Original Article

Effect of Cryoprotectants on Long-Term Storage of Oral Mucosal Epithelial Cells: Implications for Stem Cell Preservation and Proliferation Status

(oral mucosal epithelial cells / limbal stem cell deficiency / cryopreservation / cryoprotectives / cell culture / stemness)

JOAO VICTOR CABRAL[1], NATÁLIE SMORODINOVÁ[1,2], ELENI VOUKALI[1], LUKÁŠ BALOGH[1], TOMÁŠ KUČERA[2], VOJTĚCH KOLÍN[3], PAVEL STUDENÝ[4], TOMÁŠ VACÍK[1], KATEŘINA JIRSOVÁ[1]

[1]Institute of Biology and Medical Genetics, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic
[2]Institute of Histology and Embryology, First Faculty of Medicine, Charles University, Prague, Czech Republic
[3]Department of Pathology, Third Faculty of Medicine, Charles University and University Hospital Kralovske Vinohrady, Prague, Czech Republic
[4]Department of Ophthalmology, Third Faculty of Medicine, Charles University and University Hospital Kralovske Vinohrady, Prague, Czech Republic

Abstract. In this study, we tested a method for long-term storage of oral mucosal epithelial cells (OMECs) so that the cells could be expanded *in vitro* after cryopreservation and used for the treatment of bilateral limbal stem cell deficiency. The ability of suspended primary OMECs to proliferate *in vitro* after cryopreservation was compared to that of OMEC cultures that had undergone the same process. Both were preserved in standard complex medium (COM) with or without cryoprotective agents (CPAs) (glycerol at 5 % or 10 % or dimethyl sulphoxide at 10 %). We found that after cryopreservation, primary OMECs could form a confluent cell sheet only in a few samples after 22 ± 2.9 (mean ± SD) days of cultivation with 72.4 % ± 12.9 % overall viability. Instead, all *ex vivo* OMEC cultures could re-expand after cryopreservation with a comparable viability of 78.6 ± 13.8 %, like primary OMECs, but with significantly faster growth rate (adj. P < 001), forming a confluent cell sheet at 13.7 ± 3.9 days. Gene expression analyses of the *ex vivo* expansion of OMEC cultures showed that the stemness, proliferation and differentiation-related gene expression was similar before and after cryopreservation, except for *KRT13* expression, which significantly decreased after the second passage (adj. P < 0.05). The addition of CPAs had no effect on these outcomes. In conclusion, the optimal strategy for OMEC preservation is to freeze the cells that have been previously cultured, in order to maintain cell viability and the capacity to create a sizable graft even without CPAs.

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Corresponding author: Kateřina Jirsová, Laboratory of the Biology and Pathology of the Eye, Institute of Biology and Medical Genetics, First Faculty of Medicine, Charles University, Albertov 4, 128 00 Prague, Czech Republic; E-mail: katerina.jirsova@lf1.cuni.cz

Abbreviations: COM – complex medium, COMET – cultivated oral mucosal epithelial cell transplantation, CPAs – cryoprotective agents, DMSO – dimethyl sulphoxide, FDR – false discovery rate, HPRT1 – hypoxanthine phosphoribosyltransferase 1, LECs – limbal epithelial cells, LSCD – limbal stem cell deficiency, OM – oral mucosa, OMECs – oral mucosal epithelial cells, P1A – passage 1 after cryopreservation of primary OMECs, P1B – passage 1 of primary OMECs not cryopreserved, P2B – passage 2 of previously cryopreserved cultured OMECs, RPL32 – ribosomal protein L32.

Introduction

Limbal stem cell deficiency (LSCD) is a disease of the ocular surface characterized by disruption of the barrier between the avascular cornea and the vascularized conjunctiva. The limbus represents the interface bet-

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ween the cornea and the conjunctiva and contains stem cells responsible for maintaining the corneal epithelium. Damage to the limbus results in corneal conjunctivalization and vascularization, ultimately leading to vision loss and, in severe cases, blindness (Osei-Bempong et al., 2013). Limbal epithelial cells (LECs) can be transplanted from a healthy eye in the case of unilateral injury, and they can even be cultured and expanded prior to grafting (Pellegrini et al., 1997). In the case of bilateral damage, a graft comprising oral mucosal epithelial cells (OMECs), including stem cells, can be used for transplantation (Liu et al., 2011; Ma et al., 2021).

The average success rate of LSCD treatment with cultivated oral mucosal epithelial cell transplantation (COMET) is 70 % and approximately 75 % after LEC transplantation (Nakamura et al., 2004; Nishida et al., 2004; Cabral et al., 2020). These data indicate that at least 25 % of LSCD patients who have undergone cell therapy for LSCD will experience graft failure and require re-grafting (Kaufman et al., 2014). Should a further re-transplantation be required, the patient might need another tissue biopsy, which could result in irreversible damage to the limbal tissue. An alternative approach is to prepare the cells in advance along with the graft for the initial transplant (Mohamed-Noriega et al., 2011; Lužnik et al., 2016). The cells can be stored long-term and used in subsequent surgeries, thus avoiding the need for additional tissue removal. The most effective long-term solution for preserving viable cells for therapeutic applications is cryopreservation (Zhao and Fu, 2017; Yang et al., 2020).

Cryopreservation entails a method that sustains the viability and functionality of living cells even at extremely low temperatures, typically ranging from −80 °C to −196 °C (Zhao and Fu, 2017; Yang et al., 2020). This process maintains the intricate structure of cells, allowing biological material to be stored at cryogenic temperatures for extended periods (Mazur, 1970; Sambu, 2015). Cryopreserved cells or tissues offer several advantages for clinical purposes, including availability of the preserved products, enabling rigorous quality testing to confirm suitability for transplantation without the need for fresh samples (Ibars et al., 2016; Jang et al., 2017).

