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Strategies to Enhance Stability of Cryopreservation Processes for Cell-Based Products

Yuki Uno [a,b], Yusuke Hayashi [c], Hirokazu Sugiyama [c], Jun Okuda [a,d], Tetsuji Nakamura [d], Masahiro Kino-oka [a,b,d,*]

[a] Department of Biotechnology, Graduate School of Engineering, The University of Osaka, 2-1 Yamadaoka, Suita-shi, Osaka 565-0871, Japan
[b] Research Base for Cell Manufacturability, Graduate School of Engineering, The University of Osaka, 2-1 Yamadaoka, Suita-shi, Osaka 565-0871, Japan
[c] Department of Chemical System Engineering, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
[d] Joint Research Laboratory (Iwatani) for Cell Storage & Transport Technology, Graduate School of Engineering, The University of Osaka, 2-1 Yamadaoka, Suita-shi, Osaka 565-0871, Japan

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ABSTRACT

The projected expansion of the global market for cell manufacturing, which contributes to regenerative medicine and cell therapies, warrants the designing and development of scalable cryopreservation processes for cell-based products (CBPs) for use in both standard and personalized therapies. However, the change in scale causes variations in process parameters, which affects the stability of the CBP quality. Therefore, the cryopreservation process for CBPs needs to be designed based on the concept of cell manufacturability and consideration of both engineering and biological aspects. In this review, we discussed strategies to enhance the quality stability of CBPs during cryopreservation, focusing primarily on four key processes: dispensing, freezing, storage, and thawing. Additionally, we discussed the application of simulation technologies because they aid in constructing digital twins for the designing and development of the cryopreservation process and facilitate efficiency with limited time and resources.

1. Introduction

The global market for cell manufacturing, which contributes to regenerative medicine and cell therapy, has shown a notable 25.5 % compound annual growth rate between 2022 and 2025. This growth is projected to reach $558 million by 2027 (Bahari et al., 2023). This rapid increase in global demand warrants the urgent establishment of a supply chain that is capable of providing a stable supply of cell-based products (CBPs) with stable quality. CBPs encompass not only isolated cells but also three-dimensional structures such as cell aggregates and organoids. Within the cell manufacturing process, these entities may serve as starting materials, intermediates, or final products, depending on the application.

CBPs are classified into two categories based on whether they are derived from autologous or allogeneic cells (Li et al., 2021), and the scale and cost of manufacturing the CBPs and complexity of the development pathway differ depending on the category (Mason and Dunnill, 2009). Bahari et al. have predicted that the use of autologous cells would decrease from 56 % to 35 % by 2029 and that allogeneic cells would account for the majority of the global market (Bahari et al., 2023). They have made this prediction in market decline based on the high manufacturing cost per patient for autologous CBPs. In contrast, allogeneic CBPs incur lower manufacturing costs than that of autologous CBPs because they may be manufactured commercially on a large scale; however, allogeneic CBPs carry the risk of rejection after administration (Hui and Yamanaka, 2024; Kawamoto and Masuda, 2024). Based on the current situation and projections, we believe that scale-up technologies would become increasingly important for manufacturing large

Abbreviations: ACD, active cooling device; AFP, antifreeze protein; BIB, box-in-box; CAPD, computer-aided process design; CBP, cell-based product; CFD, computational fluid dynamics; CPA, cryoprotective agents; DMSO, dimethyl sulfoxide; ELS, alginate-encapsulated liver cells; IBP, ice-binding protein; INP, ice-nucleating protein; iPSCs, induced pluripotent stem cells; LN_2, liquid nitrogen; MD, molecular dynamics; MSC, mesenchymal stromal/stem cells; PBMC, peripheral blood mononuclear cells; PCD, passive cooling device.

* Corresponding author at: Department of Biotechnology, Graduate School of Engineering, The University of Osaka, 2-1 Yamadaoka, Suita-shi, Osaka 565-0871, Japan.
E-mail address: kino-oka@bio.eng.osaka-u.ac.jp (M. Kino-oka).

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quantities of stable quality CBPs for standard therapies. However, scale-out technologies are equally important for personalized therapies to enable the manufacturing of tailor-made CBPs to meet the unique requirements of each patient (Heathman et al., 2015).

The manufacturing process of CBPs can be broadly divided into three streams: upstream, downstream, and outstream (Kino-oka et al., 2019). The processes in upstream refer to a set of operations aimed at increasing the number of target cells, including media preparation, cell expansion, and differentiation. In contrast, the processes in downstream refer to a set of operations aimed at preparing the product in a form suitable for storage or transport, including separation, purification, dispensing, freezing, and packaging. Additionally, the processes in outstream refer to operations not included in upstream or downstream, such as the transportation of cells from cell banks or hospitals to cell processing facilities, or from processing facilities to hospitals, as well as in-hospital preparations (e.g., thawing and washing). Designing a scalable manufacturing process requires coordinated development of the processes in upstream, downstream, and outstream, with consideration of their interconnectivity.

Upstream processes include various scales of culturing technologies ranging from single-layer flasks to bioreactors, and these are steadily advancing toward technical maturity (Jankovic et al., 2023; Lee et al., 2022). Particularly, culturing technologies for mesenchymal stromal/stem cells (MSCs) and induced pluripotent stem cells (iPSCs), which have been validated through clinical trials (Kirkeby et al., 2025; Thanaskody et al., 2022), will be crucial. Scalable culture technologies for these cells have been developed using approaches such as culturing iPSCs in aggregates (Yamamoto et al., 2024; Yamamoto and Kino-oka, 2021; Yehya et al., 2024) and MSCs on microcarriers (Rogers et al., 2021; Zhang et al., 2022) using bioreactor systems. Furthermore, the technological maturation of upstream processes has made the development of scalable downstream processes a key challenge (Hassan et al., 2015) owing to several hurdles in achieving this goal (Pigeau et al., 2018).

The downstream processes involved in CBP manufacturing typically include cryopreservation to enable long-term preservation (Clarke and Smith, 2019). Cryopreservation is performed by obtaining a cell suspension in the upstream process and replacing it into a cryopreservation solution from culture medium, which is dispensed into containers such as cryovials or cryobags, followed by freezing and storage. Generally, CBP quality decreases during this series of processes. Thus, to establish scalable cryopreservation processes, the factors causing CBP quality degradation during each step need to be identified and controlled appropriately or technologies need to be developed to suppress their impact.

In the development of supply chains for CBPs, scaling technologies for cryopreservation play a critical role. These technologies facilitate the establishment of cell banks by enabling the long-term preservation of large quantities of raw materials with consistent quality, thereby allowing flexible responses to fluctuations in therapeutic demand. Moreover, this preservation approach helps to overcome the spatio-temporal constraints inherent in supply chains. By significantly extending the time window between manufacturing and administration, it enables greater flexibility in treatment scheduling (Tyagarajan et al., 2019). In addition, temperature-controlled long-distance transport between facilities allows for the flexible design of both centralized and decentralized supply chain models (Chang et al., 2025; Shah et al., 2023). Taken together, the advancement of cryopreservation and its scaling technologies represent a fundamental element in the strategic design of supply chains for CBPs.

This review summarizes the basic concepts of cell preservation and discusses strategies to enhance the stability of quality of CBPs across each step of the cryopreservation process, including dispensing, freezing, storage, and thawing. In addition, we explore the potential applications of simulation technologies throughout the cryopreservation process. It should be noted that the primary focus of this review is on CBPs composed predominantly of isolated cells.

2. Basic concepts of cell preservation from physical and biochemical perspectives

The primary goal of CBP preservation methods is to maintain CBP quality for the required time period. However, maintaining CBP quality is challenging because they contain living cells that change their state in a time-dependent manner via metabolic and other biochemical reactions (Ly et al., 2020). Therefore, these reactions need to be suppressed or halted to achieve preservation, and temperature is the key parameter in achieving this.

In the late 19th century, Arrhenius mathematically and phenomenologically expressed the temperature dependence of reaction rates using the Arrhenius equation, as shown in Eq.1 (Arcus and Mulholland, 2024).

$$k = A \exp\left(-\frac{Ea}{RT}\right) \tag{1}$$

where, k indicates the reaction rate constant, T indicates the absolute temperature, A indicates the pre-exponential factor, E_a indicates the molar activation energy of the reaction, and R is the universal gas constant. According to this equation, the reaction rate decreases exponentially as temperature decreases. Therefore, low temperatures suppress the progression of biochemical reactions.

CBP preservation methods involving low temperatures are broadly classified into two categories: preservation at temperatures above the ice nucleation temperature (non-cryopreservation) and preservation at temperatures below the ice nucleation temperature (cryopreservation). The CBP preservation period varies depending on the category. Fig. 1 shows a conceptual diagram of low-temperature preservation and preservable periods. Both physical (intracellular and extracellular states of matter) and biochemical (reaction rates) factors critically determine the CBP preservation period.

Most non-cryopreservation procedures are performed under environmental temperatures that are lower than that of the culture environment (typically 310 K), which suppresses the biochemical reactions to a certain extent (Roobol et al., 2009). However, the preservation period is short because both the intracellular and extracellular environments are in a liquid state, and the reaction process is continuous. The applicability of non-cryopreservation methods has been explored for several cell types including hepatocytes (Puts et al., 2015; Usta et al., 2013), peripheral blood stem cells (Hechler et al., 1996), MSCs (Huang et al., 2020), and retinal pigment epithelium cells (Kitahata et al., 2019; Pasovic et al., 2013) with preservation periods ranging from a few hours to a few days (Robinson et al., 2014). Meanwhile, protocols have been reported that allow red blood cells to be stored under non-cryopreservation conditions for up to 42 days (Shields, 1969). The non-cryopreservation solution used in these protocols typically contains additives that prevent blood coagulation and support cellular metabolism. However, as Park et al. point out, several studies have reported time-dependent degradation of blood functionality during storage, as well as a positive correlation between storage period and post-transfusion patient mortality (Park et al., 2016). Additionally, Koch et al. reported an association between the transfusion of non-cryopreserved red blood cells for more than 14 days and an increased risk of postoperative complications, as well as reduced patient survival following cardiac surgery (Koch et al., 2008). These findings suggest that red blood cells may also undergo progressive, time-dependent changes in their characteristics during storage, highlighting the need for further validation regarding their preservable period. Although the preservation period is short, non-cryopreservation may be potentially applied for CBPs comprising cells with low tolerance for freeze-thaw cycles or when the time interval between manufacturing and administration to patient is short (Kitahata et al., 2019).

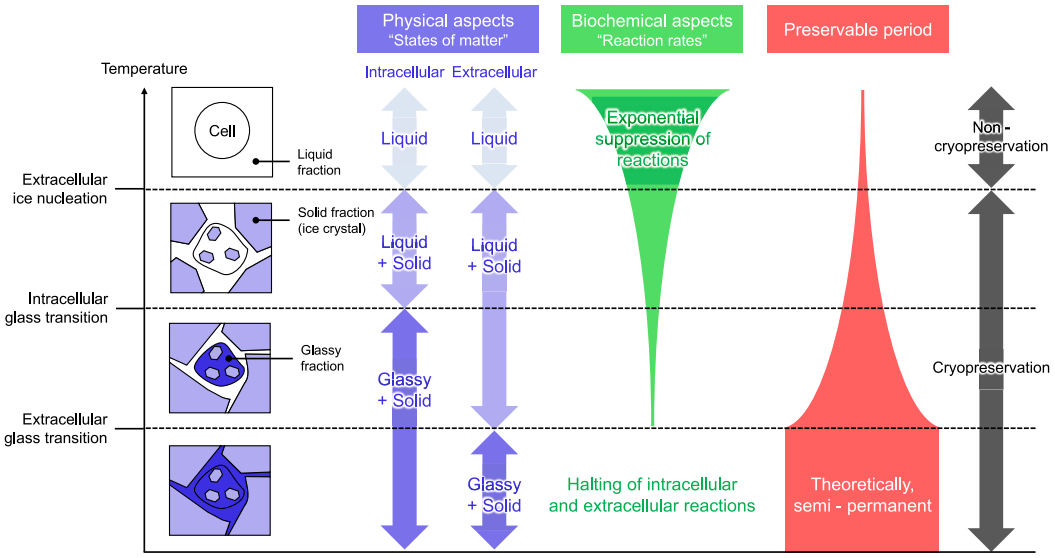

Fig. 1. Conceptual diagram of preservable period of CBPs from physical (states of matter) and biochemical (reaction rates) aspects. Theoretically, to achieve semi-permanent preservation, CBPs must be stored below the intracellular and extracellular glass transition temperatures at which biochemical reactions halt completely.

In contrast, cryopreservation enables preservation for longer time periods than that achieved using non-cryopreservation. Water molecules are involved in various biochemical reactions, and 42 % of known enzymatic reactions depend on water molecules (Frenkel-Pinter et al., 2021). As cryopreservation is performed at lower temperatures than that of non-cryopreservation, it may suppress the progression of biochemical reactions by removing water molecules by inducing ice crystal formation.

Theoretically, the halting of biochemical reactions is essential for achieving semi-permanent preservation with the intracellular and extracellular glass transitions being key factors. The glassy state is a thermodynamically metastable state that is similar to the condition between liquid and solid states (Jiang and Zhang, 2014), in which molecular motion is essentially halted while maintaining the liquid structural characteristics (Biroli and Garrahan, 2013). Thus, cryopreservation below the intracellular and extracellular glass transition temperatures virtually halts the progression of biochemical reactions and enables semi-permanent preservation.

Generally, the intracellular glass transition temperature tends to be higher than the extracellular glass transition temperature. This trend may be attributed to the higher protein concentration in the intracellular environment than in the extracellular environment (Amini and Benson, 2023). By comparing the thermograms of cell-containing and cell-free samples obtained using differential scanning calorimetry, the intracellular and extracellular glass transition temperatures can be experimentally distinguished (Meneghel et al., 2019). It should be noted, however, that this method detects transitions at the population level rather than at the single-cell scale. Moreover, the glass transition temperatures are derived from thermogram-based estimations, and the development of more precise measurement techniques is warranted.

For example, T lymphocytes (Jurkat cells) in a cryopreservation solution containing 10 % dimethyl sulfoxide (DMSO) and albumin show 226 K intracellular and 150 K extracellular temperatures (Meneghel et al., 2019); *Lactobacillus bulgaricus* in a cryopreservation solution containing 4.5 % DMSO, 5.2 % glycerol, and 20 % sucrose shows 240 K intracellular and 174 K extracellular temperatures (Fonseca et al., 2016). Thus, the glass transition temperatures vary depending on the cell type (Kilbride et al., 2021) and cryopreservation solution (Sydykov et al., 2018). Overall, the glass transition temperatures reported in studies suggest that storing CBPs in the vapor-phase section of a storage tank with liquid nitrogen (LN_2) would allow intracellular and extracellular glass transitions and theoretically achieve semi-permanent preservation.

3. Traditional cooling methods in cryopreservation

The conventional cooling methods in cryopreservation include slow cooling and vitrification, as shown in Fig. 2.

During a typical slow cooling process, the cells are suspended in a cryopreservation solution containing cryoprotective agents (CPAs) at low concentrations (e.g., 5–15 % DMSO) (Linkova et al., 2022). Then, the suspension is filled into cryovials or cryobags (volume, $\geq 10^2$ μL) and cooled using either a passive cooling device (PCD) or active cooling device (ACD) at cooling rates ranging from 10^{0} to 10^{1} K/min (Jaiswal and Vagga, 2022; Jang et al., 2017). A PCD is a cooling device used statically in an electric freezer chamber set to 193 K and designed to cool the cell suspension at a rate of approximately 1 K/min (Martinez-Madrid et al., 2004; May and Roberts, 1988). Commercially available PCDs for cryovials include Mr. Frosty (Thermo Fisher Scientific, MA, USA) and CoolCell (Corning, NY, USA). In contrast, an ACD is a programmable freezer equipped with a closed-loop control system, such as a proportional–integral–derivative controller, designed to cool the cell suspension at a desired cooling rate.

In the slow cooling method, the cell suspension often experiences supercooling, leading to the formation and growth of intracellular and extracellular ice crystals along with ice nucleation. During this process, cells may be damaged by osmotic stress and ice crystals (discussed in-depth in section 6) (Gao and Critser, 2000). Generally, the cell suspension is cooled to 193 K by slow cooling, followed by secondary cooling via vapor phase storage in a tank with LN_2 for long-term preservation (Chatzistamatiou et al., 2014; Holm et al., 2010). However, in slow cooling protocols optimized for specific combinations of cell types and cryopreservation solution, samples are sometimes transferred to the liquid or vapor phase of LN_2 storage tanks at temperatures above 193 K or subjected to stepwise cooling within this higher temperature range. For example, Ross-Rodriguez et al. developed an interrupted protocol for TF-1 cells (Ross-Rodriguez et al., 2010a, 2010b). Similarly, Marquez-Curtis et al. reported a comparable method for porcine and human corneal endothelial cells (Marquez-Curtis et al., 2017). Kashuba et al. also established an interrupted cooling approach for mouse embryonic stem cells (Kashuba et al., 2014). In addition, Hayashi et al. proposed a multi-step cooling strategy for human iPSCs (Hayashi et al., 2024). The effects of cooling rate during the freezing process and the significance of stepwise approaches are discussed in Chapter 6.

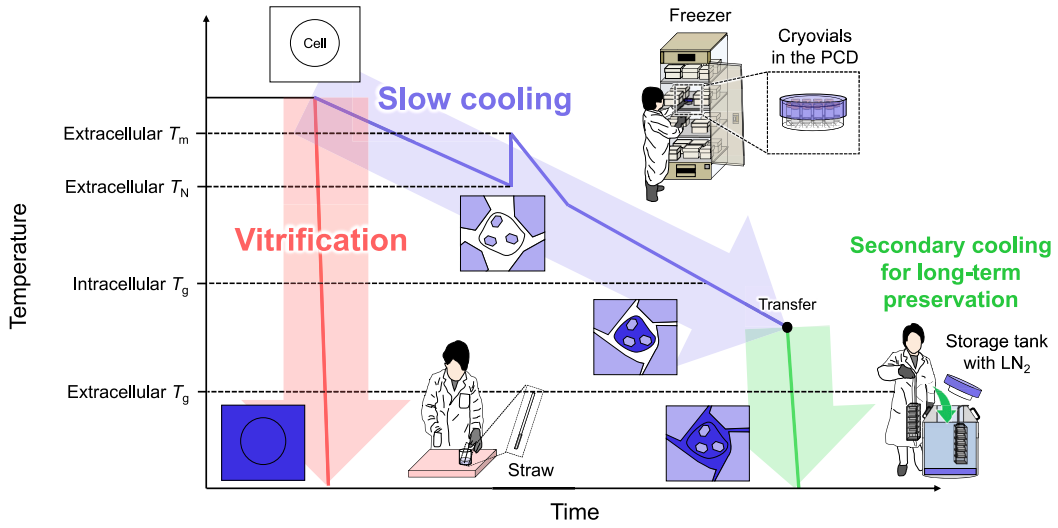

Fig. 2. Schematic drawing of difference between slow cooling and vitrification methods. In a typical slow cooling method, CBPs are cooled to 193 K using a passive cooling device or an active cooling device (blue arrow; illustration shows an example of slow cooling using a passive cooling device). For long-term preservation, CBPs are stored in an environmental temperature that is lower than the extracellular glass transition temperature (green arrow). Conventionally, storage tanks with LN_2 are used. This figure does not take into account the temporary temperature rise that occurs during transfer to the storage tanks. In a typical vitrification method, straws or similar containers are directly immersed in LN_2, and ultra rapid cooling is performed to achieve intracellular and extracellular glass transitions (red arrow; illustration shows an example of vitrification using straw). In the figure, T_m refers to the melting point, T_N to the ice nucleation temperature, and T_g to the glass transition temperature. PCD stands for passive cooling device. The colors used in the illustration of the physical phenomena experienced by the cells have the same meaning as those in Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A typical vitrification process involves suspending cells in a cryopreservation solution with a relatively high concentration of CPAs (e.g., 40-60 %), compared to the slow cooling (Jang et al., 2017). Containers such as capillary tubes and straws are frequently employed in vitrification (Amini and Benson, 2023). Cooling methods employed in vitrification include direct immersion in LN_2 and contact with a precooled solid surface (Amini and Benson, 2023; Xing et al., 2010). Vitrification is especially valuable in the cryopreservation of oocytes and embryos, which are less tolerant to the stresses associated with slow cooling (Sciorio et al., 2024; Wozniak et al., 2024). Considering the cytotoxicity of CPAs and the need for uniform ultra rapid cooling (e.g., a cooling rate on the order of 10^5 to 10^6 K/min is required to vitrify a CPA at a concentration of 1.8 M) (Lee et al., 2010), both the filling volume and the allowable handling time are limited. Therefore, slow cooling may be more advantageous than vitrification for the development of scalable cryopreservation processes. Additionally, when vitrification involves direct immersion in LN_2, there is a risk of cross-contamination because LN_2 may contain viruses, fungi, or other microorganisms (Bielanski et al., 2000; Mirabet et al., 2012).

As a variant of the vitrification method, the liquidus-tracking approach has been developed (Fu et al., 2022; Khaydukova et al., 2024; Piasecka-Belkhayat et al., 2024). In this approach, the CPA concentration is gradually increased while the biological sample is cooled and the liquidus is monitored, ensuring that the sample remains above its equilibrium melting point throughout the process (Khaydukova et al., 2024). Unlike conventional vitrification methods, this approach does not require ultra-rapid cooling and is expected to mitigate the cytotoxic effects of CPAs (Fu et al., 2022; Piasecka-Belkhayat et al., 2024). Nevertheless, systems based on liquidus monitoring and stepwise CPA addition pose significant challenges when applied to the design of scalable cryopreservation processes. Conversely, isochoric freezing method has emerged as a promising approach in recent studies (Dhanya et al., 2024). In this approach, the volumetric expansion associated with the phase transition to ice is constrained within a sealed rigid container, thereby promoting internal pressure and suppressing ice crystal growth (Solanki and Rabin, 2023). Reports on isochoric freezing of isolated cells are limited compared to three-dimensional constructs (Dhanya et al., 2024; Preciado and Rubinsky, 2018). Additionally, challenges remain in designing containers to reduce rupture risk and in controlling internal pressure. Therefore, the potential application of this approach to scalable cryopreservation processes warrants further discussion.

This review highlights the cryopreservation process based on slow cooling.

4. Cryoprotective agent

A CPA is a key component that determines the stability of CBP quality against variations of the cryopreservation process parameters. This section briefly summarizes the basic principles of CPA to provide a foundation for a CPA-based approach to handle the challenges that comprise cryopreservation. For the purpose of this review, CPA is defined as a compound that is expected to suppress the processes that affect cell quality during cryopreservation (e.g., ice crystal formation); a cryopreservation solution is defined as a solution containing one or more CPAs. Several researchers worldwide are studying CPAs, and more than 120 compounds have been identified as potential CPAs (Linkova et al., 2022).

