METHODS and TECHNIQUES for SKIN RESEARCH

# Reassessing Long-Term Cryopreservation Strategies for Improved Quality, Safety, and Clinical Use of Allogeneic Dermal Progenitor Cells

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In regenerative medicine, ongoing advancements in cell culture techniques, including isolation, expansion, banking, and transport, are crucial for clinical success. Cryopreservation ensures off-the-freezer availability of living cells, enabling long-term storage and transport. Customizing cryopreservation techniques and cryoprotective agents (CPAs) for specific cell types is crucial for cell source quality, sustainability, safety, and therapeutic intervention efficiency. As regenerative medicine progresses, it becomes imperative that the scientific community and industry provide a comprehensive, cell-specific landscape of available and effective cryopreservation techniques, preventing trial-and-error approaches and unlocking the full potential of cell-based therapies. Open-sharing data could lead to safer, more efficient cell therapies and treatments. Two decades of dermal progenitor cell use for burn wound treatment and Good Manufacturing Practice–compliant technology transfers have highlighted the need for further cryopreservation optimization in manufacturing workflows. In this paper, we present experimental data assessing 5 different cryopreservation formulae for long-term storage of clinical-grade FE002 primary progenitor fibroblasts, emphasizing the crucial difference between DMSO-based and DMSO-free CPAs. Our findings suggest that CryoOx, a DMSO-free CPA, is a promising alternative yielding cell viability similar to that of established commercial CPAs. This research highlights the importance of secure, robust, and efficient cryopreservation techniques in cell banking for maximizing quality, ensuring patient safety, and advancing regenerative medicine.

**Keywords:** Clinical safety, Cryopreservation, Good Manufacturing Practices, Long-term storage, Progenitor cells

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Cryopreservation technology is fundamental for cell-based therapy and tissue engineering to, among others, extend the Shelf-life of cells and tissues not only during storage but also during shipment. This is highly important for cell-based medicines and bioengineered tissues, given their high clinical need and temperature sensitivity, as well as advanced therapy medicinal products (ATMPs) (*eg*, for primary burn wounds, complex cutaneous wounds, etc) (Weng, 2021; Martín-López et al, 2023). Despite the large number of studies using various cryopreservation methods for human cells (Chen et al, 2023), especially primary cells (*eg*, FE002 primary progenitor dermal fibroblasts), there is still a lack of a systematic evaluation of available cryopreservation techniques and hence a need to further improve the assessment methodology of these methods (Laurent et al, 2020).

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*Abbreviations: API, active pharmaceutical ingredient; ATMP, advanced therapy medicinal product; CHUV, Centre Hospitalier Universitaire Vaudois; CPA, cryoprotective agent; DSW, donor site wound; EMA, European Medicines Agency; EOPCB, end-of-production cell bank; FBS, fetal bovine serum; FDA, Food and Drug Administration; FPC, fetal progenitor cell; GMP, Good Manufacturing Practices; MCB, Master Cell Bank; PBB, Progenitor Biological Bandage; RBC, red blood cell; TWA, Transwell Biotech; UTR, Unité de Thérapie Régénérative; WCB, Working Cell Bank*

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## SUMMARY POINTS

- Cryopreservation technology plays a fundamental role in supporting cell-based therapies and tissue engineering developments.
- Despite several studies on cryopreservation methods for human cells, particularly primary cells, there is a need for a systematic evaluation of reliable techniques.
- The lack of standardized cell cryopreservation protocols is a critical hurdle in regenerative medicine, which hampers the development and clinical implementation of cell-based therapies and forces clinical and scientific applications to rely on trial-and-error outcomes.
- Achieving standardized and transferable cryopreservation protocols under Good Manufacturing Practice standards demands open-sharing data among and between the scientific community and the industry.

Initial research in the field of cryopreservation dates to the late 1800s, when scientists focused on preserving spermatozoa (Polge et al, 1949) and red blood cells (RBCs) (Smith, 1950). In 1949, the accidental discovery of glycerol's cryoprotective properties on avian spermatozoa and later on RBCs played a crucial role in biomedical cryopreservation applications (Polge et al, 1949). These events also highlighted the need for CPAs, to avoid the mechanical and osmotic detrimental effects on cells when these undergo freezing and thawing cycles. First synthesized in 1867 (Wright and Winer, 1966), DMSO was proposed in 1959 as the first CPA that could replace glycerol and enhance cellular permeability. This was made possible once the most critical process during cryopreservation, namely the osmotic stress induced in RBCs by cryoinjury, was understood. Specifically, bovine RBCs are not permeable to glycerol; thus, the mechanisms of protection from cryoinjury of DMSO and glycerol are different (Lovelock and Bishop, 1959). Since then, DMSO has been widely investigated for numerous biomedical and biotechnological applications. However, most clinical trials relying on DMSO incorporation were halted by the Food and Drug Administration in 1965 because DMSO was perceived to be a new thalidomide case (Brobyn, 1975). Further research on DMSO revealed that its toxicity was related to the specific administered concentration (Verheijen et al, 2019).

Currently, DMSO is still widely used at relatively low concentrations for a wide spectrum of pharmacological effects, such as local analgesia, anti-inflammatory or diuretic action, vasodilation, weak bacteriostasis, and muscle relaxation (Jacob and Herschler, 1986). In cell culture applications, DMSO has been used as a cell proliferation inducer, free radical scavenger, and radioprotectant agent (Verheijen et al, 2019) but is most often utilized as the standard CPA during cell cryopreservation.

A current and widely applied methodology for long-term mammalian cell storage is based on slowly freezing cells from room temperature to −80 °C in the presence of 10% DMSO using controlled-rate freezing containers placed in a freezer, followed by transfer of the vial lot into liquid nitrogen (−196 °C) or liquid nitrogen in vapor phase (−150 °C) tanks for long-term storage. Exposing cells to subzero temperatures without any CPA addition is widely known to be fatal to processed biological materials. It should be emphasized that cells are exposed to various stresses during cryopreservation, and adding CPAs could cause osmotic shock to the cells. During the cooling phase, ice crystal formation occurs, which leads to freezing, solute concentration, and cellular dehydration. During the warming phase, ice recrystallization may occur owing to crystal growth, which causes mechanical disruption of the cell membrane, intracellular ice nucleation, and cell death (Murray and Gibson, 2022). Ultrafast vitrification is an alternative method to cryopreserve cells and tissues, but it is only limited to small samples and not applicable to cell banks for cell therapies (Heo et al, 2015).

