



# Concise Review: Considering Optimal Temperature for Short-Term Storage of Epithelial Cells

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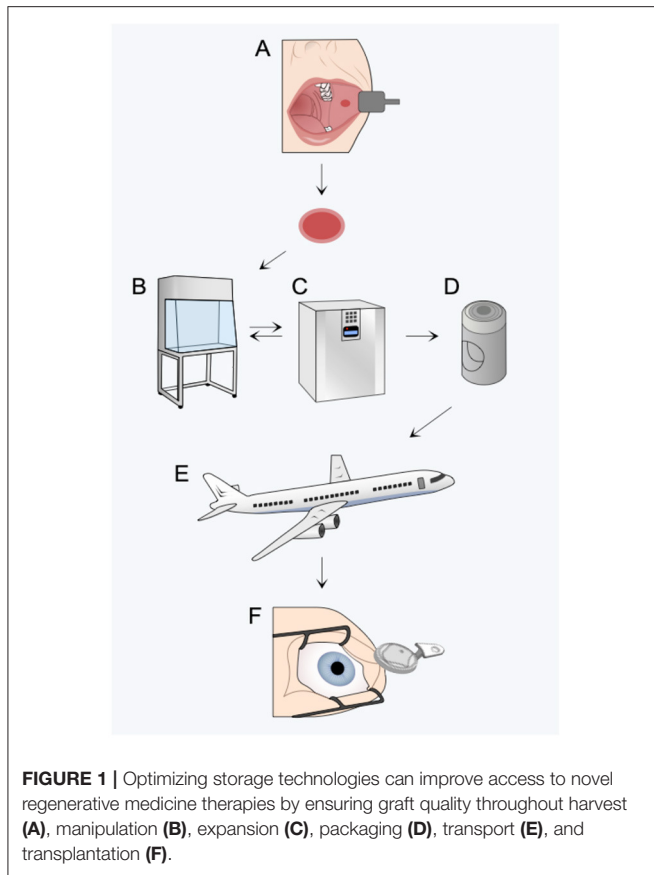
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Transplantation of novel tissue-engineered products using cultured epithelial cells is gaining significant interest. While such treatments can readily be provided at centralized medical centers, delivery to patients at geographically remote locations requires the establishment of suitable storage protocols. One important aspect of storage technology is temperature. This paper reviews storage temperature for above-freezing point storage of human epithelial cells for regenerative medicine purposes. The literature search uncovered publications on epidermal cells, retinal pigment epithelial cells, conjunctival epithelial cells, corneal/limbal epithelial cells, oral keratinocytes, and seminiferous epithelial cells. The following general patterns were noted: (1) Several studies across different cell types inclined toward 4 and 16°C being suitable short-term storage temperatures. Correspondingly, almost all studies investigating 37°C concluded that this storage temperature was suboptimal. (2) Cell death typically escalates rapidly following 7–10 days of storage. (3) The importance of the type of storage medium and its composition was highlighted by some of the studies; however, the relative importance of storage medium vs. storage temperature has not been investigated systematically. Although a direct comparison between the included investigations is not reasonable due to differences in cell types, storage media, and storage duration, this review provides an overview, summarizing the work carried out on each cell type during the past two decades.

**Keywords:** cell banking, regenerative medicine, storage temperature, cell therapy, storage technologies, transplantation

## INTRODUCTION

Transplantation of skin grafts is common in clinical practice. However, novel tissue-engineered therapies using cultured epithelial cells are gaining significant interest due to several breakthroughs during the past few decades. Applications include regeneration of burn wounds (1), corneal diseases (2), urethral reconstruction (3), and treatment of retinal dysfunction (4). Provision of these therapies at centralized medical centers has been accomplished, but delivery of such treatment opportunities to patients at geographically remote locations is equally important from a health



equity perspective. Because tissue-engineering laboratories require specialized facilities and are subject to high safety and quality standards, few laboratories are able to meet these requirements. This will likely be a barrier to widespread access to such therapies. Therefore, tissue storage technologies need to be improved in order to facilitate transportation of novel tissue-engineered products from centralized laboratories to clinics worldwide (5) (Figure 1). Optimization of tissue storage technology can also facilitate greater flexibility in surgery logistics and allow sufficient time for quality control and microbiological testing (5).

Temperature is generally considered important in transplantation medicine when transporting (1) donor tissue from the operating room to the laboratory and (2) the manufactured tissue-engineered product from the laboratory back to the operating room. While the latter step has been the subject of many investigations, the former step has received little attention. Due to this lack of evidence, this review will focus on storage temperature in general. To maintain storage temperature during transport, thermal insulation inserts, chilled coolant packs, and refrigerated transportation can be employed. Additionally, sophisticated purpose-built storage devices have been described (6, 7).