CPAs are broadly classified into penetrating and non-penetrating categories based on their ability to penetrate cell membranes (Taylor et al., 1974). Penetrating CPAs are non-ionic small-molecule compounds capable of crossing cell membranes, whereas non-penetrating CPAs are compounds such as small-molecule sugars and high-molecular-weight polymers that cannot cross cell membranes (Raju et al., 2021). Penetrating CPAs include DMSO, glycerol, and propanediol, all of which show different cell membrane permeabilities (Keros et al., 2005; Xu et al., 2014).

The CPA loading step is one of the critical processes that must consider both physical stresses, such as osmotic pressure, and chemical stresses, such as cytotoxicity (Karlsson et al., 2015). The importance of this step has been well summarized by several researchers, including Raju et al. (Raju et al., 2021) and Kangas et al. (Kangas et al., 2025). After CPA loading, cells shrink due to the water movement from the intracellular to the extracellular environment (dehydration) in response to the increased extracellular osmotic pressure. In the case of

penetrating CPAs, the agents diffuse into the cells, reversing the osmotic gradient and causing water to move back into the intracellular environment, resulting in cell swelling (Raju et al., 2021). In contrast, with non-penetrating CPAs, no water influx occurs, and thus the cells do not swell. Depending on the cell type, abrupt alterations in cell volume may lead to cellular damage (Kangas et al., 2025). To address this challenge, stepwise loading protocols for CPAs have been developed to reduce osmotic stress (Karlsson et al., 2015; Mukherjee et al., 2007). Meanwhile, it is also necessary to consider the equilibration of CPAs. As previously described, the membrane permeability varies depending on the type of CPAs (Keros et al., 2005; Xu et al., 2014). Inadequate equilibration of CPAs may compromise their cryoprotective efficacy. Therefore, considering the membrane permeability of CPAs, it is necessary to allow sufficient equilibration time. However, prolonged suspension time raise concerns about cytotoxic effects (see Chapter 5). Accordingly, the suspension time prior to freezing needs to be established by balancing adequate CPA equilibration with minimizing cytotoxicity.

DMSO has been widely used in both research and clinical applications for more than 60 years, since Lovelock and Bishop first reported its usefulness in 1959 in the cryopreservation of bull sperm (Lovelock and Bishop, 1959). Owing to its long history of use in cryopreservation, considerable physical knowledge related to crystals has been accumulated regarding the cryoprotective mechanisms of DMSO such as its suppression of ice crystal formation (Mandumpal et al., 2011) and eutectic NaCl crystallization (Klbik et al., 2022b) and prevention of direct contact between the cell membrane and ice crystals (Klbik et al., 2022a). Furthermore, several molecular dynamic (MD) simulation-based studies have led to improved biological knowledge of cryoprotective mechanisms related to the cell membrane. Consequently, DMSO has been reported to improve cell membrane permeability probably through the formation of water pores in the cell membrane (Gurtovenko and Anwar, 2007; He et al., 2012; Lin et al., 2012). Increased cell membrane permeability may suppress intracellular ice crystal formation by promoting dehydration during the freezing process (details of dehydration are discussed in section 6).

Although DMSO appears to be the most effective CPA, it exhibits cytotoxicity and has been associated with adverse effects in clinical setting. Awan et al. have summarized in detail the cytotoxicity of DMSO in various cell types, which has highlighted that fact that even at <10 % concentration, DMSO affects cell viability and gene expression patterns in several cell types including embryonic stem cells, foreskin-derived MSCs, and retinal neuronal cells (Awan et al., 2020). In 2018, Madsen et al. summarized the adverse clinical effects possibly associated with DMSO (Madsen et al., 2018). They identified adverse effects such as ventricular extrasystoles and seizures after the administration of cryopreserved cells with DMSO. However, the use of DMSO as a CPA was not necessarily harmful because these adverse effects depended on factors such as the mode of administration (e.g., intravenous or transdermal) and dose; furthermore, these effects were mostly temporary or mild (Madsen et al., 2018). Considering the above, if the final concentration of DMSO after administration is expected to cause adverse effects depending on the site or dose of administration, it may be necessary to remove or dilute the DMSO.

To avoid the adverse effects associated with DMSO-based cryopreservation solutions, alternatives such as cryopreservation solution with reduced DMSO concentration in combination with non-penetrating CPAs (Haastrup et al., 2021; Murray et al., 2020) and DMSO-free cryopreservation solutions (Weng and Beauchesne, 2020) have been developed. Countless combinations of CPAs are available for the development of cryopreservation solutions. Hence, a high-throughput screening approach is essential for exploring potential CPAs. Stubbs et al. have developed a photochemistry-based approach for screening high molecular weight CPAs using polyampholytes synthesized using photo-reversible-addition-fragmentation-chain-transfer polymerization (Stubbs et al., 2020). Hayashi et al. have developed a quantum chemistry and MD simulation-based approach for screening CPAs for CBPs (Hayashi et al., 2021b). Recently, Ahmadkhani et al. developed a high throughput screening approach by adapting the fluorescence quenching technique for use with an automated plate reader, enabling rapid evaluation of cell membrane permeability and estimation of CPA toxicity (Ahmadkhani et al., 2025). Ideally, future studies need to incorporate high-throughput screening approaches to efficiently develop cryopreservation solutions that minimize changes in CBP quality caused by changes in process parameters during the cryopreservation processes.

5. Dispensing process

A general requirement of the dispensing process is the filling of multiple containers with a predetermined volume and density of suspensions to ensure homogeneous product composition. However, additional requirements need to be incorporated in CBP dispensing processes to maintain cell characteristics.

Typically, manual dispensing is performed by trained operators; however, it is time-consuming, and the volume and density of the suspensions are subject to variations in the containers, which includes cryovials and cryobags (Abbasalizadeh et al., 2017). Therefore, mechanization and automation of the dispensing process is in high demand to establish a scalable and stable system (Moutsatsou et al., 2019; Park et al., 2024). However, the issue of prolonged suspension time in cryopreservation solutions containing potentially cytotoxic CPAs remains a challenge (Pigeau et al., 2018).

Kagihiro et al. have reported that cell decay depends on the suspension time in cryopreservation solutions containing 10 % DMSO, and CBP quality was maintained only for 1 h for iPSCs and 0.4 h for MSCs (Kagihiro et al., 2018). Similar time-dependent decay was observed in cartilage cells (Elmoazzen et al., 2007), pulmonary artery endothelial cells (Ahmadkhani et al., 2025) and polymorphonuclear leukocytes (Takahashi et al., 1985), which highlights the need for designing approaches that suppress decay in order to establish a scalable dispensing process. Furthermore, this necessitates an understanding of the underlying decay mechanisms.

Additionally, Kagihiro et al. have reported that the suspension-time-dependent decline in iPSC quality could be attributed to a redox-balance-dependent pathway (apoptosis induced by reactive oxygen species accumulation) and redox-balance-independent pathway (loss of cell adhesive ability) (Kagihiro et al., 2020). The former pathway was suppressed by the addition of *N*-acetylcysteine, which is an antioxidant, whereas both pathways were suppressed at low temperatures. These results suggest that dispensing in a low-temperature environment would suppress the impact of prolonged suspension time and produce CBPs with homogeneous quality. In fact, Nair et al. have reported that suspension in a low-temperature environment stabilized CBP quality during the dispensing process by suppressing the instability within and between batches (Nair et al., 2022). Meanwhile, it is important to consider that low temperatures increase the time required for CPA equilibration (Davidson et al., 2015). This is partly because membrane permeability is temperature dependent, and it decreases as the temperature is lowered (Fry and Higgins, 2012). Inadequate CPA equilibration may result in differences in protective efficacy, even when the same CPAs are used. Therefore, the acceptable suspension time should be determined by considering the balance between cytotoxicity and CPA equilibration. Designing a scalable and stable dispensing process requires methodologies that can be used to determine the lot size by taking into account the suspension time in the cryopreservation solution (Sugiyama et al., 2020).

6. Freezing process

The freezing process involves cooling the CBPs to a storage temperature for long-term preservation while maintaining CBP quality.

However, during freezing, the cells and cryopreservation solution undergo substantial and complex phase transition involving ice nucleation, which is a stochastic event (Deck et al., 2022), and formation and growth of ice crystals (Gao and Critser, 2000). Therefore, the proper control of process parameters that govern freezing is necessary to maintain CBP quality. Previous reviews have addressed the freezing process-associated parameter in depth (Bojic et al., 2021; Hunt, 2019; Jang et al., 2017; Murray and Gibson, 2022; Whaley et al., 2021). In this review, we summarize the contributions of process parameters, such as cooling rate and ice nucleation temperature, to biophysical events during freezing, and propose strategies to enhance the quality stability of CBPs throughout the freezing process.

6.1. Impacts of process parameters on quality of cell-based products

During the freezing process, as the temperature of CBPs decreases, the concentration of extracellular solutions increases due to the formation and growth of ice crystals, causing an osmotic imbalance between the intracellular and extracellular solutions, which leads to cell dehydration (Gao and Critser, 2000). The degree of dehydration is a crucial factor that determines the fate of cells, and it depends on the cooling rate and ice nucleation temperature.

6.1.1. Cooling rate

Mazur et al. have investigated the impact of cooling rate on the quality of Chinese hamster tissue culture cells and proposed a two-factor hypothesis, which suggests that the degree of dehydration depends on the cooling rate and that different freezing phenomena occur in response to the degree (Mazur et al., 1972). Fig. 3 shows a conceptual diagram of the freezing phenomena with respect to cooling rate based on their hypothesis.

According to this hypothesis, cooling rates that are too slow to cause excessive dehydration result in a high concentration of intracellular solutions, which cause chemical damage. However, excessively rapid cooling rates cause insufficient dehydration resulting in the formation of intracellular ice crystals, which cause physical damage. Therefore, Mazur's two-factor hypothesis is based on the premise that the degree of dehydration depends on the cooling rate, and that the degree of dehydration acts as a balancing factor between chemical and physical cellular damage. Notably, cell viability after thawing followed an inverted U-shaped curve when expressed as a function of the cooling rate (Shitzer, 2011; Woods et al., 2016). These results suggest the existence of an optimal cooling rate that achieves the "just right" degree of dehydration, which minimizes both types of damage. Furthermore, the optimal cooling rate varies depending on the cell type (Shitzer, 2011). This is because the intracellular water content, membrane permeability, and surface-area-to-volume ratio differ between various cell types (Hubel, 1997). Additionally, the optimal cooling rate depends on the CPA combination (Morris and Farrant, 1972). Some CPAs can alter membrane properties, such as DMSO, which increases membrane water permeability (Gurtovenko and Anwar, 2007; He et al., 2012; Lin et al., 2012). In addition, the membrane permeability of CPAs affects the extent of pre-dehydration during CPA equilibration prior to freezing, thereby influencing the initial intracellular water content (Huang et al., 2017; Raju et al., 2021). The suppression of ice crystal formation by CPAs (Chang and Zhao, 2021) may alter the osmotic pressure difference between the intracellular and extracellular environments, which serves as the driving force for dehydration. Thus, depending on the combination of CPAs, intracellular water content, membrane water permeability, and the osmotic pressure difference across the membrane can vary, resulting in different cellular responses to cooling rates. Therefore, in principle, the optimal cooling rate is specific to each CBP type and must be determined individually.

Furthermore, we need to understand which cooling rate in the temperature profile of the freezing process is critical to CBP quality. Meneghel et al. performed experiments by suspending Jurkat cells in a cryopreservation solution containing 10 % DMSO, followed by cooling at a rate of 1 K/min and immersion in LN_2 at various temperatures (Meneghel et al., 2019). They found that ultra rapid cooling by immersion in LN_2 exerted a significant impact on the viability and biological function of thawed Jurkat cells, whereas ultra rapid cooling below 226 K, which is the intracellular glass transition temperature of Jurkat cells, exerted little impact. A similar trend was observed for Chinese hamster ovary, liver cancer, and osteosarcoma cells suspended in a cryopreservation solution containing 10 % DMSO with intracellular glass transition temperatures ranging from 214 to 224 K (Kilbride et al., 2021). These results suggest that precisely controlling the cooling rate above the intracellular glass transition temperature is critical during freezing. Mazur et al. reported the necessity of minimizing intracellular free water in order to suppress intracellular ice formation and the

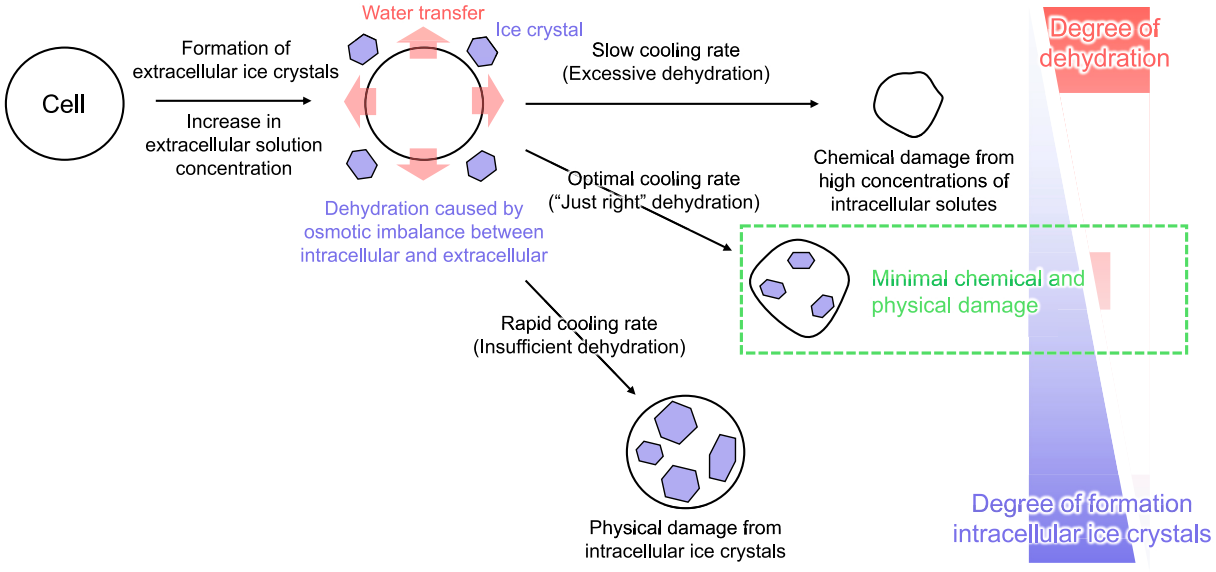

Fig. 3. Conceptual diagram of the freezing phenomena based on Mazur's two-factor hypothesis (Gao and Critser, 2000; Mazur et al., 1972). The degree of cell dehydration, which depends on the cooling rate, determines the extent of physical damage caused by intracellular ice crystals and extent of chemical damage associated with intracellular solute concentration.

associated damage (Mazur et al., 1984). Considering the concept of intracellular glass transition, the temperature range within which intracellular free water should be minimized is considered to be above the intracellular glass transition temperature. Accordingly, stabilizing the cooling rate within this temperature range is considered important for ensuring the stability of CBP quality. However, ice nucleation occurs above the intracellular glass transition, and as it is a stochastic event, it is difficult to control. Therefore, controlling ice nucleation is essential for managing the cooling rate to maintain CBP quality.

Conversely, below the intracellular glass transition temperature, cooling as rapidly as possible to shorten the process time may help improve the productivity of CBPs. Some freezing protocols employ a two-step cooling program that transitions from a slow to a rapid cooling rate (Fernandes et al., 2019; Hiramatsu et al., 2022; Kaiser et al., 2021). These programs switch to a relatively rapid cooling rate between 213 K and 233 K, with the intracellular glass transition temperature potentially considered during cooling program design (Dobruskin et al., 2024).

As mentioned above, the optimal cooling rate varies depending on the composition of CBPs, such as cell type and CPAs. Therefore, the optimal cooling rate is not fixed value and should be determined by considering multiple factors. Cell membrane permeability, as one of the cellular properties, is an important parameter in the design of an optimal cooling rate. Several methods have been proposed to determine membrane permeability, including measurements using differential scanning calorimetry (Shu et al., 2016) and calculations based on biophysical and mathematical models (Todrin et al., 2023).

Hayashi et al. divided the temperature profiles into three steps (dehydration, ice nucleation, and further cooling) and evaluated >10,000 temperature profiles using a hybrid single-cell model that predicts the quality and productivity of iPSCs (Hayashi et al., 2021a, Hayashi et al., 2020). In this numerical simulation model, the physical part consisted of heat transfer, mass transfer, and crystallization models. In the mass transfer model, the water permeability of the cell membrane was incorporated into the calculation. Subsequently, the temperature profile with the following cooling rates showed the highest-quality iPSCs: the dehydration step ranging from 277 to 233 K at 4 K/min, ice nucleation step ranging from 233 to 208 K at 2 K/min, and further cooling step ranging from 208 to 193 K at 4 K/min (Hayashi et al., 2024). However, it should be noted that in this study, cell membrane permeability was not measured directly; instead, the permeability of human pluripotent stem cells was adopted from a previously published study (Xu et al., 2014). Future integration of measurement technologies for cellular characteristics, including membrane permeability, with this simulation framework could facilitate the development of optimized cooling profiles that achieve a better balance between CBP quality and manufacturing productivity. Additionally, this study did not take the intracellular glass transition temperature into consideration but concurs with the conclusion of previous studies that switching cooling rates at certain steps may maintain CBP quality and improve its productivity. However, the extent to which strict switching of cooling rate is feasible in large-scale freezing processes remains debatable.

6.1.2. Ice nucleation temperature

Several studies have provided scientific evidence to support the fact that ice nucleation temperature affects quality in various cells such as prostate tumor cells (Wolkers et al., 2007), MSCs (Lauterboeck et al., 2015), granulosa cells (Daily et al., 2020), peripheral blood mononuclear cells (PBMCs) (Huang et al., 2021), and oocytes (Trad et al., 1998).

A higher ice nucleation temperature tends to result in higher viability in cell types such as oocytes (Trad et al., 1998), embryos, and encapsulated hepatocytes (Morris and Acton, 2013). Trad et al. have reported an increase in the number of intracellular ice crystals at low ice nucleation temperatures (Trad et al., 1998). Therefore, the lower viability at lower ice nucleation temperatures may be attributed to increased intracellular ice crystal formation. Recently, Murray and Gibson summarized the relationship between ice nucleation temperature and intracellular crystal formation in detail (Murray and Gibson, 2022). Once ice nucleation occurs, the latent heat increases the temperature of the cryopreservation solution to the melting point, which then gradually approaches the wetted-portion temperature of the container. The lower the ice nucleation temperature, the larger the gap with the melting point. Thus, the rapid cooling rate from the melting point caused by a large gap promotes insufficient cell dehydration and subsequent intracellular ice crystal formation (Murray and Gibson, 2022).

Meanwhile, it should be noted that a higher ice nucleation temperature is not always advantageous, as reported for certain cell types such as prostate tumor cells (Wolkers et al., 2007), MSCs (Lauterboeck et al., 2015), and dermal fibroblasts (Zhou et al., 2012). For example, Lauterboeck et al. evaluated the impact of ice nucleation temperature on the quality of MSCs in terms of viability and metabolic activity (Lauterboeck et al., 2015). They reported that MSCs nucleated at 263 K exhibited higher post-thaw membrane integrity and attachment efficiency than those nucleated at 269 K or 259 K, using a cryopreservation solution containing 5 % DMSO. Furthermore, their study showed that lower nucleation temperatures resulted in an increased proportion of cells exhibiting blackening, likely due to intracellular ice formation— a finding consistent with the earlier report by Trad et al. on oocytes (Trad et al., 1998). Conversely, regarding the effects of higher nucleation temperatures, Lauterboeck et al. also suggested that the formation of larger extracellular ice crystals at higher nucleation temperatures may enhance the degree of dehydration. In general, a higher ice nucleation temperature leads to the formation of larger and coarser ice crystals, whereas a lower ice nucleation temperature results in the formation of smaller and finer ice crystals (Kang et al., 2020). While the precise mechanisms underlying reduced cell viability at higher nucleation temperatures remain unclear, the degree of dehydration may be a key contributing factor.

The effects of the cooling rate and ice nucleation temperature on CBP quality have often been treated as independent phenomena. However, focusing on the degree of dehydration simplifies the phenomena and narrows down the key process parameters that need to be controlled during freezing. Moreover, if the degree of dehydration depends on the rate of cooling from the extracellular melting point (derived from the extracellular ice nucleation temperature), we propose that these phenomena may be explained by Mazur's two-factor hypothesis, as shown in Fig. 4. Together with the findings of Kilbride et al. and Meneghel et al., which emphasize the importance of precise control of the cooling rate up to the intracellular glass transition temperature (Kilbride et al., 2021; Meneghel et al., 2019), these observations suggest that maintaining the quality of CBPs can be achieved through careful regulation of the cooling rate from the extracellular melting temperature to the intracellular glass transition temperature.

To the best of our knowledge, no studies have directly reported a relationship between cooling rate and intracellular glass transition temperature. However, Meneghel et al. reported that higher concentrations of proteins and other intracellular solutes may lead to an increase in the intracellular glass transition temperature (Meneghel et al., 2019). Therefore, it is important to consider that slower cooling rates may result in greater cellular dehydration, thereby increasing intracellular solute concentrations and potentially raising the intracellular glass transition temperature.