While cryopreservation is the established method for the long-term storage of suspended cells, standardized protocols for freezing and thawing are essential to preserve the stem cell content and structural integrity of cultured tissues (Lužnik et al., 2016). The use of cryoprotective agents (CPAs) is essential for successful cryopreservation, as they maintain the tissue structural and biomechanical properties during the freezing and thawing processes (Martin-Lopez et al., 2023). CPAs, such as dimethyl sulphoxide (DMSO) or glycerol, are organic solvents commonly employed for intracellular protection and utilized to mitigate the damaging effects of ice formation (Yang et al., 2020). However, their biocompatibility is a concern (Yang et al., 2020). DMSO has been associated with various adverse effects in patients, including neurotoxicity and cardiovascular complications (Yang et al., 2016; Jang et al., 2017), while glycerol can induce severe haemolysis and renal failure (Best, 2015; Sui et al., 2019). Therefore, it is crucial to minimize their concentrations during the cryopreservation process (Martin-Lopez et al., 2023). Despite their drawbacks, DMSO and glycerol remain widely used in research and clinical applications. For example, red blood cell stocks are cryopreserved using glycerol concentrations of 20–40 wt%, and immortalized cell lines are routinely stored in 10 % (v/v) DMSO (Murray and Gibson, 2022). Haematopoietic stem cells intended for clinical transplantation are often cryopreserved in 5–10 % DMSO, among other additives (Lysak et al., 2021).

Regarding long-term storage of LECs and OMECs, several approaches were described, particularly for primary limbal cell suspension (Pellegrini et al., 2014) or already bioengineered limbal cells or OMECs with or without cell scaffolds (Hibino et al., 1996; Yeh et al., 2008). Some studies have shown that certain cell types, such as ocular epithelial cells, can be stored in liquid nitrogen for prolonged periods without compromising their morphology and phenotype (Oliva et al., 2019). In the context of long-term cryopreservation of cells derived from tissues, two main options can be considered: storage of primary cells, which are obtained directly from solid tissue, or storage of cells following *in vitro* cell expansion. It is important to note that during the cultivation process, cell differentiation occurs gradually, which may be either phenotypically desirable or undesirable. Furthermore, the differentiation process is accompanied by a reduction in stem cell numbers, which continues with the passaging of the cultured cells (Stadnikova et al., 2019).

Given the higher number of experimental studies conducted to identify suitable cryopreservation parameters for LECs, we decided to utilize an OMEC cell suspension, for which only limited data are available. Our study had two objectives: first, to determine whether primary OMECs or cultured OMECs are more suitable for storage; second, to compare the efficacy of the storage medium without cryopreservation (complex medium) with the medium containing low concentrations of glycerol (5 % or 10 %) and DMSO (10 %), to optimize the cryopreservation of OMECs.

Methods

Tissue retrieval, sample preparation

Ethical approval was obtained (No. 225/20 S-IV) and the study adhered to the principles outlined in the Helsinki Declaration. The procurement of donor tissue complied with all legal regulations in the Czech Republic, including confirmation of the absence of the donor's name in the national registry of individuals objecting to post-mortem tissue and organ retrieval. Informed consent was not required under the Czech law (Act No. 372/2011 Coll.) provided that the data were anonymized before inclusion.

Oral mucosal tissues were acquired from the Department of Pathology, University Hospital Kralovske Vinohrady, Prague within 48 hours after death (23.3 ± 15.4 hours, mean ± SD, range 5.0–44.1), (Supplementary Material, Table S1). Prior to biopsy, the collection site (20 mm posterior to the mouth's angle) underwent a 1-minute treatment with 10 % iodinated povidone (Betadine, EGIS, Pharmaceuticals, Budapest, Hungary) solution diluted in 0.9 % NaCl (B. Braun SE, Melsungen, Germany). A disposable and sterile 6-mm biopsy punch (Kai Medical, Tokyo, Japan) was utilized to create a superficial, circular incision, and the specimen was excised with a scalpel. Two samples were obtained from each side of the buccal mucosa (right and left) and preserved in BASE•128 (Alchimia, Ponte San Nicolò, Italy) at 2–8 °C until further processing.

Donors

For this study, oral mucosa (OM) biopsies retrieved from ten donors were used. The average donor (five females, five men) age was 66.8 ± 16.9 years (range 32–85 years). Causes of death included heart failure (three), haemorrhagic shock (two), septic shock (one), cardiorespiratory failure (one), pneumonia (one), and brain oedema (two); for details see Supplementary Material, Table S1.

Oral mucosal epithelial cell preparation and cultivation

Primary OMECs were prepared as described previously (Prabhasawat et al., 2016; Booranapong et al., 2022). Shortly, after tissue decontamination (BASE•128, Alchimia), OMEC samples were immersed in dispase II (STEMCELL Technologies, Vancouver, BC, Canada) at a concentration of 1.2 U/ml, prepared from a 5 U/ml solution (STEMCELL Technologies) and incubated overnight at 2–8 °C. Then, the epithelium was separated from the submucosa, transferred to 0.05 % trypsin-EDTA (Gibco, Paisley, UK) for 15 min, resuspended and filtered through a 70-μm cell strainer (PluriSelect, Leipzig, Germany). Then, the cells were centrifuged (10 min at 250 × *g*), and cell viability and cell density were analysed, see section "Cell counting and cell viability." The cell suspension was cryopreserved or cultured. The overview of the experimental design is shown in Fig. 1. OMECs were seeded in a 24-well plate (VWR, Radnor, PA) at a concentration of 4.5×10^4 cells per well pretreated with 200 μl of fibrin (Tisseel Lyo, Baxter; 5 mg/ml fibrinogen and 5 U/ml thrombin) (Brejchova et al., 2018; Trousil et al., 2024). Three to six wells were cultured per experimental group.