There are chiefly three approaches to storage of mammalian cells (8): (1) cryopreservation, (2) cell desiccation, and (3)

hypothermic cell preservation. While cryopreservation is useful in laboratory settings, it presents logistical challenges during transport (9) and is associated with low post-thaw cell viability (10) and dimethyl sulfoxide (DMSO)-associated toxicity. Desiccation involves preserving cells in a dehydrated form by either freeze-drying or using a vacuum, but concerns related to exposure of cells to severe osmotic imbalance (11, 12), free radical-mediated cell toxicity (13), and chemical treatment of cells before transplantation (14) are important disadvantages. Hypothermic cell preservation (at above freezing temperature) slows down cell metabolism without causing cellular ice damage, is practical, and is a method already widely in use (15). Whereas the limited storage time (in comparison to cryopreservation) remains a significant drawback, the use of biomaterials, such as hydrogels, has made this storage technique very relevant because it enables the delivery of cultured cells in a structurally inert way without compromising graft pliability or biocompatibility (16–18).

This study reviews the scientific literature reported during the past two decades on epithelial cell storage at above freezing point in an effort to uncover the optimal temperature for hypothermic storage of epithelial cells. Studies on the storage of donor corneas are excluded from the review, as this has been adequately covered elsewhere (19, 20).

## METHODS

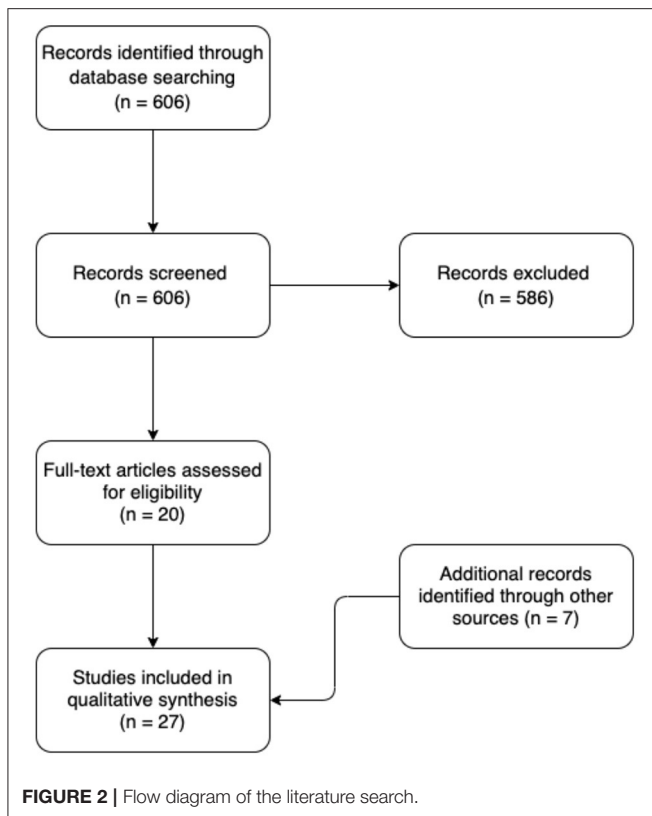
We performed a literature search on the storage of epithelial cells. With the search algorithms provided in the Appendix, the following databases were searched: Embase, Ovid MEDLINE, Cochrane Library, and Google Scholar. A total of 606 records were retrieved through database searching. These records were then screened manually. Articles were excluded according to predetermined criteria, i.e., if they were conducted on non-human tissue, if they were performed on non-epithelial cells, if they primarily were focused on storage of donor corneas, or if the storage technique was other than above-0°C storage, such as cryopreservation. Also excluded were patents, dissertations, articles in languages other than English, and papers published prior to year 2000. Twenty records remained eligible following the exclusion process. Additionally, seven records were identified based on the authors' acquaintance with the subject matter. These were not detected by the literature search. Thus, 27 English-language publications from the last two decades investigating above-freezing point storage of human epithelial cells were included in the final qualitative synthesis (Figure 2; Table 1).

## RESULTS

Results are presented in Table 1.

## DISCUSSION

Epithelium “covers or lines body surfaces and forms the functional units of secretory glands” (47). Epithelial cells are classified based on their anatomical location, the shape of the



individual cell (squamous, cuboidal, or columnar), and the arrangement of cells in one or more layers (simple epithelia or stratified epithelia) (47). In this review, we summarize work on storage temperatures for above-freezing point storage of human epithelial cells for regenerative medicine purposes. The literature search uncovered publications on epidermal cells, retinal pigment epithelial (RPE) cells, conjunctival epithelial cells, corneal/limbal epithelial cells, oral keratinocytes, and seminiferous epithelial cells (Figure 3).