As previously mentioned, the degree of dehydration is presumed to be a critical parameter in the design of freezing process. However, studies that have directly and quantitatively evaluated dehydration during freezing are limited, with most relying on indirect methods. For example, Zhang et al. assessed the dehydration of endothelial cells based on microscopic imaging (Zhang et al., 2006), while Zhao et al. estimated dehydration of red blood cells through volumetric changes observed via differential scanning calorimetry (Zhao et al., 2004). Additionally, indirect assessments of the dehydration using Fourier transform infrared spectroscopy and cryo-microscopy have also been reported (Akhoondi

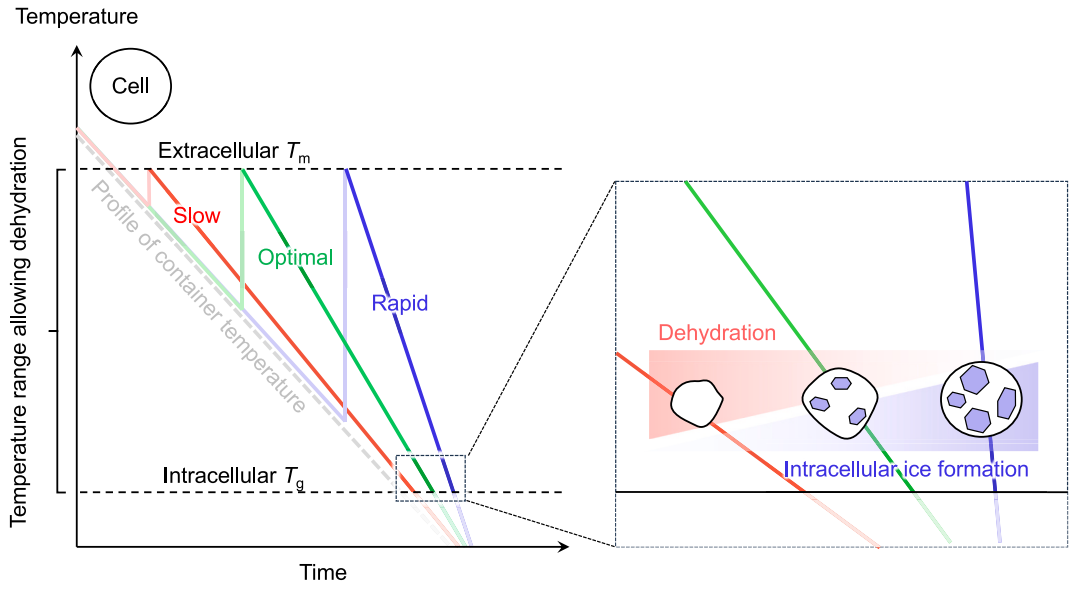

Fig. 4. Conceptual diagram illustrates the effect of the cooling rate after ice nucleation on cells, incorporating the framework of Mazur's two-factor hypothesis. The latent heat released during ice nucleation causes the temperature of the cryopreservation solution to rise to its melting point. The temperature difference between the container and the solution depends on the ice nucleation temperatures, which in turn affects the cooling rate. This figure was inspired by Fig. 5 in the review article by Murray and Gibson (Murray and Gibson, 2022) and incorporates the concepts of Mazur's two-factor hypothesis and intracellular glass transition. In the figure, T_m refers to the melting point, and T_g to the glass transition temperature.

et al., 2012). In contrast, direct evaluations include the work by Dong et al., who visualized the spatial distribution of intracellular water using cryo-Raman microscopy (Dong et al., 2010), and Huebinger et al., who employed cryo-electron microscopy and X-ray diffraction to observe the state of intracellular water (Huebinger et al., 2016). Furthermore, although not conducted under frozen conditions, approaches employing dielectric spectroscopy in conjunction with the Bruggeman-Hanai equation to assess intracellular water dynamics have been reported (Matsuura et al., 2023). Despite these advances, methodologies that enable direct and quantitative assessment of dehydration based on changes in water content before and after freezing remain limited. However, continued development of techniques for evaluating intracellular water is expected to facilitate more precise quantification of dehydration in the future.

6.2. *Stability of process parameters*

Stabilizing CBP quality during freezing requires precise control of the cooling rate between the extracellular melting point and intracellular glass transition temperature after ice nucleation, which may be achieved by understanding the factors that cause variations in cooling rate.

The freezing process may be considered a complex system composed of multiple systems. In this review, we discuss the stability of the cooling rate by classifying the freezing process into three systems based on cell manufacturability (Kino-oka et al., 2019): the cooling device system (e.g., PCDs and ACDs), container system (e.g., cryovials and cryobags), and cellular system. For the definitions of PCD and ACD, refer to Chapter 3. For example, during the cooling of cryovials using an ACD (Fig. 5), the cooling rate of the chamber in the ACD based on the predefined program is converted to the cooling rate outside the cryovials (black to blue

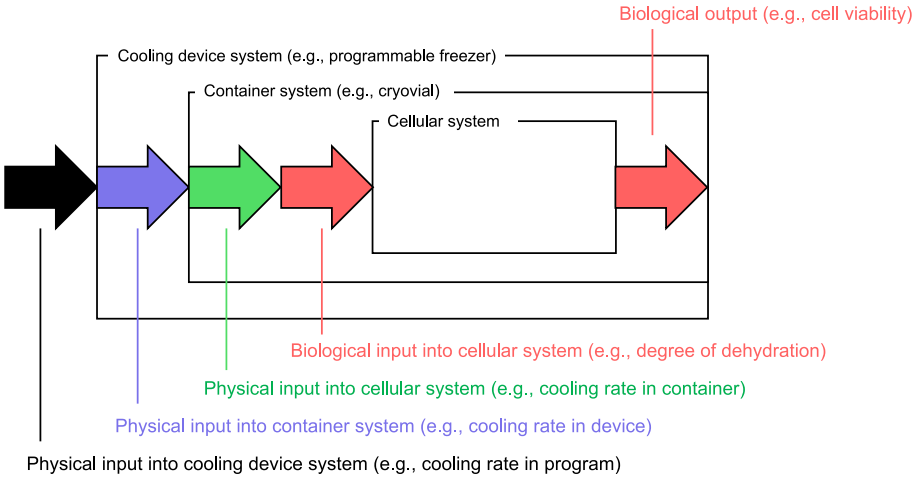

Fig. 5. Conceptual diagram of the classification of the freezing process into three systems (cooling system, container system and cellular system) based on the concept of cell manufacturability (Kino-oka et al., 2019). During the cooling of CBPs in cryovials using an active cooling device (ACD), the cooling rate of the chamber in ACD determined via the predefined program is converted to the cooling rate outside the cryovials (black to blue arrows). Then, this rate is converted to the cryopreservation solution in the cryovials (blue to green arrows), which is converted to the degree of dehydration (green to red arrows). Finally, it becomes a biological output such as cell viability. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

arrows). Then, this rate is converted to the cooling rate in the cryopreservation solution (blue to green arrows), which is further converted to the degree of dehydration (green to red arrows). Thus, the conversion of input parameters such as the cooling rate occurs across multiple systems during freezing.

To stabilize CBP quality, the physical inputs need to be controlled to maintain the stability of the biological inputs into the cellular system. In this review, the strategies for stabilizing biological inputs are discussed in the following order (Fig. 6): (1) control of physical inputs into container system and (2) control of physical inputs into cellular system.

6.2.1. Control of physical inputs into container system

Understanding the characteristics of the cooling device system is necessary to stabilize the container temperature, which is a physical input to the container system.

6.2.1.1. Passive cooling device. In a typical slow-cooling method, the CBPs are cooled using PCDs or ACDs. PCDs are relatively inexpensive, easy to handle, minimally dependent on infrastructure, and have been adopted in the freezing protocols in many laboratories (Crook et al., 2017; Nevi et al., 2017; Shibamiya et al., 2020). As the name implies, PCDs lack a cooling rate control system and cool passively from a chamber in an electric freezer, which means that the cooling rate of CBPs in PCDs is readily affected by the temperature of the freezer chamber. Hunt reported a difference in the cooling rates (the average temperature changes per minute from 263 K to 233 K, in this study) when Mr. Frosty was used alone and when the two devices were stacked (Hunt, 2019). The cooling rate was 0.98 K/min when only one Mr. Frosty was used; however, it was 0.65 K/min for the upper device and 0.91 K/min for the lower one when a double stack was used. These results indicate that the cooling rate of CBPs in PCDs is sensitive to the environment outside the PCDs. This suggests that cooling in an electric freezer chamber, where the quantity and layout of stored materials change daily, may increase the variation in CBP quality within and between batches.

Additionally, the use of PCDs for the commercial manufacture of CBPs is limited to the use of cryovials as the available containers and by the lack of a temperature-monitoring system. Shu et al. and Zhou et al. have developed a box-in-box (BIB) method as a new PCD that enables temperature monitoring and is applicable to cryobags (Shu et al., 2010; Zhou et al., 2011). In the BIB, the PCD consists of a flat outer box and inner boxes (canisters) with gaps filled with insulation and designed to cool 25 mL cryobags at a rate of 1 K/min. They have reported that the cooling rate of the CBP in the BIB depends on the thickness of the insulation, CPA concentration, and CBP liquid volume. These results suggest that BIB customization accommodates a variety of CBPs with different optimal cooling rates. However, similar to other general PCDs, the CBP cooling rate in the BIB is expected to be affected by the temperature of the chamber in the electric freezer. Therefore, one of the factors affecting the stabilization of the physical inputs into the container system is the optimization of the PCD layout and other stored materials in the electric freezer chamber. This presents a challenge in adopting PCDs for scalable freezing processes.

6.2.1.2. Active cooling device. ACDs are cooling devices that are capable of controlling the cooling rate. They comprise cooling systems such as a Stirling engine-based system and a forced convection system that uses a fan to circulate LN_2 gas.

The Stirling engine operates on the Stirling cycle to transfer heat by compressing and expanding an inert gas such as helium between hot and cold cylinders using a heat exchanger (Getie et al., 2020). Stirling engines are useful for small portable applications because of their rapid cooling and warming responses and light weight (Ismail et al., 2021). Commercially available Stirling engine-based ACDs include the CytoSaver Controlled-Rate Freezer, LN_2 Free Model FZ series (Strex, Osaka, Japan), and VIA Freeze series (Cytiva, Cambridge, UK). These ACDs are capable of cooling approximately 200 or fewer cryovials, and they are compatible with cryobags and straws. Generally, ACDs are not affected by the external environment. Therefore, the use of small ACDs is expected to better stabilize the physical input into the container system compared with the use of PCDs. Furthermore, LN_2 is not used; hence, Stirling engine-based ACDs may be installed in areas requiring higher cleanliness levels in cell-processing facilities (Morris et al., 2006), although the airflow and particles from the fans in ACDs need to be considered.

Stirling-engine-based ACDs have a limited range of controllable cooling rates due to the characteristics of the engine. However, since a cooling rate of 1 K/min is commonly employed, this limitation in cooling rate range generally does not pose a significant issue in most

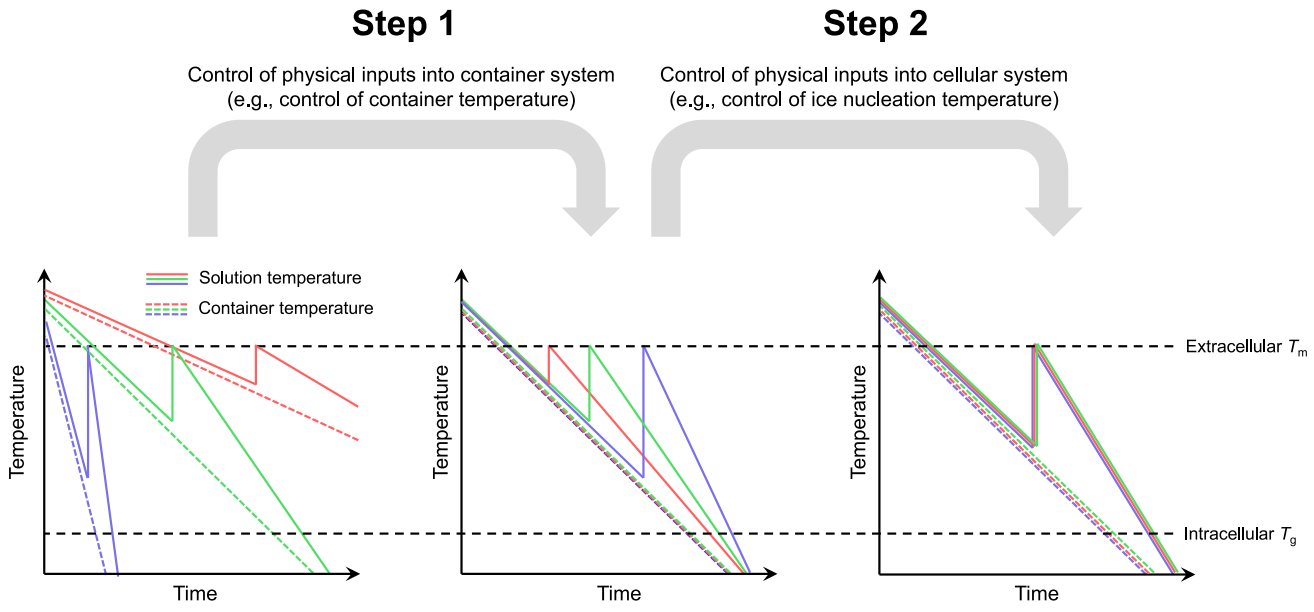

Fig. 6. Strategies for stabilizing biological inputs into the cellular system. Step 1: control of physical inputs into container system (e.g., control of container temperature). Step 2: control of physical inputs into cellular system (e.g., control of ice nucleation temperature). T_m refers to the melting point, and T_g to the glass transition temperature.

applications. Additionally, challenges remain with scalability. The challenges for scale-up include high-pressure gas sealing treatment, rapid control of gas pressure, heat and power loss, and friction in the driving parts (Ahmed et al., 2020). Additionally, the large number of driving parts require maintenance at a high frequency, which results in high running costs (Ismail et al., 2021).

In ACDs with the forced convection cooling system, the cooling rate is adjusted by adjusting the supply of LN_2 gas to the chamber containing containers (such as cryovials and cryobags) by opening or closing the solenoid valves. Commercially available ACDs with forced convection cooling systems include the CryoMed series (Thermo Fisher Scientific, MA, USA) and Kryo series (Planer, UK). These devices cool several cryovials or cryobags. However, temperature control is based on the air temperature at a specific location in the chamber or the liquid temperature in a dummy sample. Therefore, the cooling rate of the containers in a batch may vary depending on the location of the reference measurement point for temperature control. Additionally, the layout and number of containers in the chamber affect the airflow, which influences the variation in the cooling rate of the containers. In such systems, where multiple factors such as airflow and heat transfer are intricately intertwined and affect the stability of the cooling rate of containers, simulation-based analyses such as computational fluid dynamic (CFD) analysis need to be incorporated.

Recently, the spatial temperature heterogeneity of cryovials (Scholz et al., 2022) and cryobags (Chauhan et al., 2019) during the freezing process using ACDs with a forced convection cooling system was analyzed using CFD analysis. Scholz et al. used a novel simulation approach that combined a hybrid single-cell freezing model (which accounts for both the cell and cryovial layers) with a CFD model (which simulates the freezing layer) and have reported spatial heterogeneity in the quality of the iPSCs within ACDs with a forced convection cooling system (Scholz et al., 2022). Their simulation-based approach is expected to contribute to the stabilization of physical inputs into the container system and the stabilization of CBP quality by aiding in the calculation of the spatial temperature heterogeneity based on the number and layout of containers and optimizing them.

Additionally, they have developed a new simulation approach by considering ice nucleation and have proposed a continuous freezing process for iPSCs using multiple units of coupled ACDs with a forced convection cooling system (Scholz et al., 2024). A continuous freezing process may potentially address the challenges in large-scale manufacturing such as poor CBP quality due to prolonged suspension in the cryopreservation solution during the filling process. Additionally, it may mitigate spatial heterogeneity in CBP quality resulting from multiple factors such as the layout and number of CBPs in the ACDs during freezing. In future, a simulation-based approach may be necessary to design scalable and stable dispensing and freezing processes.

6.2.2. *Control of physical inputs into cellular system*

6.2.2.1. Ice nucleation. The control of ice nucleation is essential for controlling the cooling rate between the extracellular melting point and intracellular glass transition temperature after ice nucleation. However, the control of the ice nucleation temperature remains a challenge because of its nature as a stochastic event and a molecular-scale phenomenon occurring on extremely short timescales (Deck et al., 2022; Kang et al., 2020).

Ice nucleation is classified into two types: homogeneous nucleation, in which water molecules aggregate to form ice nuclei, and heterogeneous nucleation, in which water molecules aggregate with foreign substances to form ice nuclei (Maeda, 2021; Zachariassen et al., 2004). Wilson et al. have stated that ice nucleation in biological systems is always heterogeneous ice nucleation (Wilson et al., 2003). As the CBP system comprises a container, cryopreservation solution, and cells, the occurrence of heterogeneous ice nucleation may be a reasonable assumption. Unlike homogeneous ice nucleation, heterogeneous ice nucleation involves foreign substances acting as catalysts, which lower the energy barrier and facilitate the formation of ice nuclei (Maeda, 2021). Therefore, heterogeneous ice nucleation is affected by several factors such as the gas-liquid interfacial area (Kar et al., 2021; Li et al., 2014, Li et al., 2013), solution viscosity (Kimizuka et al., 2008), impurity concentration (Desnos et al., 2020), liquid volume (Daily et al., 2020), cooling rate (Li et al., 2024), and presence of shock or vibration (Wang et al., 2024).

These factors are closely associated with the dispensing and freezing processes. For example, the gas-liquid interfacial area is influenced by the shape of the container and the presence of bubbles in the container; solution viscosity is determined by the composition of the cryopreservation solution; and impurity concentration and liquid volume are related to the filled density and volume of cell suspensions during the dispensing process. Thus, ice nucleation temperature is particularly difficult to control because it is influenced by both the freezing dispensing processes and involves a complex interplay of multiple factors. Nevertheless, ice nucleation temperature may be regulated using two approaches: physical approaches that control ice nucleation via external stimuli and chemical approaches that suppress or promote supercooling through additives (Morris and Acton, 2013; Lin et al., 2023; Murray and Gibson, 2022).

6.2.2.1.1. Physical approaches. Various physical approaches to controlling ice nucleation via external stimuli include cold spots (Lauterboeck et al., 2015), voltages (Petersen et al., 2006), electromagnetic fields (Hiramatsu et al., 2022), and ultrasound (Saclier et al., 2010).

The cold-spot approach is the simplest physical method for ice nucleation control (Murray and Gibson, 2022). In this approach, a cold spot is created outside the container using the Pertier effect (Lauterboeck et al., 2015) or by manually blowing LN_2 gas (Li et al., 2018) to induce ice nucleation. Although cold-spot-based control may be used in experiments involving a small number of containers, it is not suitable for CBP manufacturing with high scalability. Particularly, the method of using a sprayer is a manual operation, which is not conducive to precise control.

The shock approach is an extension of the cold-spot method (Morris and Acton, 2013). It involves the induction of ice nucleation by temporarily spraying LN_2 gas onto the ACDs as part of a cooling program, followed by rapid cooling and immediate rapid heating (Zhou et al., 2012). The shock approach simultaneously promotes ice nucleation in several CBPs; however, uniform rapid cooling may not be possible depending on the layout of the CBPs in the ACD chamber; hence, the ice nucleation temperature may vary.

Electro-freezing has been studied for many years as an approach for active control of ice nucleation (Acharya and Bahadur, 2018). Petersen et al. have developed a device that incorporated an electro-freezing approach for cryopreservation (Petersen et al., 2006). Their novel device controlled the ice nucleation temperature of up to eight containers by applying a voltage. However, the current design needs to be improved for its application in CBP manufacturing because the equipment outside the container would be in contact with CBPs.

6.2.2.1.2. Chemical approaches. Chemical approaches aim to suppress or promote supercooling by using bioinspired compounds, minerals, or other additives (He et al., 2018; William et al., 2023).

Bio-inspired compounds are primarily ice-binding proteins (IBPs) that are synthesized by cold-adapted organisms (Dolev et al., 2016). IBPs are broadly classified into two categories: ice-nucleating proteins (INPs), which initiate ice crystal formation at high subzero temperatures, and antifreeze proteins (AFPs), which lower the freezing point and inhibit ice nucleation (Białkowska et al., 2020).

Various INPs such as plant-derived INPs (e.g., Secale leaves and Prunus wood), animal-derived INPs (e.g., *Tipula* and *Dendroides*), and fungal-derived INPs (e.g., *R. chrysoleuca* and *F. avenaceum*) have been discovered (Pummer et al., 2015). Among these, InaZ protein has been

the most effective at activating ice nucleation (Roeters et al., 2021). It is synthesized in the outer membrane of *P. syringae* (Lindow et al., 1982), and InaZ protein-based ice-nucleating agents are commercially available and been used in various studies on ice nucleation (Desnos et al., 2020; Häusler et al., 2018; Roy et al., 2021). However, several of these INPs are non-sterile and pose cost challenges, which makes their application in CBP manufacturing difficult; furthermore, their clinical adverse effects remain unknown (Wragg et al., 2020). Recently, ice-nucleating agents have been developed based on an understanding of INPs and focus on clinical applications (Hunt, 2019). Their performance has been reported in the cryopreservation of stem cells such as MSCs (Wragg et al., 2020).

AFPs derived from plants and fish have been applied for the cryopreservation of sperm and stem cells (Correia et al., 2021; Liu et al., 2021; Liu et al., 2021). However, the clinical application of AFPs has been limited by challenges such as cytotoxicity and immunogenic reactions (Ekpo et al., 2022). Biomimetics of AFPs such as oxidized quasi-carbon nitride quantum dots (Bai et al., 2017) and ε-poly-L-lysine (Matsumura et al., 2021, Matsumura et al., 2010) have been developed to overcome these problems in clinical applications, but compounds that combine sufficient efficiency with biocompatibility are not yet available (Wu et al., 2021).

Additionally, minerals have been explored as ice-nucleating agents (Marcolli et al., 2016; Soni and Patey, 2022). Previously studied ice-nucleating agents for non-bioinspired compounds include silver iodide (Marcolli et al., 2016; Soni and Patey, 2022) and crystalline cholesterol (Massie et al., 2014; Sosso et al., 2018). However, the use of these substances may not comply with current good manufacturing practices (Daily et al., 2023). Recently, Daily et al. have reported a comparative study of the ice nucleation ability of LDH1 derived from potassium feldspar, crystalline cholesterol, and K-feldspar (Daily et al., 2023). These ice-nucleating agents suppressed variability, as evidenced by the increase in ice nucleation temperatures; moreover, LDH1 completely suppressed real supercooling.

The chemical approaches for stabilizing the ice nucleation temperature through the addition of bioinspired compounds or minerals have been summarized in this review. These approaches are expected to stabilize the ice nucleation temperature without altering the existing PCDs or ACDs. In a strict sense, these chemical approaches are designed for spontaneous ice nucleation in the presence of additives and not for controlling ice nucleation temperature; therefore, it does not constitute a true control. Hence, it may be considered as an approach to suppress or promote supercooling. Nevertheless, ice-nucleating agents are constituents of cryopreservation solutions, and their biocompatibility and need to be removed after thawing (because of solubility) should be discussed in the context of clinical applications. Furthermore, considering that certain cell types exhibit reduced viability at higher ice nucleation temperatures (Lauterboeck et al., 2015; Wolkers et al., 2007; Zhou et al., 2012), the application of ice-nucleating agents may not always be suitable.

6.2.2.2. Temperature gradient within the container. Depending on the size and shape of the container, delays in internal heat transfer may arise, complicating temperature control. Massie et al. reported that, in 200 mL cryobags, the hold time at the extracellular melting point following ice nucleation was prolonged at greater distances from the heat sink (Massie et al., 2014). Furthermore, they demonstrated that artificially prolonging the hold time led to a decrease in ELS viability. These effects may be attributed to excessive cellular dehydration and the cytotoxicity of CPAs (Massie et al., 2014).

Additionally, temperature gradients within the container can affect ice crystal morphology. Kilbride et al. reported that network solidification occurs when temperature gradients within the container are small and ice nucleation is uniform, whereas progressive solidification occurs when temperature gradients are large and nucleation is non-uniform (Kilbride et al., 2014, 2016). Network solidification produces dispersed, disordered dendritic ice crystals, whereas progressive solidification results in dense, homogeneous, non-dendritic ice crystals. Although the mechanisms by which these solidifications influence cell viabilities remain unclear, cells that undergo network solidification tend to exhibit higher viability (Kilbride et al., 2014, 2016). As they noted, in cryobags where thermal equilibration within the container is not achieved prior to ice nucleation, progressive solidification may occur. To suppress this effect, minimizing temperature gradients within the cryobags is essential.