The mean interval between death and the beginning of cell cultivation was 61.16 h ± 22.9 hours. The standard complex medium (COM) was used for the cultivation: COM comprising DMEM/F12 1 : 1 GlutaMAX (Gibco) 10 % pooled human serum (HS, #HU.SE.0500, Bio&Sell, Nuremberg, Germany), 1 % antibiotic-antifungal solution (Gibco), 10 ng/ml recombinant EGF (Gibco), 5 μg/ml insulin-transferrin-selenium (Merck KGaA, Darmstadt, Germany), 0.4 μg/ml hydrocortisone (VUAB Pharma A.S., Roztoky, Czech Republic), 24 μg/ml adenine hydrochloride (Merck), 1.4 ng/ml triiodothyronine (Merck), and 8.4 ng/ml cholera toxin (Merck). The medium was supplemented with tranexamic acid (160 μg/ml, Merck) to prevent digestion of the fibrin gel during the cultivation. The medium was replaced every second day.

Ex vivo expanded OMECs were harvested once 85–95 % confluence was attained, using enzymatic treatment, as described before (Trousil et al., 2024). The resulting cell pellet was resuspended in 1 ml of DMEM/F12 media, and cell concentration was determined accordingly.

Cell counting and cell viability

Cell density and viability were analysed from 1.0–2.0 × 10^5 cells by a TC20™ Automated Cell Counter using a 1 : 1 dilution of the cell suspension with 0.4 % trypan blue dye (both from Bio-Rad, Hercules, CA).

Cryopreservation, storage and thawing of stored cells

Primary suspended OMECs or *ex vivo* expanded OMECs were prepared for cryopreservation by storage in four different media, each with a distinct composition and concentration of cryoprotectants. The control medium (COM) was prepared as described above. COM was then combined with 5 % glycerol (GLYO.ON, Alchimia), 10 % glycerol, or 10 % DMSO (CRYO.ON, Alchimia). The cell samples were spun for eight minutes in the Spectrafuge mini (Labnet Intl., Inc., Edison, NJ), maximum RMP/RCF: 6,000/2,000 × *g*, and the cell pellet was resuspended in pre-cooled freezing media to achieve a concentration of 5.0×10^6 cells/ml.

The cell suspensions in the freezing media were then transferred to cell culture cryogenic storage vials (NuncTM 374081, Rochester, New York), which had been pre-cooled. The vials were then placed in an insulated box and stored at –90 °C for 24–48 hours. The temperature drop was measured with a thermocouple data logger (EasyLog EL-USB-TC-LCD, Lascar Electronics, Salisbury, UK). The cooling rate was calculated to be 1.27 (temperature difference: from 24 °C to –90 °C; this decrease took 90 minutes, i.e., 114/90 = 1.27). Following the initial storage period, the samples were transferred to liquid nitrogen and stored for an average of 21 days. The thawing of all groups from the same specimen was conducted on the same day, followed by cultivation.

To initiate the thawing process, the cryovials were removed from the liquid nitrogen and placed into a water bath maintained at 37 °C. Subsequently, the vials were transferred to a biohazard hood to ensure sterility of the subsequent procedures. Then, a pre-warmed complete growth medium was introduced into the centrifuge tube containing the thawed cells. Subsequently, the cells were subjected to centrifugation at approximately 200 × *g* for