## Epidermal Cells

Refrigeration remains the preferred method for the short-term storage of skin grafts. A survey performed among plastic surgery centers across Europe confirmed that split-thickness skin grafts are routinely stored for up to 10 days at 4°C in saline-moisturized gauze (26). Histologic evaluations have shown no major macroscopic or microscopic alterations within skin grafts stored for 7 days at 4°C (26). Seet et al. (28) successfully stored a tissue-engineered skin construct composed of keratinocytes and fibroblasts at 4°C for 3 days without observing a major reduction in cell viability. However, cell viability appears to decline with increased storage duration (26). Li et al. (27) also reported that skin cell viability declines with prolonged storage. Despite testing four different storage media, they showed that viability was reduced to 50% by storage day 14. By storage day 28, viability was <5% across all storage groups (27). Closely related to viability of the skin graft is the colony-forming efficiency of keratinocytes, which was inversely correlated with storage time (27). Hence,

the prevailing evidence on skin graft storage at 4°C indicates a storage time of about 7 days, with decreasing viability with increasing storage time.

In our literature search, the earliest report deviating from skin storage at 4°C was a publication by Robb et al. (29) in 2001. In this study, the authors reported better preservation of tissue anatomy when skin grafts were stored at room temperature compared to 4°C for up to 21 days. Importantly, they replaced the storage media every 3 days and thus provided the cells continuous nutrition, which is impractical when transporting grafts. Therefore, we consider this study closer to organ culture rather than cell storage.

When discussing skin storage, a distinction between skin grafts and cultured cells appears to be reasonable, as these two tissues differ considerably in origin, handling, and tissue anatomy.

Whereas clinical experience and scientific evidence agree on 4°C storage as the preferred storage temperature for short-term storage of skin grafts, this is not the case when considering cultured cells. Jackson et al. (23–25) have published three reports on the short-term hypothermic storage of cultured epidermal cell sheets (CES). In the first study, they stored cells for 14 days and reported a tendency of better viability in cells stored at higher temperatures (24, 28, 32, and 37°C) compared to cells stored at lower temperatures (4, 8, 12, 16, and 20°C) (25). However, cell death was most prominent at the extremes of the storage temperatures studied, i.e., 4 and 37°C. In preserving morphology, storage at 12 and 16°C appeared superior compared to other temperatures. In their second study, cells were stored for the same time period and at the same storage temperatures, but differentiation was studied more thoroughly (24). The authors concluded that the undifferentiated phenotype, which is desirable in the case of transplantation, was best maintained at the lower end of the abovementioned temperature spectrum, particularly 12°C. In the third study, cells were stored for 7 days at five different storage temperatures (4, 8, 12, 16, and 24°C) (23). This study concluded, based on morphological, phenotypical, cytokine, viability, and reactive oxygen species assays, that storage at 12°C uniquely provided optimal morphology and undifferentiated phenotype. Interestingly, storage at 12°C yielded the highest post-storage viability in the 1-week study compared to the 2-week storage experiments, suggesting a possible 1-week “shelf life” of cultured epidermal cells stored at 12°C. Reppe et al. (22) achieved a post-storage viability higher than unstored control when storing CES in minimum essential medium (MEM) at 12°C for a week. The increase in viability was attributed to cell proliferation during storage, which may suggest that the storage medium formula is of importance. Similarly, Ringstad et al. (21) stored CES at 12°C but for up to 15 days. They reported superior viability when the cells were stored at a pre-confluent stage (i.e., storage was initiated when cell cultures covered 80% of the culture dish). Collectively, data from these reports are in favor of 12°C as the optimal storage temperature for short-term storage of cultured epidermal cells.

Hence, based on the reported literature, we advise a storage temperature of 4°C for skin grafts and 12°C for cultured epidermal cells.

