



REVIEWS

Off the shelf cellular therapeutics: Factors to consider during cryopreservation and storage of human cells for clinical use

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Abstract

The field of cellular therapeutics has immense potential, affording an exciting array of applications in unmet medical needs. One of several key issues is an emphasis on getting these therapies from bench to bedside without compromising safety and efficacy. The successful commercialization of cellular therapeutics will require many to extend the shelf-life of these therapies beyond shipping “fresh” at ambient or chilled temperatures for “just in time” infusion. Cryopreservation is an attractive option and offers potential advantages, such as storing and retaining patient samples in case of a relapse, banking large quantities of allogeneic cells for broader distribution and use and retaining testing samples for leukocyte antigen typing and matching. However, cryopreservation is only useful if cells can be reanimated to physiological life with negligible loss of viability and functionality. Also critical is the logistics of storing, processing and transporting cells in clinically appropriate packaging systems and storage devices consistent with quality and regulatory standards. Rationalized approaches to develop commercial-scale cell therapies require an efficient cryopreservation system that provides the ability to inventory standardized products with maximized shelf life for later on-demand distribution and use, as well as a method that is scientifically sound and optimized for the cell of interest. The objective of this review is to bridge this gap between the basic science of cryobiology and its application in this context by identifying several key aspects of cryopreservation science in a format that may be easily integrated into mainstream cell therapy manufacture.

Key Words: *cell manufacturing, cellular shelf life, cellular stability, cellular therapeutics, cryopreservation, stem cell therapy*

Introduction

Cryopreservation is a process of preserving biological function by freezing and storing material below -80°C , typically at or near the temperature of liquid nitrogen (LN_2 ; -196°C). Other methods of biopreservation could include vitrification, which refers to avoiding ice all together through ultra-fast cooling rates and high solute concentrations to form a metastable, amorphous glassy state; lyophilization, in which cells may be “freeze dried” to sublimate water and allow for stability at higher temperatures; or even vacuum drying without the freeze step to achieve a similar result. Although much research is being done on these

methods, cryopreservation via slow cooling methods remains the industry standard at this time and will be the focus of this review.

One of the most consistent findings from slow-cooling cryopreservation research on cells is the evidence-practice gap due to the failure to translate research into practice. As a result, cells are routinely cryopreserved using traditional, non-optimized methods while many scientific advances in basic cryopreservation science are not fully integrated into practice. As cellular therapy emerges, there are many challenges to its successful development and widespread use. Challenges in developing these products include product consistency, safety and potency as well as reliable

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storage and transport with high efficacy at reasonable economic cost.

Fundamental cryobiological research focuses on revealing the underlying physical and biological mechanisms related to cell injury occurring during the processes of preparing cells for preservation, bringing them down to a freezing point where biologically stable mode is reached with cessation of enzymatic and metabolic activity, and finally bringing the cells back to physiologically relevant temperatures. This methodology was developed as a way to define the discrete challenges a cell faces during the process, with an understanding that the greatest challenge lies not within their ability to withstand storage at ultra-low temperatures but with the transitional phases (ice nucleation, ice propagation during cooling to -60°C or lower, thawing) during which they experience potentially lethal physicochemical events, specifically those associated with phase change of water in both the extra- and intracellular environments. However, the apparent discrepancy between the concept of cryopreservation and experimental findings suggests that there are other numerous lethal events (often poorly defined and not fully recognized) in a preservation cycle that can inflict damage to the cells. There exists injury to the cells due to osmotic intolerance [1] or due to the toxicity of the cryoprotectants [2] or chilling or cold-shock injury associated with the reduction of temperature from room temperature to nucleation temperature [3] and cooling injury associated with water-to-ice phase change until

a glassy (i.e., ice crystal-free) state is achieved [4] (Figure 1). There also exists a hypothermic continuum and significant array of cellular stress events during warming/thawing or unexpected temperature excursions that may affect survival and function. A cumulative understanding of these injury mechanisms on cells immediate and delayed post-thaw survival and potency have become critical in development and implementation of optimized cryopreservation protocols for efficient manufacturing and banking of cellular therapeutics.

Another unavoidable fact is that not all therapeutic cells cryopreserve “equally.” Cells from various tissue sources maintain varying physical and biological properties and could be expected to react differently to cryopreservation. Although not practical to deal with, there will be further differences in cryopreservation outcomes when the cells are from varying donors of the same species. Additionally the metabolic state of the cells (e.g., lag vs. log phase; end of passage-interval vs. early or mid-interval) cell culture density, highly metabolic, cytokine driven populations vs. non-stimulated cultures, and cell age in an expanded primary cell culture with limited doubling potential could also play roles in cryopreservation outcomes [5,6]. Consequently, to optimize survival after cryopreservation, the protocols used need to be designed specifically for the particular cell type to be stored, rather than simply borrowing protocols that have been successfully used for cells derived from

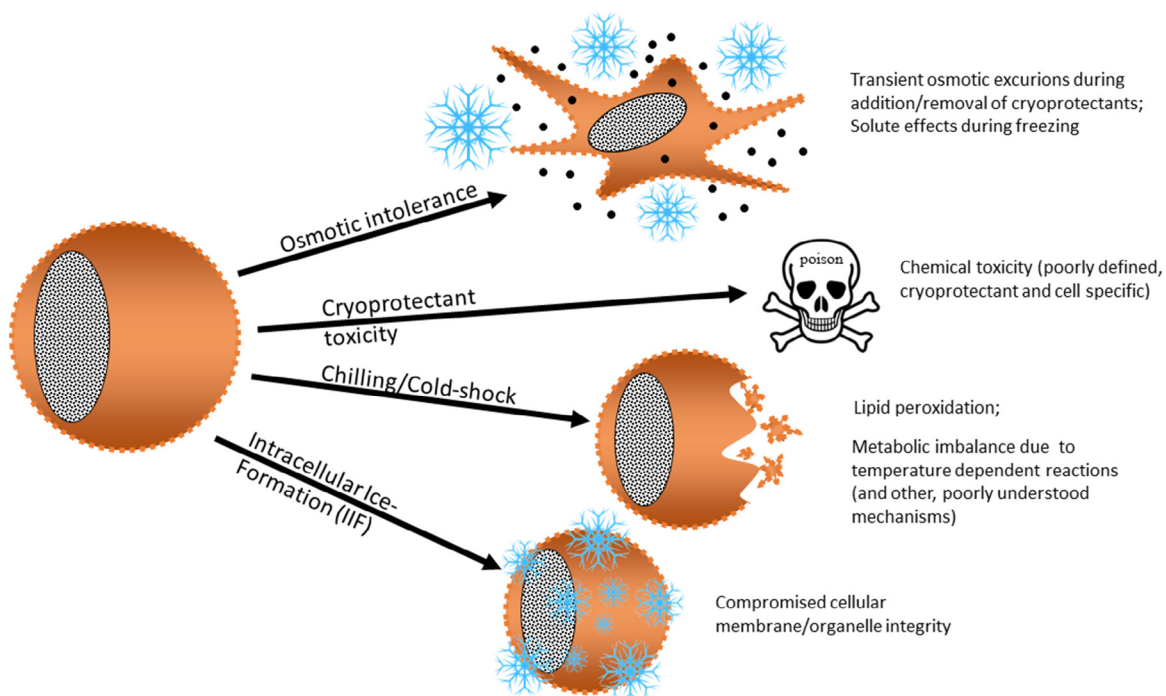


Figure 1. This representation depicts potential mechanisms of damage that can occur during cryopreservation processing.

other tissue sources. Other variables that can affect cryopreservation outcome may include type and concentration of cryoprotectants, concentration and cryopreservation container used for storage. This article reviews and discusses the modes of cryo-injuries associated with several phases of the preservation cycle to help the reader to understand and develop optimized procedures for long-term cryopreservation of therapeutic cells.

Critical elements and considerations for successful cryopreservation

Although many cells have been shown to respond similarly to the same cryopreservation process, a post-thaw cell loss of as much as 40% is still reported for some therapeutic cells [7–9]. Products such as umbilical cord blood may have a fair amount of neutrophils, and as such, overall viabilities may be much lower compared with frozen/thawed peripheral blood-derived mononuclear cells. Although loss of these cells is considered somewhat acceptable because they do not contribute to engraftment, non-optimally cryopreserved cells can result in significant post-thaw cell loss and limit the total dose available for administration. This is of particular concern in situations such as umbilical cord blood hematopoietic stem cells in which harvested volumes are small and the total number of therapeutic (CD34⁺) cells/kg body mass is crucial to ensuing engraftment in the recipient.

It is known that a given optimal cooling rate for any cell type depends on the following: (i) cell size or cell surface to volume ratio, (ii) cell membrane permeability (to water and cryoprotectant) and its temperature dependence, and (iii) osmotic limits of the cell. Additionally, the volume of the cell that does not respond to osmotic forces (osmotically inactive volume) is also cell dependent and can play a critical role in cryopreservation outcome [10,11]. Variations in any of these attributes exist not only among species but also between primary cells and cell lines of the same type. Hence cells with differences in these factors can be expected to have different optimal cooling rates. Empirical studies combined with theoretical modeling have been extensively published to define optimal cooling rates for multiple biological systems [1,12–16]. Defining optimal cooling conditions based on quantitative evaluation of critical cryobiological parameters is therefore important.