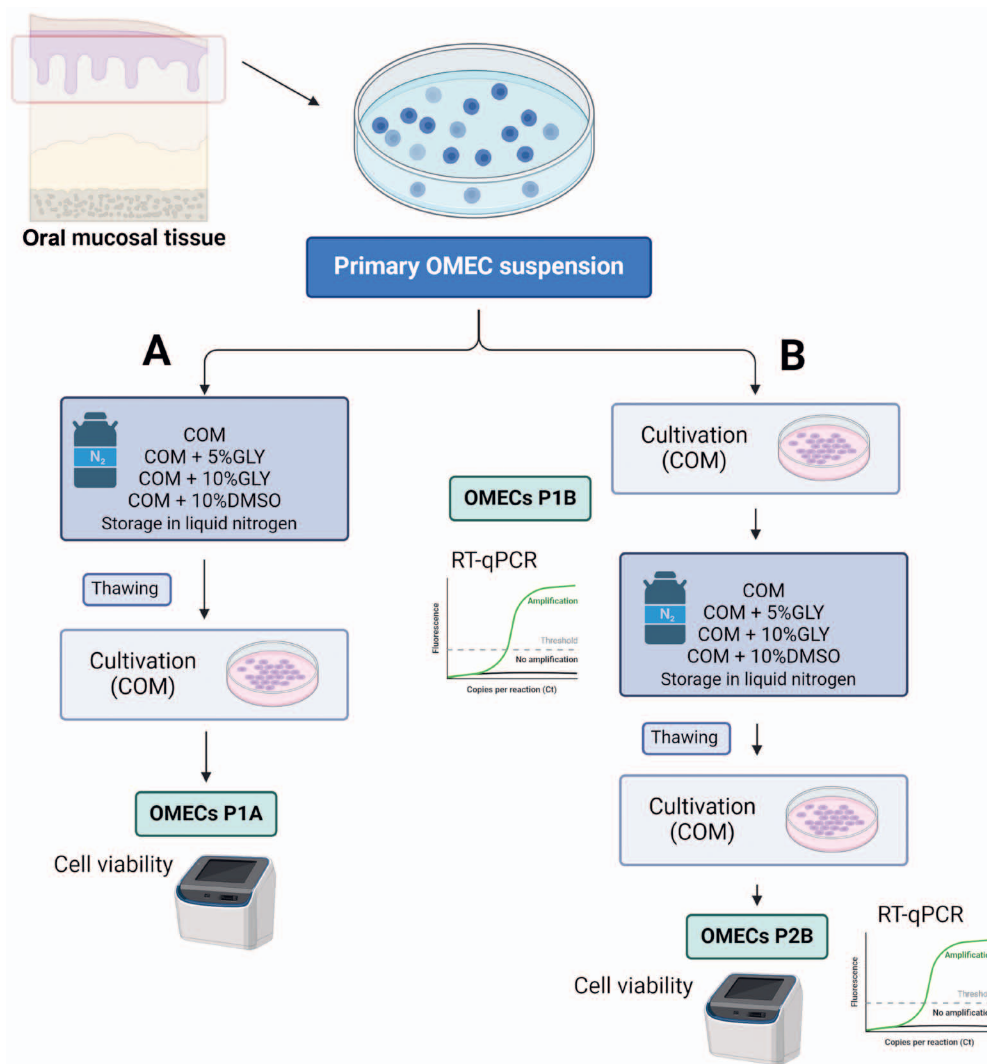

Fig. 1. Overview of the study design. The suspension of oral mucosal epithelial cells (OMECs) derived from solid oral mucosa tissue was subjected to cryopreservation (line A) or cultured in complex medium (line B). (**A**) OMECs were stored in four different solutions: complex medium (COM) without cryoprotectant; COM + 5 % glycerol, COM + 10 % glycerol, and COM + 10 % dimethyl sulphoxide (DMSO) and cryopreserved in liquid nitrogen. Following cryopreservation, the cells were thawed, cultured, and subsequently evaluated for cell confluence and viability (P1A – OMECs passaged once). (**B**) The primary OMECs were cultured and when they reached 85–95 % confluence, they were harvested (P1B – OMECs passaged once). One part was processed for gene expression analysis and the other part was cryopreserved in four different solutions as in (**A**) and cryopreserved in liquid nitrogen. After cryopreservation, the cultured cells were thawed and cultured again. Their confluence and viability were assessed and OMECs were harvested for PCR analysis (P2B – OMECs passaged twice).

5–10 minutes, which facilitated separation of the cells from any residual debris. Following centrifugation, the supernatant was removed without disturbing the cell pellet. The cell pellet was then gently resuspended with a growth medium. Approximately 4.0×10^4 cells were seeded per well, and the culture proceeded with media changes until confluence, as previously described (Trousil et al., 2024). Upon reaching confluence, the cells were harvested, the cell density was calculated, and OMECs were collected in lysis buffer for qPCR analysis.

Real-time quantitative polymerase chain reaction gene expression analysis

Cell samples after primary OMEC culture (N = 4 donors) (P1B) were compared with samples that were subsequently maintained in the COM medium without CPA or in the COM medium with 10 % glycerol and COM with 10 % DMSO, then re-cultured and harvested (P2B). Total RNA extraction was performed utilizing the RNeasy® Micro Kit, including DNase digestion, in accordance with the manufacturer's protocol (Qiagen,

Hilden, Germany). RNA quantity and purity were assessed using a spectrophotometer (Eppendorf BioPhotometer Model #6131), with additional evaluation of RNA quality via agarose gel electrophoresis. Subsequently, cDNA synthesis was done using the iScript cDNA synthesis kit (Bio-Rad). RT-qPCR was performed in a Hard Shell 96-well PCR plate (Bio-Rad) employing the SsoAdvanced Universal SYBR Green Supermix RT-qPCR Kit (Bio-Rad). Specific primers targeting stemness, proliferation and differentiation-related genes, along with two housekeeping genes hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and ribosomal protein L32 (*RPL32*), were used (Supplementary Material, Table S2). The results were evaluated using a Bio-Rad detection system (CFX Connect Real-Time PCR Detection System; Bio-Rad). Negative controls lacking template DNA were included for each primer pair to detect potential contaminants. Additionally, melting (dissociation) curves of qPCR reactions were scrutinized to confirm the presence of a single qPCR product and the absence of primer dimers, serving as an additional quality control measure.

Statistical analysis

The statistical analyses were performed in R software (v.4.4.0) (RCoreTeam, 2024). We tested for normality and homoscedasticity in the data before running the main analysis. The Shapiro-Wilk test was used to check whether the response variable (days to confluence or percentage of cell viability upon harvesting) followed a normal distribution. To check for equal variance (homoscedasticity), we performed the Breusch-Pagan test and examined a plot of residuals versus fitted values. For the main analysis, using lme4 and lmerTest R packages, a linear mixed-effects model was fit using the cryopreservation group as a fixed effect and donor as a random effect. An ANOVA was performed to evaluate the significance of storage conditions. Post-hoc (Tukey) pairwise comparisons were conducted using the R package emmeans to compare the storage groups, with significance levels set at adj. $P < 0.05$.

For RT-qPCR results, Ct values were calculated relative to *RPL32* and log-transformed to normalize their distributions. A heatmap after bidirectional hierarchical cluster analysis was constructed to explore the expression relationships between the samples with the pheatmap R package. Then, *limma* linear models with multiple testing comparisons and False Discovery Rate (FDR) adj. $P \leq 0.05$ were used. F-tests were calculated for multiple groups. The effect of within-patient correlation was also estimated. Ggplot2 was used for data visualization.

Results

Cell growth and viability

Primary OMECs (P1A) cryopreserved in the COM medium without a CPA did not proliferate to reach full confluence after thawing. P1A cells after storage in COM with CPA showed only a low degree of cell adhesion and proliferation even after 12 days of cultivation. Confluence higher than 80 % was achieved by only two cultures out of four that grew in the COM medium with 5 % glycerol (N = 2), 10 % glycerol (N = 1) and 10 % DMSO (N = 2) at 22 ± 3.2 days (mean ± SD) with overall viability 72.4 % ± 12.9 % (Fig. 2; Supplementary Material, Table S3). Given that the majority of cultures did not reach confluence, it was not feasible to conduct a statistical analysis across the P1A groups (COM, COM + CPAs). Consequently, the samples from the limited number of growing cultures (1–2 per condition that reached at least 80 % confluence, solely COM + CPA) were aggregated into a single group (P1A, N = 5).