**TABLE 1** | Non-freezing storage of epithelial cells.

| References             | Type of epithelial tissue | Transplant type  | Storage duration | Storage medium                                  | Storage temperature(s) investigated    | Viability  | Morphology   | Phenotype  |
|------------------------|---------------------------|--|------------------|---|--|--|--|--|
| <b>Epidermal cells</b> |                           |  |                  |   |  |  |  |  |
| Ringstad et al. (21)   | Epidermal cells           | CCS  | 15 days          | MEM-based                                       | 12°C                                   | Viability deteriorates by storage day 11.  | Morphology is disrupted during extended storage but improves with reincubation   | Reincubated CES stored for 15 days retained proliferative function and the ability to differentiate.   |
| Reppe et al. (22)      | Epidermal cells           | CCS  | Up to 11 days    | CnT Prime and MEM-based                         | 12°C                                   | MEM-based storage media showed better viability compared to CnT Prime. Mathematic simulations suggested glycerol and fenoldopam mesylate as viability-promoting storage media additives. | Carnosine, fenoldopam mesylate, and glycerol had a beneficial effect on morphology when used as storage media additives. | NA   |
| Jackson et al. (23)    | Epidermal cells           | CCS  | 7 days           | MEM-based                                       | 4, 8, 12, 16, and 24°C                 | Optimal storage temperatures for viability: 12 and 16°C  | Optimal storage temperature for morphology: 12°C.  | 12°C storage demonstrated best preservation of undifferentiated cell phenotype.  |
| Jackson et al. (24)    | Epidermal cells           | CCS  | 14 days          | MEM-based                                       | 4, 8, 12, 16, 20, 24, 28, 32, and 37°C | NA   | NA   | Lower storage temperatures, and in particular 12°C, were optimal in preserving an undifferentiated phenotype during storage.                 |
| Jackson et al. (25)    | Epidermal cells           | CCS  | 14 days          | MEM-based                                       | 4, 8, 12, 16, 20, 24, 28, 32, and 37°C | Optimal storage temperature for viability: 24°C. However, almost 60% cell viability was conserved at 12, 28, 32, and 37°C.   | Optimal storage temperatures for morphology: 12 and 16°C   | Storage at 12 and 20°C preserved proliferative function at a similar level as the non-stored control.  |
| Knapik et al. (26)     | Human skin                | Split-thickness skin grafts  | Up to 7 days     | Saline-moisturized gauze                        | 4°C                                    | Viability dropped to 44% after 3 days of storage and remained at this level during the subsequent days.  | No changes observed.   | NA   |
| Li et al. (27)         | Human skin                | Split-thickness skin grafts and keratinocytes                              | Up to 28 days    | Saline, Hartmann's solution, DMEM, and DMEM/F12 | 4°C                                    | Viability decreased proportionally with storage time.  | NA   | Compared to DMEM-based media, storage in saline and Hartmann's solution resulted in better post-storage keratinocyte proliferative capacity. |
| Seet et al. (28)       |                           | Tissue-engineered skin construct composed of keratinocytes and fibroblasts | 3 days           | DMEM/F12  | 4°C                                    | Viability was 95% before storage, 91% after 24 h storage, 92% after 48 h storage, and 91% after 72 h storage.  | No change in morphology during storage.  | No significant difference was found in gene expression.  |

(Continued)

TABLE 1 | Continued

| References                              | Type of epithelial tissue     | Transplant type                               | Storage duration | Storage medium | Storage temperature(s) investigated     | Viability   | Morphology  | Phenotype   |
|---|-------------------------------|---|------------------|----------------|---|---|---|---|
| Robb et al. (29)                        | Human skin                    | Split-thickness skin grafts and skin biopsies | Up to 21 days    | Saline and MEM | 4°C or room temperature                 | NA  | Skin stored in MEM-based media maintained better histologic anatomy than skin stored in saline.   | NA  |
| <b>Retinal pigment epithelial cells</b> |                               |   |                  |                |   |   |   |   |
| Islam et al. (30)                       | ARPE-19                       | CCS   | 21 days          | MEM-based      | 4, 16, and 37°C                         | Optimal storage temperature for viability: 16°C   | Morphology was best preserved at 16°C.  | Dedifferentiation was noted in all storage temperatures.  |
| Kitahata et al. (31)                    | hiPSC-RPE                     | Cell suspensions                              | Up to 120 h      | DMEM-based     | 4, 16, 25, and 37°C                     | Following 120 h of storage, highest viability was achieved at 16°C storage  | Cells preserved at 4°C were damaged <i>via</i> microtubule fragility  | Surviving cells proliferated and secreted key proteins normally   |
| Khan et al. (32)                        | Human fetal RPE               | CCS   | 7 days           | MEM-based      | 4, 16, 20, 24, 28, and 37°C             | The three lowest storage temperatures generally showed fewer dead cells compared to the three highest storage temperatures. | 4 and 16°C storage resulted in best morphology. Membrane blebbing, intercellular distance, and loss of intercellular contact was seen at higher storage temperatures. | No storage temperature expressed differentiation markers in a consistently favorable fashion.                                     |
| Pasovic et al. (33)                     | ARPE-19                       | CCS   | 7 days           | MEM-based      | 4, 16, and 37°C                         | Gene expression analysis showed that 16°C storage resulted in highest expression of cell survival genes.                    | NA  | Gene expression analysis suggested that 37°C resulted in cell cycle arrest. This was not observed in the 4 and 16°C groups.       |
| Pasovic et al. (34)                     | ARPE-19                       | CCS   | 7 days           | MEM-based      | 4, 16, and 37°C                         | NA  | NA  | Expression of genes related to pigmentation, ion transport, and visual cycle was almost similar among the various storage groups. |
| Pasovic et al. (35)                     | ARPE-19                       | CCS   | 7 days           | MEM-based      | 4, 8, 12, 16, 20, 24, 28, 32, and 37°C  | 16 and 20°C were superior for cell survival.  | Optimal storage temperatures for morphology: 12, 16, and 20°C   | 12, 16, and 20°C were superior in maintaining differentiated phenotype compared to other temperatures.                            |
| <b>Conjunctival epithelial cells</b>    |                               |   |                  |                |   |   |   |   |
| Eidet et al. (36)                       | Conjunctival epithelial cells | CCS   | 4–7 days         | MEM-based      | 4, 8, 12, 16, 20, 24, 28, 32, and 37 °C | Viability was best preserved at 12°C storage.   | For longer storage periods, i.e., 7 days, storage temperatures below 12°C appeared more suitable.   | Storage temperatures above 12°C showed higher metabolic consumption compared to lower storage temperatures.                       |