Cell concentration

To meet the high growth demand of mesenchymal-based cell therapy in particular, cell manufacturers are adopting new high-volume culture technology platforms such as microcarrier-based bioreactors to significantly increase output to hundreds of billions

to trillions of cells [17,18]. These high harvest volumes and cell numbers will eventually need to be successfully cryopreserved and stored. Cryopreservation of the large cell numbers frequently used in therapeutic procedures generates large product volumes requiring immense storage space that can potentially be impractical and uneconomical. Moreover, infusion-related toxicities are partially attributed to the total infusion volume and the cryopreservatives in the solution. The investigation of higher cell concentrations with a smaller corresponding storage volume has been widely investigated in blood product cryopreservation.

Previously, the recommended nucleated cell concentration in cryopreserved hematopoietic stem cells was suggested to be not more than 20 million cells per milliliter, leading to a total storage space of 7 L of cryopreservation volume per patient [7,19]. This prompted researchers to focus on minimizing the cryopreservation volume per each patient, and as a result, several subsequent preclinical studies have shown that higher nucleated cell concentrations (up to 200 million cells/mL or 0.7 L of cryopreservation volume per patient) in the cryopreservative are well tolerated and result in good clinical outcome [7,20]. However, with non-hematopoietic adult stem cells such as mesenchymal stromal cells, no comprehensive study has yet reported the effects of increasing cell concentration during cryopreservation and the resulting clinical outcome [21].

Several earlier studies reported that the probability of intracellular ice formation (IIF), which is considered a major contributor to cell damage, will increase during freezing with increasing cell concentration. When the cells are tightly packed to a point analogous to interconnecting cellular structures such as compact tissue configurations, the consequential freezing lethality can arise from the stresses on intercellular tight junctions, the reduced ability of cells to repair sub-lethal damage after cryopreservation or the increased intracellular ice formation via cell-cell contact [22,23]. Furthermore, densely packed cells are likely to be damaged by undue mechanical stresses when the channels within which they are sequestered decrease in size as a result of decreasing unfrozen fraction and thus, increasing potential cell-cell interaction. Another major concern about freezing large numbers of highly concentrated cells is the risk of cell clumping occurring immediately before freezing or after thawing. Several products such as EDTA, ACD, DNase and others at varying concentrations have been suggested to prevent cellular clumping. Such materials may be acceptable for cell banking or frozen intermediates; however, for the final drug product, the unwanted side effects of these as excipients are not well studied in clinical applications [7] (although ACD does have a track record in clinical applications).

Despite some of these potential downsides, freezing highly concentrated cells is beneficial if not compulsory in large-scale therapeutic cell banking. For allogeneic cell therapies, increasing cell concentration during cryopreservation has several undeniable benefits. It effectively reduces the storage space and associated costs and potentially eliminates the cell washing post-thaw, preventing cell loss due to additional centrifugation steps. By storing the final drug product in a reduced overall volume, the amount of dimethyl sulfoxide (DMSO) infused (directly or following dilution) is correspondingly reduced to potentially mitigate infusion-related toxicities. Additionally, inoculating large bioreactors for cell lot expansions often requires seeding a large number of cells from a working cell bank. Thawing large number of cryovials from a working cell bank is time-consuming and labor intensive. Increasing the viable cell density post-thaw per vial will reduce or eliminate the need for handling multiple frozen vials, thus increasing process flexibility for large-scale expansion.

Cold shock and chilling injury

Most cryopreservation protocols begin with random cooling from normal physiological temperature to room temperature (20–25°C) and then to a few degrees below nucleation temperature of the solution. During this hypothermic range, cells may be damaged by cooling rate (cold shock) or damaged by low temperature per se (chilling injury). Cold shock is essentially caused by rapid cooling and is usually mitigated by reducing the cooling rate. On the other hand, chilling injury is poorly understood and is predominantly dependent on the absolute temperature to which the cells are cooled and usually associated with extended periods of exposure to low temperatures. There have been studies on the biochemistry of chilling injury in natural systems, but not much is known about the sources of chilling injury in cellular systems [24]. Decisive evidence of injury occurrence due to chilling or cold shock on biological systems has implicated chilling injury as one of the limiting factors for achieving optimal cryopreservation [25]. Nevertheless, there has been limited awareness on the implications of chilling or cold shock injury during therapeutic cell cryopreservation. It is evident that temperature drop will reduce metabolism and, in turn, cause biochemical imbalances [26,27]. Chilling causes the membrane lipids that are normally in a liquid crystalline state at physiological temperature to transform to a solid-gel phase with lateral separation of the membrane proteins. This membrane phase transition can change their function and induce changes in ion homeostasis and transmembrane imbalances, causing leakage of lysosomal and lipoprotein hydrolases [28,29]. A sequence

of damaging events may follow, such as the production of reactive oxygen species that increase damage to membranes, reduced rates of protein synthesis, the release of free fatty acids, activation of apoptotic events and disruption of the cytoskeletal matrix [29–33]. The accumulation of the multiple cell stress mechanisms can result in cell damage and death via necrosis, apoptosis and secondary necrosis [34–36].

Apparently cooling does provide “short-term” *in vitro* and *ex vivo* survival mainly through the decrease in metabolism and the reduction in oxygen and nutrient demand, which works to minimize the amount of ATP depletion and free radical production that occurs during hypoxia. However, without manipulative intervention, this survival is only temporary, and a progressive injury is inevitable. The biological phenomena at chilling temperatures have been extensively studied and used as a basis to develop solutions for improved stability and hypothermic preservation of cells, tissues and organs [26,35]. However, little is known on the compounding effect of hypothermia and subsequent freezing when added to warming/thawing effects on adult stem cell survival and function (or thawing and subsequent hypothermic storage). The ability to enable improved stability of non-frozen cells and tissues has been shown to support the progression of a number of cellular therapeutic products without cryopreservation [37–40] and is actively being investigated as a method to support improved post-thaw stability to allow transport from a cryopreservation processing site to patient delivery without clinical onsite product thaw/manipulation (unpublished observation, A.J. Mathew, BioLife Solutions, 2015). It has also been well recognized that non-frozen stability limitations “would not only limit the locations a commercial product could be distributed, it would also affect the logistics of manufacturing and potentially reduce the capacity to manufacture the product” (CMC Review, BLA 125197, Sipuleucel-T [Provenge®], Dendreon Corporation).

Cooling injury

Supercooling. Theoretically, the thermodynamic equilibrium freezing point (aka melting point) of an isotonic solution is -0.5°C , and the freezing point depresses further with the addition of protective solutes. However, during cooling the solution can remain liquid below its freezing point with an increasing probability of ice formation with decreasing temperatures. The reason the solution can remain liquid below its freezing point is because the creation of a thermodynamically stable crystal is both temperature and energy dependent. At temperatures close to the freezing point, ice nuclei can form, but at these relatively high temperatures, the nuclei must be very large to be stable, which requires relatively

large amounts of energy. At lower temperatures, the size of stable ice nuclei is smaller and thus requires less energy to form. For these reasons, the likelihood of spontaneous freezing increases with decreasing temperature. This temperature dependent liquid–crystal interface is associated with the interfacial free energy barrier [41]. In many cases, spontaneous ice nucleation will not occur near the melting point, and further supercooling is inevitable, unless deliberately initiated [42]. Spontaneous nucleation under excessive supercooling has a substantial effect on viability and recovery of cells. For example, in a cell suspension supercooled to -8°C , spontaneous nucleation will result in a latent heat release that will push the temperature of the sample back to its melting point. Subsequently, the temperature of the sample changes more rapidly to its surrounding temperature, however, because the temperature difference between the sample and its surroundings is relatively small, the cooling rate is still slow and non-lethal. By contrast, if a sample is supercooled to -15°C , spontaneous nucleation will result in a large amount of latent heat release followed by a rapid rate of cooling to “catch up” to its surrounding temperature. This rapid rate of cooling over a larger temperature difference between the melting point and the environment can be damaging to cells and possibly result in cellular damage through intracellular crystallization. Another problem with supercooling-associated latent heat release is that when samples of large volume with low surface-to-volume

ratios are frozen, the release of latent heat may cause a long isothermal plateau in the sample being frozen. This extended isothermal plateau will increase the temperature difference between the sample and the surroundings, resulting in rapid non-optimal sample cooling (Figure 2). The rapidness of cooling is further intensified by the higher conductivity of ice than supercooled water. So from a practical perspective, the cells undergoing supercooling during cryopreservation have to withstand a series of large and abrupt temperature changes. Therefore, initiation of ice crystallization in a controlled manner near the melting point is a key factor to avoid supercooling-connected cellular damage.