To unlock and harness their vast therapeutic potential, recent research on cell-based and biological-sourced regenerative therapies requires the development and validation of DMSO-free cryoprotectants (Yamatoya et al, 2023). Indeed, various adverse effects, such as epigenetic changes in hepatic microtissues (Verheijen et al, 2019); tonic−clonic seizures and cardiac arrest (Maral et al, 2018); growth inhibition; and, occasionally, cell dysfunction (Yamatoya et al, 2023), have been associated with the use of DMSO. Another major bottleneck in the development and clinical implementation of cell-based regenerative medicine workflows is the lack of optimized and standardized cell cryopreservation protocols because the functional outcome is largely based on trial and error. To establish standardized and transferable protocols under Good Manufacturing Practice (GMP) standards, open-sharing API stability data and results are required from the scientific community and the industry.

In this paper, we present a retrospective functional evaluation of 5 CPAs by analyzing the stability attributes (*ie*, cell viability, time of cell recovery, and cell proliferation) of clinical-grade FE002 primary progenitor fibroblasts after long-term cryopreservation. Specifically, the strength of this study resides in the use of a standardized therapeutic primary cell source, which has been globally manufactured under GMPs and is used in multiple clinical trials for cutaneous repair promotion. Our findings highlighted distinct behaviors of the cell batches between DMSO-based and DMSO-free cryopreservation media (Table 1). Such data provided enhanced knowledge on the quality and stability attributes of the FE002 primary progenitor fibroblast cell source, which are crucial to assess for risk mitigation in topical cell therapy clinical applications.

## ESTABLISHMENT AND TESTING OF AN END-OF-PRODUCTION FE002-SK2 GMP CELL BANK

After the validation processes and GMP requirements of the GMP BioReliance facility, an end-of-production cell bank (EOPCB) of the FE002-SK2 cell type (Laurent et al, 2020) was generated from a skin progenitor cell bank.

After a consented organ donation after voluntary termination of pregnancy in 2009 and under written and informed

**Table 1. Overview of the Composition of Cryopreservation Medium, Cell Culture, and Freezing Conditions Used at Each Facility**

| Facility/Site | Initial Cell Seeding Density | Frequency of Medium Changes | Centrifugation Conditions | Cell Passage at Time of Freezing | Live-Cell Density at Time of Freezing | Freezing Medium | Freezing Period | Freezing Conditions | Cell Counting Method |
|---|---|---|---|---|---|---|---|---|---|
| **BioReliance, Glasgow, Scotland** | $2 \times 10^4$/ $cm^2$ ($3.5 \times 10^6$ cells/T175) EOPCB | 2 times per wk | 230 g ± 10 g 5 min | P4 | $1.0 \times 10^7$ cells/ml | DMEM + FBS + 10% DMSO | 9 mo | Liquid nitrogen in vapor phase | KPMP1021 Procedure for determining total and viable cells counts from BioReliance/ Merck |
| | $1.5 \times 10^3$ cells/$cm^2$ ($2.5–2.7 \times 10^5$/T175) EOPCB | | 230 g ± 10 g 5 min | P12 | $1.0 \times 10^7$ cells/ml | DMEM + FBS + 10% DMSO | 2 d | | |
| **TWA, Taipei, Taiwan** | $7.67 \times 10^3$ cells/$cm^2$ ($13.2 \times 10^6$/ HYPERFlask)[1] Tier-2 WCB | | 300 g 10 min | P6 | $1.0 \times 10^7$ cells/ml | DMEM + FBS + 5% DMSO | 2 y | Liquid nitrogen | Automatic cell analyzer |
| | $3–6 \times 10^3$ cells/$cm^2$ ($5.16–10.32 \times 10^6$/ HYPERFlask)[1] TWB-102 cells | | 300 g 10 min | P7 | $1.5 \times 10^7$ cells/ml | DMEM + Trehalose + HSA + Glycerol | 4 y | | |
| **GMP Platform, Lausanne, Switzerland** | $1.5 \pm 0.5 \times 10^3$ cells/ $cm^2$ (T75) WCB | | 230 g ± 10 g 5 min | P7 | $2.0 \times 10^6$ cells/ml | CryoSoFree (Sigma-Aldrich) | 7–70 d | Liquid nitrogen | Trypan Blue + Neubauer Hemocytometer |
| **UTR, Lausanne, Switzerland** | $1.5 \pm 0.5 \times 10^3$ cells/ $cm^2$ (T75) WCB | | 230 g ± 10 g 5 min | P6 | $2.0 \times 10^6$ cells/ml | DMEM + FBS + 10% DMSO | 2-3 y | Liquid nitrogen in vapor phase | Neubauer Hemocytometer |
| | $1.5 \pm 0.5 \times 10^3$ cells/ $cm^2$ (T75) WCB | | 230 g ± 10 g 5 min | P6 | $2.0 \times 10^6$ cells/ml | CryoSoFree (Sigma-Aldrich) | 2-3 y | | |
| **CryoOx, Oxford, England** | $4-6 \times 10^3$ cells/$cm^2$ (T175) | | 200g ± 10g 5 min | P6 | $1.1 \times 10^6$ cells/ml | CryoOx medium | 1 mo | Liquid nitrogen | Automatic Cell Counter, using Trypan Blue |

Abbreviations: FBS, fetal bovine serum; GMP, Good Manufacturing Practices; min, minute; P, passage; UTR, Unit of Regenerative Therapy.
[1]HYPERFlask: 10 layers of 172 $cm^2$.

consent, multiple tissues, including the skin, were isolated. This enabled the establishment of different allogeneic sources of primary fetal progenitor cells (FPCs), according to GMP practices and under the ethical commission number 62/07 (Laurent et al, 2022, 2020). Progenitor cells were obtained from fetal organ donations under a validated protocol, approved by the local State Ethics Committee (*ie*, Centre Hospitalier Universitaire Vaudois [CHUV], Ethics Committee Protocol number 62/07: "Development of fetal cell banks for tissue engineering," August 2007). All cell sources used for research were also anonymized, stored, and logged in a Departmental Biobank (Biobank DAL, Biobank of the Musculoskeletal Medicine Department of the Lausanne University Hospital, CHUV), complying with internal regulations and following an approved protocol (Vaud State Ethics Committee, reference CHUV [BB_029_DAL]). Parental cell banks were first established after mechanical cell isolation and subsequent serial adherent in vitro cultures. The FE002-SK2 cell source was then characterized and qualified in GMP settings (BioReliance, Merck Group, Glasgow, Scotland), and cells were cryopreserved in a freezing medium containing 10% DMSO. Industrial development with the establishment of multitiered FPC-cryopreserved progeny banks (*ie*, master and working cell banks [WCBs]) and product registration have then been undertaken by a licensee of the technology (*ie*, Transwell Biotechnology [TWA], Taipei, Taiwan). Regulatory approval for phase I and phase II clinical studies (www.clinicaltrials.gov, identifiers NCT02737748 and NCT03624023) was obtained in Taiwan (*ie*, Taiwan Food and Drug Administration). TWA sponsors these trials for managing donor site wounds (DSWs) and diabetic lower limb ulcers, respectively, using the active pharmaceutical ingredient (API) formulated in a proprietary hydrogel (*ie*, finished cell therapy product, TWB-103) (Laurent et al, 2020). In 2019, part of the same FE002-SK2 cells was donated by Lee Ann Applegate (Head of the Unit of Regenerative Therapy of the CHUV, Lausanne, Switzerland) to the CHUV Burn Center in the context of a clinical study (www.clinicaltrials.gov, identifier NCT05339490) required by the Swiss regulatory authorities (Swissmedic, Bern, Switzerland). Using the same source of FE002-SK2 cells, extensive multitiered FPC-cryopreserved progeny banks were produced in line with GMP requirements. After transferring the manufacturing technology to the Cell Production Centre, Progenitor Biological Bandages (PBBs) are produced upon request from plastic surgeons at the Burn Centre (CHUV, Lausanne, Switzerland) using the API (FE002-SK2, derived WCB). PBBs consist of FE002-SK2 progenitor cells seeded on an equine collagen matrix (Collagen resorb, RESORBA Medical GmbH, Nürnberg, Germany). These can be rapidly transported to the patient's bedside and applied as early covers for skin wounds, including deep or superficial second-degree burns or DSWs. PBBs, prepared for cutaneous wound coverage and repair promotion, have been manufactured and used by the Lausanne University Hospital Burn Center for the past 30 years (Abdel-Sayed et al, 2019; Al-Dourobi et al, 2021; Laurent et al, 2020). Historically, FE002-SK2 cells were cryopreserved in a medium containing 10% DMSO, but the composition was shifted to a commercial freezing medium, the CryoSoFree (Sigma-Aldrich, St. Louis, MO), in 2021 upon internal GMP platform decisions, as depicted in Table 2.