(Continued)

TABLE 1 | Continued

| References                             | Type of epithelial tissue                             | Transplant type          | Storage duration | Storage medium                         | Storage temperature(s) investigated            | Viability   | Morphology  | Phenotype   |
|--|---|--------------------------|------------------|--|--|---|---|---|
| Vasania et al. (37)                    | Conjunctival epithelial cells                         | Cell suspensions         | 2–4 days         | DMEM/F12-based                         | 2–8°C  | Viability decreased gradually as storage time increased—from 92% viability following 6 h storage to 82% viability after 48 h storage.           | Morphology was maintained throughout the storage period.  | Specific phenotypic markers were not studied; however, cell attachment was described as “good” in all storage durations.                                  |
| Eidet et al. (38)                      | Conjunctival epithelial cells                         | CCS on amniotic membrane | 4–7 days         | HEPES-MEM and Optisol-GS               | 23°C   | Viability was well-preserved in both storage media.   | Ultrastructure integrity was well-preserved during 4-day storage. Epithelial detachment was observed following 7-day storage. | Expression of key phenotypic markers remained unchanged in both storage media.  |
| <b>Corneal/limbal epithelial cells</b> |   |                          |                  |  |  |   |   |   |
| Jackson et al. (39)                    | Limbal epithelial cells                               | CCS                      | 4 days           | Optisol-GS                             | 4 vs. 23°C                                     | 23°C storage was better in maintaining cell viability compared to storage at 4°C.   | Detachment of basal cells from the underlying membrane was observed in cells stored at 4°C.                                   | In contrast to 4°C storage, expression of stem cells and proliferation markers was maintained at pre-storage levels during storage at 23°C.               |
| Utheim et al., (40)                    | Limbal epithelial cells                               | CCS                      | Up to 7 days     | Optisol-GS                             | 4°C  | Genes associated with cell death and necrosis were upregulated following 4 and 7 days of storage.   | NA  | Gene expression deviated from the control group as storage duration increased.  |
| Utheim et al., (41)                    | Limbal explants                                       | CCS                      | Up to 7 days     | Quantum 286 medium or MEM-based medium | 23°C   | Cell viability was preserved during storage in both media.  | No substantial loss of cell layer thickness was observed during storage.  | Albeit somewhat reduced, the immature phenotype of cells was preserved during storage.  |
| Utheim et al., (42)                    | Limbal epithelial cells cultured on amniotic membrane | CCS                      | Up to 21 days    | DMEM-based medium                      | 23°C   | Viability was 88% after 14 days storage and 53% after 21 days of storage compared to unstored control.  | Multilayered tissue anatomy was preserved in 70% of cultures following 14 days of storage but lost after 21 days of storage.  | A less differentiated phenotype was maintained throughout the storage period.   |
| Raeder et al. (43)                     | Limbal epithelial cells cultured on amniotic membrane | CCS                      | 7 days           | Optisol-GS or DMEM-based medium        | Optisol-GS 5°C. DMEM-based medium 23 and 31°C. | Although the differences were statistically insignificant, the number of apoptotic cells appeared to correlate with higher storage temperature. | Storage in a DMEM-based medium at 23°C was superior in preserving the original layered structure of the stored cells.         | Cells remained undifferentiated in all storage conditions.  |
| <b>Oral keratinocytes</b>              |   |                          |                  |  |  |   |   |   |
| Islam et al. (44)                      | Oral keratinocytes                                    | CCS                      | 7 days           | MEM-based                              | 4, 8, 12, 16, 20, 24, 28, 32, and 37°C         | Storage at 12°C maintained the highest number of live cells.  | The midrange temperature groups of 12, 16, and 20°C resulted in the best morphology.  | Storage temperatures between 4 and 24°C resulted in better preservation of phenotypical markers. Cell metabolism was proportional to storage temperature. |