This can be achieved by leveraging the flexibility of the container, for example, by maximizing the surface-area-to-volume ratio. In less flexible container such as vials, designing a temperature profile that includes a hold time at a uniform temperature prior to ice nucleation may promote thermal equilibration and help reduce ice-structure-related damage. However, the hold time must be determined with consideration of CPA cytotoxicity.

7. Storage

7.1. Storage temperature

The basic concept of storage temperature is described in Section 2. It has been discussed in more detail in this section. Storage temperature is a critical parameter for maintaining CBP quality. CBPs should be stored in an environmental temperature that is lower than the intracellular and extracellular glass transition temperatures to maintain CBP quality for a long duration. Below the glass transition temperature, viscosity is very high and exceeds 10^{13} P (10^{14} Pa s in the SI unit system), which almost halts molecular motion and arrests of the progress of biochemical reactions (Mazur, 1984). Pure water shows a glass transition temperature of approximately 138 K (Angell, 2002), whereas it is approximately 153 K for aqueous solutions containing 10 % DMSO (Sydykov et al., 2018). Therefore, CBPs may be reasonably stored in the vapor phase of a LN_2 storage tank, which exhibits an environmental temperature of $<$123 K, or in an ultra-low-temperature electric freezer, which exhibits an environment temperature of $<$143 K (Meneghel et al., 2020).

Yang et al. investigated the impact of storing PBMC at 123 K or 193 K for 14 months on PBMC viability, apoptosis induction, and gene expression (Yang et al., 2016) and found that storage at 193 K resulted in a higher ratio of post-thaw apoptosis induction than storage at 123 K, although the post-thaw PBMC membrane integrity was at the same level, and the expression patterns of 18 genes associated with stress-related pathways, such as stress responses, immune activation, and cell death, were altered. Massie et al. investigated the impact of storing alginate-encapsulated liver cell spheroids (ELS) at 103 K or 193 K for 12 months on ELS viability and albumin secretory function (Massie et al., 2013). They have reported that storage at 193 K resulted in a slight decrease in ELS viability at 1 month and decrease in viability to 15 % in 12 months compared with that observed during storage at 103 K. Additionally, storage at 193 K reduced albumin secretion by 40 % after 1 month, and it was almost undetectable at 12 months.

These results suggest that storing CBPs in an environment above the glass transition temperature results in storage time-dependent heterogenization of the cell population in the CBPs; furthermore, CBP quality and therapeutic effect after CBP administration to patients may vary depending on the shipping timing even for CBPs from the same batch. Although these evaluations are based on a storage time of approximately 1 year, these studies have provided compelling evidence that storage below the glass transition temperature contributes to the maintenance of CBP quality for a longer duration.

In Chapter 6, we discussed how appropriate control of the degree of dehydration during the freezing process contributes to the maintenance of CBP quality. To the best of our knowledge, few studies have directly reported the relationship between storage temperature and the degree of dehydration. Here, we propose a hypothesis combining Ostwald ripening of ice crystals and the intracellular glass transition

temperature. It has been reported that ice crystals can grow over time through Ostwald ripening even under isothermal conditions (Pronk et al., 2005). Therefore, at temperatures above the extracellular glass transition temperature, extracellular solute concentration may increase over time due to time-dependent ice crystal growth. Conversely, below the intracellular glass transition temperature, cells are osmotically inactive, and dehydration is not expected to occur (Meneghel et al., 2019). Taken together, within the temperature range above the intracellular glass transition temperature, dehydration may increase in a time-dependent manner due to the osmotic gradient caused by Ostwald ripening of extracellular ice crystals. However, since intracellular ice crystals may also undergo Ostwald ripening, the time dependency of dehydration under isothermal conditions could vary depending on the extent of intracellular ice formation. As no studies have reported on these phenomena, further detailed investigations are warranted. However, several studies have reported on the isothermal crystallization kinetics in solution systems (Kharatyan et al., 2022; Westen and Groot, 2018), which are expected to provide valuable insights into the Ostwald ripening of ice crystals both intracellularly and extracellularly.

7.2. *Temperature fluctuations*

Multiple warming and re-cooling cycles (temperature fluctuations) in the cold chain of CBPs caused by shipping operations during storage impact CBP quality (Okuda et al., 2024; Pogozhykh et al., 2017). The impact of temperature fluctuations caused by shipping operations on CBP quality is shown in Fig. 7. During shipping operations, CBP temperature asymptotically increases to the environmental temperature outside the storage tank, and after the operation, the temperature of the unshipped CBP asymptotically decreases to the environmental temperature inside the storage tank. This fluctuation occurs each time the CBPs are shipped, resulting in concerns regarding the impact of the temperature range and number of fluctuations on CBP quality. In addition, opening the storage tank and removing CBPs can generate temperature fluctuations in the vapor phase. Under high-frequency usage, the vapor phase temperature is often elevated, resulting in a higher starting point for temperature excursions and consequently greater fluctuation amplitudes. These fluctuations may further compromise the quality of CBPs.

Pogozhykh et al. evaluated the impact of the temperature range (77-193 K) and number of fluctuations ($\leq$ 50 cycles) during the storage of MSCs on post-thaw quality indicators such as MSCs viability, metabolic activity, and differential potential (Pogozhykh et al., 2017). They found that viability and metabolic activity decreased as the number of fluctuations increased, and the rate of decrease was more pronounced as the fluctuation range increased. Notably, the differentiation potential of mesenchymal (adipogenic, chondrogenic, and osteogenic) lineages was not lost in response to the fluctuations, but differences in gene expression patterns were observed after differentiation; these differences were more pronounced in the adipogenic lineage than in the other lineages. These results suggest that temperature fluctuations cause cell decay, leading to the heterogenization of cell populations in CBPs. This highlights the need for potency assays such as differentiation and therapeutic potential assays for the target disease (Kim et al., 2023). Additionally, they performed flow cytometric analysis and found an increase in Annexin V-positive cells due to temperature fluctuations, which suggests a relationship between decreased viability and apoptosis. Although decreased viability has been reported during temperature fluctuations below 123 K, the induction of apoptosis was not observed. Furthermore, the cause of this decrease in viability remains unclear because neither solid-liquid phase transitions nor glass-liquid transitions occur below 123 K.

At present, the mechanisms by which temperature fluctuations occurring entirely below the glass transition temperature affect biological sample quality remain unclear. Meanwhile, Carrell et al. demonstrated that human spermatozoa frozen in 7.5 % glycerol exhibited a

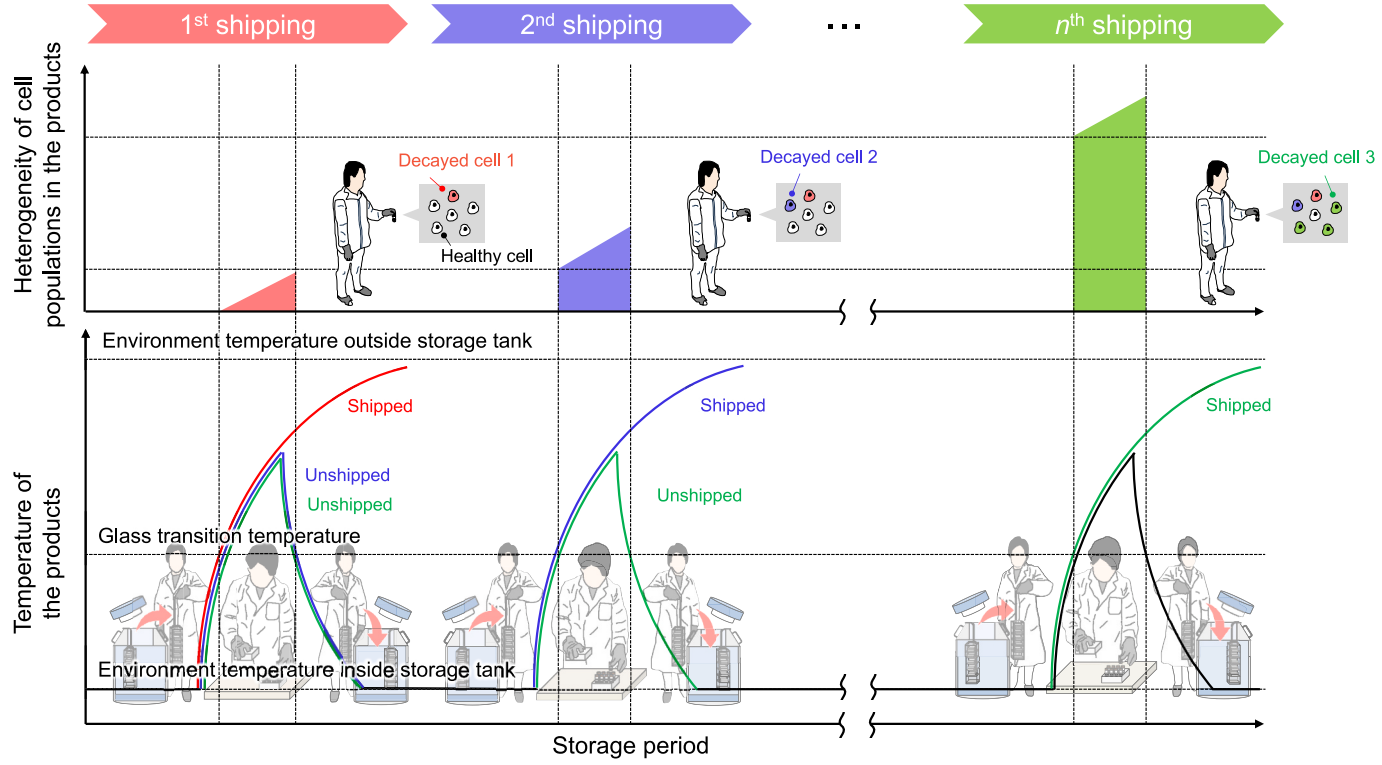

Fig. 7. Schematic diagram of the heterogenization of cell populations in CBPs caused by temperature fluctuations due to shipping from the storage tank. During the shipping operation, the temperature of the CBPs asymptotically increases to the environment temperature outside the storage tank. After the operation, the temperature of the unshipped CBPs asymptotically decreases to the environment temperature inside the storage tank. Particularly, temperature fluctuations across the glass transition temperature potentially led to heterogenization of cell populations in CBPs.

significant decrease in motility after only 1 min of exposure to room temperature, during which the sample temperature rose to approximately 138 K, remaining below the estimated extracellular glass transition temperature (Carrell et al., 1996). Furthermore, a vitrification study has shown that the thermomechanical stress resulting from differential thermal expansion is as a major factor that affects the structural integrity of biological specimens. Temperature gradients within the sample and mismatches in thermal expansion between the tissue, cryopreservation solution, and container generate mechanical stress during the fluctuations, which potentially leads to structural damage such as fractures or plastic deformation, especially in large or compositionally heterogeneous samples (Solanki and Rabin, 2022). In this way, the fluctuations below the extracellular glass transition temperature are presumed to impose intracellular and extracellular physical stress; however, reports of the resulting damage remain limited (Carrell et al., 1996; Pogozhykh et al., 2017), and no direct scientific evidence has yet been reported to elucidate the underlying mechanisms. Further studies are required to better understand this phenomenon, including research that bridges thermodynamic behavior with biological responses. Despite the lack of direct scientific evidence in the context of cryopreservation, these findings suggest that thermally induced mechanical stress may contribute to the loss of cell viability even at temperatures below the glass transition temperature.

Regarding the range of temperature fluctuations, Vysekantsev et al. have mentioned the possibility that fluctuations across the glass transition temperature could cause a phase transition between glass and liquid, thereby promoting ice recrystallization and affecting CBP quality (Vysekantsev et al., 2005). As mentioned earlier, the glass transition temperature differs between the intracellular and extracellular environments. Measurements using differential scanning calorimetry in Jurkat cells have shown that the intracellular glass transition temperature is approximately 226 K, which is higher than the extracellular glass transition temperature of approximately 150 K (Meneghel et al., 2019). Although the composition of the cryopreservation solution differed, the ice recrystallization observed during the temperature fluctuations below 153 K in the study by Vysekantsev et al. may have originated from water molecules contained in the extracellular liquid phase. However, the mechanisms underlying cell decay caused by repeated phase transitions remain unclear.

Recently, Okuda et al. evaluated the impact of the temperature range (123–193 K) and number of fluctuations during storage on post-thawing viability, mitochondrial membrane potential, and attachment efficiency after re-seeding iPSCs; additionally, they attempted to elucidate the underlying mechanism using cryogenic Raman spectroscopy (Okuda et al., 2024). They have reported that fluctuations across the glass transition temperature of the cryopreservation solution containing 10 % DMSO caused an increase in cell populations with low mitochondrial membrane potential and a decrease in attachment efficiency in a fluctuation number-dependent manner. Additionally, the Raman spectroscopic observations showed an increase in the intracellular DMSO signal as the number of fluctuations increased and loss of the cytochrome signal immediately after thawing owing to fluctuations across the glass transition temperature. Based on these results, they have proposed that the diffusion of DMSO in the liquid state into iPSCs mediated by the transient phase transition caused by fluctuations across the glass transition temperature leads to post-thaw cytochrome *c* oxidation and a subsequent increase in the cell population with low mitochondrial membrane potential and apoptosis.

Fig. 8 shows a summary of these study results, which implies that the temperature fluctuation-induced damage during storage results from different physical phenomena depending on the temperature. While the discussion so far has focused on temperature fluctuations below the intracellular glass transition temperature, exceeding this threshold may induce intracellular ice recrystallization, potentially leading to mechanical stress and cell damage (Vysekantsev et al., 2005; Xu et al., 2021). Biard et al., reported a positive correlation between the inhibition of annealing-driven intracellular ice recrystallization and increased post-thaw viability of human red blood cells (Briard et al., 2016). These studies have contributed to a better understanding of the heterogenization of cell populations in CBPs because of cell decay caused by temperature fluctuations. Furthermore, these findings could contribute to the establishment of a supply system with stable CBP quality.

7.3. *Strategies for establishing cold chains to withstand temperature fluctuations*

The data from various study findings shown in Table 1 emphasize the critical impact of temperature fluctuations across the glass transition

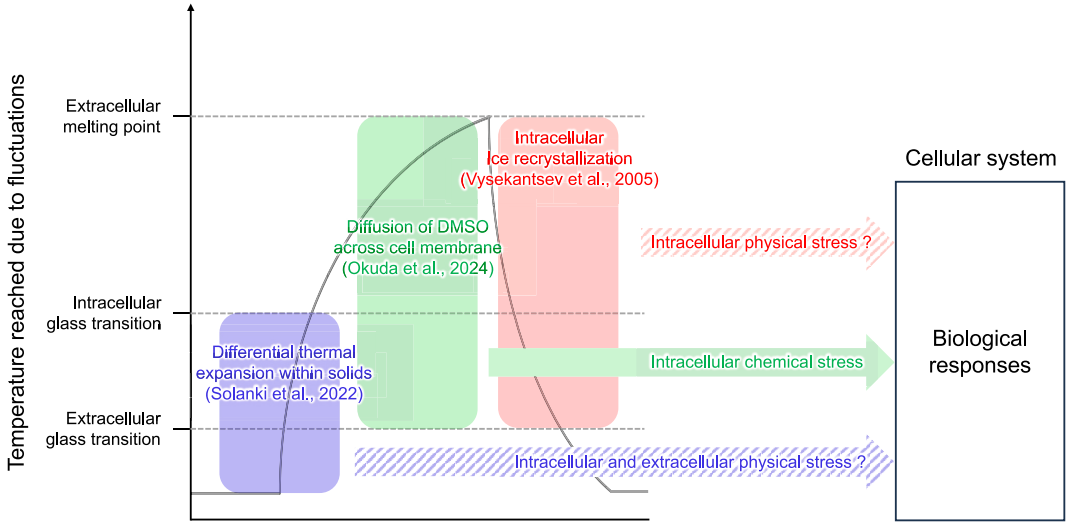

Fig. 8. Conceptual summary of physical and chemical phenomena caused by temperature fluctuations. In systems where glass and solids coexist, thermal strain is generated owing to temperature fluctuation (Solanki and Rabin, 2022). According to our hypothesis, this thermal strain may induce intracellular and extracellular physical stress, although no direct scientific evidence is available to evaluate this stress (striped, blue arrow). When the temperature exceeds the extracellular glass transition temperature, DMSO diffuses into the cell, leading to intracellular chemical stress (green arrow) (Okuda et al., 2024). Furthermore, if the temperature exceeds the extracellular glass transition temperature, recrystallization of intracellular ice crystals may occur, leading to intracellular physical stress (red arrow) (Vysekantsev et al., 2005). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Summary of studies investigating the impacts of temperature fluctuations on cells during cryogenic storage.

Cell type	Range of temperature fluctuation	Number of fluctuation cycles	Observed impacts on cells	Type of cryopreservation solution	Reference
Embryonic liver and neurotissue cell	77–173 K	≤ 10	Cell viability was significantly reduced at 5 and 10 cycles from 77 to 173 K	Cryopreservation solution with 10 % DMSO	(Vysekantsev et al., 2005)
PBMC	138–213 K	400	Viability, recovery, and antigen-specific immune response reduced at 400 cycles	Xeno-free cryomedium IBMT I (Procryotect, Ruedingen, Switzerland)	(Germann et al., 2013)
PBMC	143–213 K	≤ 350	Viability, recovery, and T-cell functionality decreased as number of cycles increased	Cryomedium IBMT I (Fraunhofer IBMT, Sulzbach, Germany)	(Angel et al., 2016)
PBMC	123–193 K	98	Viability declined because of increased apoptosis in Media-based cocktails and CS7.5; caspase inhibitor mitigated effects of temperature cycling	Media-based cocktail, CryoStor® CS5 (BioLife Solutions, Inc., Bothell, WA, U.S.), CryoStor 7.5 + caspase inhibitors	(Cosentino et al., 2007)
Placental-derived MSC	77–193 K	≤ 50	Viability, metabolic function, and apoptotic induction as number of cycles increased; altered adhesion and impacted gene regulation	Culture medium with 10 % DMSO and 10 % FBS	(Pogozhykh et al., 2017)
Umbilical cord-derived MSC	77–213 K	≤ 400	Viability, recovery, adherence, and adipogenic differentiation declined from 200 cycles; mitochondrial dysfunction from 100 cycles; proliferation and osteogenic differentiation from 50 cycles	CELLBANKER® 2 (ZENOAQ, Japan)	(Xu et al., 2021)
iPSC	123–193 K	≤ 70	Reduced attachment and mitochondrial membrane potential as number of cycles increased; increased intracellular DMSO and cytochromes oxidation	STEM-CELLBANKER® GMP grade (ZENOAQ, Japan)	(Okuda et al., 2024)
Umbilical cord blood units	93–193 K	10	Numbers of colony forming units decreased by 8 %	CryoSure-Dex40 (WAK-Chemie Medical GmbH, Germany)	(Mrowiec et al., 2012)

temperature on CBP quality. Therefore, shipping operation designs need to consider temperature fluctuation across the glass transition temperature, as shown in Fig. 9.

Most previous studies on temperature fluctuations have assumed that the same fluctuation patterns occur repeatedly. However, the temperature profiles during actual shipping operations vary depending on factors such as the number and arrangement of CBPs in storage containers (Hunt, 2019). Moreover, factors such as container size and fill volume of CBPs may also contribute to variations in temperature fluctuation patterns within the container (Pasha et al., 2020). Notably, not all fluctuations occurring under expected storage configurations can be identified using manual temperature measurements. Therefore, a technology that calculates the temperature profiles through thermodynamic simulations would be effective for predicting their impact on CBP quality. Methods to suppress temperature fluctuations include performing shipping operations on a cryogenic workbench (e.g., Cryocart) using LN_2 (Benson et al., 2013), shortening the time required for the operation, implementing mechanization and automation to suppress temperature fluctuations such as storage equipment that picks only containers with shipped CBPs (Fink et al., 2016) and transportation equipment that could be connected to the storage equipment (Xu et al., 2021). However, these are expensive and difficult to install in all cell storage facilities despite aiding in suppressing temperature fluctuations. In such cases, the upper limit of the number of fluctuations must be determined after identifying the range of the temperature fluctuations.

Considering the challenge of the temperature fluctuations from the viewpoint of cryopreservation solutions, the development or utilization of cryopreservation solutions with high glass transition temperatures to

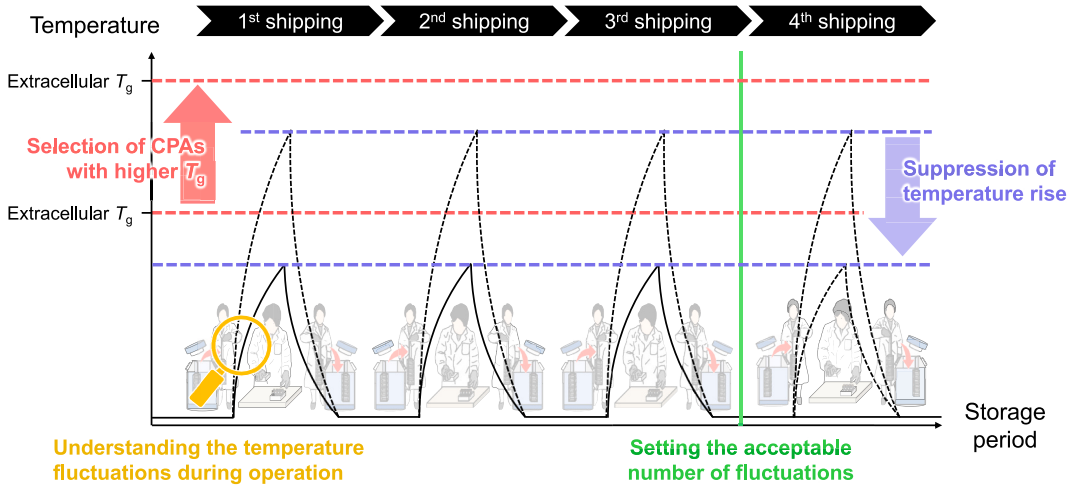

Fig. 9. Schematic drawing of strategies for establishing stable cold chains to withstand temperature fluctuation. The temperature fluctuation trends are identified for each expected shipping case. Then, appropriate shipping procedures are constructed based on their impact on CBP quality with respect to the range and number of temperature fluctuations. Additionally, the development or use of cryopreservation solutions with high glass transition temperatures may be effective in improving the robustness of cryopreservation solutions against temperature fluctuations. In this figure, T_g refers to the glass transition temperature.

increase the gap between the glass transition and storage temperatures would design shipping operations to withstand temperature fluctuations. Sydykov et al. have reported a sucrose concentration-dependent change in glass transition temperature after the addition of sucrose to an aqueous solution containing 10 % DMSO (Sydykov et al., 2018) from approximately 153 K to 172 K or 196 K after the addition of 0.5 or 1.0 M sucrose. These findings demonstrate the possibility of developing cryopreservation solutions that are robust against temperature fluctuations. To the best of our knowledge, no commercial cryopreservation solutions that focus on the glass transition temperature are yet available; however, glass transition temperature may become an important design factor in the future development of cryopreservation solutions to withstand temperature fluctuations due to shipping operations. In addition, additives that inhibit ice recrystallization have attached increasing attention. Low-molecular-weight ice recrystallization inhibitors have been reported to suppress recrystallization during both freezing and thawing, potentially protecting red blood cells from damage caused by transient temperature increases (Briard et al., 2016). These findings suggest that ice recrystallization inhibitors may serve as a complementary strategy to mitigate the detrimental effects of temperature fluctuations during the storage and transport of CBPs.