## CELL RECOVERY UNDER GMP-LIKE CONDITIONS

### BioReliance (Glasgow, Scotland [GMP conditions])

A vial of FE002-SK2 cells (passage 4), containing $1.0 \times 10^7$ live cells/ml, was stored in liquid nitrogen in vapor phase for 9 months. Cells were preserved in a freezing medium containing 10% DMSO (DMEM:fetal bovine serum [FBS]:DMSO; DMSO, catalog number D2438, Sigma-Aldrich), after which cells were rapidly thawed in sterile conditions and counted to measure cell viability. Then, cell expansion was initiated by seeding $3.5 \times 10^6$ live cells in a T-175 flask (Nunc). Cells (passage 5) reached full confluency after 4 days of culture, prompting detachment by trypsinization, cell counting, and subsequent reseeding into T-175 flasks ($3.45 \times 10^6$ live cells per flask). This process was repeated until passage 11 was reached. Cells harvested at passage 11 were counted and distributed into 30 T-175 flasks, at a seeding density of $3.00 \times 10^5$ viable cells per flask. After 4 days of culture, cells at passage 12 were detached, pooled, and counted to be conditioned into 70 cryovials, each containing $1.0 \times 10^7$ viable cells/ml in 10% DMSO-based cryopreservation medium, as previously described. This set of 70 cryovials constituted the EOPCB, slated for further analysis at BioReliance. After 2 days of storage after cryopreservation, 3 vials (*ie*, numbers 4, 37, and 67) were removed from liquid nitrogen, rapidly thawed, and counted to validate the cell viability rate.

Cells preserved after the BioReliance protocol, which comprises DMSO, exhibited a morphologically healthy phenotype upon initiation, displaying an elongated fibroblast shape, which was consistent with defined specifications across successive passages. Our findings show that cell recovery remained high and stable (ranging 98.2−100%) throughout passages 4−12 (Figure 1a). Three vials (numbers 4, 37, and 67) from an EOPCB (passage 12) stored in liquid nitrogen in vapor phase for 2 days after manufacture were used to assess cell recovery and growth (Figure 1b). At the cell culture initiation, the 3 vials randomly selected from the EOPCB showed comparable recovery rates of around 98% upon thawing (Figure 1b). Furthermore, these cells maintained their proliferative capacity when expanded until passage 13 at day 8 after seeding (Figure 1c). The material from EOPCB vial 4 was lost in culture owing to cell contamination.

### TWA (Taipei, Taiwan [GMP conditions])

After the industrial scale-up and transposition of the original FE002-SK2 banking workflow to GMP standards, as carried out in collaboration with BioReliance, part of the FE002-SK2 cell bank was transferred to TWA in Taipei, Taiwan (referred to as TWA in the remaining parts of this paper) (Laurent et al, 2020). From the same original stock of biological materials (FE002-SK2 parental cell bank), several cell banks were created. Briefly, the original FE002-SK2 cells (passage 4) obtained from BioReliance were cultured and then cryopreserved at passage 6 for small batch production using DMEM/PhenolRed + FBS + DMSO as freezing medium. From these materials, TWA produced API batches at

**Table 2. Summary of Assessed Strategies for Long-Term Cell Storage in GMP Conditions**

| Long-Term Cryopreservation Strategy | Site | Cell p | Cryopreservation Period | Type of CPA | Freezing Medium | Live Cell Density at Time of Freezing |
|---|---|---|---|---|---|---|
| **BioReliance** | Glasgow, Scotland | P4 | 9 mo | CPA with DMSO | DMEM + FBS + 10% DMSO | $1.0 \times 10^7$ cells/ml |
| | | P12 | 2 d | | | |
| **UTR** | Lausanne, Switzerland | P6 | 2-3 y | CPA with DMSO | CryoSoFree (Sigma-Aldrich) | $2.0 \times 10^6$ cells/ml |
| **TWA** | Taipei, Taiwan | P7 | 4 y | DMSO-free CPA | DMEM + Phenol Red + Glutamine + Trehalose + Human serum albumin + Glycerol | $1.5 \times 10^7$ cells/ml |
| **GMP Platform** | Lausanne, Switzerland | P7 | 7–70 d | DMSO-free CPA | CryoSoFree (Sigma-Aldrich) | $2.0 \times 10^6$ cells/ml |
| **CryoOx** | Oxford, England | P6 | 1 mo | DMSO-free CPA | CryoOx medium | $1.1 \times 10^6$ cells/ml |

Abbreviations: CPA, cryoprotective agent; FBS, fetal bovine serum; GMP, Good Manufacturing Practices; p, passage; TWA, Transwell Biotech; UTR, Unit of Regenerative Therapy.

passage 7, to be included in the finished therapeutic product, which is currently used in clinical trials. The final API frozen did not contain DMSO. After a 4-year cryopreservation period in liquid nitrogen tanks, vials from 4 different batches of the API were removed throughout the storage period and for up to 56 months. Each vial was thawed by placing it inside a 37 °C water bath, and then the cells were diluted, stained, and then counted using an automatic cell counter.