The attachment and proliferation of post-thawed OMECs were more efficient when the cells were first cultured before cryopreservation (P2B) than when the cells were immediately cryopreserved (P1A). P2B cells

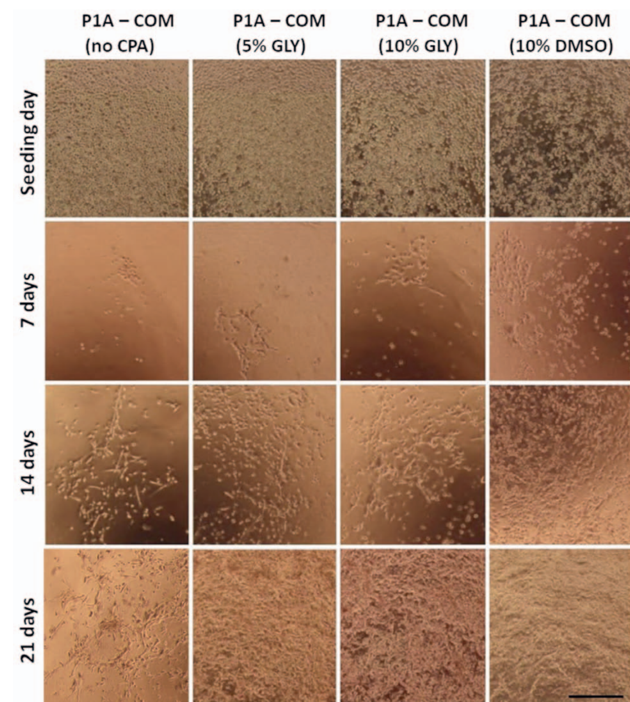

Fig. 2. Inverted phase-contrast microscopy of cultures of primary oral mucosal epithelial cells (OMECs) in complex (COM) medium after storage of cells in liquid nitrogen (P1A). The primary OMECs were cryopreserved in: COM, COM + 5 % glycerol, COM + 10 % glycerol, and COM + 10 % dimethyl sulphoxide (DMSO). After thawing, cells were cultured in COM. Representative images of successful cultures at the seeding day, day 7, day 14, and day 21 of cultivation. Cells cultured without cryoprotectant (CPA) did not achieve confluence, while cells cultured with CPA (COM + 5 % glycerol, COM + 10 % glycerol, and COM + 10 % DMSO) reached 85–95 % confluence. Scale bar: 500 μm.

that were cultured, stored in COM only, with no CPA, frozen, and subsequently re-cultured reached confluence after 13.4 ± 3.8 days (range 7–16 days), exhibiting viability of 84.8 ± 8.2. Immediately following thawing, P2B cells stored in COM + CPA exhibited high viability (Supplementary Material, Table S3). Upon reaching the second passage, the P2B cells in COM + CPA exhibited confluence after 13.8 ± 4.1 days (range 8–21 days), with the viability of 77.1 ± 14.6 % (combined data). Notably, the viability of OMECs stored in COM + 5 %, 10 % glycerol, and 10 % DMSO reached 73.0 ± 14.5 %, 77.2 ± 16.6 %, and 82.0 ± 15.7 %, respectively (Fig. 3; Supplementary Material, Table S3).

While accounting for the effect of donor, fitting a linear mixed effects model following ANOVA with Tukey post hoc test, we confirmed that the cells of P1A reached confluence significantly more slowly than P2B-COM (adj. $P < 0.001$) or P2B-COM+CPAs (adj. $P < 0.0001$, for all CPAs: 5 % or 10 % glycerol or DMSO) (Fig. 3A). There were no significant differences between the P2B groups. However, despite these differences in the cell growth rate among the P1A and P2B groups, the cell viability was comparable across the groups (ANOVA with Tukey post hoc test, adj. $P > 0.05$ for all groups, Supplementary Material, Tables S4–5). Although non-significantly, P2B cells in COM tended to be more viable than other groups (Fig. 3B).

OMECs in P2B showed cobblestone-like morphology with a high nucleus-to-cytoplasm ratio and noticeable cell-to-cell contacts. After the first week of cultivation, formation of epithelial layer stratification was observed (Fig. 4).

Gene expression alterations resulting from sequential culturing

Using RT-qPCR, we followed the changes in expression for twelve preselected genes regulating stemness, proliferation and differentiation between matched samples when cultured (P1B, N = 4) and when cultured for a second time after storage (P2B, N = 4 per group) with or without CPA (10 % glycerol or 10 % DMSO). Cluster analysis of the relative mRNA expression values revealed that these genes were classified into three groups