Recently, technical guidelines on the storage of cells used in regenerative medicine and cell therapy have been issued, underscoring the importance of proper cell storage from a regulatory perspective (Uno et al., 2025). Strategies that account for temperature fluctuations are expected to play a key role in supporting the development of these guidelines and the corresponding storage procedures.

8. Preparation of stored cell-based products for use

As part of the preparation for culturing or administration, stored CBPs are thawed and, if necessary, subjected to the CPA removal process. This chapter discusses strategies for maintaining the quality of CBPs, during thawing and CPA removal process.

8.1. Thawing process

During the thawing process, the warming rate is one of the key parameters that affect the quality of CBPs. Although the response to warming rates varies depending on the type of CPAs and other components constituting CBPs, rapid warming at rates of 60 K/min or higher is generally recommended to minimize the effects of both intracellular and extracellular ice recrystallization (Baust et al., 2017; Yu and Hubel, 2020). The gold standard for thawing is immersion in a water bath at a temperature between 310 K and 315 K (Gurina et al., 2016).

It is important to consider that the temperature profile during thawing is nonlinear. For example, when thawing a cryotube containing 1 mL of cryopreservation solution in the water bath at 310 K, the average warming rates are approximately 348 K/min from 123 K to 193 K, 117 K/min from 193 K to 253 K, and 14 K/min from 253 K to 273 K (Hunt, 2019). Poisson et al. reported that a reduction in warming rate at temperature above 233 K led to an increase in the average size of intracellular ice crystals (Poisson et al., 2019). In their study, warming rates of 2 K/min and 25 K/min were tested, revealing that slower warming significantly promoted intracellular ice crystal growth. This temperature range is assumed to be above the intracellular glass transition temperature, as discussed in the previous chapters (Kilbride et al., 2021; Meneghel et al., 2019). Taken together, these findings suggested that slower warming rates may promote intracellular ice recrystallization and consequently compromise the quality of CBPs. As noted by Baust et al., the reduced warming rate in this temperature range may be addressed by increasing the water bath temperature (Baust et al., 2017). However, caution must be exercised to avoid overheating, as elevated temperatures may exacerbate the cytotoxicity of CPAs.

Due to the risk of microbial contamination, the use of water baths is generally avoided in clinical applications to maintain sterility (Muyldermans et al., 1998; Yu and Hubel, 2020). In addition, variations in operator technique, such as differing immersion depth in the water bath, can lead to inconsistent warming rates. Recently, automated dry-warming devices have been developed (Kilbride et al., 2020), offering a promising solution to these challenges. However, compared to water baths that utilize it as a fluid and effective heat conductor, dry-warming devices tend to result in slower warming rates (Hunt, 2019). Additionally, for larger-volume containers such as bags, warming rates may vary spatially within the container, with slower warming occurring at certain locations. This spatial heterogeneity should be taken into account, as it can lead to variability in warming rates within the same unit. In the case of bags, designing the container to be thin can help maximize the surface-area-to-volume ratio, potentially reducing internal variability in warming rates (Baboo et al., 2019).

Ice recrystallization inhibitors may have the potential to mitigate the adverse effects of reduced warming rates (Poisson et al., 2019). Therefore, combining a chemical approach using ice recrystallization inhibitors with an engineering approach employing automated dry-warming devices may enable the maintenance of CBP quality during the thawing process. Baboo et al. reported that when the cooling rate during the freezing process was slow (1.0, 1.7 and 2.7 K/min in their study), the viability of T cells and Chinese hamster ovary cells was less affected by variations in the warming rate (Baboo et al., 2019). In contrast, when faster cooling rates were used (10 and 100 K/min in their study), they observed enhanced non-equilibrium crystallization, which increased the likelihood of recrystallization during thawing process, making the cells more sensitive to the warming rate. These findings suggest a synergistic effect between freezing and thawing parameters, highlighting the importance of designing cryopreservation processes in an integrated manner rather than treating each process in isolation.

When assessing the quality of CBPs after thawing, it is essential to establish evaluation systems that account for delayed cell death and functional decline (Baust et al., 2017). For example, trypan blue staining enables rapid and straightforward detection of cells that have lost membrane integrity (Strober, 2019), and it is commonly used to determine the number of live cells after thawing. However, this method does not allow for the prediction of delayed cell death, such as apoptosis, which may be induced by the cryopreservation process. Indeed, it has been reported that the effects of cryopreservation-related process parameters may manifest in a delayed manner (Kagihiro et al., 2020; Kagihiro et al., 2018; Nair et al., 2022; Okuda et al., 2024). Therefore, the quality assessment of CBPs after thawing should be conducted at multiple time points following thawing to ensure accurate and reliable evaluation.

8.2. Removal process of cryoprotective agents

In cases where CPAs exert adverse effects during post-thaw culture or administration, their removal becomes necessary. Typically, thawed cell suspensions are first diluted with fresh medium, followed by centrifugation and resuspension in fresh medium (Glass et al., 2008). Similar to CPA loading, CPA dilution should be designed with consideration of the osmotic balance between the intracellular and extracellular environments. During dilution, water enters the cells, causing them to swell; however, if this volumetric change occurs too rapidly, it may lead to cellular damage (Penninckx et al., 1984). Strategies to mitigate this risk include stepwise dilution protocols (Wang et al., 2010) and the addition of osmolytes such as betaine (Sui et al., 2019).

However, CPA removal processes involving centrifugation require operator intervention, and studies have reported that 27 % to 30 % of cells can be lost depending on the operator's technical skill (Glass et al., 2008). This underscores the need for operator-independent methods. For instance, microfluidic devices for DMSO removal (Glass et al., 2008; Hanna et al., 2019) and dilution–filtration systems for glycerol removal (Qiao et al., 2014) have been developed. The application of these devices is expected to contribute to maintaining CBP quality. Moreover,

operating them as closed systems offers advantages in ensuring sterility.

9. Application of simulation technologies for establishing stable cryopreservation processes

Generally, several parameters need to be defined in cryopreservation processes, which implies that considerable cost and time is required to optimize these parameters based on cellular experiments. Computer-aided process design (CAPD) has been studied for several decades in the field of process systems engineering (Bogusch et al., 2001; Frutiger et al., 2019; Gani, 2004; Lee et al., 2023; Liu et al., 2019; Qi et al., 2025; Simasatitkul et al., 2013). These approaches may accelerate the optimization of cryopreservation processes. This section introduces the applications of simulation technologies in establishing stable cryopreservation processes.

Cell freezing has long been the subject of modeling studies. Mazur et al. have developed a mathematical model for assessing the mass transfer due to cell dehydration (Mazur et al., 1972; Mazur, 1984; Mazur, 1970; Mazur, 1963). Toner et al. have constructed an ice-nucleation-limited model to assess the mass transfer due to intracellular ice formation during freezing (Toner et al., 1990). Subsequently, these models to have been applied to various cells in various studies (Anderson et al., 2019; Fadda et al., 2011; Traversari and Cincotti, 2021; Tsuruta et al., 1998; Yi et al., 2014). Xu et al. proposed a prediction method of an optimal cooling rate based on cell membrane permeability (Xu et al., 2014). Recently, models of cell damage during freezing have been developed to enable the design of freezing processes by incorporating the heat transfer during freezing (Hayashi et al., 2020). The extended model estimates cell damage as a function of process conditions such as cooling rate, cryovial material and size, or CPA. Additionally, the freezing process model was improved by incorporating the process-condition data (Hayashi et al., 2021a). Based on simulations using the improved model, a promising temperature profile that can contribute to both quality and productivity was proposed for 16,206 candidate temperature profiles, and the validity of the cooling temperature profile was confirmed experimentally (Hayashi et al., 2024). Thus, temperature profiling could be a new standard for industrial cell manufacturing.

Additionally, the modeling of cell thawing has been investigated in several recent studies. Weng et al. have modified models of cell damage during freezing to estimate the cell volume during thawing (Weng et al., 2010). Hopkins et al. have extended the ice-nucleation-limited model to assess intracellular recrystallization (Hopkins et al., 2012). Len et al. have found that the exposure of cells to high temperatures causes the intracellular accumulation of reactive oxygen species during thawing (Len et al., 2019). Kagihiro et al. have indicated that reactive oxygen species accumulation induces apoptosis in cells in a cryopreservation solution (Kagihiro et al., 2020). Subsequently, a mathematical model has been developed to evaluate the impact of reactive oxygen species accumulation on cell quality by incorporating the heat transfer during thawing (Hayashi et al., 2022). The optimal thawing temperature and cryovial diameter for productivity change significantly depending on the acceptable quality specifications and cell demand. Recently, intracellular recrystallization during thawing has been mathematically modeled (Yuan et al., 2025). Recrystallization models may be used to evaluate the relationship between the heating rate during thawing and cell damage caused by recrystallization, which could aid in optimizing the heating temperature profile during thawing.

Large-scale freezing processes must be established to accommodate the growing demand for CBPs (Li and Ma, 2012). Furthermore, rigorous simulation of heat and mass transfer in large-scale freezing using CFD-based models is quite useful to scale up freezing processes. A thermo-mechanical stress analysis of cryopreservation in cryobags was conducted (Solanki et al., 2017). It was demonstrated in the work that while the level of stress may generally increase with the increasing amount of CPA filled in the cryobags, the ratio between width and length of the cryobags would play a significant role. Recently, a CFD-based model was applied to investigate the scale-up of freezing processes using a forced-convection-based batch freezer that accommodated 235 cryovials (Scholz et al., 2022). The results showed that an appropriate selection of the inlet coolant velocity could achieve both high cell quality and low heterogeneity of cell quality in the freezer. Furthermore, a continuous freezing process was designed using a CFD-based model (Scholz et al., 2024), which confirmed that the process could achieve both high cell quality and productivity. Moreover, the freezing process did not require complicated equipment or operations because it comprised four forced convection-based batch freezers and a conveyor belt that could achieve a rapid scale-up of freezing processes.

DMSO continues to be widely used in cell manufacturing (Elliott et al., 2017; Hubálek, 2003). However, DMSO and glycerol affect cell function because of their cytotoxicity (Best, 2015; Hunt, 2019; Iwatani et al., 2006; Miyamoto et al., 2012; Young et al., 2004). Therefore, it is preferable to avoid them—particularly in the case of CBPs—since cells or products are implanted directly into the human body. Various experimental approaches have attempts to identify CPA alternative to DMSO (Matsumura et al., 2021; Matsumura et al., 2013; Pi et al., 2019; Wen et al., 2016). However, the time and resources required for the experimental search for alternative CPAs among the various candidates has been challenging. To overcome this problem, several studies have applied simulations—particularly MD simulations. For example, Posokhov and Kyrychenko investigated the effects of acetone accumulation on the structures and dynamics of lipid membranes (Posokhov and Kyrychenko, 2013). Weng et al. have calculated the effects of temperature and concentration on the hydrogen-bonding abilities of ethylene glycerol and glycerol as candidate CPAs (Weng et al., 2011). Hughes et al. have shown interactions between DMSO and the phospholipid bilayer of cell membranes (Hughes et al., 2012). Recently, 40 compounds were subjected to computational screening as CPAs for CBPs using quantum chemistry and MD simulations (Hayashi et al., 2021b). Based on the screening results, formamide, thiourea, and urea were selected as the first candidates for further investigation. Furthermore, a screening support method for CPAs and CBPs was developed based on their environmental impacts (Hayashi et al., 2023). According to the evaluation results, the environmental impacts of the agricultural- and livestock-origin groups were higher than those of the natural-resource-origin group. Recently, a computer-aided molecular design-based approach was used to search for comprehensive compounds like CPAs for stem cells (Tamaki et al., 2025). The results showed that 1-methylimidazole and pyridazine were selected as promising CPA candidates for establishing novel DMSO-free cryopreservation solutions. Furthermore, a computer-aided high throughput method for simultaneous screening of membrane permeability was developed (Ahmadkhani et al., 2025), which would contribute to a more efficient search for alternative CPAs.

Thus, computer-aided processing approaches have been used to resolve individual issues in cryopreservation processes. Consequently, solutions for each individual issue may be proposed by appropriately utilizing the experimental data. However, future studies must develop models that can comprehensively address the entire process and resolve its challenges. Such models are expected to support the design of cryopreservation process aimed at enhancing the quality stability of CBPs.

10. Conclusion and future perspectives

In this review, we have summarized the fundamental concepts of cell preservation and provided technical insights, from both experimental and simulation perspectives, to enhance the quality stability of CBPs during the cryopreservation process. As the global market for CBPs is projected to grow, scalable cryopreservation processes need to be urgently developed, which warrants the introduction of the concept of cell manufacturability, involving the understanding of process aspects such as variations in process parameters and biological aspects such as cell characteristics. This fundamental understanding must be enhanced to

efficiently optimize the cryopreservation processes through the use of simulation technologies, which could help reduce the burden on both time and resources. Furthermore, the accumulation of experimental data and application of simulation technologies would enable the future construction of digital twins, which may enable faster and more efficient design and development of scalable cryopreservation processes.

This review has primarily focused on CBPs composed of isolated cells. However, the development of stable cryopreservation processes for three-dimensional structures such as cell aggregates and organoids is expected to become increasingly important in various fields, including regenerative medicine, cell therapy, drug screening, and alternatives to animal testing (Han et al., 2024). Because of the differences in heat and mass transfer characteristics between isolated cells and three-dimensional structures, cryopreservation process optimized for isolated cells may not be directly applicable to more complex systems. This indicates the need to redesign the composition of CPAs and process parameters to account for the specific properties of these structures.

For example, Moisieiev et al. reported that the permeability of DMSO across the cell membrane significantly decreases toward the center of cell aggregates (Moisieiev et al., 2021). In addition, Gordiyenko et al. pointed out the possibility that dense cell packing within aggregates may restrict water transport across the cell membrane driven by osmotic gradients (Gordiyenko et al., 2024). These observations indicate that mass transfer limitations, such as reduced CPA permeability and restricted water transport, may influence key steps in the cryopreservation process, including CPA loading and elution, as well as dehydration during freezing. Moreover, temperature gradients that can arise inside three-dimensional structures may lead to slower cooling and warming rates. Therefore, it is challenging to directly apply the cryopreservation process developed for isolated cells to three-dimensional structures.

Nevertheless, the cryopreservation process design strategies discussed in this review for isolated cells may be applicable to three-dimensional structures, provided that their distinct thermal and mass transfer characteristics are properly considered. In the future, a deeper understanding of heat and mass transfer in three-dimensional structures will be crucial for the successful development of their cryopreservation processes.

Author contribution

Yuki Uno: Conceptualization, Methodology, Data curation, Writing–original draft, Writing–review and editing, and Visualization; **Yusuke Hayashi**: Conceptualization, Methodology, Data curation, Writing–original draft, Writing–review and editing, Resources, Project administration, and Funding acquisition; **Hirokazu Sugiyama**: Conceptualization, Writing–review and editing, Resources, Supervision, Project administration, and Funding acquisition; **Jun Okuda**: Conceptualization, Methodology, Data curation, Writing–review and editing, and Visualization; **Tetsuji Nakamura**: Conceptualization, and Writing–review and editing; **Masahiro Kino-oka**: Conceptualization, Writing–review and editing, Resources, Supervision, Project administration, and Funding acquisition.

Declaration of competing interest

Jun Okuda and Tetsuji Nakamura are employed by Iwatani Corporation. Masahiro Kino-oka received grants for collaborative research from the Iwatani Corporation. The following authors have no conflicts to declare: Yuki Uno, Yusuke Hayashi, and Hirokazu Sugiyama.

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Data availability

No datasets were generated or analyzed in this study.

References

Abbasalizadeh, S., Pakzad, M., Cabral, J.M.S., Baharvand, H., 2017. Allogeneic cell therapy manufacturing: process development technologies and facility design options. Expert. Opin. Biol. Ther. 17, 1201–1219. https://doi.org/10.1080/14712598.2017.1354982.

Acharya, P.V., Bahadur, V., 2018. Fundamental interfacial mechanisms underlying electrofreezing. Adv. Colloid Interf. Sci. 251, 26–43. https://doi.org/10.1016/j.cis.2017.12.003.

Ahmadkhani, N., Benson, J.D., Eroglu, A., Higgins, A.Z., 2025. High throughput method for simultaneous screening of membrane permeability and toxicity for discovery of new cryoprotective agents. Sci. Rep. 15, 1862. https://doi.org/10.1038/s41598-025-85509-x.

Ahmed, F., Huang, H., Ahmed, S., Wang, X., 2020. A comprehensive review on modeling and performance optimization of Stirling engine. Int. J. Energy Res. 44, 6098–6127. https://doi.org/10.1002/er.5214.

Akhoondi, M., Oldenhof, H., Sieme, H., Wolkers, W.F., 2012. Freezing-induced cellular and membrane dehydration in the presence of cryoprotective agents. Mol. Membr. Biol. 29, 197–206. https://doi.org/10.3109/09687688.2012.699106.

Amini, M., Benson, J.D., 2023. Technologies for vitrification based cryopreservation. Bioengineering 10, 508. https://doi.org/10.3390/bioengineering10050508.

Anderson, D.M., Benson, J.D., Kearsley, A.J., 2019. Numerical solution of inward solidification of a dilute ternary solution towards a semi-permeable spherical cell. Math. Biosci. 316, 108240. https://doi.org/10.1016/j.mbs.2019.108240.

Angel, S., Von Briesen, H., Oh, Y.J., Baller, M.K., Zimmermann, H., Germann, A., 2016. Toward optimal cryopreservation and storage for achievement of high cell recovery and maintenance of cell viability and T cell functionality. Biopreserv. Biobank. 14, 539–547. https://doi.org/10.1089/bio.2016.0046.

Angell, C.A., 2002. Liquid fragility and the glass transition in water and aqueous solutions. Chem. Rev. 102, 2627–2650. https://doi.org/10.1021/cr000689q.

Arcus, V.L., Mulholland, A.J., 2024. Temperature, dynamics, and enzyme-catalyzed reaction rates. Annu. Rev. Biophys. 49, 163–180. https://doi.org/10.1146/annurev-biophys-121219.

Awan, M., Buriak, I., Fleck, R., Fuller, B., Goltsev, A., Kerby, J., Lowdell, M., Mericka, P., Petrenko, A., Petrenko, Y., Rogulska, O., Stolzing, A., Stacey, G.N., 2020. Dimethyl sulfoxide: a central player since the dawn of cryobiology, is efficacy balanced by toxicity? Regen. Med. 15, 1463–1491. https://doi.org/10.2217/rme-2019-0145.

Baboo, J., Kilbride, P., Delahaye, M., Milne, S., Fonseca, F., Blanco, M., Meneghel, J., Nancekievill, A., Gaddum, N., Morris, G.J., 2019. The impact of varying cooling and thawing rates on the quality of cryopreserved human peripheral blood T cells. Sci. Rep. 9, 3417. https://doi.org/10.1038/s41598-019-39957-x.

Bahari, M., Mokhtari, H., Yeganeh, F., 2023. Stem cell therapy, the market, the opportunities and the threat. Int. J. Mol. Cell. Med. 12, 310–319. https://doi.org/10.22088/IJMCM.BUMS.12.3.310.

Bai, G., Song, Z., Geng, H., Gao, D., Liu, K., Wu, S., Rao, W., Guo, L., Wang, J., 2017. Oxidized quasi-carbon nitride quantum dots inhibit ice growth. Adv. Mater. 29, 1606843. https://doi.org/10.1002/adma.201606843.

Baust, J.M., Campbell, L.H., Harbell, J.W., 2017. Best practices for cryopreserving, thawing, recovering, and assessing cells. In Vitro Cell. Dev. Biol. Anim. 53, 855–871. https://doi.org/10.1007/s11626-017-0201-y.

Benson, E.E., Betsou, F., Fuller, B.J., Harding, K., Kofanova, O., 2013. Translating cryobiology principles into trans-disciplinary storage guidelines for biorepositories and biobanks: a concept paper. CryoLetters 34, 277–312.

Best, B.P., 2015. Cryoprotectant toxicity: facts, issues, and questions. Rejuvenation Res. 18, 422–436. https://doi.org/10.1089/rej.2014.1656.

Białkowska, A., Majewska, E., Olczak, A., Twarda-clapa, A., 2020. Ice binding proteins: diverse biological roles and applications in different types of industry. Biomolecules 10, 274. https://doi.org/10.3390/biom10020274.

Bielanski, A., Nadin-Davis, S., Sapp, T., Lutze-Wallace, C., 2000. Viral contamination of embryos cryopreserved in liquid nitrogen. Cryobiology 40, 110–116. https://doi.org/10.1006/cryo.1999.2227.

Biroli, G., Garrahan, J.P., 2013. Perspective: the glass transition. J. Chem. Phys. 138. https://doi.org/10.1063/1.4795539.

Bogusch, R., Lohmann, B., Marquardt, W., 2001. Computer-aided process modeling with MODKIT. Comput. Chem. Eng. 25, 963–995. https://doi.org/10.1016/S0098-1354(01)00626-3.

Bojic, S., Murray, A., Bentley, B.L., Spindler, R., Pawlik, P., Cordeiro, J.L., Bauer, R., de Magalhães, J.P., 2021. Winter is coming: the future of cryopreservation. BMC Biol. 19, 1–20. https://doi.org/10.1186/s12915-021-00976-8.

Briard, J.G., Poisson, J.S., Turner, T.R., Capicciotti, C.I., Acker, J.P., Ben, R.N., 2016. Small molecule ice recrystallization inhibitors mitigate red blood cell lysis during freezing, transient warming and thawing. Sci. Rep. 6, 23619. https://doi.org/10.1038/srep23619.

Carrell, D.T., Wilcox, A.L., Urry, R.L., 1996. Effect of fluctuations in temperature encountered during handling and shipment of human cryopreserved semen. Andrologia 28, 315–319. https://doi.org/10.1111/j.1439-0272.1996.tb02808.x.

Chang, T., Zhao, G., 2021. Ice inhibition for cryopreservation: materials, strategies, and challenges. Adv. Sci. 8, 2002425. https://doi.org/10.1002/advs.202002425.

Chang, Y.H., Fujimori, Y., Chen, C.C., Urabe, M., Karaki, T., Izumi, Y., Kaneko, K., Mochiki, K., 2025. Practice of cryopreservation of cellular starting materials from the Asia-Pacific region: an industrial perspective. Ther. Innov. Regul. Sci. 59, 1107–1116. https://doi.org/10.1007/s43441-025-00808-9.