The cell viability and stability data, gathered throughout a period of 56 months of storage in liquid nitrogen tanks, showcased the stability of 4 batches of API (Figure 2). At the time of vial thawing, cell viability ranged from 81 to 93.4%. It is relevant to highlight the long-term scope of API cryopreservation addressed in this study: 2 batches were stored for 4 years, whereas the remaining 2 batches were stored for periods exceeding 2 and 3 years, respectively. These results validated the stability of the API when cryopreserved without DMSO.

**GMP Platform (Lausanne, Switzerland [GMP conditions])**

Several vials of the FE002-SK2 cell source were donated by Prof. Lee Ann Applegate to the Burn Center at the CHUV in Lausanne to be able to continue clinical application of PBBs for severe burns and to conduct clinical trials. In parallel, manufacturing protocols were transposed to the GMP Platform according to standard operating procedures for future clinical trials.

From the original FE002-SK2 cell source, the FE002-SK2 Master Cell Bank (MCB) and a derived WCB were produced in 2021 by the GMP Platform. Microscopic assessment and cell confluency were monitored throughout the entire GMP cell bank production process. At the final stage of the WCB production and once FE002-SK2 cultures attained optimal banking confluency (*ie*, >95 %, generally reached after 12 ± 3 days), the cells were harvested after detachment by trypsinization, counted, and resuspended in the CryoSoFree DMSO-free Cryopreservation Medium (Sigma-Aldrich). The conditioned cryovials of FE002-SK2 cells contained 2.0 ± 0.2 × $10^6$ cells (passage 7), corresponding to the WCB. These vials were cooled and frozen overnight in a controlled-rate freezing container placed in a freezer at −80 °C, before being transferred to a liquid nitrogen in vapor phase. These cells were defined in their cryopreserved form as the FE002-SK2 API, which should be further used for the production of PBBs in a clinical trial designed for the treatment of standardized wounds. The conditioned materials from the production batch were stored in liquid nitrogen in vapor phase until required for clinical use. To gain insights into cell recovery potential after a cryopreservation period of 7–70 days, the cell viability at the time of thawing of 41 vials was monitored. Total and viable cell counts were determined using Trypan blue exclusion dye.

Our results (Figure 3) showed that over 50% (*ie*, 27 of 41 vials) of the thawed FE002-SK2 vials from WCB maintained in CryoSoFree exhibited cell recovery <80% after cryopreservation. This outcome suggests that the cryopreservation and/or storage conditions led to suboptimal poststorage cell recovery. These findings showed the lack of homogeneity in the subsequent production of a standardized cell therapy product

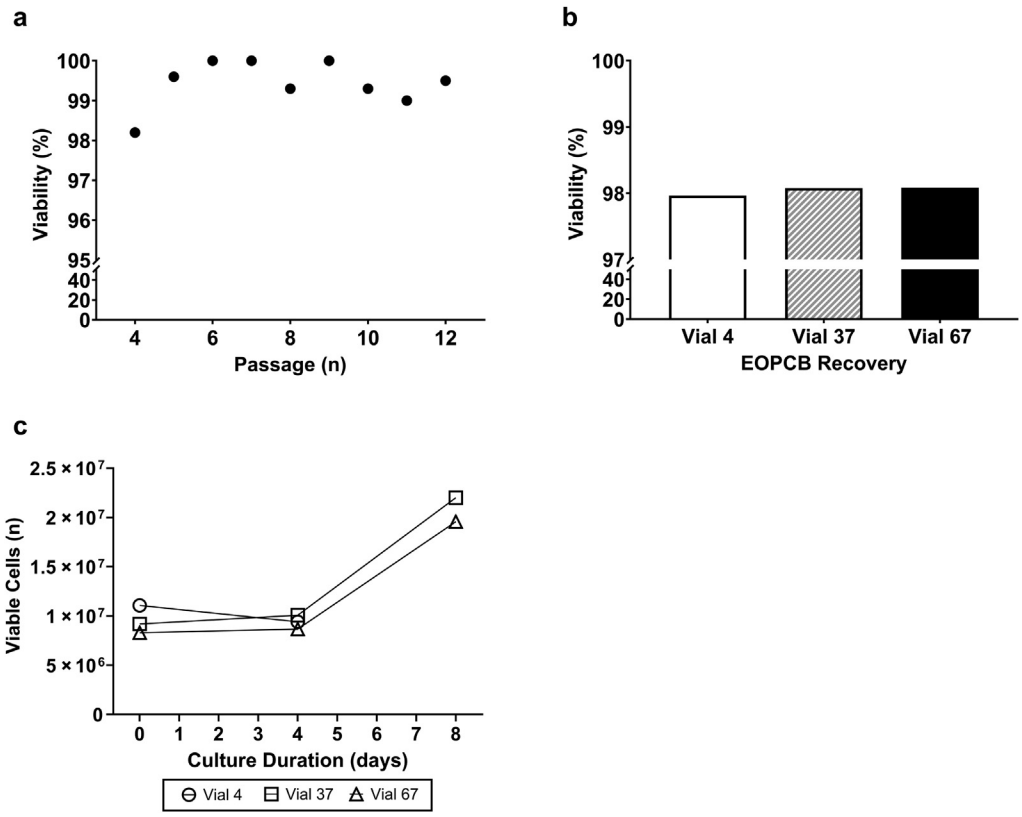


**Figure 1. Postcryopreservation data of FE002-SK2 cells cryopreserved with the BioReliance protocol.** Assessment of (**a**) cell recovery and growth after several passaging iterations; (**b**) EOPCB (p12) viability after thawing of 3 vials, taking into consideration the total population frozen; and (**c**) recovery and viable cell number up to 8 days of culture after cryopreservation (n = 3), representing cellular behavior after thaw. Viability was calculated as the ratio of the total number of cells in each vial to the initially frozen cell number. EOPCB, end-of-production cell bank; p12, passage 12.

intended for clinical use, for instance, standardized wound treatment for burn patients. As such, a cryopreservation strategy solely relying on CryoSoFree is assessed as being suboptimal because FE002-SK2 cells would be later used to produce PBBs in a clinical trial designed for the treatment of standardized wounds.