During large-scale cryopreservation, ice crystallization around the freezing point is critical for several reasons. The spontaneous ice formation in solutions far below their freezing points can occur at random, unpredictable temperatures, and survival rates will be highly variable among samples frozen using the same freezing protocol. Extremely rapid crystallization due to severe supercooling can also damage cells by inducing mechanical stresses from uneven expansion and contraction, leading to unfavorable post-thaw results [43]. Furthermore, ice nucleation near the freezing point is required to initiate membrane transport for vital cellular dehydration (see the next section for the significance of cellular dehydration for optimal cell survival) at slow cooling rates. Because cell membrane permeability decreases with lowering temperature,

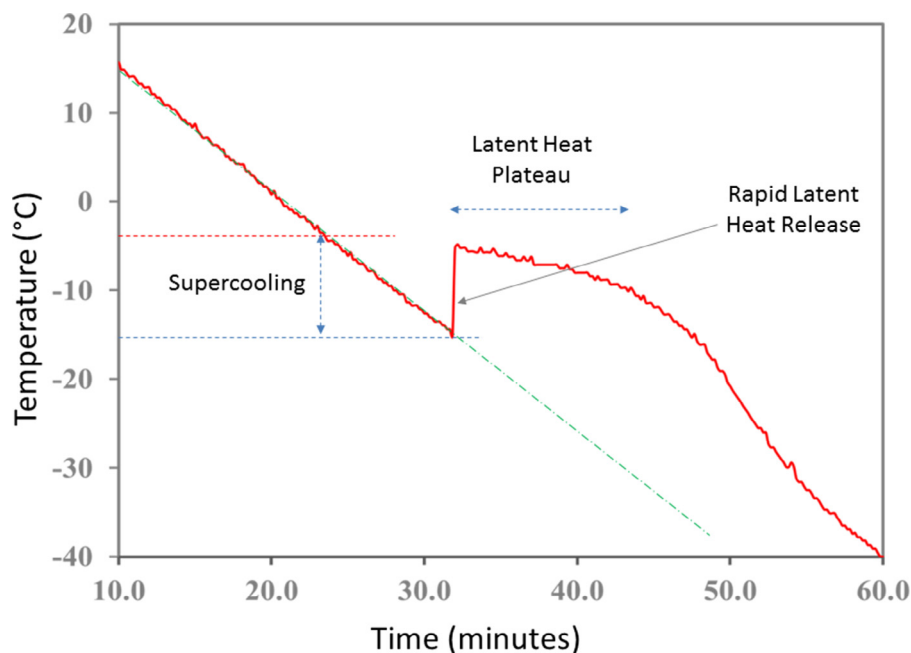


Figure 2. This schematic indicates how sample supercooling can be observed on a time temperature profile (solid line). The equilibrium melting point of the sample is shown as a red dashed line. When the supercooled solution nucleates, the release of latent heat of crystallization will result in an increase in sample temperature toward the equilibrium melting.

cellular dehydration can only occur within a certain range of subzero temperatures near the freezing point. The sum of these effects support the concept that ice nucleation near the freezing point is a critical parameter to consider in designing an optimal freezing protocol for cells.

Slow or Rapid Cooling Injury. As ice forms near the phase change temperature, the partially frozen extracellular solution becomes lower in chemical potential than the supercooled intracellular solution [44]. This results in a thermodynamically non-equilibrium state that provides a driving force for the two biophysical processes during freezing, cellular dehydration and the formation of intracellular ice [4]. The rate of cooling has a dramatic effect on these two biophysical processes (Figure 3).

Slow cooling supports the steady growth of extracellular ice, which allows for the osmotic efflux of water from the cell while driving up the intracellular viscosity toward forming glass. As ice crystals grow, solutes are excluded and the extracellular solution becomes more concentrated and prolonged exposure of cells to these concentrated solutes can have a detrimental effect. On the other hand, if the cell is cooled too rapidly, adequate cellular dehydration will not occur, and water gets trapped inside the cells. The trapped water continues to supercool while the probability of lethal intracellular ice formation increases. Cooling rates that are either “too slow” or “too fast” reduce the post thaw survival of cells. Therefore, a

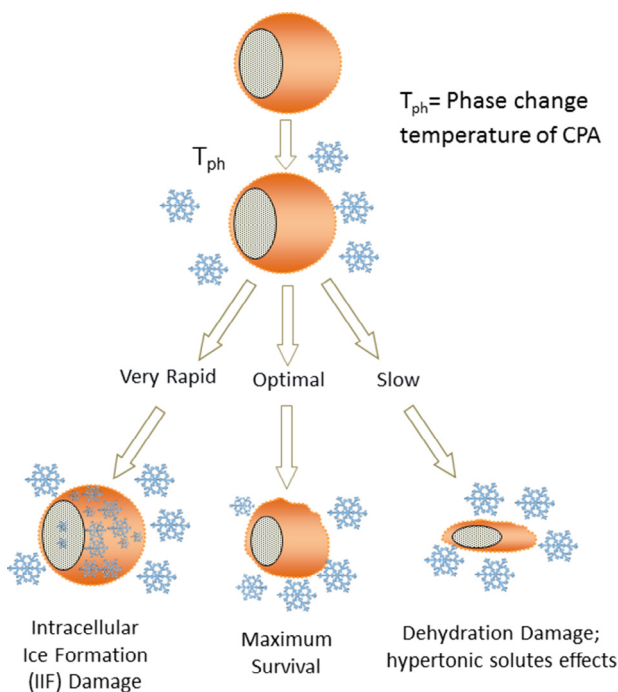


Figure 3. Cooling rate dependent injury mechanisms after extracellular ice formation are shown. Adapted from Mazur [44].

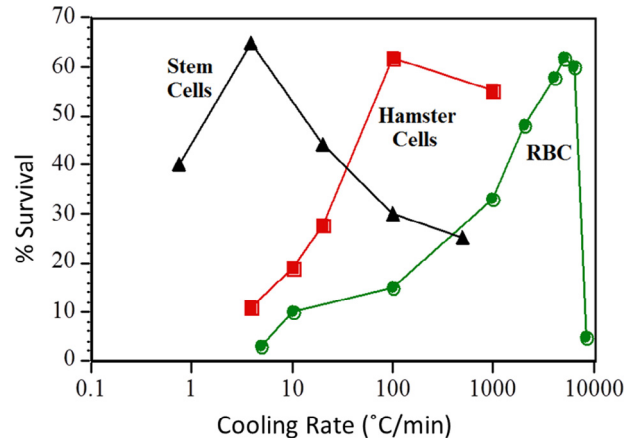


Figure 4. Peak survival of cells over varying cooling rates.

cooling rate for maximum cell survival exists between the “high” and “low” rates [45]. This has been confirmed experimentally for a variety of cells, and the curve of cell survival plotted as a function of the cooling rate has a characteristic peak, as shown in Figure 4 [46]. Permeability of the membrane indicates how fast the water can leave the cell at a given cooling rate. Therefore, whether a prescribed cooling rate is too “slow” or too “fast” is a function of cell membrane permeability and the probability that any water remaining trapped within the cell at a given subzero temperature will nucleate and turn to ice.

Because the difference in membrane permeability and probability of IIF results in different optimal cooling rates for different cells, quantitative understanding of water transport across the cell membrane during cryopreservation is critical for the success of a respective protocol. Water transport during freezing of cell suspensions has been extensively studied using various methods [45,47–49]. Furthermore, with the addition of cryoprotectants, the membrane permeability reduces [50], and therefore the ability to predict membrane permeability in the presence of cryoprotective agents (CPA) is also important.

Storage injury

Shelf life and stability. Stability of preserved cellular therapeutics is very relevant from a clinical perspective. In general, the liquid phase of the nitrogen (LN_2) should be considered the most stable storage temperature and is considered the ultimate method of choice for long-term storage. However, storage in liquid nitrogen can potentially be undesirable because LN_2 can act because a vehicle for cross-contamination [51], forcing cell banks to primarily rely on storage in LN_2 vapor phase (it should, however, be noted that risk of cross-contamination has also been reported with LN_2 vapor storage from environmental contaminants) [52].

Once a cell is taken to cryogenic temperatures, biological time all but ceases. Liquid water does not exist below -130°C ; the only states that do exist are crystalline or glassy, and in both cases, the viscosity is so high ($>10^{13}$ as opposed to 10^{-4} poises at 20°C) that diffusion is not significant over less than geological time spans [53]. Ordinary chemical reactions (e.g., those requiring molecular motion) simply do not occur [54]. The only types of reactions that can occur in aqueous systems at cryogenic temperatures are photophysical events as a result of background ionizing radiation. Ionization occurs when the absorbed radiation has enough energy to eject one or more orbital electrons from an atom/molecule. This can cause direct damage when a target molecule itself (such as DNA) reacts with radiation. Indirectly, in aqueous systems, radiolysis of water through ionization can lead to generation of free radicals, which can then form radicals with other molecules causing damage [54–56]. Background ionizing radiation has been estimated to be around 1.0–2.0 mSv/yr (or 0.1–0.2 rad/yr) depending on location on the planet, which arise primarily through natural causes such as cosmic rays and terrestrial radiation sources [57].

Given that background ionizing radiation is the only potential for damage to a properly cryopreserved cell, analysis of the effects of this becomes relevant in the context of determining shelf life. Historically, this has been studied more thoroughly in microorganisms such as yeast (e.g., *Saccharomyces cerevisiae*) and bacteria (e.g., *Escherichia coli*) [54]. Stapleton and Dington (1956) and Wood and Taylor (1957) showed that yeast and *E. coli* could be exposed to about three times the tolerable dose of x-rays at room temperature when the cells were frozen to less than -30°C [53,58,59]. Wood and Taylor also showed that yeast frozen in the presence of oxygen is somewhat less sensitive to x-rays than yeast irradiated at room temperature under anoxic conditions at high dose rates before significant repair can be initiated, and in that sense, it is analogous to the long-duration, low-rate irradiation that cells are subjected to at liquid nitrogen (LN_2) temperature (-196°C) [54].