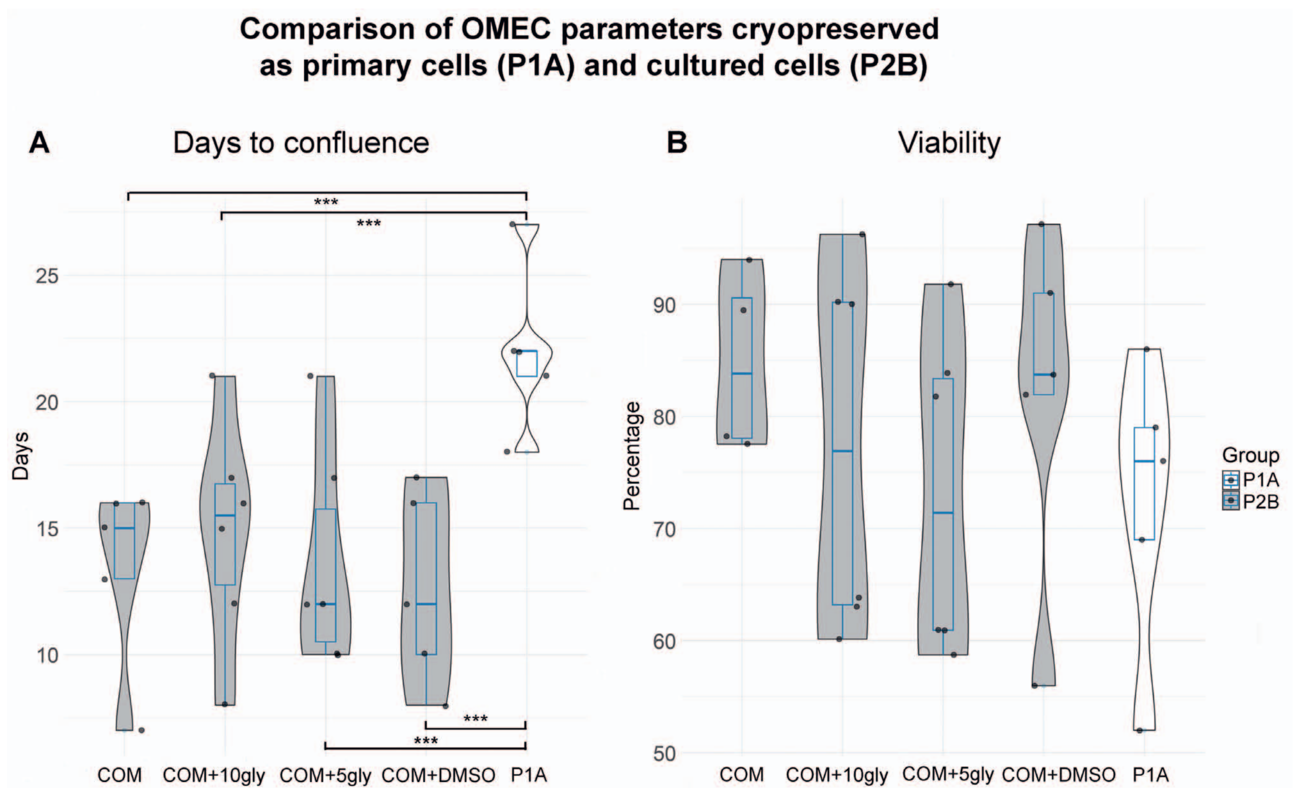

Fig. 3. Days to confluence and viability of oral mucosal epithelial cells (OMECs) when cryopreserved, thawed, and cultured (P1A) and when cultured, cryopreserved, thawed, and cultured (P2B). The time required to reach cell confluence was significantly shorter in P2B (**A**), while cell viability was comparable in both conditions (**B**).The P1A group includes the few successful samples (N = 5) derived from two donors. The violin plots include the individual values and boxplots indicating the median for each group. Asterisks on the horizontal line above every two boxes indicate statistical significance of adj. $P < 0.001$. COM, P2B stored in complex medium (COM) without cryoprotectant; COM+10gly (P2B stored in COM and 10 % glycerol); COM+5gly (P2B stored in COM and 5 % glycerol); COM+DMSO (P2B stored in COM and DMSO).

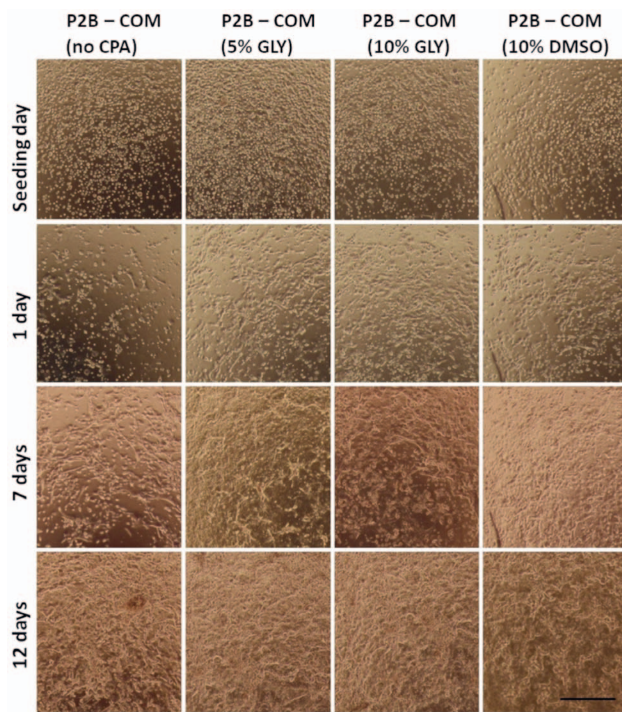

Fig. 4. Inverted phase-contrast microscopy of re-cultured oral mucosal epithelial cells (OMECs P2B) in complex medium (COM). The primary OMECs were cultured, cryopreserved (COM alone, COM + 5 % glycerol, COM + 10 % glycerol, and COM + 10 % DMSO), thawed, and re-cultured again. Representative images on the seeding day, day 5, day 7, and day 12 of cultivation. Scale bar: 500 μm.

based on their relative level of expression (Fig. 5A). Particularly highly expressed genes included the keratins *KRT7* and *KRT14*. Highly expressed genes were also *KRT13*, *KLF4*, *dNp63a* and *PCNA*. Low levels of expression were found for *KRT3*, *ABCG2*, *KRT12*, Ki-67 and *SOX2*. The mRNA expression of these genes was compared between the P1B and P2B groups with or without CPA, while estimating the within-patient correlations. Using the *limma* linear model, F-tests with multiple testing comparisons revealed that the expression of the tested genes remained at similar levels after storage, except for *KRT13* expression that decreased significantly in P2B cultures (adj. $P < 0.05$) (Fig. 5B). Noticeably, there was a lower trend in *SOX2* expression in P2B groups, but this effect was insignificant (adj. $P > 0.05$) (Fig. 5B). The presence of CPA had no further effect on the expression of any of the tested genes (adj. $P > 0.05$). There was a positive within-donor correlation (correlation coefficient = 0.54).