**Unit of Regenerative Therapy (Lausanne, Switzerland [GMP-like conditions])**

To compare the impact of cryopreservation of skin progenitor cells (FE002-SK2) using CPAs with or without DMSO, a batch of cells at passage 6 (MCB) was transferred from the Cell Production Centre to the Regenerative

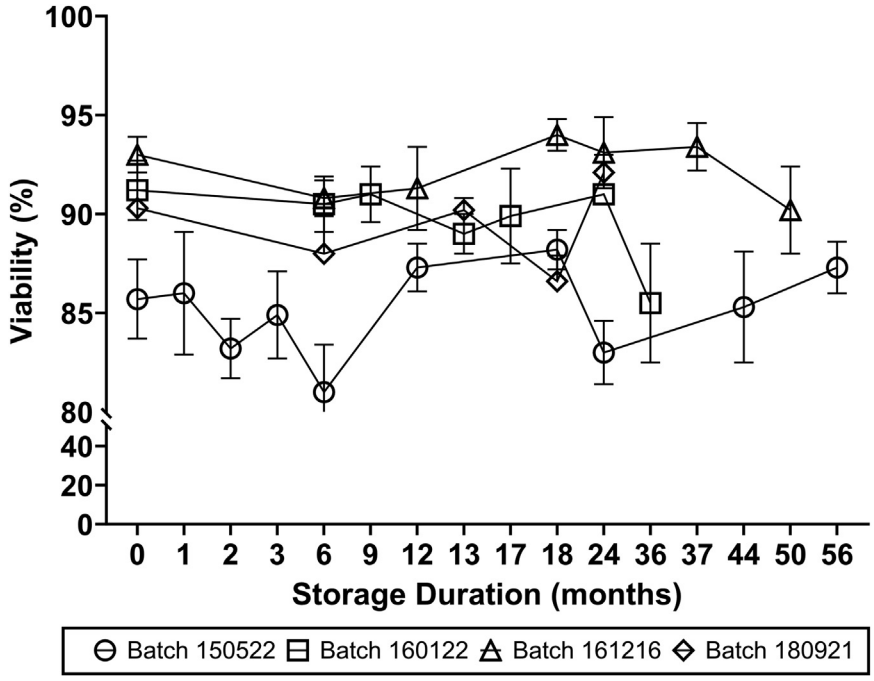


**Figure 2. Average cell viability throughout a 56-month cryostorage period of 4 independent batches of API manufactured by TWA.** Error bars indicate SD (n = 3–6). API, active pharmaceutical ingredient; TWA, Transwell Biotech.

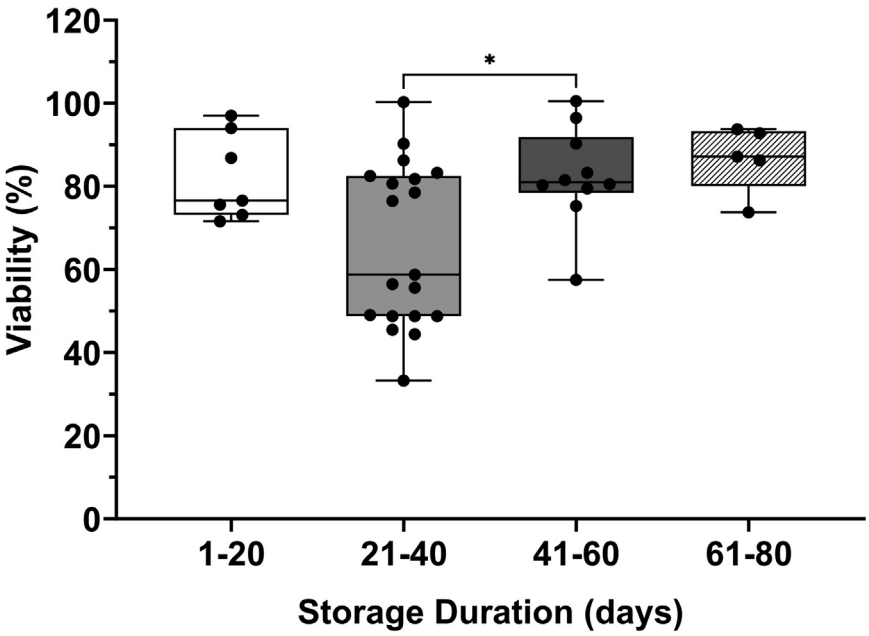


**Figure 3. Cell recovery (%) after cryopreservation of FE002-SK2 cells in CryoSoFree, by storage duration (days).** Data are grouped by number of days during which the samples were cryostored as whisker plots highlighting the average (middle line) and minimum and maximum (error bars) cell viability. All data points (n = 41) are also plotted. Only statistically significant differences are shown (*) (1-way ANOVA/Tukey honest significant difference, $P < .05$).

Therapy Unit (Unité de Thérapie Régénérative [UTR]) during the API manufacturing process. Cells were cryopreserved by UTR by dividing cells into several cryovials, each with $2 \times 10^6$ FE002-SK2 cells (passage 6) frozen in CryoSoFree medium (6 vials) or in DMEM−FBS−10% DMSO medium (6 vials). After 2 and 3 years of cryopreservation, the cryovials were quickly thawed following GMP standards, and cell viability was assessed by counting cells in a Neubauer Hemocytometer.

After thawing and counting, the individual contents of the cryovials of FE002-SK2 cells were centrifuged (1200 r.p.m., 10 minutes) to remove all traces of the freezing medium. Each thawed vial was split into 3 T-75 flasks and seeded at a density of $1.5 \times 10^3$ cells/cm$^2$. Cells were then cultured in a humidified incubator (37 °C, 5% carbon dioxide) until confluency (>95%) was reached. The medium was exchanged 1−2 times per week. After complete cell detachment, cells were resuspended in a complete medium and counted. Cell growth of each cryovial was obtained.

Our results (Figure 4b) showed that FE002-SK2 cells, preserved for 2 and 3 years in DMEM + FBS + 10% DMSO, achieved near-complete recovery after thawing, with 93% (n = 4) of measured viability. In contrast, the FE002-SK2 cells maintained in the CryoSoFree-freezing medium for the same periods displayed a significant decline in the recovery of viable cells after thawing with a mean of 61% of viability. These data confirmed that DMSO is more suitable for the extended cryopreservation of FE002-SK2 primary progenitor fibroblasts than CryoSoFree. Figure 4b outlines cell recovery over a culture period of 11−18 days for cells cryopreserved in 10% DMSO or CryoSoFree for 3 years. These results show that FE002-SK2 cells maintained for 3 years in 10% DMSO are able to grow more rapidly than cells maintained in CryoSoFree, with a percentage of growth increase at day 18 more than double between both cryopreservation conditions.

**CryoOx (Oxford, England [GMP-like conditions])**

Samples from the original stock of FE002-SK2 primary progenitor cells (passage 4) were cultured following GMP protocols. Briefly, each cell vial was thawed in sterile conditions and centrifuged (1200 r.p.m., 10 minutes) before cell counting. Cells were then expanded using an initial seeding density of $1.0 \times 10^6$ live cells per flask (T-75 flask, Corning). Once full confluency was reached, cells were passaged onto 6-well plates at a density of $1.0 \times 10^6$ live cells per well and cultured until 85% confluency was reached (*i.e.,* usually achieved in 1 day). Then, cells were treated with CryoOx medium for 1 day before being washed with DMEM, harvested with TrypLE (Gibco), centrifuged (1200 r.p.m., 5 minutes), and counted. Cells were suspended in a DMSO-free cryopreservation medium, CryoOx, at a density of $7.2 \times 10^5$ viable cells/ml and stored in cryovials. These vials were cooled and frozen overnight in a controlled-rate freezing container placed in a freezer at −80 °C, before being transferred to a liquid nitrogen tank in which they were stored for 1 month. After this cryopreservation period, cells were thawed, diluted, stained, and counted (Figure 5).