In studies of mammalian cells, the D_0 (dose that gives an average of one hit per target, yielding 37% survival over the exponential portion of a survival curve) at physiological temperatures ranges from 10 rads for the early mouse oocyte [60] to 30 rads for mouse spermatogonia [61], to somewhat less than 100 rads for 1-cell fertilized mouse embryos in the pronuclear stage [62], to 100 and 150 rads for various cultured cells [63]. These data apply to cells in ambient air. Under anoxic conditions, the D_0 for cultured cells is 2 to 2.5 times higher, or some 200 to 400 rads [63]. Burki and Okada (1970) reported that the D_0 for frozen mouse leukemic cells irradiated at 400 rads/min at -79°C is 300 rads [53,64]. This is in agreement with

the concept that irradiating frozen cells is somewhat analogous to irradiating anoxic cells at physiological temperatures. On the basis of this information and considering background radiation of 0.1 rad/yr, Mazur (1976) estimated the attainment of a dose equal to D_0 in mammalian cells stored at -196°C to be between 200 and 3000 years [53].

From a practical standpoint, Whittingham et al. (1977) first proposed what amounted to a method of accelerated age testing of cryopreserved cells using increased radiation exposure to simulate extended, long-term cryostorage [65]. For these studies, frozen mouse embryos were stored in liquid nitrogen freezers and placed at three distances from a small radium source. The liquid nitrogen levels were kept constant and the freezers were partially rotated weekly to allow uniform exposure. The radiation doses investigated amounted to 1.8 \times , 9 \times and 84 \times the background dose level at the experimental site. The investigators regarded the 1.8 \times dose as the control to account for variation among background radiation level from different geographic locations around the world. When the background level of radiation was increased ninefold, no adverse effect was found on embryo storage for up to 2.5 years (equivalent to approximately 20 years of storage in ambient background radiation). Additionally, while implantation rates were lower at 84 \times the background dose for the longest exposure time (equivalent to approximately 184 years of storage under ambient radiation), survival immediately upon thawing did not seem to be affected [65].

Another more recent study investigating the consequences of irradiating cryopreserved peripheral blood mononuclear cells (PBMC) revealed that frozen cells actually tolerated irradiation better than those exposed at room temperature [56]. This has been observed previously and some radiation protection from low temperature directly has been described for cultured human kidney cells [66] and HeLa cells [67,68]. In this case, the frozen state reduces or eliminates the secondary chemical consequences of x-rays arising from diffusible substances and leaves only the effects of direct ionizations. There are also potential chemical protective effects of CPAs such as DMSO and glycerol [69] that further increase radiation tolerance. Consistent with the previously mentioned studies on microorganisms, investigators studying irradiation of cryopreserved HeLa Cells or Chinese hamster cells cryopreserved in DMSO to LN_2 temperatures observed 3.3 to 3.5 times the tolerable radiation dose as compared with 22°C exposure [69,70]. Results of these studies indicated that it would require approximately 30,000 years of storage of cells in 10% DMSO at -196°C before and accumulated x-ray dose from background radiation would reach a level that, upon cellular rehydration and resurrection, would result in

the equivalent lethal and chromosomal damage of an acute (e.g., 90% dead, or D_{10}) dose [69].

With the preponderance of scientific data pointing towards the ability for cryopreserved cells to remain stable hundreds if not tens of thousands of years, the question remains as to why some literature reports have indicated drops in survival after extended periods of cryostorage time [71]. The most likely reason for this apparent phenomenon is inappropriate maintenance of storage. Cell pre-cryo preparation, cryopreservation procedures, and post-thaw treatment are normally controlled; however, sample storage can be undefined and uncontrolled, with temperature fluctuations occurring during sample transfer to LN_2 tanks, sample storage, sample sorting and sample removal [72]. This is particularly the case in clinical environments not purpose-designed for long-term cryostorage. To ensure the long-term viability of stored cells, it is important to ensure that storage areas are well controlled with regular checks on LN_2 levels and vessel-filling rotations as well as staff training [73].

When cells are stored in frames (for bags) or racks (in the case of cryo-vials), a frame or rack containing several to potentially hundreds of samples is transiently removed from storage for removal of the desired sample and then returned to storage. This exposes some samples to several if not hundreds of transient warming events. To better understand the impact of such multiple temperature fluctuations on cells, a recent study investigating peripheral blood stem cell (PBMC) storage evaluated the results of 400 such cycles [72]. In this study, the investigators compared ideal LN_2 vapor storage (reported as $-135^\circ C$), cycling to $+20^\circ C$ for 5 min, and cycling under a protective hood to mitigate temperature fluctuations. In this study, the samples removed from storage without protection reached a temperature of $-60^\circ C$ before being placed back into LN_2 vapor. Results of this study indicated a significant reduction both in gross viability as measured by dye exclusion assay as well as T-cell-specific response in the samples that were cycled without protection. Repeated temperature shifts led to a decrease in all measured parameters [72].

For these reasons, when developing a shelf-life estimate for cryopreserved cells, if cryopreservation processing methods are optimized and have been validated, and assuming a storage temperature of $<130^\circ C$, it is more appropriate to base the shelf life on how many transient events will occur (either due to LN_2 storage tank level or sample removal) than on time itself.

Thawing/warming injury

Cells that have survived cooling and extended storage at low subzero temperatures are still challenged during

thawing, which can have effects as profound as the effects of cooling rate [4]. Suboptimal warming is often a root cause of cryoinjury in cryopreserved cells and tissues. Although there are several potential mechanisms of cellular damage that may occur during thawing from a frozen state, cryoinjury may result in part from recrystallization (also known as Ostwald ripening). This phenomenon occurs when innocuous extra- or intracellular ice formed during freezing melts and coalesces into larger, more damaging crystals during a temperature excursion or suboptimal warming procedures (typically slow warming). Although the amount of ice stays constant, change in ice shape and size can cause injury to cells.

The relationship between the cooling and warming rates and the effects of thawing or warming depends on whether the prior rate of cooling has induced cellular dehydration or IIF. Previous research has shown that the thawing response to slowly cooled cells is often complex and highly variable and is more difficult to predict *a priori* [74]. In some studies, no differences have been observed between slow and rapid thawing, whereas in other studies, slow thawing is either better or worse than rapid thawing depending on the freezing conditions and/or cell type [75].

Thermodynamically, slow cooling favors the formation of fewer extracellular ice nuclei, from which a smaller number of ice crystals grow to a larger size. Sufficiently slow cooling will prevent IIF by dehydration and causes progressive freeze concentration leading to increase in viscosity, reduction in diffusion coefficients and ultimately a glassy matrix when no more water is available to crystallize. At slower cooling rates, the extent of dehydration and the subsequent end temperature at which the cells are plunged into LN_2 following slow cooling are influential on the optimal thawing rate used. For example, when cells are slowly cooled to $-40^\circ C$ followed by a plunge into LN_2 , a rapid thawing rate may be optimal. This is because at higher end temperatures, the cells still maintain residual freezable water that does not crystallize during subsequent plunge into liquid nitrogen but undergoes what is known as vitrification to form metastable glass. During this vitrification step, rather than the phase changing from liquid to solid by crystallization (ice), an amorphous solidified liquid is formed with little to no crystallization. Because the recrystallization process is time dependent, during slow thawing rates, the metastable vitrified water will exhibit devitrification and have sufficient time for re-growth to large intracellular crystals causing damage to the cells. On the other hand, during rapid thawing the probability of substantial devitrification and regrowth of damaging ice crystals is relatively low, due to the high speed and short time of thawing. When slow cooling is continued to $-60^\circ C$ or lower, cells will lose most if not

all freezable water and shrink in volume considerably. The intracellular freeze concentrate will therefore form a more stable glass following a plunge in LN₂ and consequently may not undergo devitrification or recrystallization regardless of the warming rate. In this case, the optimal warming rate will be more dependent on the balance between osmotic and solute effects. Rapid thawing of the cells may cause considerable differences in solute concentrations and rapid influx of the solvent (water) may induce lethal osmotic shock to the extensively dehydrated cells. However, slow thawing may cause longer exposure to concentrated solutes introducing solute related toxicity to the cells during the transition from a glassy state to a liquid phase. Therefore, for slow cooling cryopreservation, the thawing rate employed is dependent on the cell tolerance to osmotic and solute effect, which is cell type- and/or species-specific.

If the cells experience rapid cooling rates during cryopreservation, the probability of ice formation increases both inside and outside the cell, resulting in numerous ice crystals of smaller size. In this case, rapid thawing is almost exclusively required to avoid recrystallization of small intracellular ice crystals formed during the prior cooling step. The smaller ice crystals maintain high surface energies and tend to grow into harmful large ice crystals by recrystallization given sufficient time, as is the case with slow thawing rates. Therefore, a slow thaw following the use of rapid cooling would be expected to have detrimental effect on cells.