(Continued)



TABLE 1 | Continued

| References                           | Type of epithelial tissue     | Transplant type  | Storage duration | Storage medium   | Storage temperature(s) investigated | Viability  | Morphology  | Phenotype  |
|--------------------------------------|-------------------------------|------------------|------------------|--|-------------------------------------|--|---|--|
| Oie et al. (7)                       | Oral mucosal epithelial cells | CCS              | 12 h             | HBSS-based   | 35°C                                | Viability, morphology, and key phenotypical markers were adequately preserved during transportation.   |   |  |
| Lee et al. (45)                      | Oral keratinocytes            | CCS              | Up to 72 h       | Keratinocyte growth medium, saline, and temperature DMEM with or without albumin supplementation | 4°C or room temperature             | Highest cell viability was obtained in saline or DMEM both supplemented with 10% serum albumin at 4°C or at room temperature.                                | NA  | NA   |
| <b>Seminiferous epithelial cells</b> |                               |                  |                  |  |                                     |  |   |  |
| Faes and Goossens (46)               | Seminiferous epithelium       | Biopsy specimens | 3 days           | DMEM/F12   | 4°C, room temperature, and 37°C     | No significant difference was observed in cell viability; however, storage for 3 days at 37°C showed a higher number of apoptotic cells compared to control. | No significant morphological changes were observed. | Storage temperature did not appear to affect the number of spermatogonia in samples. |

The table is sectioned based on cell type, and the rows are sorted by year of publication (descending). CCS, cultured cell sheets; MEM, minimum essential medium; CES, cultured epidermal cell sheets; CrT Prime, a commercially available, fully defined, animal component-free culture medium; DMEM, Dulbecco's modified Eagle medium; DMEM/F12, 1:1 mixture of DMEM and Ham's F-12 medium; ARPE-19, adult human retinal pigment epithelial cell line 19; hiPSC-RPE, human induced pluripotent stem cell-derived retinal pigment epithelium; RPE, retinal pigment epithelium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Optisol-GS, a commercially available corneal storage medium containing gentamicin and streptomycin; HBSS, Hanks' Balanced Salt Solution.

## Retinal Pigment Epithelial Cells

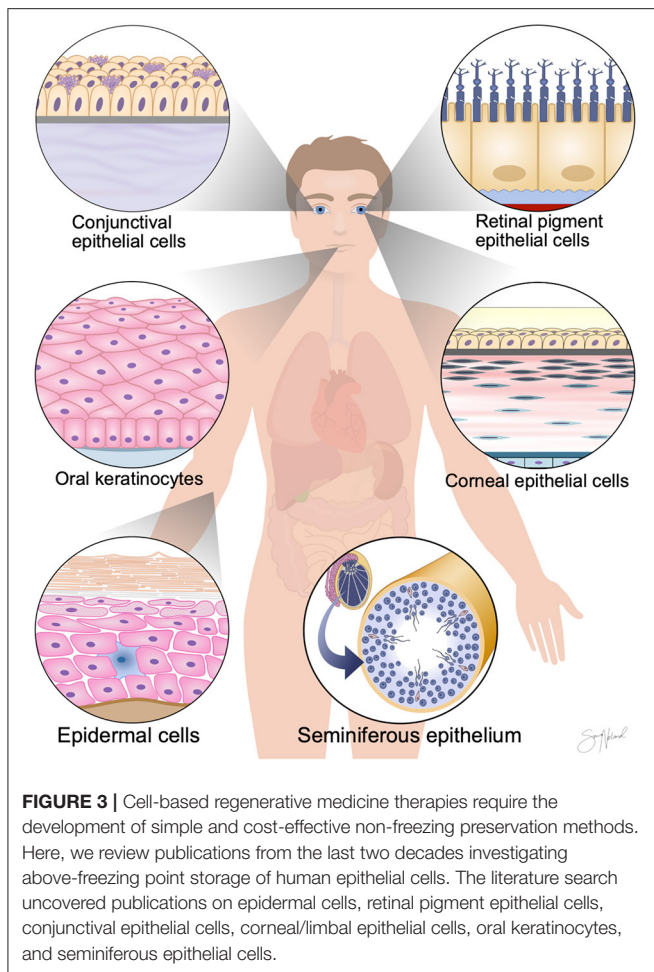
Transplantation of RPE is emerging as a promising treatment alternative for sight-threatening eye diseases such as age-related macular degeneration, Stargardt macular dystrophy, and some forms of retinitis pigmentosa (48). Results from three important clinical trials have been reported in recent years (49–51).