Chatzistamatiou, T.K., Papassavas, A.C., Michalopoulos, E., Gamaloutsos, C., Mallis, P., Gontika, I., Panagouli, E., Koussoulakos, S.L., Stavropoulos-Giokas, C., 2014. Optimizing isolation culture and freezing methods to preserve Wharton's jelly's mesenchymal stem cell (MSC) properties: an MSC banking protocol validation for the Hellenic cord blood Bank. Transfusion (Paris) 54, 3108–3120. https://doi.org/10.1111/trf.12743.

Chauhan, A., Trembley, J., Wrobel, L.C., Jouhara, H., 2019. Experimental and CFD validation of the thermal performance of a cryogenic batch freezer with the effect of loading. Energy 171, 77–94. https://doi.org/10.1016/j.energy.2018.12.149.

Clarke, D., Smith, D., 2019. Managing starting material stability to maximize manufacturing flexibility and downstream efficiency. Cell Gene Ther. Insights 5, 303–314. https://doi.org/10.18609/cgti.2019.033.

Correia, L.F.L., Alves, B.R.C., Batista, R.I.T.P., Mermillod, P., Souza-Fabjan, J.M.G., 2021. Antifreeze proteins for low-temperature preservation in reproductive medicine: a systematic review over the last three decades. Theriogenology 176, 94–103. https://doi.org/10.1016/j.theriogenology.2021.09.025.

Cosentino, L.M., Corwin, W., Baust, J.M., Diaz-Mayoral, N., Cooley, H., Shao, W., Van Buskirk, R., Baust, J.G., 2007. Preliminary report: evaluation of storage conditions and cryococktails during peripheral blood mononuclear cell cryopreservation. Cell Preserv. Technol. 5, 189–204. https://doi.org/10.1089/cpt.2007.9987.

Crook, J.M., Tomaskovic-Crook, E., Ludwig, T.E., 2017. Cryobanking pluripotent stem cells. Methods Mol. Biol. 1590, 151–164. https://doi.org/10.1007/978-1-4939-6921-0_11.

Daily, M.I., Whale, T.F., Partanen, R., Harrison, A.D., Kilbride, P., Lamb, S., Morris, G.J., Picton, H.M., Murray, B.J., 2020. Cryopreservation of primary cultures of mammalian somatic cells in 96-well plates benefits from control of ice nucleation. Cryobiology 93, 62–69. https://doi.org/10.1016/j.cryobiol.2020.02.008.

Daily, M.I., Whale, T.F., Kilbride, P., Lamb, S., John Morris, G., Picton, H.M., Murray, B. J., 2023. A highly active mineral-based ice nucleating agent supports in situ cell cryopreservation in a high throughput format. J. R. Soc. Interface 20, 20020682. https://doi.org/10.1098/rsif.2022.0682.

Davidson, A.F., Glasscock, C., McClanahan, D.R., Benson, J.D., Higgins, A.Z., 2015. Toxicity minimized Cryoprotectant addition and removal procedures for adherent endothelial cells. PLoS One 10, e0142828. https://doi.org/10.1371/journal.pone.0142828.

Deck, L.T., Ochsenbein, D.R., Mazzotti, M., 2022. Stochastic ice nucleation governs the freezing process of biopharmaceuticals in vials. Int. J. Pharm. 625, 122051. https://doi.org/10.1016/j.ijpharm.2022.122051.

Desnos, H., Bruyère, P., Louis, G., Buff, S., Baudot, A., 2020. Ice induction using Snomax® in the dimethyl-sulfoxide-containing aqueous solution for DSC experiments. Thermochim. Acta 692, 178734. https://doi.org/10.1016/j.tca.2020.178734.

Dhanya, R., Panoth, A., Venkatachalapathy, N., 2024. A comprehensive review on isochoric freezing: a recent technology for preservation of food and non-food items. Sustainable Food Technol. 2, 9–18. https://doi.org/10.1039/D3FB00146F.

Dobruskin, M., Toner, G., Kander, R., 2024. Optimizing cryopreservation strategies for scalable cell therapies: a comprehensive review with insights from iPSC-derived therapies. Biotechnol. Prog. 40, e3504. https://doi.org/10.1002/btpr.3504.

Dolev, M.B., Braslavsky, I., Davies, P.L., 2016. Ice-binding proteins and their function. Annu. Rev. Biochem. 85, 515–542. https://doi.org/10.1146/annurev-biochem-060815-014546.

Dong, J., Malsam, J., Bischof, J.C., Hubel, A., Aksan, A., 2010. Spatial distribution of the state of water in frozen mammalian cells. Biophys. J. 99, 2453–2459. https://doi.org/10.1016/j.bpj.2010.08.035.

Ekpo, M.D., Tan, S., Xie, J., Hu, Y., Liu, X., Liu, F., Xiang, J., Zhao, R., Wang, B., 2022. Antifreeze proteins: novel applications and navigation towards their clinical application in Cryobanking. Int. J. Mol. Sci. 23, 2639. https://doi.org/10.3390/ijms23052639.

Elliott, G.D., Wang, S., Fuller, B.J., 2017. Cryoprotectants: a review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. Cryobiology 76, 74–91. https://doi.org/10.1016/j.cryobiol.2017.04.004.

Elmoazzen, H.Y., Poovadan, A., Law, G.K., Elliott, J.A.W., McGann, L.E., Jomha, N.M., 2007. Dimethyl sulfoxide toxicity kinetics in intact articular cartilage. Cell Tissue Bank. 8, 125–133. https://doi.org/10.1007/s10561-006-9023-y.

Fadda, S., Cincotti, A., Cao, G., 2011. Rationalizing the equilibration and cooling stages of cryopreservation: the effect of cell size distribution. AICHE J. 57, 1075–1095. https://doi.org/10.1002/aic.12320.

Fernandes, S., Khan, N., Kale, V., Limaye, L., 2019. Catalase incorporation in freezing mixture leads to improved recovery of cryopreserved iPSC lines. Cryobiology 90, 21–29. https://doi.org/10.1016/j.cryobiol.2019.09.003.

Fink, J., Hawkins, B.J., Abazari, A., Albert, M., O'Donnell, K., 2016. Next generation technology procedures and products facilitate biopreservation Best practices and increased viability for cellular therapy. Cytotherapy 18, S45–S46. https://doi.org/10.1016/j.jcyt.2016.03.106.

Fonseca, F., Meneghel, J., Cenard, S., Passot, S., Morris, G.J., 2016. Determination of intracellular vitrification temperatures for unicellular micro organisms under conditions relevant for cryopreservation. PLoS One 11, E0152939. https://doi.org/10.1371/journal.pone.0152939.

Frenkel-Pinter, M., Rajaei, V., Glass, J.B., Hud, N.V., Williams, L.D., 2021. Water and life: the medium is the message. J. Mol. Evol. 89, 2–11. https://doi.org/10.1007/s00239-020-09978-6.

Frutiger, J., Cignitti, S., Abildskov, J., Woodley, J.M., Sin, G., 2019. Computer-aided molecular product-process design under property uncertainties – a Monte Carlo based optimization strategy. Comput. Chem. Eng. 122, 247–257. https://doi.org/10.1016/j.compchemeng.2018.08.021.

Fry, A.K., Higgins, A.Z., 2012. Measurement of Cryoprotectant permeability in adherent endothelial cells and applications to cryopreservation. Cell. Mol. Bioeng. 5, 287–298. https://doi.org/10.1007/s12195-012-0235-x.

Fu, Y., Dang, W., He, X., Xu, F., Huang, H., 2022. Biomolecular pathways of cryoinjuries in low-temperature storage for mammalian specimens. Bioengineering 9, 545. https://doi.org/10.3390/bioengineering9100545.

Gani, R., 2004. Chemical product design: challenges and opportunities. Comput. Chem. Eng. 28, 2441–2457. https://doi.org/10.1016/j.compchemeng.2004.08.010.

Gao, D., Critser, A.K., 2000. Mechanisms of cryoinjury in living cells. ILAR J. 41. https://doi.org/10.1093/ilar.41.4.187, 187–19.

Germann, A., Oh, Y.J., Schmidt, T., Schön, U., Zimmermann, H., von Briesen, H., 2013. Temperature fluctuations during deep temperature cryopreservation reduce PBMC recovery, viability and T-cell function. Cryobiology 67, 193–200. https://doi.org/10.1016/j.cryobiol.2013.06.012.

Getie, M.Z., Lanzetta, F., Bégot, S., Admassu, B.T., Hassen, A.A., 2020. Reversed regenerative Stirling cycle machine for refrigeration application: a review. Int. J. Refrig. 118, 173–187. https://doi.org/10.1016/j.ijrefrig.2020.06.007.

Glass, K.K.F., Longmire, E.K., Hubel, A., 2008. Optimization of a microfluidic device for diffusion-based extraction of DMSO from a cell suspension. Int. J. Heat Mass Transf. 51, 5749–5757. https://doi.org/10.1016/j.ijheatmasstransfer.2008.04.018.

Gordiyenko, O.I., Kovalenko, I.F., Rogulska, O.Y., Trufanova, N.A., Gurina, T.M., Trufanov, O.V., Petrenko, O.Y., 2024. Theory-based cryopreservation mode of mesenchymal stromal cell spheroids. Cryobiology 115, 104906. https://doi.org/10.1016/j.cryobiol.2024.104906.

Gurina, T.M., Pakhomov, A.V., Polyakova, A.L., Legach, E.I., Bozhok, G.A., 2016. The development of the cell cryopreservation protocol with controlled rate thawing. Cell Tissue Bank. 17, 303–316. https://doi.org/10.1007/s10561-015-9533-6.

Gurtovenko, A.A., Anwar, J., 2007. Modulating the structure and properties of cell membranes: the molecular mechanism of action of dimethyl sulfoxide. J. Phys. Chem. B 111, 10453–10460. https://doi.org/10.1021/jp073113e.

Haastrup, E.K., Munthe-Fog, L., Ballesteros, O.R., Fischer-Nielsen, A., Svalgaard, J.D., 2021. DMSO (Me_2SO) concentrations of 1-2% in combination with pentaisomaltose are effective for cryopreservation of T cells. Transfus. Apher. Sci. 60, 103138. https://doi.org/10.1016/j.transci.2021.103138.

Han, H., Zhan, T., Guo, N., Cui, M., Xu, Y., 2024. Cryopreservation of organoids: strategies, innovation, and future prospects. Biotechnol. J. 19, 2300543. https://doi.org/10.1002/biot.202300543.

Hanna, J., Hubel, A., Lemke, E., 2019. Diffusion-based extraction of DMSO from a cell suspension in a three stream. Vertical Microchannel. Biotechnol. Bioeng. 109, 2316–2324. https://doi.org/10.1002/bit.24499.

Hassan, S., Simaria, A.S., Varadaraju, H., Gupta, S., Warren, K., Farid, S.S., 2015. Allogeneic cell therapy bioprocess economics and optimization: downstream processing decisions. Regen. Med. 10, 591–609. https://doi.org/10.2217/rme.15.29.

Häusler, T., Witek, L., Felgitsch, L., Hitzenberger, R., Grothe, H., 2018. Freezing on a Chip-a new approach to determine heterogeneous ice nucleation of micrometer-sized water droplets. Atmosphere (Basel) 9, 140. https://doi.org/10.3390/atmos9040140.

Hayashi, Y., Horiguchi, I., Kino-oka, M., Sugiyama, H., 2020. Slow freezing process design for human induced pluripotent stem cells by modeling intracontainer variation. Comput. Chem. Eng. 132, 106597. https://doi.org/10.1016/j.compchemeng.2019.106597.

Hayashi, Y., Horiguchi, I., Kino-oka, M., Sugiyama, H., 2021a. Model-based assessment of temperature profiles in slow freezing for human induced pluripotent stem cells. Comput. Chem. Eng. 144, 107150. https://doi.org/10.1016/j.compchemeng.2020.107150.

Hayashi, Y., Nakajima, Y., Sugiyama, H., 2021b. Computational screening of cryoprotective agents for regenerative medical products using quantum chemistry and molecular dynamics simulations. Cryobiology 100, 101–109. https://doi.org/10.1016/j.cryobiol.2021.03.002.

Hayashi, Y., Kino-oka, M., Sugiyama, H., 2022. Hybrid-model-based design of fill-freeze-thaw processes for human induced pluripotent stem cells considering productivity and quality. Comput. Chem. Eng. 156, 107566. https://doi.org/10.1016/j.compchemeng.2021.107566.

Hayashi, Y., Shigeyama, A., Sugiyama, H., 2023. A screening support method of cryoprotective agents for regenerative medical products considering environmental impacts. Chem. Eng. Res. Des. 191, 83–92. https://doi.org/10.1016/j.cherd.2023.01.021.

Hayashi, Y., Uno, Y., Kino-oka, M., Sugiyama, H., 2024. Computer-aided exploration of multiobjective optimal temperature profiles in slow freezing for human induced pluripotent stem cells. Cryobiology 115, 104885. https://doi.org/10.1016/j.cryobiol.2024.104885.

He, F., Liu, W., Zheng, S., Zhou, L., Ye, B., Qi, Z., 2012. Ion transport through dimethyl sulfoxide (DMSO) induced transient water pores in cell membranes. Mol. Membr. Biol. 29, 107–113. https://doi.org/10.3109/09687688.2012.687460.

He, Z., Liu, K., Wang, J., 2018. Bioinspired materials for controlling ice nucleation, growth, and recrystallization. Acc. Chem. Res. 51, 1082–1091. https://doi.org/10.1021/acs.accounts.7b00528.

Heathman, T.R., Nienow, A.W., McCall, M.J., Coopman, K., Kara, B., Hewitt, C.J., 2015. The translation of cell-based therapies: clinical landscape and manufacturing challenges. Regen. Med. 10, 49–64. https://doi.org/10.2217/rme.14.73.

Hechler, G., Weide, R., Heymanns, J., Köppler, H., Havemann, K., 1996. Storage of noncryopreserved peripheral blood stem cells for transplantation. Ann. Hematol. 72, 303–306. https://doi.org/10.1007/s002770050176.
Hiramatsu, S., Morizane, A., Kikuchi, T., Doi, D., Yoshida, K., Takahashi, J., 2022. Cryopreservation of induced pluripotent stem cell-derived dopaminergic Neurospheres for clinical application. J. Parkinsons Dis. 12, 871–884. https://doi.org/10.3233/JPD-212934.
Holm, F., Ström, S., Inzunza, J., Baker, D., Strömberg, A.M., Rozell, B., Feki, A., Bergström, R., Hovatta, O., 2010. An effective serum-and xeno-free chemically defined freezing procedure for human embryonic and induced pluripotent stem cells. Hum. Reprod. 25, 1271–1279. https://doi.org/10.1093/humrep/deq040.
Hopkins, J.B., Badeau, R., Warkentin, M., Thorne, R.E., 2012. Effect of common cryoprotectants on critical warming rates and ice formation in aqueous solutions. Cryobiology 65, 169–178. https://doi.org/10.1016/j.cryobiol.2012.05.010.
Huang, H., Zhao, G., Zhang, Y., Xu, J., Toth, T.L., He, X., 2017. Predehydration and ice seeding in the presence of Trehalose enable cell cryopreservation. ACS Biomater Sci. Eng. 3, 1758–1768. https://doi.org/10.1021/acsbiomaterials.7b00201.
Huang, H., Rey-Bedón, C., Yarmush, M.L., Usta, O.B., 2020. Deep-supercooling for extended preservation of adipose-derived stem cells. Cryobiology 92, 67–75. https://doi.org/10.1016/j.cryobiol.2019.11.004.
Huang, Z., Liu, W., Liu, B., He, X., Guo, H., Xue, S., Yan, X., Jaganathan, G.K., 2021. Cryopreservation of human T lymphocytes under fast cooling with controlled ice nucleation in cryoprotective solutions of low toxicity. Cryobiology 103, 92–100. https://doi.org/10.1016/j.cryobiol.2021.09.002.
Hubálek, Z., 2003. Protectants used in the cryopreservation of microorganisms. Cryobiology 46, 205–299. https://doi.org/10.1016/S0011-2240(03)00046-4.
Hubel, A., 1997. Parameters of cell freezing: implications for the cryopreservation of stem cells. Transfus. Med. Rev. 11, 224–233. https://doi.org/10.1053/tmrv.1997.0110224.
Huebinger, J., Han, H.M., Hofnagel, O., Vetter, I.R., Bastiaens, P.I.H., Grabenbauer, M., 2016. Direct measurement of water states in cryopreserved cells reveals tolerance toward ice crystallization. Biophys. J. 110, 840–849. https://doi.org/10.1016/j.bpj.2015.09.029.
Hughes, Z.E., Mark, A.E., Mancera, R.L., 2012. Molecular dynamics simulations of the interactions of DMSO with DPPC and DOPC phospholipid membranes. J. Phys. Chem. B 116, 11911–11923. https://doi.org/10.1021/jp3035538.
Hui, K.K., Yamanaka, S., 2024. iPS cell therapy 2.0: preparing for next-generation regenerative medicine. BioEssays 46, 240072. https://doi.org/10.1002/bies.202400072.
Hunt, C.J., 2019. Technical considerations in the freezing, low-temperature storage and thawing of stem cells for cellular therapies. Transfus. Med. Hemother. 46, 134–150. https://doi.org/10.1159/000497289.
Ismail, M., Yebiyo, M., Chaer, I., 2021. A review of recent advances in emerging alternative heating and cooling technologies. Energies (Basel) 14, 502. https://doi.org/10.3390/en14020502.
Iwatani, M., Ikegami, K., Kremenska, Y., Hattori, N., Tanaka, S., Yagi, S., Shiota, K., 2006. Dimethyl sulfoxide has an impact on epigenetic profile in mouse Embryoid body. Stem Cells 24, 2549–2556. https://doi.org/10.1634/stemcells.2005-0427.
Jaiswal, A.N., Vagga, A., 2022. Cryopreservation: a review article. Cureus 14, e31564. https://doi.org/10.7759/cureus.31564.
Jang, T.H., Park, S.C., Yang, J.H., Kim, J.Y., Seok, J.H., Park, U.S., Choi, C.W., Lee, S.R., Han, J., 2017. Cryopreservation and its clinical applications. Integr. Med. Res. 6, 12–18. https://doi.org/10.1016/j.imr.2016.12.001.
Jankovic, M.G., Stojkovic, M., Bojic, S., Jovicic, N., Kovacevic, M.M., Ivosevic, Z., Juskovic, A., Kovacevic, V., Ljujic, B., 2023. Scaling up human mesenchymal stem cell manufacturing using bioreactors for clinical uses. Curr. Res. Transl. Med. 71, 103393. https://doi.org/10.1016/j.retram.2023.103393.
Jiang, Z.H., Zhang, Q.Y., 2014. The structure of glass: a phase equilibrium diagram approach. Prog. Mater. Sci. 61, 144–215. https://doi.org/10.1016/j.pmatsci.2013.12.001.
Kagihiro, M., Fukumori, K., Aoki, T., Ungkulpasvich, U., Mizutani, M., Viravaidya-Pasuwat, K., Kino-oka, M., 2018. Kinetic analysis of cell decay during the filling process: application to lot size determination in manufacturing systems for human induced pluripotent and mesenchymal stem cells. Biochem. Eng. J. 131, 31–38. https://doi.org/10.1016/j.bej.2017.11.019.
Kagihiro, M., Fukumori, K., Horiguchi, I., Kim, M.H., Kino-oka, M., 2020. Suppression of time-dependent decay by controlling the redox balance of human induced pluripotent stem cells suspended in a cryopreservation solution. Biochem. Eng. J. 155, 107465. https://doi.org/10.1016/j.bej.2019.107465.
Kaiser, D., Otto, N.M., McCallion, O., Hoffmann, H., Zarrinrad, G., Stein, M., Beier, C., Matz, I., Herschel, M., Hester, J., Moll, G., Issa, F., Reinke, P., Roemhild, A., 2021. Freezing medium containing 5% DMSO enhances the cell viability and recovery rate after cryopreservation of regulatory T cell products ex vivo and in vivo. Front. Cell Dev. Biol. 9, 750286. https://doi.org/10.3389/fcell.2021.750286.
Kang, T., You, Y., Jun, S., 2020. Supercooling preservation technology in food and biological samples: a review focused on electric and magnetic field applications. Food Sci. Biotechnol. 29, 303–321. https://doi.org/10.1007/s10068-020-00750-6.
Kangas, J.R., Hogan Jr., C.J., Bischof, J.C., 2025. Eliminating osmotic stress during cryoprotectant loading: a mathematical investigation of solute-solvent transport. Cryobiology 118, 105198. https://doi.org/10.1016/j.cryobiol.2025.105198.
Kar, A., Bhati, A., Lokanathan, M., Bahadur, V., 2021. Faster nucleation of ice at the three-phase contact line: influence of interfacial chemistry. Langmuir 37, 12673–12680. https://doi.org/10.1021/acs.langmuir.1c02044.
Karlsson, J.O.M., Szurek, E.A., Higgins, A.Z., Lee, S.R., Eroglu, A., 2015. Optimization of cryoprotectant loading into murine and human oocytes. Cryobiology 68, 18–28. https://doi.org/10.1016/j.cryobiol.2013.11.002.
Kashuba, C.M., Benson, J.D., Critser, J.K., 2014. Rationally optimized cryopreservation of multiple mouse embryonic stem cell lines: II–Mathematical prediction and experimental validation of optimal cryopreservation protocols. Cryobiology 68, 176–184. https://doi.org/10.1016/j.cryobiol.2013.12.003.
Kawamoto, H., Masuda, K., 2024. Trends in cell medicine: from autologous cells to allogeneic universal-use cells for adoptive T-cell therapies. Int. Immunol. 36, 65–73. https://doi.org/10.1093/intimm/dxad051.
Keros, V., Rosenlund, B., Hultenby, K., Aghajanova, L., Levkov, L., Hovatta, O., 2005. Optimizing cryopreservation of human testicular tissue: comparison of protocols with glycerol, propanediol and dimethylsulphoxide as cryoprotectants. Hum. Reprod. 20, 1676–1687. https://doi.org/10.1093/humrep/deh797.
Kharatyan, T., Gopireddy, S.R., Ogawa, T., Kodama, T., Nishimoto, N., Osada, S., Scherließ, R., Urbanetz, N.A., 2022. Quantitative analysis of glassy state relaxation and ostwald ripening during annealing using freeze-drying microscopy. Pharmaceutics 14, 1176. https://doi.org/10.3390/pharmaceutics14061176.
Khaydukova, I.V., Ivannikova, V.M., Zhidkov, D.A., Belikov, N.V., Peshkova, M.A., Timashev, P.S., Tsiganov, D.I., Pushkarev, A.V., 2024. Current state and challenges of tissue and organ cryopreservation in biobanking. Int. J. Mol. Sci. 25, 11124. https://doi.org/10.3390/ijms252011124.
Kilbride, P., Morris, G.J., Milne, S., Fuller, B., Skepper, J., Selden, C., 2014. A scale down process for the development of large volume cryopreservation. Cryobiology 69, 367–375. https://doi.org/10.1016/j.cryobiol.2014.09.003.
Kilbride, P., Lamb, S., Milne, S., Gibbons, S., Erro, E., Bundy, J., Selden, C., Fuller, B., Morris, J., 2016. Spatial considerations during cryopreservation of a large volume sample. Cryobiology 73, 47–54. https://doi.org/10.1016/j.cryobiol.2016.05.013.
Kilbride, P., Meneghel, J., Creasey, G., Masoudzadeh, F., Drew, T., Creasey, H., Bloxham, D., Morris, G.J., Jestice, K., 2020. Automated dry thawing of cryopreserved haematopoietic cells is not adversely influenced by cryostorage time, patient age or gender. PLoS One 15, e0240310. https://doi.org/10.1371/journal.pone.0240310.
Kilbride, P., Meneghel, J., Fonseca, F., Morris, J., 2021. The transfer temperature from slow cooling to cryogenic storage is critical for optimal recovery of cryopreserved mammalian cells. PLoS One 16, e0259571. https://doi.org/10.1371/journal.pone.0259571.
Kim, M.H., Tan, S.Y., Yamahara, K., Kino-oka, M., 2023. An in vitro culture platform to study the extracellular matrix remodeling potential of human mesenchymal stem cells. Acta Biomater. 170, 376–388. https://doi.org/10.1016/j.actbio.2023.08.035.
Kimizuka, N., Viriyarattanasak, C., Suzuki, T., 2008. Ice nucleation and supercooling behavior of polymer aqueous solutions. Cryobiology 56, 80–87. https://doi.org/10.1016/j.cryobiol.2007.10.179.
Kino-oka, M., Mizutani, M., Medcalf, N., 2019. Cell manufacturability. Cell Gene. Ther. Insights. 5, 1347–1359. https://doi.org/10.18609/cgti.2019.140.
Kirkeby, A., Main, H., Carpenter, M., 2025. Pluripotent stem-cell-derived therapies in clinical trial: a 2025 update. Cell Stem Cell 32, 10–37. https://doi.org/10.1016/j.stem.2024.12.005.
Kitahata, S., Tanaka, Y., Hori, K., Kime, C., Sugita, S., Ueda, H., Takahashi, M., 2019. Critical functionality effects from storage temperature on human induced pluripotent stem cell-derived retinal pigment epithelium cell suspensions. Sci. Rep. 9, 2891. https://doi.org/10.1038/s41598-018-38065-6.
Klbik, I., Čechová, K., Maťko, I., Lakota, J., Šauša, O., 2022a. On crystallization of water confined in liposomes and cryoprotective action of DMSO. RSC Adv. 12, 2300–2309. https://doi.org/10.1039/d1ra08935h.
Klbik, I., Čechová, K., Milovská, S., Rusnák, J., Vlasáč, J., Melicherčík, M., Mat'Ko, I., Lakota, J., Šauša, O., 2022b. Cryoprotective mechanism of DMSO induced by the inhibitory effect on eutectic NaCl crystallization. J. Phys. Chem. Lett. 13, 11153–11159. https://doi.org/10.1021/acs.jpclett.2c03003.
Koch, C.G., Li, L., Sessler, D.I., Figueroa, P., Hoeltge, G.A., Mihaljevic, T., Blackstone, E. H., 2008. Duration of red-cell storage and complications after cardiac surgery. N. Engl. J. Med. 358, 1229–1239. https://doi.org/10.1056/nejmoa070403.
Lauterboeck, L., Hofmann, N., Mueller, T., Glasmacher, B., 2015. Active control of the nucleation temperature enhances freezing survival of multipotent mesenchymal stromal cells. Cryobiology 71, 384–390. https://doi.org/10.1016/j.cryobiol.2015.10.145.
Lee, H.J., Elmoazzen, H., Wright, D., Biggers, J., Rueda, B.R., Heo, Y.S., Toner, M., Toth, T.L., 2010. Ultra-rapid vitrification of mouse oocytes in low cryoprotectant concentrations. Reprod. Biomed. Online 20. https://doi.org/10.1016/j.rbmo.2009.11.012.
Lee, B., Jung, S., Hashimura, Y., Lee, M., Borys, B.S., Dang, T., Kallos, M.S., Rodrigues, C. A.V., Silva, T.P., Cabral, J.M.S., 2022. Cell culture process scale-up challenges for commercial-scale manufacturing of allogeneic pluripotent stem cell products. Bioengineering 9, 92. https://doi.org/10.3390/bioengineering9030092.
Lee, Y.S., Galindo, A., Jackson, G., Adjiman, C.S., 2023. Enabling the direct solution of challenging computer-aided molecular and process design problems: chemical absorption of carbon dioxide. Comput. Chem. Eng. 174, 108204. https://doi.org/10.1016/j.compchemeng.2023.108204.
Len, J.S., Koh, W.S.D., Tan, S.X., 2019. The roles of reactive oxygen species and antioxidants in cryopreservation. Biosci. Rep. 39. https://doi.org/10.1042/BSR20191601. BSR20191601.
Li, Y., Ma, T., 2012. Bioprocessing of cryopreservation for large-scale banking of human pluripotent stem cells. Biores Open Access 1, 205–214. https://doi.org/10.1089/biores.2012.0224.
Li, T., Donadio, D., Galli, G., 2013. Ice nucleation at the nanoscale probes no man's land of water. Nat. Commun. 4, 1887. https://doi.org/10.1038/ncomms2918.
Li, K., Xu, S., Chen, J., Zhang, Q., Zhang, Y., Cui, D., Zhou, X., Wang, J., Song, Y., 2014. Viscosity of interfacial water regulates ice nucleation. Appl. Phys. Lett. 104, 101605. https://doi.org/10.1063/1.4868255.