As discussed earlier, for slowly cooled cells, a wide range of thawing rates can be used depending on the conditions the cells experienced during the initial cooling step. Over the years, research on adult stem cells has revealed slow cooling combined with rapid thawing with agitation is optimal. However, in a large-scale bio-banking, where large batches of cells are normally cryopreserved, often at large volumes per vial or bag, achieving rapid thawing rates is particularly difficult because the thermal mass of the specimen dictates the warming rate that can be achieved. Additionally, where cells are densely packed for cryopreservation, the modified ice kinetics that occurs during cooling will have an effect on the thawing rate required for optimal survival.

Cryoprotective agents

The addition of CPAs is a common practice during cryopreservation to reduce the freezing and thawing-induced damage to the cells. The CPAs protect slowly frozen cells through colligative effects by mitigating the solute damage via suppression of salt concentration in the unfrozen fraction at a given temperature. It is also presumed that the increasingly high viscosity of CPAs during lowering of the temperature may

inhibit or retard damaging ice crystal growth. There is a large divergence in choice of CPAs, ranging from low molecular weight solutes (permeating) such as DMSO and glycerol, to sugars such as sucrose and trehalose (non-permeating), to high molecular weight polymers such as polyvinylpyrrolidone and hydroxyethyl starch. The effectiveness of a given CPA for a given cell type usually depends on the permeability of that cell to that CPA and its toxicity [76]. Currently the most widely used CPA is DMSO because it is known to permeate most cells well and has historically yielded better results post-thaw than other CPAs for most cell types, and it is also available in pharmaceutical- and pharmacopeia-grade preparations.

The base media used to prepare the CPA can also be important. Typically, an isotonic electrolyte solution approved for infusion, such as Isolyte (B. Braun Medical, Inc.) or PlasmaLyte (Baxter Healthcare, Ltd), may be used as the solvent to prepare a 5–10% concentration of DMSO. Although this system may be straightforward from a regulatory perspective, these isotonic salt solutions are devoid of proteins, fats and cholesterol and may not provide for the best cryopreservation outcomes. Historically, fetal bovine serum has been used either as a supplement or the base media for cryopreservation with good success; however, safety concerns have made this impractical for clinical use. Recent work has been done to replace fetal bovine serum with clinically reasonable protein sources, such as human serum albumin; however, many of the other factors present in more complex preparations are likely contributory for cryosurvival, such as lipids and cholesterol. This has prompted investigation into other animal sources, such as buffalo [77], and other human sources, such as human platelet lysates, which contain human albumin as well as lipids, cholesterol, cytokines and anti-inflammatory factors (unpublished observation, E.J. Woods, Cook Regentec, 2014).

Cytotoxic effect of CPAs

Despite the cryoprotection they offer, some CPAs can also be harmful to cells. For instance, DMSO is potentially cytotoxic and can have negative systemic effects *in vivo* when infused. This complicates direct use of thawed cells in clinical applications using previously cryopreserved cells with this compound. The clinical use of frozen-thawed hematopoietic stem cells (HSC) treated with DMSO has been shown to cause mild to serious adverse reactions in animals as well as in humans [78–83]. On the basis of these toxicity considerations, the removal of DMSO from frozen-thawed cells is often considered prudent before clinical use if large volumes of DMSO are included in the preparation to be delivered. International Conference on Harmonisation guideline Q3C defines DMSO as a

class 3 solvent (solvents with low toxic potential), and as such residual solvent levels of 50 mg per day or less would be accepted by regulators without justification; quantities greater than this would need to be evaluated for safety [84]. Cell-washing methods using isotonic salt solutions and centrifugation for DMSO removal are somewhat effective, particularly at laboratory scale, but introduce mechanical forces and osmotic stress, causing cell packing/clumping and potentially significant cell loss as well as putting burden on clinical sites and creating a potential break in aseptic conditions [8,85]. Taken together, this evidence suggests that for clinical applications, it is highly desirable to develop cryopreservation protocols either with lower concentrations of DMSO or with non-toxic alternatives to DMSO. Several studies have investigated, with promising results, either freezing with minimal concentrations of DMSO or replacing DMSO with non-toxic alternatives [86–88].

Osmotic effects of CPA

In addition to toxicity considerations, CPAs can also play a direct role in producing cryoinjury if they are not optimally added and removed in a cryopreservation process [67]. This is because CPAs can potentially induce osmotic injury to the cells during their addition and removal, and the higher the CPA concentration, the greater the likelihood of damage. While permeating CPAs do indeed penetrate cells, none to date have been identified that cross their membranes as quickly as water. The addition of a permeating CPA therefore causes the cell to undergo extensive initial dehydration due to osmotic efflux of water followed by rehydration due to influx of the CPA and water. During removal of a CPA, the cells at first swell due to the osmotic influx of water and then slowly return to initial isotonic volume as the CPA and water leave the cell. These repeated volumetric changes can result in significant loss of functional integrity and even cell death [89].

The rate at which a permeable CPA diffuses into the cells varies between the cryoprotectants and is also temperature and concentration dependent. For this reason, impermeable solutes such as sugars are often added to dilution media to prevent excessive osmotic swelling during post-thaw CPA removal. As mentioned earlier, CPA diffusion across the membrane is also a function of concentration and temperature. The higher the concentration, the faster and more extensive the osmotic shock. This may be overcome either by gradual or stepwise addition and dilution of CPAs before freezing and after warming [90–92]. Temperature also has a profound effect on permeation because CPAs tend to penetrate more rapidly at higher temperatures. CPAs also reduce the temperature at which ice crystallization first occurs, thereby extending the

osmotically driven dehydration time during freezing. Therefore, procedures for the addition and removal of a CPA must be optimized according to specific cell characteristics to ensure successful cryopreservation. The dynamics of cell volume changes must be maintained within the tolerable limits by carefully selecting the optimal type and concentration of a CPA along with a cooling rate that yields the optimal time necessary for diffusion of a CPA in and out of the cell during cooling in the presence of ice. For this, information is required regarding the osmotic tolerance limits of cells, defined as the extent of volume excursions cells can withstand before irreversible loss of function occurs. In addition, to optimize addition and removal of CPAs, it is also necessary to define other osmotic properties, such as osmotically inactive cell volume, hydraulic conductivity and solute permeability of the cell.

Factors pertaining to systems and devices

Although overlooked at times, especially earlier in the development process, the freezing/storage systems and devices used for cell therapies can play a critical role. A variety of aspects within the cold chain process should be considered for clinical and commercial development. The freezing equipment, product storage containers and transport logistics can vary significantly and effect product success. As products move from clinical trials into true manufacturing, scale becomes significant. Laboratory and even clinical trial scale is often orders of magnitude lower than what will be encountered as products reach mainstream clinical production. Many solutions have been proposed and are under development, some of which rely on technology initially conceived for food preservation, such as high throughput tunnel freezers and spiral freezers produced by Praxir, Linde and others. Such high-throughput controlled rate and storage devices will become crucial for the ultimate success of many cell-based products.

As discussed earlier, the use of cryogenic temperatures (below -150°C) is often applied for cell therapies. Liquid nitrogen or LN_2 vapor phase is the standard method for achieving cryogenic temperatures for storing stem cell products. These cell-based products are usually frozen down using a controlled-rate step-wise process using both manual and automated systems. Cooling rates between -0.5°C and -2.0°C per minute are often employed to minimize temperature shock and optimize cell viability and recovery. Traditional low-cost manual methods often use devices such as the Mr. Frosty container (Thermo Scientific) or the CoolCell device (BioCision). These systems are widely used and accepted within the research community. Such devices are typically maintained in a refrigerated environment before use. Cryovials are placed

into the chilled device, and then the entire device is moved to a -80°C freezer. They remain in the freezer for a minimum of 16 h or until the samples reach -80°C . The vials are then transferred from the device to an LN_2 tank for long-term storage. For clinical applications, larger and more expensive automated controlled-rate freezing systems are typically used. A number of systems are available, including solenoid driven LN_2 vapor systems (Planer, Planer PLC; CryoMed, Thermo Fisher Scientific) and sterling cycle driven systems (VIA Freeze, Asymptote). Many of these systems can be used for freezing contents in cryovials or cryobags. The automated systems are programmable, and the cooling chambers are typically cooled by LN_2 . As with the manual devices, the vials or bags are often chilled/frozen to -80°C and then transferred to LN_2 tanks or vapor storage systems for long-term storage.

A variety of container options exist for frozen storage of cell therapy products, but users should consider their requirements when determining the appropriate container. Frozen storage container consideration should include type of application (research or clinical), fill volume, temperature, aseptic filling and removal access, sterile containment, container biocompatibility, possible regulatory requirements and scalability options. Small volume products (5 mL or less) are typically best suited to be contained in vials because some product loss can occur in the attached tubing or ports if using cryobags. The use of cryovials for cryopreservation storage is standard practice, especially for research and development uses. However, traditional screw-cap cryovial containers are not ideal where clinical and larger scale applications are intended as they rely on open processing steps. If clinical applications are anticipated with small volumes, disposable closed-system cell freezing containers such as the CellSeal closed-system cryogenic vial (Cook Medical) or the Crystal closed vial (Aseptic Technologies) should be considered.