## CELL VIABILITY AFTER CRYOPRESERVATION UNDER DIFFERENT CPAs

Data on cell recovery after cryopreservation from BioReliance, TWA, University Hospital GMP Platform, and UTR are included in Figure 6, alongside the DMSO-free cryopreservation media CryoOx. Our results suggest that FE002-SK2 cells exhibit a superior recovery performance when cryopreserved in a standard freezing medium containing DMSO (*i.e.,* 98 and 93% of cell viability after thawing for BioReliance and UTR groups, respectively), particularly in contrast with alternatives such as CryoSoFree (*i.e.,* 73.9% (± 17.5) and 92.7% (± 4.6) of cell viability after thawing for GMP Platform of the University Hospital and UTR group, respectively). Although DMSO-based CPAs resulted in significantly higher cell survival rates than the DMSO-free

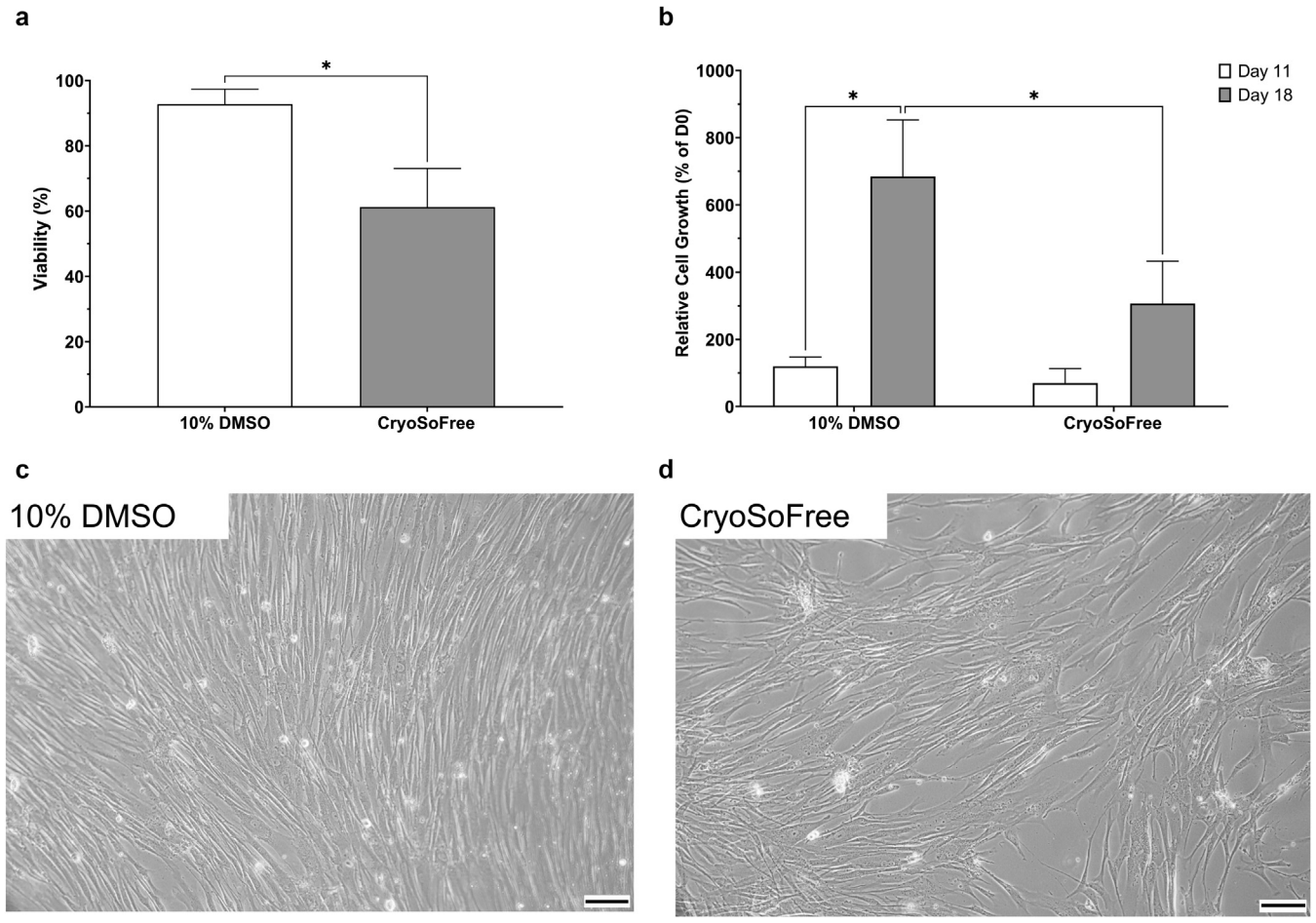


**Figure 4. Postcryopreservation data of FE002-SK2 cells stored in 1 of 2 cryoprotective agents, with DMSO (DMEM–FBS–10% DMSO) or without DMSO (CryoSoFree).** (**a**) Cell viability evaluation after 2 or 3 years of cryopreservation (n = 4 for each condition) and (**b**) cell recovery after 11 and 18 days of cell culture as relative cell growth against the respective values at day 0. (**c, d**) Photographs of cells in culture were taken after cryopreservation at day 18 after thawing of cells cryopreserved with either (**c**) 10% DMSO or (**d**) CryoSoFree (*t*-test, $P < .05$). Only statistically significant differences are shown. Scale bar = 100 μm.

CPA CryoSoFree, the high variability in CryoSoFree's cryopreservation capacity results in no statistically significant difference. Therefore, cryopreservation with such heterogeneity should be avoided.

There was indeed the necessity to create the WCB with DMSO for the TWA group, and all cell banking was done with DMSO (Figure 6). However, the API was then developed without DMSO, and the stability of these preparations is shown (Figure 6).

However, CryoOx, a DMSO-free cryoprotectant developed by the Tissue Engineering Group at the University of Oxford, shows results comparable to those of DMSO-containing CPA counterparts. This is of considerable significance and promise, given the importance of utilizing DMSO-free cryopreservation media, particularly for clinical use (*i.e.,* such as treating burn patient wounds), in an effort to minimize the risk of toxicity associated with DMSO.

## STATISTICAL ANALYSIS

Where relevant, data were evaluated using 1-way ANOVA with Tukey's honest significant difference as a posthoc test, where $P < .05$ was deemed to be statistically significant. Data analysis and graphing were performed using GraphPad Prism (version 10.1.2, Dotmatics, Boston, MA). Unless otherwise specified, data are presented as mean values with SDs. The graphical abstract was created using BioRender.