Discussion

Cryopreservation is an essential tool for the prolonged storage of stem cells, ensuring their availability for future therapeutic interventions (Jaiswal and Vagga, 2022). In this study, we demonstrated that cryopreservation is a suitable method for the long-term preservation of OMECs intended for use as a graft for the management of bilateral LSCD. Our goal was to evaluate the viability, proliferation and stemness of the cell grafts made from primary cells that had been cryopreserved and then re-cultured (P1A), or primary cells previously cultivated (P1B), cryopreserved and re-cultured (P2B).

Our findings demonstrate that primary cells frozen without a CPA fail to proliferate and form cell sheets (P1A) in most of the examined samples. In contrast, cultured cells that had been cryopreserved without CPA and re-cultured exhibited faster growth rates. The time required for primary cells P1A to attain confluence after thawing and seeding was almost twice as long as the typical 12- to 14-day culture period for OMECs to maintain their stemness (Cabral et al., 2024), irrespective of the CPA use.

Primary cells could grow following storage with CPA in the COM media in less than 50 % of cases (P1). The prolonged time (from 18 up to 27 days) to reach confluence might drive cells toward a differentiated phenotype, reducing the stem cell count, which is undesirable (Stadnikova et al., 2019). As most of the samples in this condition did not form a confluent cell sheet, we were unable to get enough OMECs for gene expression analysis. These results indicate that the process is incompatible with the method's possible application in clinical practice, in which cultures should result in a cell graft ideally in all cases. It has been previously reported that successful cultivation was accomplished when primary human (Xiong et al., 2010) or rabbit tissue (Promprasit et al., 2015) was employed as the source of cells, rather than primary dissociated cells.

Compared to P1A, P2B OMECs had faster cell growth, reaching full confluence about ten days earlier. Intriguingly, although not significantly, the highest viability was observed in cells stored in COM without a CPA. Human oral keratinocytes that had already been cultivated were effectively cryopreserved and re-cultured on a variety of cell surfaces including bovine collagen (Leelahavanichkul and Gutkind, 2013), decellularized porcine dermal matrix (Xiong et al., 2010), temperature-responsive poly(N-isoproprylacrylamide) polymer (Morino et al., 2019), the latter material being acceptable for clinical use. While the first two studies (Xiong et al., 2010; Leelahavanichkul and Gutkind, 2013) used 10 % DMSO as a CPA, another study (Morino et al., 2019) used a commercial medium (CELLBANKER1, Zenoaq, Fukushima, Japan) containing serum, but the type of CPA had not been disclosed. DMSO (10 %) was also part of the cryopreservation medium for storage of cell sheets containing rabbit OMECs (Oliva et al., 2019), and a loss of ΔNp63 expression was reported when the storage solution comprised ethylene glycol and DMSO. In our study, we did not observe *dNp63* loss in any of the groups achieving confluence post thawing and culture, including groups stored in the media containing 10 % DMSO. When