Standard bags and vials on the market are all produced using single-use thermoplastic materials (COC, EVA based, FEP, etc.) designed to withstand cryogenic temperatures. It should be noted that although bags are rated to cryogenic temperatures, the tubing (PVC, C-Flex) and connectors often used for connecting and filling them are not rated for use and storage at cryogenic temperatures. These components are commonly used for filling only and removed before freezing and storage. In addition, for clinical products, a full validation package or regulatory approvals may be warranted. Several bag systems currently commercially available include the CryoStore (Origen Biomedical), CryoMACS (Miltenyi Biotec) and Cell Freeze (Charter Medical) frozen storage containers.

In addition to frozen storage, transport logistics should also be considered when developing a frozen

storage protocol. If products are required to be transported from one location to another (i.e., from production site to patient), the optimal container, temperature and shipper, need to be considered, along with the proper testing and validation. For frozen products, two standard methods exist, dry ice or liquid nitrogen (LN_2) dry shippers. Both options are fairly common, but dry ice shippers maintain a temperature at approximately -80°C , whereas liquid nitrogen shippers maintain products temperatures at approximately -196°C . The length of transit time is important because dry ice shippers can generally maintain temperatures for 24–48 h, whereas LN_2 dry shippers can maintain temperatures for 10 days or more. Upon receipt of the cells, clinical sites need to be prepared to use the cells quickly or store them for some period of time before use. This can be accomplished through mechanical ultra-low freezers, liquid nitrogen vapor storage units, or potentially using protocols in which cells are warmed to -80°C and stored for some period before use. In the latter case, those protocols must be validated and may be cell- and system-specific and time dependent. It is important to note that traversing the T_g of a system creates the potential for ice nucleation, and the more nucleation intracellularly, the more potential for recrystallization upon thaw and subsequent cell damage. This is a time dependent process, that is, the longer the sample is held above the T_g , the more likely nucleation is to occur, but molecular motion at such temperatures is still slow. Proper secondary storage containers and packaging are also necessary to ensure the frozen storage containers and products are not damaged during transport.

A best practices systems approach to improved biopreservation

When developing methods for improved cryopreservation of cells, the focus tends to be on the ice-inhibiting cryoprotectant(s), the cooling rate steps, the storage temperature, and thawing. As mentioned earlier, there are potential stresses that can negatively precondition the cells before cryopreservation, and then mechanisms of cell damage or death that may manifest for hours to days post-thaw. Therefore, best practices to support a “systems” or risk-based approach to reduce the potential stresses that can result from biopreservation or stability intervals would likely benefit from focus. The focus should start with the source biologic material, including cell/tissue processing, non-frozen and/or frozen storage, packaging, thermal cycling or adverse warming events, transport integrity, and stability through patient application. Cells isolated and manufactured from the cellular starting material may require different cryopreservation conditions at different stages of product manufacturing (e.g., cell bank, frozen

intermediate, retained samples, final drug product) as their characteristics are altered.

Cellular therapies and regenerative medicine use cell and tissue products sourced from blood, bone marrow and various tissues. The clinical and commercial success of these products is potentially affected by stability limitations, which include transport of the source material before cell processing. This focus on potential upstream impact is consistent with the lessons learned from hypothermic organ preservation for transplant [26,93], which led to the development of improved hypothermic preservation solutions that were intracellular-like in chemical composition rather than isotonic-based [35,93].

Following isolation and processing, cells are prepared for storage in combination with a media formulation. As described earlier, for cryopreservation, the formulation includes a cryoprotectant agent (CPA) with the non-CPA components of the cryo-cocktail traditionally based on isotonic-based media such as serum, culture media or balanced salt solutions, with the potential addition of a protein. Recent focus on the hypothermic continuum (both during freezing and also thawing) as in organ preservation, and further design of the non-CPA components of the cryopreservation media with targeted inhibition of cellular stress, have resulted in intracellular-like

(non-isotonic) formulations with promising results and also the elimination of serum or protein [94,95].

Cell type-specific optimization of the cooling rate and the temperature of storage in relation to the glass transition temperature may improve the biopreservation outcome. Furthermore, an oft-neglected aspect of frozen storage is the limitation of thermal cycling or transient warming events. The potential stresses from this thermal variability have been shown to have a negative impact on cell stability [96]. In addition to the events of thawing detailed earlier, consideration should be given to the positive/negative impact of post-thaw wash steps (both in consideration to the cell product as well as to the manufacturing or clinical delivery workflow). Cells may be osmotically buffered (or not) by the media environment during and after thawing, they may be sensitive to post-thaw exposure to cryoprotectants, and they may also be significantly affected by potential stresses introduced during wash steps. All of these accumulated and isolated stresses subsequently impact post-thaw stability (yield, viability, cell/molecular integrity, and functionality).

Finally, in consideration to a risk-based approach to product development, it is advised to evaluate the Quality/Regulatory footprint of all components within the biopreservation system (media formulations, packaging, shipping containers, labeling, etc.) for

Table I. Summary of definitions of damage mechanisms and factors to consider associated with a cryopreservation process.

Damage mechanism	Description and factors to consider
Osmotic injury or toxic injury	Injury due to the addition and removal of cryoprotective agents. Cell-specific characteristics such as biophysical parameters (size, shape, membrane permeability to water and cryoprotectants, osmotically inactive water, osmotic and volumetric tolerance limits) should be considered.
Cold-shock injury	Injury due to an abrupt change in temperature. Cooling rate should be considered, with very slow (<0.5°C/min) cooling rates applied to cold shock-sensitive cells.
Chilling injury	Injury due to prolonged exposure to cold (but above cryogenic) temperatures. Absolute exposure time is the most critical factor to consider. If cells appear to be chilling sensitive but are tolerant of a cryoprotectant such as DMSO at warmer temperatures, strategies can be employed to perform cryoprotectant additions at or near room temperature and reduce the amount of time “chilled.”
Cooling injury	Injury associated with extracellular and intracellular ice formation. Factors to consider can be cell type-specific and include cooling rate, ice nucleation regimen, supercooling, end temperature before transfer to storage, cellular dehydration, intracellular ice formation and hypertonic solute toxicity.
Storage injury	Injury due to unwanted thermal fluctuations (transient warming events), cosmic rays and free radical formation. Factors to consider include the glass transition temperature of the cryoprotectant and careful maintenance of the storage temperature at all times. Properly cryopreserved and stored cells are viable indefinitely. Although practically challenging, if at all possible a sample should never be removed from cryostorage until it is to be used; otherwise temperature of the sample should be monitored throughout any temporary removal (such as removing a rack of vials or frame of bags). Additional considerations should include the use of closed system containers for storage (in vapor or liquid).
Thawing injury	Injury associated with warming sample from LN ₂ storage temperature to above phase change temperature. Potential recrystallization during warming should be considered. If slow cooling is used, a wide range of warming rates are likely acceptable; however, faster warming generally may result in less intracellular recrystallization.
Post-cryopreservation processing	Upon thaw, cells are in a potentially compromised state. Care must be given to appropriately prepare them for use. If a permeable cryoprotectant is used (such as DMSO), knowledge of cell-specific osmotic characteristics is important. Cells swell and may lyse upon removal of permeable cryoprotectants and may not survive one-step dilution. If cells are administered directly from thaw without dilution or a washing step, this is effectively a one-step dilution and may result in significant cell loss <i>in vivo</i> .

qualification within a Good Manufacturing Practice process and/or for clinical application. All of these potential stress points within the life cycle of the cell product (including packaging and cold chain management) from source material to patient application, affect the clinical therapeutic efficacy and commercial viability of the cell or tissue product. Additionally, becoming dependent on a sole supplier of a key excipient used in cryopreservation is a significant risk, which is why it is often necessary to identify multiple suppliers or have escrow agreements in place that provide the list of ingredients in case the vendor goes out of business so that the excipients can then be made for the end user by an alternative manufacturer.

In conclusion, successful cryopreservation is a critical and potentially overlooked component of successful cellular therapeutic development. A strong, science-driven approach to defining the optimal cryopreservation, storage, shipping and thaw parameters should be a central part of any such program. Some of the key damage mechanisms and associated factors to consider described here are summarized in Table I. It is important also to consider that in developing a cryopreservation process, variables should be adjusted not just on the basis of total cell recovery and gross viability, but on *functional* recovery, with the specific product efficacy requirements in mind to define what “functional” means to that specific cellular therapeutic product. If a robust cryopreservation and post-thaw processing system is developed, the product shelf life is essentially indefinite so long as temperature is maintained.