Li, R., Yu, G., Azarin, S.M., Hubel, A., 2018. Freezing responses in DMSO-based cryopreservation of human iPS cells: aggregates versus single cells. Tissue Eng. Part C Methods 24, 289–299. https://doi.org/10.1089/ten.tec.2017.0531.
Li, C., Zhao, H., Cheng, L., Wang, B., 2021. Allogeneic vs. autologous mesenchymal stem/stromal cells in their medication practice. Cell Biosci. 11, 1–21. https://doi.org/10.1186/s13578-021-00698-y.
Li, P., Dong, C., Zhang, L., 2024. Mechanistic investigation of nucleation kinetics in heterogeneous ice crystallization: the role of cooling rate, surface energy, surface nanostructure, and wetting state. Int. J. Heat Mass Transf. 232, 125939. https://doi.org/10.1016/j.ijheatmasstransfer.2024.125939.
Lin, J., Novak, B., Moldovan, D., 2012. Molecular dynamics simulation study of the effect of DMSO on structural and permeation properties of DMPC lipid bilayers. J. Phys. Chem. B 116, 1299–1308. https://doi.org/10.1021/jp208145b.
Lin, M., Cao, H., Li, J., 2023. Control strategies of ice nucleation, growth, and recrystallization for cryopreservation. Acta Biomater. 155, 35–56. https://doi.org/10.1016/j.actbio.2022.10.056.
Lindow, S.E., Arny, D.C., Upper, C.D., 1982. Bacterial ice nucleation: a factor in frost injury to plants. Plant Physiol. 70, 1084–1089. https://doi.org/10.1104/pp.70.4.1084.
Linkova, D.D., Rubtsova, Y.P., Egorikhina, M.N., 2022. Cryostorage of mesenchymal stem cells and biomedical cell-based products. Cells 11, 2691. https://doi.org/10.3390/cells11172691.
Liu, Q., Zhang, L., Liu, L., Du, J., Tula, A.K., Eden, M., Gani, R., 2019. OptCAMD: an optimization-based framework and tool for molecular and mixture product design. Comput. Chem. Eng. 124, 285–301. https://doi.org/10.1016/j.compchemeng.2019.01.006.
Liu, X., Peng, H., Xie, J., Hu, Y., Liu, F., Wang, X., Yang, S., Liu, Z., Zhu, Q., Tan, S., 2021. Methods in biosynthesis and characterization of the antifreeze protein (AFP) for potential blood cryopreservation. J. Nanomater. 2021, 9932538. https://doi.org/10.1155/2021/9932538.
Lovelock, J.E., Bishop, M.W.H., 1959. Prevention of freezing damage to living cells by dimethyl Sulphoxide. Nature 183, 1394–1395. https://doi.org/10.1038/1831394a0.
Ly, C.H., Lynch, G.S., Ryall, J.G., 2020. A metabolic roadmap for somatic stem cell fate. Cell Metab. 31, 1052–1067. https://doi.org/10.1016/j.cmet.2020.04.022.
Madsen, B.K., Hilscher, M., Zetner, D., Rosenberg, J., 2018. Adverse reactions of dimethyl sulfoxide in humans: a systematic review. F1000Res 7, 1746. https://doi.org/10.12688/f1000research.16642.1.
Maeda, N., 2021. Brief overview of ice nucleation. Molecules 26, 392. https://doi.org/10.3390/molecules26020392.
Mandumpal, J.B., Kreck, C.A., Mancera, R.L., 2011. A molecular mechanism of solvent cryoprotection in aqueous DMSO solutions. Phys. Chem. Chem. Phys. 13, 3839–3842. https://doi.org/10.1039/c0cp02326d.
Marcolli, C., Nagare, B., Welti, A., Lohmann, U., 2016. Ice nucleation efficiency of AgI: review and new insights. Atmos. Chem. Phys. 16, 8915–8937. https://doi.org/10.5194/acp-16-8915-2016.
Marquez-Curtis, L.A., McGann, L.E., Elliott, J.A.W., 2017. Expansion and cryopreservation of porcine and human corneal endothelial cells. Cryobiology 77, 1–13. https://doi.org/10.1016/j.cryobiol.2017.04.012.
Martinez-Madrid, B., Dolmans, M.-M., Van Langendonckt, A., Defrère, S., Donnez, J., 2004. Freeze-thawing intact human ovary with its vascular pedicle with a passive cooling device. Fertil. Steril. 82, 1390–1394. https://doi.org/10.1016/j.fertnstert.2004.
Mason, C., Dunnill, P., 2009. Assessing the value of autologous and allogeneic cells for regenerative medicine. Regen. Med. 4, 835–853. https://doi.org/10.2217/rme.09.64.
Massie, I., Selden, C., Hodgson, H., Fuller, B., 2013. Storage temperatures for cold-chain delivery in cell therapy: a study of alginate-encapsulated liver cell spheroids stored at-80°C or-170°C for up to 1 year. Tissue Eng. Part C Methods 19, 189–195. https://doi.org/10.1089/ten.tec.2012.0307.
Massie, I., Selden, C., Hodgson, H., Fuller, B., Gibbons, S., Morris, G.J., 2014. GMP cryopreservation of large volumes of cells for regenerative medicine: active control of the freezing process. Tissue Eng. Part C Methods 20, 693–702. https://doi.org/10.1089/ten.tec.2013.0571.
Matsumura, K., Bae, J.Y., Hyon, S.H., 2010. Polyampholytes as cryoprotective agents for mammalian cell cryopreservation. Cell Transplant. 19, 691–699. https://doi.org/10.3727/096368910X508780.
Matsumura, K., Hayashi, F., Nagashima, T., Hyon, S.H., 2013. Long-term cryopreservation of human mesenchymal stem cells using carboxylated poly-l-lysine without the addition of proteins or dimethyl sulfoxide. J. Biomater. Sci. Polym. Ed. 24, 1484–1497. https://doi.org/10.1080/09205063.2013.771318.
Matsumura, K., Hayashi, F., Nagashima, T., Rajan, R., Hyon, S.H., 2021. Molecular mechanisms of cell cryopreservation with polyampholytes studied by solid-state NMR. Commun. Mater. 2, 15. https://doi.org/10.1038/s43246-021-00118-1.
Matsuura, H., Takano, K., Shirakashi, R., 2023. Slow water dynamics in dehydrated human Jurkat T cells evaluated by dielectric spectroscopy with the Bruggeman-Hanai equation. RSC Adv. 13, 20934–20940. https://doi.org/10.1039/d3ra02892e.
May, S.R., Roberts, A.D.P., 1988. Development of a passive device for freezing large amounts of transplantable skin at one time in a-70°C mechanical Refrigeratoriv2. Cryobiology 25, 186–196. https://doi.org/10.1016/0011-2240(88)90025-9.
Mazur, P., 1963. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. J. Gen. Physiol. 47, 347–369. https://doi.org/10.1085/jgp.47.2.347.
Mazur, P., 1970. Cryobiology: the freezing of biological systems. Science 168, 939–949. https://doi.org/10.1126/science.168.3934.939.
Mazur, P., 1984. Freezing of living cells: mechanisms and implications. Am. J. Phys. Cell Phys. 247, 125–142. https://doi.org/10.1152/ajpcell.1984.247.3.C125.
Mazur, P., Leibo, S.P., Chu, E.H.Y., 1972. A two-factor hypothesis of freezing injury evidence from Chinese Hamster tissue-culture cells. Exp. Cell Res. 72, 345–355. https://doi.org/10.1016/0014-4827(72)90303-5.
Mazur, P., Rall, W.F., Leibo, S.P., 1984. Kinetics of water loss and the likelihood of intracellular freezing in mouse ova. Influence of the method of calculating the temperature dependence of water permeability. Cell Biophys. 6, 197–213. https://doi.org/10.1007/bf02788619.
Meneghel, J., Kilbride, P., Morris, J.G., Fonseca, F., 2019. Physical events occurring during the cryopreservation of immortalized human T cells. PLoS One 14, e0217304. https://doi.org/10.1371/journal.pone.0217304.
Meneghel, J., Kilbride, P., Morris, G.J., 2020. Cryopreservation as a key element in the successful delivery of cell-based therapies—a review. Front. Med. (Lausanne) 7, 592242. https://doi.org/10.3389/fmed.2020.592242.
Mirabet, V., Alvarez, M., Solves, P., Ocete, D., Gimeno, C., 2012. Use of liquid nitrogen during storage in a cell and tissue bank: contamination risk and effect on the detectability of potential viral contaminants. Cryobiology 64, 121–123. https://doi.org/10.1016/j.cryobiol.2011.12.005.
Miyamoto, Y., Noguchi, H., Yukawa, H., Oishi, K., Matsushita, K., Iwata, H., Hayashi, S., 2012. Cryopreservation of induced pluripotent stem cells. Cell Med. 3, 89–95. https://doi.org/10.3727/215517912x639405.
Moisieiev, A.I., Kovalenko, I.F., Kovalenko, S.Y., Bozhok, G.A., Gordiyenko, O.I., 2021. Dynamics of dimethyl sulfoxide penetration into L929 cells and L929-based spheroids. Probl. Cryobiol. Cryomed. 31, 316–325. https://doi.org/10.15407/cryo31.04.316.
Morris, G.J., Acton, E., 2013. Controlled ice nucleation in cryopreservation - a review. Cryobiology 66, 85–92. https://doi.org/10.1016/j.cryobiol.2012.11.007.
Morris, G.J., Farrant, J., 1972. Interactions of cooling rate and protective additive on the survival of washed human erythrocytes frozen to-196°C. Cryobiology 9, 173–181. https://doi.org/10.1016/0011-2240(72)90029-6.
Morris, G.J., Acton, E., Faszer, K., Franklin, A., Yin, H., Bodine, R., Pareja, J., Zaninovic, N., Gosden, R., 2006. Cryopreservation of murine embryos, human spermatozoa and embryonic stem cells using a liquid nitrogen-free, controlled rate freezer. Reprod. Biomed. Online 13, 421–426. https://doi.org/10.1016/S1472-6483(10)61448-4.
Moutsatsou, P., Ochs, J., Schmitt, R.H., Hewitt, C.J., Hanga, M.P., 2019. Automation in cell and gene therapy manufacturing: from past to future. Biotechnol. Lett. 41, 1245–1253. https://doi.org/10.1007/s10529-019-02732-z.
Mrowiec, Z.R., Angelina, A., Laluf, J., 2012. Transient warming events and cryogenic storage of cord blood mononuclear cells for stem cell transplantation. J. Transfus. Med. 4, 153–158. https://doi.org/10.5603/jtm.28356.
Mukherjee, I.N., Song, Y.C., Sambanis, A., 2007. Cryoprotectant delivery and removal from murine insulinomas at vitrification-relevant concentrations. Cryobiology 55, 10–18. https://doi.org/10.1016/j.cryobiol.2007.04.002.
Murray, K.A., Gibson, M.I., 2022. Chemical approaches to cryopreservation. Nat. Rev. Chem. 6, 579–593. https://doi.org/10.1038/s41570-022-00407-4.
Murray, K.A., Tomás, R.M.F., Gibson, M.I., 2020. Low DMSO cryopreservation of stem cells enabled by macromolecular Cryoprotectants. ACS Appl. Bio Mater. 3, 5627–5632. https://doi.org/10.1021/acsabm.0c00638.
Muyldermans, G., de Smet, F., Pierard, D., Steenssens, L., Stevens, D., Bougatef, A., Lauwers, S., 1998. Neonatal infections with *Pseudomonas aeruginosa* associated with a water-bath used to thaw fresh frozen plasma. J. Hosp. Infect. 39, 309–314. https://doi.org/10.1016/s0195-6701(98)90296-1.
Nair, A., Horiguchi, I., Fukumori, K., Kino-oka, M., 2022. Development of instability analysis for the filling process of human-induced pluripotent stem cell products. Biochem. Eng. J. 185, 108506. https://doi.org/10.1016/j.bej.2022.108506.
Nevi, L., Cardinale, V., Carpino, G., Costantini, D., Di Matteo, S., Cantafora, A., Melandro, F., Brunelli, R., Bastianelli, C., Aliberti, C., Monti, M., Bosco, D., Berloco, P.B., Panici, P.B., Reid, L., Gaudio, E., Alvaro, D., 2017. Cryopreservation protocol for human biliary tree stem/progenitors, hepatic and pancreatic precursors. Sci. Rep. 7, 6080. https://doi.org/10.1038/s41598-017-05858-0.
Okuda, J., Watanabe, N., Nakamura, T., Mizushima, K., Xi, H., Kumamoto, Y., Fujita, K., Kino-Oka, M., 2024. The impact of repeated temperature cycling on cryopreserved human iPSC viability stems from cytochrome redox state changes. Front. Bioeng. Biotechnol. 12, 1443795. https://doi.org/10.3389/fbioe.2024.1443795.
Park, H.J., Lee, S.Y., Ji, M., Kim, K., Son, Y.H., Jang, S., Park, Y.K., 2016. Measuring cell surface area and deformability of individual human red blood cells over blood storage using quantitative phase imaging. Sci. Rep. 6, 34257. https://doi.org/10.1038/srep34257.
Park, M., Safford, M., Scheers, J., Hammill, L., Pleitez, D., Jerbi, T., Koudji, E.M., Yelity, S., Campion, S., Miller, M.M., Gibb, S.L., Sargent, A., 2024. Automation preserves product consistency and quality for the formulation, fill, and finish of T cell-based therapies. Cytotherapy 12, 1566–1570. https://doi.org/10.1016/j.jcyt.2024.07.006.
Pasha, R., Howell, A., Turner, T.R., Halpenny, M., Elmoazzen, H., Acker, J.P., Pineault, N., 2020. Transient warming affects potency of cryopreserved cord blood units. Cytotherapy 22, 690–697. https://doi.org/10.1016/j.jcyt.2020.04.039.
Pasovic, L., Utheim, T.P., Maria, R., Lyberg, T., Messelt, E.B., Aabel, P., Chen, D.F., Chen, X., Eidet, J.R., 2013. Optimization of storage temperature for cultured ARPE-19 cells. J. Ophthalmol. 2013, 216359. https://doi.org/10.1155/2013/216359.
Penninckx, F., Poelmans, S., Kerremans, R., Loecker, W.D., 1984. Erythrocyte swelling after rapid dilution of cryoprotectants and its prevention. Cryobiology 21, 25–32. https://doi.org/10.1016/0011-2240(84)90019-1.
Petersen, A., Schneider, H., Rau, G., Glasmacher, B., 2006. A new approach for freezing of aqueous solutions under active control of the nucleation temperature. Cryobiology 53, 248–257. https://doi.org/10.1016/j.cryobiol.2006.06.005.

Pi, C.H., Yu, G., Dosa, P.I., Hubel, A., 2019. Characterizing modes of action and interaction for multicomponent osmolyte solutions on Jurkat cells. Biotechnol. Bioeng. 116, 631–643. https://doi.org/10.1002/bit.26880.

Piasecka-Belkhayat, A., Skorupa, A., Paruch, M., 2024. Determining Thermophysical parameters of cryopreserved articular cartilage using evolutionary algorithms and experimental data. Materials 17, 5703. https://doi.org/10.3390/ma17235703.

Pigeau, G.M., Csaszar, E., Dulgar-Tulloch, A., 2018. Commercial scale manufacturing of allogeneic cell therapy. Front. Med. (Lausanne) 5, 233. https://doi.org/10.3389/fmed.2018.00233.

Pogozhykh, D., Pogozhykh, O., Prokopyuk, V., Kuleshova, L., Goltsev, A., Blasczyk, R., Mueller, T., 2017. Influence of temperature fluctuations during cryopreservation on vital parameters, differentiation potential, and transgene expression of placental multipotent stromal cells. Stem Cell Res Ther 8, 1–16. https://doi.org/10.1186/s13287-017-0512-7.

Poisson, J.S., Acker, J.P., Briard, J.G., Meyer, J.E., Ben, R.N., 2019. Modulating intracellular ice growth with cell-permeating small-molecule ice recrystallization inhibitors. Langmuir 35, 7452–7458. https://doi.org/10.1021/acs.langmuir.8b02126.

Posokhov, Y.O., Kyrychenko, A., 2013. Effect of acetone accumulation on structure and dynamics of lipid membranes studied by molecular dynamics simulations. Comput. Biol. Chem. 46, 23–31. https://doi.org/10.1016/j.compbiolchem.2013.04.005.

Preciado, J., Rubinsky, B., 2018. The effect of isochoric freezing on mammalian cells in an extracellular phosphate buffered solution. Cryobiology 82, 155–158. https://doi.org/10.1016/j.cryobiol.2018.04.004.

Pronk, P., Ferreira, C.A.I., Witkamp, G.J., 2005. A dynamic model of Ostwald ripening in ice suspensions. J. Cryst. Growth 275, e1355–e1361. https://doi.org/10.1016/j.jcrysgro.2004.11.173.

Pummer, B.G., Budke, C., Augustin-Bauditz, S., Niedermeier, D., Felgitsch, L., Kampf, C. J., Huber, R.G., Liedl, K.R., Loerting, T., Moschen, T., Schauperl, M., Tollinger, M., Morris, C.E., Wex, H., Grothe, H., Pöschl, U., Koop, T., Fröhlich-Nowoisky, J., 2015. Ice nucleation by water-soluble macromolecules. Atmos. Chem. Phys. 15, 4077–4091. https://doi.org/10.5194/acp-15-4077-2015.

Puts, C.F., Berendsen, T.A., Bruinsma, B.G., Ozer, S., Luitje, M., Usta, O.B., Yarmush, M. L., Uygun, K., 2015. Polyethylene glycol protects primary hepatocytes during supercooling preservation. Cryobiology 71, 125–129. https://doi.org/10.1016/j.cryobiol.2015.04.010.

