nucleus after H&E staining, *t*-test; spss output.

| Group name       | Mean | SD | Significance (2-tailed) |
|------------------|------|----|------------------------|
| PCNA             |      |    |                        |
| Optisol GS       | 66.7 | 33.4| 0.022                  |
| Optisol GS + OC  | 87.6 | 15.9|                        |
| Ki-67            |      |    |                        |
| Optisol GS       | 7.2  | 7.7 | 0.077                  |
| Optisol GS + OC  | 18.3 | 23.3|                        |
| p63              |      |    |                        |
| Optisol GS       | 77.1 | 12.5| 0.857                  |
| Optisol GS + OC  | 77.9 | 14.6|                        |
| H&E              |      |    |                        |
| Optisol GS       | 65.6 | 29.2| 0.001                  |
| Optisol GS + OC  | 91.1 | 7.6 |                        |

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**Fig. 3.** Immunostaining of DNA base oxidation and cell proliferation/DNA repair–associated markers 8-OHdG (A, B) and PCNA (E, F) and apoptosis marker Caspase 3 (C, D) after storage in Optisol GS and after Optisol GS + EBOC. EBOC, Eye Bank Organ Culture.

**Fig. 4.** Transmission electron micrographs demonstrating human corneal epithelial cells after storage in Optisol GS (A) and after Optisol GS + EBOC (B–D). Loss of superficial cells along with nuclear shrinkage, disorganized cytoplasm and membrane ruptures is seen after Optisol GS. Notice cubical basal cells and layers of horizontal superficial cell in (B–D) after Optisol GS + EBOC. Desmosomes (**) and hemidesmosomes (****) are seen in both systems. In addition, there are advanced foot processes (*) in the limbal area before and after the transfer to EBOC. S, superficial epithelial cells; B, basal cells; BM, basement membrane; EBOC, Eye Bank Organ Culture.
proliferative activity (Hjortdal et al. 1993; Kabosova et al. 2003; Chang et al. 2008), and Ki67-negative epithelium on EBOC corneas shows a transient proliferative increase when transferred to a complex medium commonly used to expand epithelium for transplant purposes (Joseph et al. 2004). In the latter study, density of cells positive for progenitor/stem cell marker p63 also showed a decrease after 2 weeks in culture. This is in line with our findings where we in addition show a decrease in the expression of progenitor/stem cell genes p63 and ABCG2. Considering the potential use of Eye Bank donor tissue in various limbal epithelial transplant procedures (Pellegrini et al. 1997; Stenevi et al. 2002), the effect of commonly used Eye Bank storage systems on the preservation of 'stemness' deserves further investigation.

Ex vivo storage conditions are known to exert a continuous stress on the donor tissue. Eye Bank Organ Culture has been shown to affect the expression of Bcl2 and heat shock proteins involved in cellular defence against oxidative stress and apoptosis (Gain et al. 2001), and 'cold' storage is associated with an increase in nitrated proteins (Meisler et al. 2004). Malondialdehyde, a potentially cytotoxic lipid peroxidation breakdown product, is known to accumulate in a range of disorders including bullous keratopathy, keratoconus, diabetic retinopathy and retinitis pigmentosa (Buddi et al. 2002; Johnsen-Soriano et al. 2008). DNA damage may act to destabilize cell function through an increased level of transcriptional errors (Ishibashi et al. 2005). Although some increase after EBOC was observed, we found a downregulation of p53, a sensor for cell and DNA damage and effector of cell cycle arrest and also of apoptosis. Cell death because of suprathermal damage is seen with increasing time during 'cold' storage and also in EBOC (Komuro et al. 1999; Crewe & Armitage 2001). Such studies point at programmed cell death (apoptosis) as the main subtype. Caspase-3 is a commonly used marker for apoptosis although not specific, and the TUNEL reaction may in addition to apoptosis also be positive for necrotic cells. We could not detect any prominent increase in Caspase-3 immunostaining after subsequent EBOC of epithelium previously stored in a 'cold' system. However, the organ culture period in the present experiment was relatively brief. Also, a time-dependent detachment of apoptotic as well as necrotic cells from the epithelium may be facilitated by regenerative activity during EBOC.

In conclusion, the main purpose of our study was to investigate donor corneolimbal epithelium maintained in Optisol GS for alterations associated with a secondary incubation in a regular EBOC medium. After such a dual storage procedure, the epithelium demonstrated a layered and polarized morphology and a retained proliferative potential, although the expression of some genes related to differentiation and stemness was reduced. Some increase in oxidative stress was evident; however, there was a downregulation of the damage sensor P53 during EBOC. Our findings lend some support to the feasibility of EBOC pooling of donor tissue after storage in a 'cold system' with the benefits provided by such an option. There is an obvious need for further studies, in particular on the endothelium, in order to explore the potential and the safety of such a dual storage procedure.

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