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References

- [1] Woods EJ, Liu J, Pollok K, Hartwell J, Smith FO, Williams DA, et al. A theoretically optimized method for cord blood stem cell cryopreservation. *J Hematother Stem Cell Res* 2003;12(3):341–50.
- [2] Fahy GM. The relevance of cryoprotectant “toxicity” to cryobiology. *Cryobiology* 1986;23(1):1–13.
- [3] Zenzes MT, Bielecki R, Casper RF, Leibo SP. Effects of chilling to 0 degrees C on the morphology of meiotic spindles in human metaphase II oocytes. *Fertil Steril* 2001;75(4):769–77.
- [4] Mazur P. Freezing of living cells: mechanisms and implications. *Am J Physiol* 1984;247:C125–42.
- [5] Mamidi MK, Nathan KG, Singh G, Thrichelvam ST, Mohd Usof NA, Fakharuzi NA, et al. Comparative cellular and molecular analyses of pooled bone marrow multipotent mesenchymal stromal cells during continuous passaging and after successive cryopreservation. *J Cell Biochem* 2012;113:3153–64.
- [6] Choi JS, Lee BJ, Park HY, Song JS, Shin SC, Lee JC, et al. Effects of donor age, long-term passage culture, and cryopreservation on tonsil-derived mesenchymal stem cells. *Cell Physiol Biochem* 2015;36:85–99.
- [7] Rowley SD, Bensinger WI, Gooley TA, Buckner CD. Effect of cell concentration on bone marrow and peripheral blood stem cell cryopreservation. *Blood* 1994;83:2731–6.
- [8] Berz D, McCormack EM, Winer ES, Colvin GA, Quesenberry PJ. Cryopreservation of hematopoietic stem cells. *Am J Hematol* 2007;82(6):463–72.
- [9] Lee S, Kim S, Kim H, Baek EJ, Jin H, Kim J, et al. Post-thaw viable CD34(+) cell count is a valuable predictor of haematopoietic stem cell engraftment in autologous peripheral blood stem cell transplantation. *Vox Sang* 2008;94(2):146–52.
- [10] Mazur P. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *J Gen Physiol* 1963;47:347–69.
- [11] Levin RL, Cravalho EG, Huggins CG. A membrane model describing the effect of temperature on the water conductivity of erythrocyte membranes at subzero temperatures. *Cryobiology* 1976;13:415–29.
- [12] Liu J, Zieger MAJ, Lakey JRT, Woods EJ, Critser JK. The determination of membrane permeability coefficients of canine pancreatic islet cells and their application to islet cryopreservation. *Cryobiology* 1997;35:1–13.
- [13] Woods EJ, Liu J, Gilmore JA, Reid TJ, Gao DY, Critser JK. Determination of human platelet membrane permeability coefficients using the Kedem-Katchalsky formalism: estimates from two- vs. three-parameter fits. *Cryobiology* 1999;38:200–8.
- [14] Thirumala S, Gimble JM, Devireddy RV. Transport phenomena during freezing of adipose tissue derived adult stem cells. *Biotechnol Bioeng* 2005;92(3):372–83.
- [15] Thirumala S, Ferrer MS, Al-Jarrah A, Eilts BE, Paccamonti DL, Devireddy RV. Cryopreservation of canine spermatozoa: theoretical prediction of optimal cooling rates in the presence and absence of cryoprotective agents. *Cryobiology* 2003;47:109–24.
- [16] Gilmore JA, Liu J, Woods EJ, Peter AT, Critser JK. Cryoprotective agent and temperature effects on human sperm membrane permeabilities: convergence of theoretical and empirical approaches for optimal cryopreservation methods. *Hum Reprod* 2000;15:335–43.
- [17] Pattasseril J, Varadaraju H, Lock L, Rowley JA. Downstream technology landscape for large-scale therapeutic cell processing. *Bioprocess Int* 2013;11:38–47.
- [18] Chen AK, Reuveny S, Oh SK. Application of human mesenchymal and pluripotent stem cell microcarrier cultures in cellular therapy: achievements and future direction. *Biotechnol Adv* 2013;31:1032–46.
- [19] Galmes A, Besalduch J, Bargay J, Novo A, Morey M, Guerra JM, et al. Long-term storage at -80°C of hematopoietic progenitor cells with 5% dimethyl sulfoxide as the sole cryoprotectant. *Transfusion* 1999;39:70–3.
- [20] Villalon L, Odriozola J, Ramos P, Ramos ML, Herrera P, de Oteyza JP. Cryopreserving with increased cellular

- concentrations of peripheral blood progenitor cells: clinical results. *Haematologica* 2002;87:ELT06.
- [21] Thirumala S, Fearnot E, Goebel WS, Woods EJ. A cryopreservation system for direct clinical use of MSC. *Cytotherapy* 2013;15(4):S46–7.
- [22] Mazur P, Cole KW. Influence of cell concentration on the contribution of unfrozen fraction and salt concentration to the survival of slowly frozen human erythrocytes. *Cryobiology* 1985;22(6):509–36.
- [23] Pegg DE, Diaper MP. The packing effect in erythrocyte freezing. *Cryo-Lett* 1983;4:129–36.
- [24] Morris GJ, Watson PF. Cold shock injury—a comprehensive bibliography. *Cryo-Lett* 1984;5:352–72.
- [25] Fahy GM, Wowk B, Wu J, Phan J, Rasch C, Chang A, et al. Cryopreservation of organs by vitrification: perspectives and recent advances. *Cryobiology* 2004;48(2):157–78.
- [26] Belzer FO, Southard JH. Principles of solid-organ preservation by cold storage. *Transplantation* 1988;45(4):673–6.
- [27] Toledo-Pereyra LH, Paez-Rollins AJ, Palma-Vargas JM. Science of organ preservation. In: Toledo-Pereyra LH, editor. *Organ procurement and preservation for transplantation*. Landes Bioscience; 1997. p. 1–16.
- [28] Wilson JM, McMurdo AC. Chilling injury in plants. In: Morris GJ, Clarke A, editors. *Effects of low temperature on biological membranes*. London: Academic Press; 1973. p. 285–309.
- [29] Baust JG, Gao D, Baust JM. Cryopreservation: an emerging paradigm change. *Organogenesis* 2009;5(3):90–6.
- [30] Raison JK. The influence of temperature-induced phase changes on the kinetics of respiratory and other membrane-associated enzyme systems. *J Bioenerg* 1973;4:285–309.
- [31] Stefanovich P, Ezzell RM, Sheehan SJ, Tompkins RG, Yarmush ML, Toner M. Effects of hypothermia on the function, membrane integrity and cytoskeletal structure of hepatocytes. *Cryobiology* 1995;32:389–403.
- [32] Russotti G, Brieva TA, Toner M, Yarmush ML. Induction of tolerance to hypothermia by previous heat shock using human fibroblasts in culture. *Cryobiology* 1996;33:567–80.
- [33] Clarke DM, Baust JM, Van Buskirk RG, Baust JG. Addition of anticancer agents enhances freezing-induced prostate cancer cell death: implications of mitochondrial involvement. *Cryobiology* 2004;49:45–61.
- [34] Mathew AJ, Hollister WR, Addona T, Baust JG, Van Buskirk RG. Vitamin E and EDTA Improve the Efficacy of Hypothermosol-Implication of Apoptosis. *In Vitro Mol Toxicol* 1999;12(3):163–72.
- [35] Mathew AJ, Baust JM, Van Buskirk RG, Baust JG. Cell preservation in reparative and regenerative medicine: evolution of individualized solution composition. *Tissue Eng* 2004;10(11–12):1662–71.
- [36] Baust JM. Molecular mechanisms of cellular demise associated with cryopreservation failure. *Cell Preserv Technol* 2002;1:17–31.
- [37] Bartunek J, Atta B, Dariouch D, Vanderheyden M, Ostojic M, Dens J, et al. Cardiopoietic stem cell therapy in heart failure. *J Am Coll Cardiol* 2013;61(23):2329–38.
- [38] Powell RJ, Comerota AJ, Berceci SA, Guzman R, Henry TD, Tzeng E, et al. Interim analysis results from the RESTORE-CLI, a randomized, double-blind multicenter phase II trial comparing expanded autologous bone marrow-derived tissue repair cells and placebo in patients with critical limb ischemia. *J Vasc Surg* 2011;54(4):1032–41.
- [39] Ginis I, Grinblat B, Shirvan MH. Evaluation of bone marrow-derived mesenchymal stem cells after cryopreservation and hypothermic storage in clinically safe medium. *Tissue Eng Part C Methods* 2012;18(6):453–63.
- [40] Povsic TJ, O'Connor CM, Henry T, Taussig A, Kereiakes DJ, Fortuin FD. A double-blind, randomized, controlled, multicenter study to assess the safety and cardiovascular effects of skeletal myoblast implantation by catheter delivery in patients with chronic heart failure after myocardial infarction. *Po Am Heart J* 2011;162(4):654–62, e1.
- [41] Mullen SF, Critser JK. The science of cryobiology. *Cancer Treat Res* 2007;138:83–109.
- [42] MacKenzie AP. Non-equilibrium freezing behaviour of aqueous systems. *Philos Trans R Soc Lond B Biol Sci* 1977;278(959):167–89.
- [43] Amir A, Natan Y. Transplantation of whole frozen-thawed ovaries. In: Ri-Cheng C, Quinn P, editors. *Fertility cryopreservation*. Cambridge: Cambridge University Press; 2010. p. 241–7.
- [44] Mazur P. Cryobiology: the freezing of biological systems. *Science* 1970;168(3934):939–49.
- [45] McGrath JJ. Membrane transport properties. In: McGrath JJ, Diller KR, editors. *Low Temperature biotechnology: emerging applications and engineering contributions*. New York: ASME Press; 1988. p. 273–330.
- [46] Mazur P. The role of intracellular freezing in the death of cells cooled at supraoptimal rates. *Cryobiology* 1977;14:251–72.
- [47] Diller KR, Cravalho EG. A cryomicroscope for the study of freezing and thawing processes in biological cells. *Cryobiology* 1970;7(4):191–9.
- [48] Pazhayannur PV, Bischof JC. Measurement and simulation of water transport during freezing in mammalian liver tissue. *J Biomech Eng* 1997;119(3):269–77.
- [49] Devireddy RV, Bischof JC. Measurement of water transport during freezing in mammalian liver tissue: Part II—The use of differential scanning calorimetry. *J Biomech Eng* 1998;120(5):559–69.
- [50] Karlsson JO, Cravalho EG, Borel Rinkes IH, Tompkins RG, Yarmush ML, Toner M. Nucleation and growth of ice crystals inside cultured hepatocytes during freezing in the presence of dimethyl sulfoxide. *Biophys J* 1993;65(6):2524–36.
- [51] Fountain D, Ralston M, Higgins N, Gorlin JB, Uhl L, Wheeler C, et al. Liquid nitrogen freezers: a potential source of microbial contamination of hematopoietic stem cell components. *Transfusion* 1977;37:585–91.
- [52] Grout BWW, Morris GJ. Contaminated liquid nitrogen vapour as a risk factor in pathogen transfer. *Theriogenology* 2009;71:1079–82.
- [53] Mazur P. Freezing and low-temperature storage of living cells. *Proceedings of the workshop on basic aspects of freeze preservation of mouse strains*. Stuttgart: Gustav Fischer Verlag; 1976.
- [54] Mazur P, Merryman HT, editor. *Cryobiology*. New York: Academic Press; 1966. p. 213–315.
- [55] Rice FO, Bass AM, Broida HP, editors. *Formation and trapping of free radicals*. New York: Academic Press; 1960. p. 7.
- [56] Cugia G, Centis F, Del Zotto G, Lucarini A, Argazzi E, Zini G, et al. High survival of frozen cells irradiated with gamma radiation. *Radiat Prot Dosimetry* 2011;143:237–40. doi:10.1093/rpd/ncq541.
- [57] Bibbo G, Piotta L. Background ionising radiation: a pictorial perspective. *Australas Phys Eng Sci Med* 2014;37(3):575–81. doi:10.1007/s13246-014-0286-5. [Epub 2014 Jun 28].
- [58] Stapleton GE, Edington CW. Temperature dependence of bacterial inactivation by X-rays. *Radiat Res* 1956;5(1):39–45.
- [59] Wood TH, Taylor AL. X-ray inactivation of yeast at freezing temperatures. *Radiat Res* 1957;7(2):99–106.
- [60] Oakberg RF. Gamma-ray sensitivity of oocytes of immature mice. *Proc Soc Exp Biol Med* 1962;109:763–7.
- [61] Oakberg EF. Gamma-ray sensitivity of spermatogonia of the mouse. *J Exp Zool* 1957;134(2):343–56.