Qi, Y., Zhao, L., Tang, H., Zhang, L., Gani, R., 2025. Computer aided formulation design based on molecular dynamics simulation: detergents with fragrance. Comput. Chem. Eng. 192, 108919. https://doi.org/10.1016/j.compchemeng.2024.108919.

Qiao, H., Ding, W., Sun, S., Gong, L., Gao, D., 2014. Theoretical optimization of the removal of cryoprotective agents using a dilution-filtration system. Biomed. Eng. Online 13, 120. https://doi.org/10.1186/1475-925x-13-120.

Raju, R., Bryant, S.J., Wilkinson, B.L., Bryant, G., 2021. The need for novel cryoprotectants and cryopreservation protocols: insights into the importance of biophysical investigation and cell permeability. Biochim. Biophys. Acta Gen. Subj. 1, 129749. https://doi.org/10.1016/j.bbagen.2020.129749.

Robinson, N.J., Picken, A., Coopman, K., 2014. Low temperature cell pausing: an alternative short-term preservation method for use in cell therapies including stem cell applications. Biotechnol. Lett. 36, 201–209. https://doi.org/10.1007/s10529-013-1349-5.

Roeters, S.J., Golbek, T.W., Bregnhøj, M., Drace, T., Alamdari, S., Roseboom, W., Kramer, G., Šantl-Temkiv, T., Finster, K., Pfaendtner, J., Woutersen, S., Boesen, T., Weidner, T., 2021. Ice-nucleating proteins are activated by low temperatures to control the structure of interfacial water. Nat. Commun. 12, 1183. https://doi.org/10.1038/s41467-021-21349-3.

Rogers, R.E., Haskell, A., White, B.P., Dalal, S., Lopez, M., Tahan, D., Pan, S., Kaur, G., Kim, H., Barreda, H., Woodard, S.L., Benavides, O.R., Dai, J., Zhao, Q., Maitland, K. C., Han, A., Nikolov, Z.L., Liu, F., Lee, R.H., Gregory, C.A., Kaunas, R., 2021. A scalable system for generation of mesenchymal stem cells derived from induced pluripotent cells employing bioreactors and degradable microcarriers. Stem Cells Transl. Med. 10, 1650–1665. https://doi.org/10.1002/sctm.21-0151.

Roobol, A., Carden, M.J., Newsam, R.J., Smales, C.M., 2009. Biochemical insights into the mechanisms central to the response of mammalian cells to cold stress and subsequent rewarming. FEBS J. 276, 286–302. https://doi.org/10.1111/j.1742-4658.2008.06781.x.

Ross-Rodriguez, L.U., Elliott, J.A.W., McGann, L.E., 2010a. Characterization of cryobiological responses in TF-1 cells using interrupted freezing procedures. Cryobiology 60, 106–116. https://doi.org/10.1016/j.cryobiol.2009.09.007.

Ross-Rodriguez, L.U., Elliott, J.A.W., McGann, L.E., 2010b. Investigating cryoinjury using simulations and experiments: 2. TF-1 cells during graded freezing (interrupted slow cooling without hold time). Cryobiology 61, 46–51. https://doi.org/10.1016/j.cryobiol.2010.04.005.

Roy, P., House, M.L., Dutcher, C.S., 2021. A microfluidic device for automated high throughput detection of ice nucleation of snomax®. Micromachines (Basel). 12, 1–18. https://doi.org/10.3390/mi12030296.

Saclier, M., Peczalski, R., Andrieu, J., 2010. Effect of ultrasonically induced nucleation on ice crystals' size and shape during freezing in vials. Chem. Eng. Sci. 65, 3064–3071. https://doi.org/10.1016/j.ces.2010.01.035.

Scholz, B.X., Hayashi, Y., Udugama, I.A., Kino-oka, M., Sugiyama, H., 2022. A multilayered approach to scale-up forced convection-based freezing of human induced pluripotent stem cells. Comput. Chem. Eng. 163, 107851. https://doi.org/10.1016/j.compchemeng.2022.107851.

Scholz, B.X., Hayashi, Y., Higashi, Y., Uno, Y., Gaddem, M.R., Kino-oka, M., Sugiyama, H., 2024. Computational fluid dynamics model-based design of continuous forced convection freezing processes for human induced pluripotent stem cells considering supercooling of extracellular solutions. Chem. Eng. Res. Des. 208, 674–682. https://doi.org/10.1016/j.cherd.2024.07.037.

Sciorio, R., Tramontano, L., Campos, G., Greco, P.F., Mondrone, G., Surbone, A., Greco, E., Talevi, R., Pluchino, N., Fleming, S., 2024. Vitrification of human blastocysts for couples undergoing assisted reproduction: an updated review. Front. Cell Dev. Biol. 12, 1398049. https://doi.org/10.3389/fcell.2024.1398049.

Shah, M., Krull, A., Odonnell, L., de Lima, M.J., Bezerra, E., 2023. Promises and challenges of a decentralized CAR T-cell manufacturing model. Front. Transplant. 2, 1238535. https://doi.org/10.3389/frtra.2023.1238535.

Shibamiya, A., Schulze, E., Krauß, D., Augustin, C., Reinsch, M., Schulze, M.L., Steuck, S., Mearini, G., Mannhardt, I., Schulze, T., Klampe, B., Werner, T., Saleem, U., Knaust, A., Laufer, S.D., Neuber, C., Lemme, M., Behrens, C.S., Loos, M., Weinberger, F., Fuchs, S., Eschenhagen, T., Hansen, A., Ulmer, B.M., 2020. Cell banking of hiPSCs: a practical guide to cryopreservation and quality control in basic research. Curr. Protoc. Stem Cell Biol. 55, e127. https://doi.org/10.1002/cpsc.127.

Shields, C.E., 1969. Effect of adenine on stored erythrocytes evaluated by autologous and homologous transfusions. Transfusion 9, 115–119. https://doi.org/10.1111/j.1537-2995.1969.tb05528.x.

Shitzer, A., 2011. Cryosurgery: analysis and experimentation of cryoprobes in phase changing media. J. Heat Transf. 133, 011005. https://doi.org/10.1115/1.4002302.

Shu, Z., Kang, X., Chen, H., Zhou, X., Purtteman, J., Yadock, D., Heimfeld, S., Gao, D., 2010. Development of a reliable low-cost controlled cooling rate instrument for the cryopreservation of hematopoietic stem cells. Cytotherapy 12, 161–169. https://doi.org/10.3109/14653240903377037.

Shu, Z., Hughes, S.M., Fang, C., Hou, Z., Zhao, G., Fialkow, M., Lentz, G., Hladik, F., Gao, D., 2016. Determination of the membrane permeability to water of human vaginal mucosal immune cells at subzero temperatures using differential scanning calorimetry. Biopreserv. Biobank 14, 307–313. https://doi.org/10.1089/bio.2015.0079.

Simasatitkul, L., Arpornwichanop, A., Gani, R., 2013. Design methodology for bio-based processing: biodiesel and fatty alcohol production. Comput. Chem. Eng. 57, 48–62. https://doi.org/10.1016/j.compchemeng.2013.01.018.

Solanki, P.K., Rabin, Y., 2022. Perspective: temperature-dependent density and thermal expansion of cryoprotective agents. Cryo-Letters 43, 1–9. https://doi.org/10.54680/fr22110110112.

Solanki, P.K., Rabin, Y., 2023. Is isochoric vitrification feasible? Cryobiology 111, 9–15. https://doi.org/10.1016/j.cryobiol.2023.03.007.

Solanki, P.K., Bischof, J.C., Rabin, Y., 2017. Thermo-mechanical stress analysis of cryopreservation in cryobags and the potential benefit of nanowarming. Cryobiology 76, 129–139. https://doi.org/10.1016/j.cryobiol.2017.02.001.

Soni, A., Patey, G.N., 2022. Ice nucleation by the primary prism face of silver iodide. J. Phys. Chem. C 126, 6716–6723. https://doi.org/10.1021/acs.jpcc.1c10227.

Sosso, G.C., Whale, T.F., Holden, M.A., Pedevilla, P., Murray, B.J., Michaelides, A., 2018. Unravelling the origins of ice nucleation on organic crystals. Chem. Sci. 9, 8077–8088. https://doi.org/10.1039/c8sc02753f.

Strober, W., 2019. Trypan blue exclusion test of cell viability. Curr. Protoc. Immunol. 111. https://doi.org/10.1002/0471142735.ima03bs111. A3.B.1–A3.B.3.

Stubbs, C., Murray, K.A., Ishibe, T., Mathers, R.T., Gibson, M.I., 2020. Combinatorial biomaterials discovery strategy to identify new macromolecular Cryoprotectants. ACS Macro Lett. 9, 290–294. https://doi.org/10.1021/acsmacrolett.0c00044.

Sugiyama, H., Shiokaramatsu, M., Kagihiro, M., Fukumori, K., Horiguchi, I., Kino-oka, M., 2020. Apoptosis-based method for determining lot sizes in the filling of human-induced pluripotent stem cells. J. Tissue Eng. Regen. Med. 14, 1641–1651. https://doi.org/10.1002/term.3127.

Sui, X., Wen, C., Yang, J., Guo, H., Zhao, W., Li, Q., Zhang, J., Zhu, Y., Zhang, L., 2019. Betaine combined with membrane stabilizers enables solvent-free whole blood cryopreservation and one-step Cryoprotectant removal. ACS Biomater Sci. Eng. 5, 1083–1091. https://doi.org/10.1021/acsbiomaterials.8b01286.

Sydykov, B., Oldenhof, H., Sieme, H., Wolkers, W.F., 2018. Storage stability of liposomes stored at elevated subzero temperatures in DMSO/ sucrose mixtures. PLoS One 13, e0199867. https://doi.org/10.1371/journal.pone.0199867.

Takahashi, T., Bross, J.B., Shaber, R.E., Williams, R.J., 1985. Effect of cryoprotectants on the viability and function of unfrozen human polymorphonuclear cells. Cryobiology 22, 336–350. https://doi.org/10.1016/0011-2240(85)90181-6.

Tamaki, R., Hayashi, Y., Uno, Y., Kino-oka, M., Sugiyama, H., 2025. A circular exploration of cryoprotective agents for stem cells using computer-aided molecular design approaches. Digit. Chem. Eng. 16, 100248. https://doi.org/10.1016/j.dche.2025.100248.

Taylor, R., Adams, G.D.J., Boardman, C.F.B., Wallis, R.G., 1974. Cryoprotection-permeant vs nonpermeant additives. Cryobiology 11, 430–438. https://doi.org/10.1016/0011-2240(74)90110-2.

Thanaskody, K., Jusop, A.S., Tye, G.J., Wan Kamarul Zaman, W.S., Dass, S.A., Nordin, F., 2022. MSCs vs. iPSCs: potential in therapeutic applications. Front. Cell Dev. Biol. 10, 1005926. https://doi.org/10.3389/fcell.2022.1005926.

Todrin, A.F., Kovalenko, I.F., Smolyaninova, Y.I., Timofeyeva, O.V., Popivnenko, L.I., Gordiyenko, O.I., 2023. Determination of cell membrane permeability coefficients: comparison of models in the case of oocytes. Cryobiology 113, 104789. https://doi.org/10.1016/j.cryobiol.2023.104789.

Toner, M., Cravalho, E.G., Karel, M., 1990. Thermodynamics and kinetics of intracellular ice formation during freezing of biological cells. J. Appl. Phys. 67, 1582–1593. https://doi.org/10.1063/1.345670.

Trad, F.S., Toner, M., Biggers, J.D., 1998. Effects of cryoprotectants and ice-seeding temperature on intracellular freezing and survival of human oocytes. Hum. Reprod. 14, 1569–1577. https://doi.org/10.1093/humrep/14.6.1569.

Traversari, G., Cincotti, A., 2021. Insights into the model of non-perfect osmometer cells for cryopreservation: a parametric sweep analysis. Cryobiology 100, 193–211. https://doi.org/10.1016/j.cryobiol.2020.11.013.

Tsuruta, T., Ishimoto, Y., Masuoka, T., 1998. Effects of glycerol on intracellular ice formation and dehydration of onion epidermis, in: annals of the new York Academy of Sciences. Ann. N. Y. Acad. Sci. 858, 217–226. https://doi.org/10.1111/j.1749-6632.1998.tb10155.x.

Tyagarajan, S., Schmitt, D., Acker, C., Rutjens, E., 2019. Autologous cryopreserved leukapheresis cellular material for chimeric antigen receptor-T cell manufacture. Cytotherapy 21, 1198–1205. https://doi.org/10.1016/j.jcyt.2019.10.005.

Uno, Y., Nakamura, K., Tobita, M., Mizutani, M., Watanabe, M., Kawai, K., Kino-oka, M., 2025. Basic points to consider for cell storage under the act on the safety of regenerative medicine in Japan. Regen. Ther. 30, 252–258. https://doi.org/10.1016/j.reth.2025.06.011.

Usta, O.B., Kim, Y., Ozer, S., Bruinsma, B.G., Lee, J., Demir, E., Berendsen, T.A., Puts, C. F., Izamis, M.L., Uygun, K., Uygun, B.E., Yarmush, M.L., 2013. Supercooling as a viable non-freezing cell preservation method of rat hepatocytes. PLoS One 8, e0069334. https://doi.org/10.1371/journal.pone.0069334.

Vysekantsev, I.P., Gurina, T.M., Martsenyuk, V.F., Petrenko, T.F., Kudokotseva, E.V., Koshchiy, S.V., Groshevoy, M.I., 2005. Probability of lethal damages of cryopreserved biological objects during storage. CryoLetters 26, 401–408 (PMID: 16547552).

Wang, X., Naib, A.A.I., Sun, D.W., Lonergan, P., 2010. Membrane permeability characteristics of bovine oocytes and development of a step-wise cryoprotectant adding and diluting protocol. Cryobiology 61, 58–65. https://doi.org/10.1016/j.cryobiol.2010.05.001.

Wang, L., Meng, H., Wang, F., Liu, H., 2024. Ice nucleation mechanisms and the maintenance of supercooling in water under mechanical vibration. Results Phys 59, 107581. https://doi.org/10.1016/j.rinp.2024.107581.

Wen, Y.Z., Su, B.X., Lyu, S.S., Hide, G., Lun, Z.R., Lai, D.H., 2016. Trehalose, an easy, safe and efficient cryoprotectant for the parasitic protozoan Trypanosoma brucei. Acta Trop. 164, 297–302. https://doi.org/10.1016/j.actatropica.2016.09.024.

Weng, L., Beauchesne, P.R., 2020. Dimethyl sulfoxide-free cryopreservation for cell therapy: a review. Cryobiology 94, 9–17. https://doi.org/10.1016/j.cryobiol.2020.03.012.

Weng, L., Li, W., Zuo, J., 2010. Kinetics of osmotic water flow across cell membranes in non-ideal solutions during freezing and thawing. Cryobiology 61, 194–203. https://doi.org/10.1016/j.cryobiol.2010.07.004.

Weng, L., Chen, C., Zuo, J., Li, W., 2011. Molecular dynamics study of effects of temperature and concentration on hydrogen-bond abilities of ethylene glycol and glycerol: implications for cryopreservation. J. Phys. Chem. A 115, 4729–4737. https://doi.org/10.1021/jp111162w.

Westen, T.V., Groot, R.D., 2018. Predicting the kinetics of ice recrystallization in aqueous sugar solutions. Cryst. Growth Des. 18, 2405–2416. https://doi.org/10.1021/acs.cgd.8b00038.

Whaley, D., Damyar, K., Witek, R.P., Mendoza, A., Alexander, M., Lakey, J.R.T., 2021. Cryopreservation: an overview of principles and cell-specific considerations. Cell Transplant. 30. https://doi.org/10.1177/0963689721999617, 0963689721999617.

William, N., Mangan, S., Ben, R.N., Acker, J.P., 2023. Engineered compounds to control ice nucleation and recrystallization. Annu. Rev. Biomed. Eng. 25, 333–362. https://doi.org/10.1146/annurev-bioeng-082222.

Wilson, P.W., Heneghan, A.F., Haymet, A.D.J., 2003. Ice nucleation in nature: Supercooling point (SCP) measurements and the role of heterogeneous nucleation. Cryobiology 46, 88–98. https://doi.org/10.1016/S0011-2240(02)00182-7.

Wolkers, W.F., Balasubramanian, S.K., Ongstad, E.L., Zec, H.C., Bischof, J.C., 2007. Effects of freezing on membranes and proteins in LNCaP prostate tumor cells. Biochim. Biophys. Acta Biomembr. 1768, 728–736. https://doi.org/10.1016/j.bbamem.2006.12.007.

Woods, E.J., Thirumala, S., Badhe-Buchanan, S.S., Clarke, D., Mathew, A.J., 2016. Off the shelf cellular therapeutics: factors to consider during cryopreservation and storage of human cells for clinical use. Cytotherapy 18, 697–711. https://doi.org/10.1016/j.jcyt.2016.03.295.

Wozniak, K., Reichelderfer, R., Ghaemi, S., Hupp, D., Fuzesi, P., Ringler, G., Marrs, R.P., Schiewe, M.C., 2024. Ultra-fast vitrification and rapid elution of human oocytes: part II À verification of blastocyst development from mature oocytes. Reprod. Biomed. Online 49, 104690. https://doi.org/10.1016/j.

Wragg, N.M., Tampakis, D., Stolzing, A., 2020. Cryopreservation of mesenchymal stem cells using medical grade ice nucleation inducer. Int. J. Mol. Sci. 21, 1–16. https://doi.org/10.3390/ijms21228579.

Wu, X., Yao, F., Zhang, H., Li, J., 2021. Antifreeze proteins and their biomimetics for cell cryopreservation: mechanism, function and application-a review. Int. J. Biol. Macromol. 192, 1276–1291. https://doi.org/10.1016/j.ijbiomac.2021.09.211.

Xing, W., Zhou, C., Bian, J., Montag, M., Xu, Y., Li, Y., Li, T., 2010. Solid-surface vitrification is an appropriate and convenient method for cryopreservation of isolated rat follicles. Reprod. Biol. Endocrinol. 8. https://doi.org/10.1186/1477-7827-8-42.

Xu, Y., Zhang, L., Xu, J., Wei, Y., Xu, X., 2014. Membrane permeability of the human pluripotent stem cells to me 2SO, glycerol and 1,2-propanediol. Arch. Biochem. Biophys. 550–551, 67–76. https://doi.org/10.1016/j.abb.2014.04.010.

Xu, Y., Chan, L.L.Y., Chen, S., Ying, B., Zhang, T., Liu, W., Guo, H., Wang, J., Xu, Z., Zhang, X., He, X., 2021. Optimization of UC-MSCs cold-chain storage by minimizing temperature fluctuations using an automatic cryopreservation system. Cryobiology 99, 131–139. https://doi.org/10.1016/j.cryobiol.2020.11.010.

Yamamoto, R., Kino-oka, M., 2021. Design of suspension culture system with bubble sparging for human induced pluripotent stem cells in a plastic fluid. J. Biosci. Bioeng. 132, 190–197. https://doi.org/10.1016/j.jbiosc.2021.04.010.

Yamamoto, R., Sakakibara, R., Kim, M.H., Fujinaga, Y., Kino-oka, M., 2024. Growth prolongation of human induced pluripotent stem cell aggregate in three-dimensional suspension culture system by addition of botulinum hemagglutinin. J. Biosci. Bioeng. 137, 141–148. https://doi.org/10.1016/j.jbiosc.2023.11.010.

Yang, J., Diaz, N., Adelsberger, J., Zhou, X., Stevens, R., Rupert, A., Metcalf, J.A., Baseler, M., Barbon, C., Imamichi, T., Lempicki, R., Cosentino, L.M., 2016. The effects of storage temperature on PBMC gene expression. BMC Immunol. 17, 1–15. https://doi.org/10.1186/s12865-016-0144-1.

Yehya, H., Raudins, S., Padmanabhan, R., Jensen, J., Bukys, M.A., 2024. Addressing bioreactor hiPSC aggregate stability, maintenance and scaleup challenges using a design of experiment approach. Stem Cell Res Ther 15, 191. https://doi.org/10.1186/s13287-024-03802-4.

Yi, J., Liang, X.M., Zhao, G., He, X., 2014. An improved model for nucleation-limited ice formation in living cells during freezing. PLoS One 9 (9), e98132. https://doi.org/10.1371/journal.pone.0098132.

Young, D.A., Gavrilov, S., Pennington, C.J., Nuttall, R.K., Edwards, D.R., Kitsis, R.N., Clark, I.M., 2004. Expression of metalloproteinases and inhibitors in the differentiation of P19CL6 cells into cardiac myocytes. Biochem. Biophys. Res. Commun. 322, 759–765. https://doi.org/10.1016/j.bbrc.2004.07.178.

Yu, G., Hubel, A., 2020. The role of preservation in the variability of regenerative medicine products. Regen. Eng. Transl. Med. 5, 323–331. https://doi.org/10.1007/s40883-019-00110-9.

Yuan, P., Dong, X., Wang, H., Wang, X., Gong, M., 2025. Modeling and typical cases analyze at the cell-scale of transmembrane transport and intracellular crystallization and recrystallization during the freeze-thaw process. Cryobiology 118, 105201. https://doi.org/10.1016/j.cryobiol.2025.105210.

Zachariassen, K.E., Kristiansen, E., Pedersen, S.A., Hammel, H.T., 2004. Ice nucleation in solutions and freeze-avoiding insects - homogeneous or heterogeneous? Cryobiology 48, 309–321. https://doi.org/10.1016/j.cryobiol.2004.02.005.

Zhang, A., Xu, L.X., Sandison, G.A., Cheng, S., 2006. Morphological study of endothelial cells during freezing. Phys. Med. Biol. 51, 6047–6060. https://doi.org/10.1088/0031-9155/51/23/007.

Zhang, Y., Na, T., Zhang, K., Yang, Y., Xu, H., Wei, L., Xu, L., Yan, X., Liu, W., Liu, G., Wang, B., Meng, S., Du, Y., 2022. GMP-grade microcarrier and automated closed industrial scale cell production platform for culture of MSCs. J. Tissue Eng. Regen. Med. 16, 934–944. https://doi.org/10.1002/term.3341.

Zhao, G., He, L., Zhang, H., Ding, W., Liu, Z., Luo, D., Gao, D., 2004. Trapped water of human erythrocytes and its application in cryopreservation. Biophys. Chem. 107, 189–195. https://doi.org/10.1016/S0301-4622(03)00211-4.

Zhou, X., Shu, Z., Ding, W., Heimfeld, S., Chung, J., Du, P., Liu, C., Gao, D., 2011. Heat transfer analysis for the design and application of the passive cooling rate controlled device - box-in-box. Int. J. Heat Mass Transf. 54, 2136–2143. https://doi.org/10.1016/j.ijheatmasstransfer.2010.12.014.

Zhou, Y., Fowler, Z., Cheng, A., Sever, R., 2012. Improve process uniformity and cell viability in cryopreservation. Bioprocess Int. 10, 70–76.