Storage temperature for short-term preservation of RPE cells has been investigated by six studies. One report showed favorable results with storage at 4°C (32), while three studies concluded that 16°C is the most suitable above-0°C storage temperature (30, 31, 35). In addition, two studies suggested that both 4 and 16°C were suitable storage temperatures (33, 34).

In support of 4°C, Khan et al. (32) stored cultured human fetal RPE sheets for 7 days at six different storage temperatures (4, 16, 20, 24, 28, and 37°C). After 7 days of storage, cell viability, morphology, pH, and phenotypic expression of differentiation markers were assessed. No single storage temperature consistently outperformed other storage temperatures across all investigated parameters. However, storage at 4°C best preserved tissue morphology (in comparison to non-stored control cells). A tendency of higher cell death in the three highest storage temperatures (24, 28, and 37°C) was noted.

In support of 16°C, Kitahata et al. (31) investigated the effect of storage temperature on storage of human induced pluripotent stem cell-derived retinal pigment epithelial (hiPSC-RPE) cell suspensions. They tested four different storage temperatures, 4, 16, 25, and 37°C, and demonstrated best viability when using a 16°C storage temperature. They showed that storage at 4°C resulted in microtubule fragility, while 37°C caused cell death due to hypoxia secondary to elevated cell metabolism. Likewise, a study on cultured ARPE-19 cell sheets stored at 4, 8, 12, 16, 20, 24, 28, 32, and 37°C in a MEM-based medium for 7 days also concluded 16°C to be the optimal storage temperature (35). Importantly, this study was carried out on cultured cell sheets and an immortalized RPE cell line, in contrast to Kitahata et al. (31), who stored cell suspensions and hiPSC-RPE, respectively. In a microarray analysis, Pasovic et al. (34) compared gene expression profiles of ARPE-19 cells stored for 1 week in a MEM-based medium at 4, 16, and 37°C. Storage at 4 and 16°C resulted in gene expression most similar to non-stored control, while storage at 37°C significantly altered gene expression. Furthermore, in a later study, they again showed that gene expression following storage at 4°C was closest to control cultures that were not stored (33). Cultures stored at 16 and 37°C displayed much greater change in gene expression. At 37°C, activation of vascular endothelial growth factor (VEGF) was discovered, which is considered disadvantageous in an RPE graft.

Testing a wide range of temperatures has shown that increments in temperature can alter storage outcome. Although both 4 and 16°C storage show favorable results, only one study investigated a storage temperature between these two temperatures. Hence, the true optimal hypothermic storage temperature may hide in this uninvestigated interval. Finally, a direct comparison between the reported studies is not reasonable due to differences in cell types, storage media, and storage duration.



## Conjunctival Epithelial Cells

Transplantation of cultured conjunctival epithelial cells has been reported to improve vision in patients with limbal stem cell deficiency (LSCD) (52), a disorder characterized by deficient or dysfunctional stem cells in the limbal region (53). Three papers describe storage of conjunctival epithelial cells. The first study stored conjunctival epithelial cells cultured on amniotic membrane in MEM containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES-MEM) and Optisol-GS at 23°C for 4 and 7 days (38). In this paper, viability and phenotype were maintained for at least 4 days of storage at 23°C (in both media). The second study was a prospective, open-label, single-arm, multicentric clinical trial in which 25 patients underwent autologous conjunctival epithelial cell transplantation (37). The clinical outcome was reported to be satisfactory and without serious adverse effects. The cell grafts were stored and transported for a 48-h period at a temperature interval of 2–8°C before surgery. Investigation of parallel cultures showed that cell attachment and morphology were acceptable throughout the storage period. Cell viability was adversely affected as storage time increased, dropping to 95, 90, 88, and 82% after storage at 6, 12, 24, and 48 h, respectively. The third study evaluated the effects of

storage temperature on morphology, viability, cell number, and metabolism of cultured human conjunctival epithelial cells (36). Cells were stored for 4 and 7 days. The following temperatures were investigated: 4, 8, 12, 16, 20, 24, 28, 32, and 37 °C. Here, 12°C storage appeared optimal, as this was the only storage temperature at which viability was preserved following a 7-day storage period. Moreover, total cell number had decreased in all groups, except 12°C. Furthermore, cell morphology was also maintained at this temperature. The authors suggested temperature-related effects on cell metabolism as the primary reason for their findings. Among the three cited studies, only the latter compared different storage temperatures, making it difficult to conclude on an optimal storage temperature.

## Corneal/Limbal Epithelial Cells

A number of reports have been published on the storage of limbal epithelial cells (39, 41–43). Although most are in favor of 23°C storage in an Optisol-GS or a DMEM-based medium, it must be noted that the mentioned studies have only tested 4, 23, and 37°C temperatures. As the preceding subsections discussing other cell types suggest, the temperature interval between 4 and 23°C is highly relevant and should be investigated in future studies.