## THE NEED FOR UPDATED GMP CRYOPRESERVATION PROTOCOLS

Establishing and updating standardized protocols, particularly under GMP standards, requires a transparent exchange of data and results from the development and implementation of different cryopreservation strategies within the scientific community (Bormann et al, 2023). By collaboratively tackling cryopreservation challenges, we can collectively enhance our understanding of best practices in our laboratories and in clinical use to drive continuous improvement, which is crucial to ensure preclinical and clinical efficacy, reliability, reproducibility, and patient safety, which remain a paramount concern.

In the past 15 years, there has been a growing interest in Europe and in the United States in establishing clearer regulations for the utilization of innovative cell therapies for the treatment of patients with various medical conditions. In response to this need, Europe introduced specific regulations (*i.e.,* regulation [EC] number 2007/1394) through the

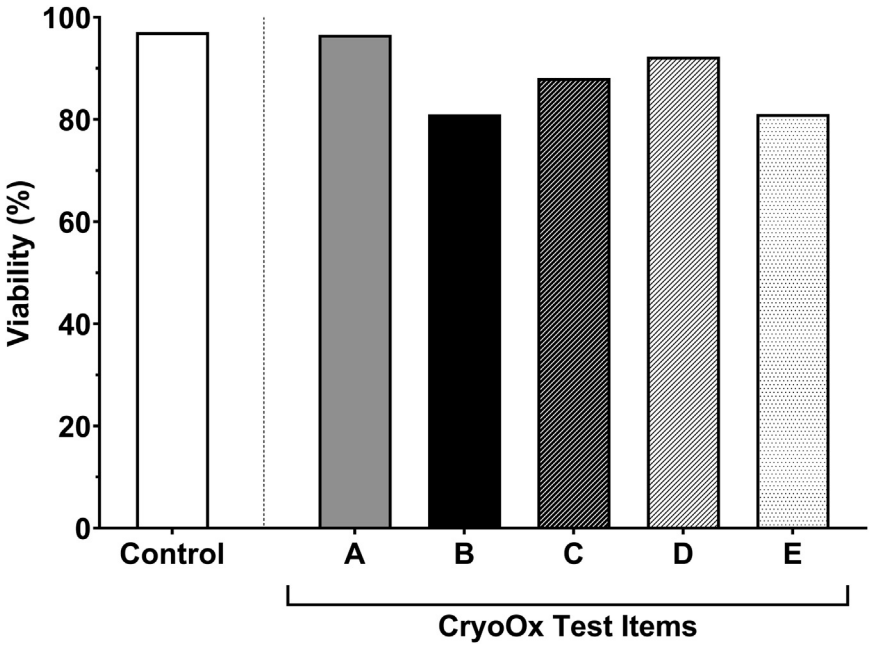


**Figure 5. Postcryopreservation data of FE002-SK2 cells stored in a DMSO-free cryoprotectant agent (CryoOx) or DMEM–FBS–10% DMSO (control) showing cell viability after 1 month of cryopreservation.**

European Medicines Agency (EMA) for ATMPs. These regulations pertain to ATMPs intended for commercial distribution and those prepared or manufactured using industrial processes (Iglesias-Lopez et al, 2020).

To enable patients in Switzerland to access and benefit from advanced and high-quality cell therapies, the country is undertaking the task of integrating European regulations into its own legal framework. This initiative aims to ensure the utilization of products with a comparable level of safety and quality as well as to guarantee compatibility between the Swiss and European markets. As part of this effort, Switzerland is currently in the process of revising the Therapeutic Products Act to explicitly include the concept of ATMPs, previously referred to as Standardized Transplants, for more precise regulation in the innovation-driven Swiss context.

It is easy to envision the numerous barriers between the approval of a therapy and its application in the clinic, including compliance with legal requirements and adherence to GMP manufacturing standards. Currently, over 500 studies involving ATMPs have been conducted under EMA regulations. However, the products and protocols used in only 23 of these studies have received marketing authorizations, accounting for <5% of the total (Hennessy et al, 2023).

The manufacturing of cell therapy products must adhere to numerous GMP requirements, encompassing the selection of cell type and origin, procedures for cell expansion, processes for cell preservation, transportation of the cell therapy product

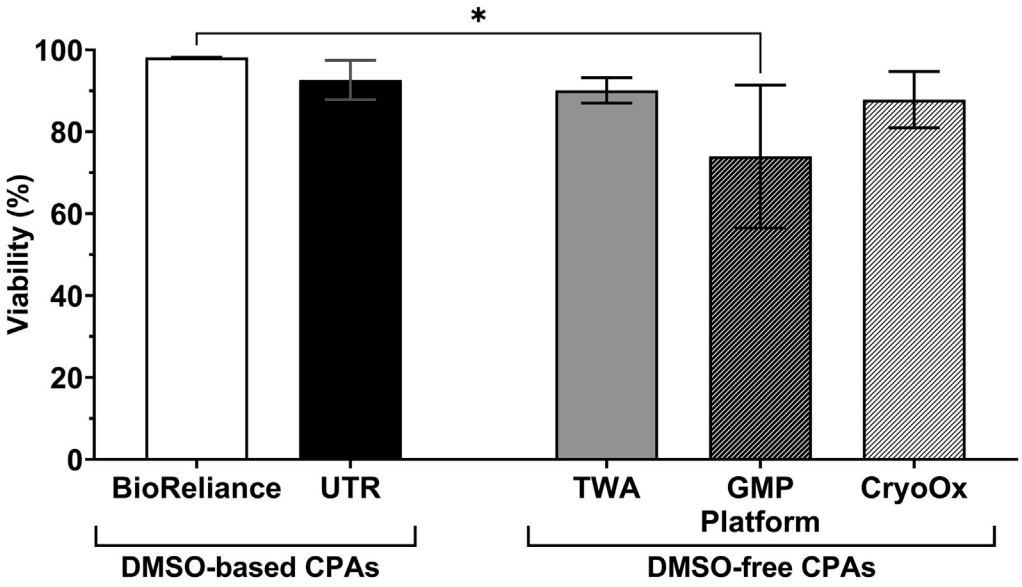


**Figure 6. Postcryopreservation cell viability of FE002-SK2 primary progenitor cells, cryopreserved using 5 different cryopreservation agents.** The associated cell viability of 5 different cryopreservation strategies is presented here: BioReliance (n = 4), Taiwan (TWA) (n = 4), UTR (n = 4), and 2 DMSO-free alternatives CryoSoFree (n = 45) and CryoOx (n = 5). Only statistically significant (*) differences are shown. There are significant differences in cell viability after thawing (%) between the CryoSoFree group and each of the other 4 CPAs (BioReliance, TWA, UTR, and CryoOx), which do not display significant differences between them (1-way ANOVA/Tukey honest significant difference, $P < .05$). CPA, cryoprotective agent; TWA, Transwell Biotech; UTR, Unit of Regenerative Therapy.

to the patient, and the product's stability attributes. Cryopreservation is a critical step in the manufacturing process of the API comprised in a cell therapy product. The specific processing workflow must provide secure and stable storage of the APIs at temperatures below −130 °C to prevent adverse metabolic changes and functional losses (Meneghel et al, 2020). The requirements for achieving optimized cryopreservation, ensuring a high level of cell recovery after thawing (*i.e.,* especially for clinical-grade material lots), should be considered at an early stage in the development of any specific cell therapy (Meneghel et al, 2020).