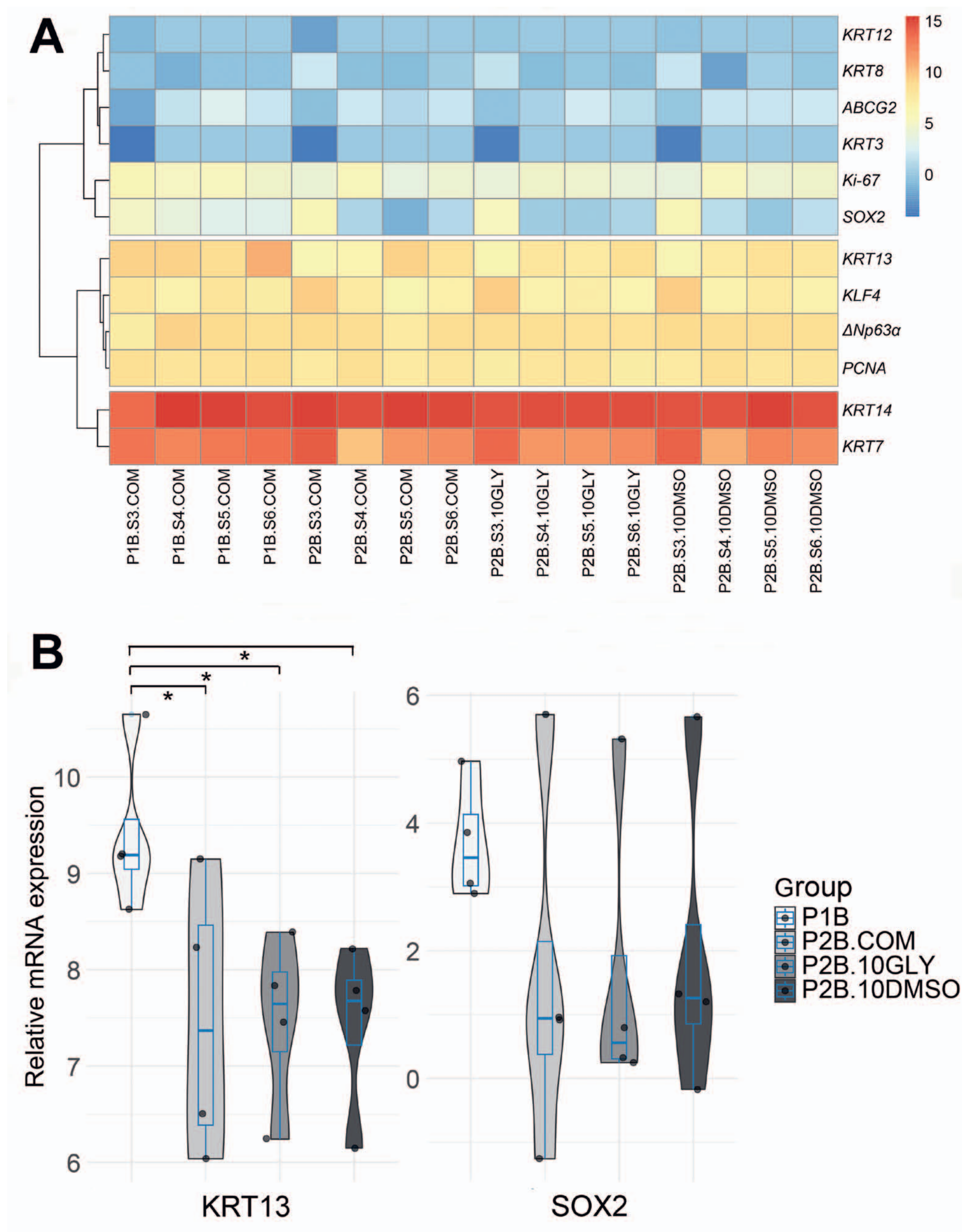

Fig. 5. Differentiation, proliferation and stemness-related gene expression in oral mucosal epithelial cells (OMECs) when cultured before storage (P1B) and when cultured again after storage without cryoprotectant (P2B) or in 10 % glycerol (P2B-10GLY) or 10 % DMSO (P2B-10DMSO). **(A)** Hierarchical clustering; a heatmap was generated to provide an overview of the relative mRNA expression for the tested genes (rows), with the samples (columns). The colour scale on the right side of the heatmap represents higher expression in red and lower expression in blue. **(B)** Altered gene expression of *KRT13* and *SOX2* in all P2B groups compared to P1B. Asterisks on the horizontal line above every two boxes indicate statistical significance of adj. $P < 0.05$.

OMECs were cultured on a 3T3 cell feeder, more favourable results in terms of cell morphology were observed after subsequent cryopreservation with glycerol than with DMSO (Hibino et al., 1996).

Regarding gene expression changes between P1B and P2B OMECs resulting from sequential culturing, we observed a consistent pattern of expression for stemness, proliferation and differentiation markers, with one exception, *KRT13.* Keratin 13 is the most characteristic keratin of oral mucosal epithelium (Jones and Klein, 2013). In comparison to P1B, *KRT13* expression significantly dropped following cryopreservation (P2B) in all groups. This may be explained by the composition of the medium, which promotes OMEC development into

a phenotype that is shared by the oral mucosal epithelium and the corneal/conjunctival epithelium (K14, K15/K19), but not entirely distinctive for OMECs (K13) (Jones and Klein, 2013) or for the cornea (K3/12) (Zieske et al., 1994). *SOX2* has been proposed as a potential biomarker for oral mucosal cells, with the aim of distinguishing them from corneal cells (Attico et al., 2022). Although not significant, we observed a decrease in *SOX2* gene expression in P2B compared to P1B, which was donor specific but could also be related to the sequential culture of OMECs. Similar to the decrease in *KRT13* expression, this could reflect a phenotype more relevant to the corneal epithelium or indicate initiation of the differentiation process and loss of stemness.

Some studies have demonstrated that CPAs can impact gene expression (Sumida et al., 2011; Cordeiro et al., 2015). Particularly, DMSO affected the expression of pluripotency genes in human embryonic stem cells, resulting in decreased stem cell marker expression (Czysz et al., 2015). To our knowledge, storage of OMECs in such diverse storage media, including those devoid of CPAs and xenobiotic-free media, has not been previously shown, rendering direct comparisons unfeasible. P2B also displayed a notable acceleration in OMEC attachment and proliferation, occurring just after 24 hours. This is a remarkably shorter period than the 4–5 days typically observed during primary OMEC cultivation under the same conditions.

The implications of cryopreserving OMECs extend beyond ocular surface regeneration to broader applications in tissue engineering and regenerative medicine. The ability to store previously cultured OMECs with minimal loss of viability and stemness without the need for high concentrations of CPAs holds promise for enhancing graft availability in clinical settings, particularly for conditions such as bilateral LSCD. This approach could streamline the preparation of cell grafts, reducing patient waiting times and improving the practicality of personalized therapies. Furthermore, given that OMECs share key characteristics with other epithelial tissues, these findings may serve as a foundation for optimizing cryopreservation techniques for various epithelial cell types used in wound healing and tissue regeneration (Miyamoto, 2023). The potential to store and re-culture these cells efficiently could facilitate advancements in regenerative treatments for burn injuries, chronic wounds, and even oral mucosal reconstruction. Thus, cryopreservation of epithelial cells without CPA or with low concentrations of CPA not only advances ocular therapies but also holds broader relevance for epithelial tissue engineering across multiple medical fields. We have shown that not only cultured but also frozen OMECs can be cultured in sufficient quality and number on a fibrin carrier and then applied directly to the patient's eye (Hirayama et al., 2012).

In conclusion, it is preferable to freeze previously *ex vivo* expanded OMECs in terms of cell viability and the capacity to form a large multi-layered graft. We found no significant difference in the quality of the cell sheet depending on the type and concentration of CPA used (glycerol vs DMSO) or a difference in cases where CPA was not applied. The present study is limited by the small number of samples included in the analysis due to the restrictions caused by the relatively small amount of tissue obtained from a single donor. Nevertheless, our findings suggest that storing cells after the first passage preserves their growth potential, and this can also be accomplished without the use of CPAs. Moreover, the long-term effects of cryopreservation on comprehensive cell functionality and genetic stability merit further study. Thus, these findings indicate that a recommended approach would be to initially culture OMECs in COM, considering cell sheet grafting. Simultaneously, a secondary parallel culture would be harvested (first passage cells) and stored for potential re-grafting. Based on the data on long-term storage, either COM or COM with low concentrations of CPA (glycerol or DMSO) would preserve the most important phenotypical characterization in terms of transplantation – their stemness.

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