Transplantation of cultured limbal epithelial cells is a therapy for LSCD (54). The ability of cultured limbal epithelial grafts to proliferate and generate a healthy population of cells is therefore critical. None of the reported publications investigated whether storage temperature affects stemness potential or the percentage of holoclones, meroclones, and paraclones that can be isolated from a biopsy and propagated *in vitro*. To conduct a correlation between storage temperature and stemness potential is therefore currently not possible. However, Jackson et al. (39) reported that expression of the stem cell marker ABCG2 was significantly reduced in the 4°C storage group compared to 23°C storage. Utheim et al. (41) also demonstrated that 23°C storage retained an immature phenotype in cultured limbal epithelial cells.

## Oral Keratinocytes

Cultured oral keratinocytes can be used to reconstruct damaged corneas and thus restore vision (55). Hypothermic storage of oral keratinocytes has been reported by Lee et al. (45), Oie et al. (7), and Islam et al. [2015]. Lee et al. (45) stored cell suspensions of primary human oral keratinocytes for 24, 48, and 72 h at 4°C and at room temperature. No significant decline in viability was observed for at least 48 h. Oie et al. (7) described a container that can maintain sterility, temperature stability, and air pressure during cell transportation. Using this custom-made container, they transported cultured oral mucosal epithelial cells by air for a transportation period of 12 h. Cell viability, morphology, phenotype, and sterility parameters were maintained during transportation. This investigation on both storage and transportation of cell products (considering practical challenges) is an excellent example of how future studies could be designed. Islam et al. (44) tested the following temperatures, 4, 8, 12, 16, 20, 24, 28, 32, and 37°C, over a storage period of 7 days. Relative to non-stored control cells, a high percentage of viable cells was retained only in the groups stored at 12 and 16°C. Morphology was preserved at 12, 16, and 20°C storage.



**TABLE 2 |** The table summarizes findings of the literature review of storage temperature for above-freezing point short-term storage of human epithelial cells.

**Temperature recommendations for the short-term storage of various epithelial cells**

|                                  |                               |
|----------------------------------|-------------------------------|
| Skin grafts                      | 4°C                           |
| Cultured epidermal cells         | 12°C                          |
| Retinal pigment epithelial cells | Between 4 and 16°C            |
| Conjunctival epithelial cells    | Insufficient evidence         |
| Corneal/limbal epithelial cells  | 23°C                          |
| Oral keratinocytes               | Insufficient evidence         |
| Seminiferous epithelial cells    | Both room temperature and 4°C |

## Other Epithelia

Faes and Goossens (46) studied how temperature affects storage of testicular tissue, including seminiferous epithelium. The tissue samples were stored at 4°C, room temperature, and 37°C. Tissue quality (judged by histology, immunohistochemistry, and apoptosis) was maintained at all temperatures following a 3-day storage period. However, in contrast to 4°C and room temperature, they found that 37°C storage caused a significant increase in apoptotic cells.

## CONCLUSION AND FUTURE PERSPECTIVES

In this review, we summarized the work investigating storage temperature for above-freezing point storage of human epithelial cells such as epidermal cells, RPE, conjunctival epithelial cells, corneal/limbal epithelial cells, oral keratinocytes, and seminiferous epithelial cells (Table 2). Such a summary is challenging for several reasons. First, the epithelial cells examined not only reside in different anatomical locations, they also differ in state (cultured cells vs. grafts; cell lines vs. primary cells; cell sheets vs. cell suspensions). Second, each study employed its own distinct set of cell culture media and storage media. Third, parameters of interest varied from study to study, e.g., while some focused on viability, others emphasized phenotypic and functional characterization. In sum, these variations do not allow for a fair comparison between the studies.

Nevertheless, some general tendencies can be observed. For instance, several studies across different epithelial cell

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types incline toward temperatures between 4 and 16°C being suitable as short-term storage temperatures. Correspondingly, almost all studies that investigated 37°C concluded that this storage temperature was suboptimal. Another common observation (when analyzing studies investigating storage duration) is that storage time typically should not exceed 7–10 days, as viability tends to decrease dramatically after this duration. Finally, the importance of the type of storage medium and its composition was highlighted by some of the studies. The topic of storage media deserves a separate review.

Future efforts should be directed toward investigation of clinical outcomes after transplantation of stored cell products.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

AK performed the literature review and drafted the manuscript. TU, CJ, KT, and JE edited the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmed.2021.686774/full#supplementary-material>

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**Conflict of Interest:** JE and TU hold a patent on the use of sericin in culture media (European Patent Number EP3317404), filed by Inven2 (the technology transfer office of the University of Oslo and Oslo University Hospital).

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer DT declared a past co-authorship with several of the authors to the handling editor.

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