Any subsequent modification to the manufacturing processes that could impact the product's quality (*i.e.,* stability) and the reproducibility of the process must be validated according to GMP requirements outlined in EudraLex (volume 4, GMP guidelines [European Commission, 2023]) and International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use Q5E and Q10 guidelines in Europe (European Medicines Agency, 2014) as well as Food and Drug Administration Regulation 21 CFR Part 211 in the United States (U.S. States Food and Drug Administration, 2023). Risk management procedures should be employed to assess planned changes in any given critical manufacturing process phase and anticipate their effects on product quality, thereby avoiding unintended consequences.

## BRIDGING THE GAP BETWEEN REGULATORY REQUIREMENTS AND COMMON PRACTICES: CHALLENGES FOR IMPLEMENTATION

The mismatch between the reference texts mentioned earlier and real-world practices in API cryopreservation is being increasingly criticized by the scientific community because this is a clearly underaddressed aspect in translational medicine. Importantly, the efficacy of a CPA is hard to comprehensively assess because different cells (*i.e.,* primary cells or cell lines) survive cryopreservation differently. Specifically, the described discrepancy lies in the contrasting methods used to report cryoprotective outcomes (*i.e.,* functional assessment of the CPA by analysis of the stability attributes of the APIs). Typically, post-thaw cell viability, which refers to the ratio of live to total cells evaluated immediately after thawing, is the most frequently reported method. In this study, the live cell indicator is merely an assessment of whether the cell membrane remains intact (*i.e.,* Trypan blue staining). On the other hand, the total number of cells recovered, represented by the ratio of the live cells cultured to grow to initial thawed cells, is less often reported (Murray and Gibson, 2020). Notably, the former method tends to yield higher values than the latter, which is clearly not enough to assess the efficacy of a CPA.

Many other DMSO-free CPAs have been comprehensively reviewed by Awan et al (2020), although most of these have not been disclosed. Currently, commercially available DMSO-free CPAs include Bambanker DMSO-free (Nippon Genetics), Biofreeze (Biochrom GmbH), CryoSoFree (Sigma-Aldrich), CryoNovo P24 & X12 (Akron Biotech), FreezeStem (BioLamina), Ibidi Freezing Medium DMSO-free (Ibidi), pZerve (Sigma-Aldrich), Repro Cryo RM (ReproCell), Stem-CellBanker (Zenoaq Resource), and StemCell Keep (Funakoshi). However, the quality and ability of these products to maintain cell functions during cryopreservation remain underinvestigated, which is a critical step before clinical application (Arantes et al, 2021; Ekpo et al, 2022; Ueno et al, 2021).

Another important aspect to consider is manual cryocontainer filling, which is feasible for small API lot sizes but may not be applicable at industrial scales. Therein, evaluating lag times in the manufacturing workflow is assessed as being critical for larger vial numbers because these may potentially compromise the API's viability and functionality. In addition, manual filling poses a risk of variability between lot units, leading to the potential for inconsistent therapeutic effects if the lot is released (Meneghel et al, 2020). Generally, publications rarely provide details on cell cryopreservation methods, yet this information is critical from a quality standpoint. Despite often being present in low concentrations, most of the clinical work still relies on DMSO-based cryoprotectants. Moreover, the freezing temperature profile, largely dependent on the controlled rate freezing containers, could vary from one operator to another, leading to poor reproducibility. Overall, cryopreservation processes in the field of cell-based regenerative medicine urgently require further optimization and validation to maintain cell viability and functionality, particularly for clinical applications.

## CONCLUSION

Cryopreservation technologies are pivotal for the development and wide-range implementation of cell therapies, tissue engineering, regenerative medicine, and other bioengineering applications. Despite the significant amounts of data generated on cell cryopreservation, many details are often not described in the scientific literature, rendering it impossible to systematically evaluate existing cryopreservation techniques. To address this issue and establish universally applicable protocols adhering to GMP standards, it is crucial for the scientific community and industry to openly share data and results regarding the stability of APIs.

In this study, we present experimental data assessing 5 different cryopreservation formulae for long-term storage of clinical-grade FE002 primary progenitor fibroblasts. The focus is placed on emphasizing the crucial difference between DMSO-based and DMSO-free CPAs. Our findings suggest that CryoOx, a DMSO-free CPA, is a promising alternative yielding cell viability similar to that of established commercial CPAs. This research emphasizes the importance of secure, robust, and efficient cryopreservation techniques in cell banking to maximize quality, ensure patient safety, and advance regenerative medicine.

In the future, prospective, better-controlled studies looking at the differences in cell viability and other measures of cell health, stability, and functionality with different DMSO-containing and DMSO-free cryoprotectants would be helpful.

## DATA AVAILABILITY STATEMENT

All data associated with this study are presented in this manuscript. Datasets related to this article can be found at https://github.com/carla-fuenteslopez/Long-Term-Cryopreservation-Strategies, hosted at the GitHub repository Long-Term-Cryopreservation-Strategies.

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## CONFLICT OF INTEREST

LAA has developed the clinical cell banks in BioReliance with funding from her spin-off company and is the owner of the FE002-SK2 cell sources. AL has been working on an industrial thesis with the University of Lausanne during this work. The remaining authors state no conflict of interest.

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Conceptualization: MF, AL, CVF, HY, ZC, CS, NH-B, LAA, VP; Data Curation: MF, AL, CVF, CS, NH-B, LAA, VP; Formal Analysis: MF, AL, CVF, NH-B, LAA, VP; Funding Acquisition: CY, ZC, LAA, VP; Investigation: MF, AL, CVF, CS, NH-B, LAA, VP; Methodology: MF, AL, NH-B, LAA, VP; Project Administration: LAA, VP; Resources: AL, ZC, LAA, VP; Supervision: AL, CY, NH-B, LAA, VP; Visualization: MF, CVF, VP; Validation: MF, AL, ZC, NH-B, LAA, VP; Writing − Original Draft Preparation: MF, CVF, VP; Writing − Review and Editing: MF, AL, CVF, HY, ZC, CS, NH-B, LAA, VP.

## SUPPLEMENTARY MATERIAL

Supplementary material is linked to this paper. Teaching slides are available as supplementary material.

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