- [62] Russell LB, Montgomery CS. Radiation-sensitivity differences within cell-division cycles during mouse cleavage. *Int J Radiat Biol Relat Stud Phys Chem Med* 1966;10(2):151–64.
- [63] Elkind MM, Whitmore GF. The radiobiology of cultured mammalian cells. Gordon and Breach. New York: Science Publishers; 1967.
- [64] Burki HJ, Okada S. Killing of cultured mammalian cells by radioactive decay or tritiated thymidine at -196°C . *Radiat Res* 1970;41(2):409–24.
- [65] Whittingham DG. Re-establishment of breeding stocks of mutant and inbred strains of mice from embryos stored at -196°C for prolonged periods. *Genet Res* 1977;30:287–99.
- [66] Vos O, Kaalen MCAC, Budke L. Radiation protection by a number of substances preventing freezing damage I. Protection of mammalian cells *in vitro*. *Int J Radiat Biol* 1965;9(2):133–42.
- [67] Belli JA, Bonte FJ. Influence of temperature on the radiation response of mammalian cells in tissue culture. *Radiat Res* 1963;18:272–6.
- [68] Dawson KB, Field EO, Jones HM. Indirect action of x-radiation on mammalian cells. *Nature* 1962;195:1012–13.
- [69] Ashwood-Smith MJ, Friedmann GB. Lethal and chromosomal effects of freezing, thawing, storage time, and x-irradiation on mammalian cells preserved at -196 degrees in dimethyl sulfoxide. *Cryobiology* 1979;16(2):132–40.
- [70] Nias AHW, Ebert M. Effects of single and continuous irradiation of HeLa cells at -196°C . *Int J Rad Biol* 1969;16:31–41.
- [71] Desrosiers P, Legare C, Lecler P, Sullivan R. Membranous and structural damage that occur during cryopreservation of human sperm may be time-related events. *Fertil Steril* 2006;85(6):1744–52.
- [72] Germann A, Oh Y-J, Schmidt T, Schon U, Zimmermann H, von Briesen H. Temperature fluctuations during deep temperature cryopreservation reduce PBMC recovery, viability and T-cell function. *Cryobiology* 2013;67:193–200.
- [73] Stacey GN, Day JG. Cryopreservation and freeze-drying protocols. In: Day JG, Stacey GN, editors. *Methods in molecular biology*, vol. 368. Second ed. 2007.
- [74] Gao D, Critser JK. Mechanisms of cryoinjury in living cells. *ILAR J* 2000;41(4):187–96.
- [75] Varma VP, Devi L, Venna NK, Murthy CLN, Idris MM, Goel S. Ocular fluid as a replacement for serum in cell cryopreservation media. *PLoS ONE* 2015;10:e0131291. doi:10.1371/journal.pone.0131291.
- [76] Dong Q, Hill D, VandeVoort CA. Interactions among pre-cooling, cryoprotectant, cooling, and thawing for sperm cryopreservation in rhesus monkeys. *Cryobiology* 2009;59(3):268–74.
- [77] Fuller BJ. Cryoprotectants: the essential antifreezes to protect life in the frozen state. *Cryo-Lett* 2004;25(6):375–88.
- [78] Alessandrino P, Bernasconi P, Caldera D, Colombo A, Bonfichi M, Malcovati L, et al. Adverse events occurring during bone marrow or peripheral blood progenitor cell infusion: analysis of 126 cases. *Bone Marrow Transplant* 1999;23:533–7.
- [79] Benekli M, Anderson B, Wentling D, Bernstein S, Czuczman M, McCarthy P. Severe respiratory depression after dimethylsulphoxide-containing autologous stem cell infusion in a patient with AL amyloidosis. *Bone Marrow Transplant* 2000;25:1299–301.
- [80] Higman MA, Port JD, Beauchamp NJ Jr, Chen AR. Reversible leukoencephalopathy associated with re-infusion of DMSO preserved stem cells. *Bone Marrow Transplant* 2000;26:797–800.
- [81] Windrum P, Morris TCM. Severe neurotoxicity because of dimethyl sulphoxide following peripheral blood stem cell transplantation. *Bone Marrow Transplant* 2003;31:315.
- [82] Windrum P, Morris TC, Drake MB, Niederwieser D, Ruutu T. Variation in dimethyl sulfoxide use in stem cell transplantation: a survey of EBMT centres. *Bone Marrow Transplant* 2005;36:601–3.
- [83] Zenhäusern R, Tobler A, Leoncini L, Hess OM, Ferrari P. Fatal cardiac arrhythmia after infusion of dimethyl sulfoxide-cryopreserved hematopoietic stem cells in a patient with severe primary cardiac amyloidosis and end-stage renal failure. *Ann Hematol* 2000;79(9):523–6.
- [84] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline, Impurities: Guideline for Residual Solvents Q3C(R5). Available at: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3C/Step4/Q3C_R5_Step4.pdf.
- [85] Thirumala S, Goebel WS, Woods EJ. Manufacturing and banking of mesenchymal stem cells. *Expert Opin Biol Ther* 2013;13(5):673–91.
- [86] Thirumala S, Gimble JM, Devireddy RV. Cryopreservation of stromal vascular fraction of adipose tissue in a serum-free freezing medium. *J Tissue Eng Regen Med* 2010;4(3):224–32.
- [87] Thirumala S, Wu X, Gimble JM, Devireddy RV. Evaluation of polyvinylpyrrolidone as a cryoprotectant for adipose tissue-derived adult stem cells. *Tissue Eng Part C Methods* 2010;16(4):783–92.
- [88] Thirumala S, Gimble JM, Devireddy RV. Evaluation of methylcellulose and dimethyl sulfoxide as the cryoprotectants in a serum-free freezing media for cryopreservation of adipose-derived adult stem cells. *Stem Cells Dev* 2010;19(4):513–22.
- [89] Mazur P, Schneider U. Osmotic responses of preimplantation mouse and bovine embryos and their cryobiological implications. *Cell Biophys* 1986;8(4):259–85.
- [90] Leibo SP. A one-step method for direct nonsurgical transfer of frozen-thawed bovine embryos. *Theriogenology* 1984;21(5):767–90.
- [91] Hunt CJ, Armitage SE, Pegg DE. Cryopreservation of umbilical cord blood: 2. Tolerance of CD34(+) cells to multimolar dimethyl sulphoxide and the effect of cooling rate on recovery after freezing and thawing. *Cryobiology* 2003;46:76–87.
- [92] Woods EJ, Benson JD, Agca Y, Critser JK. Fundamental cryobiology of reproductive cells and tissues. *Cryobiology* 2004;48:146–56.
- [93] Taylor MJ. Biology of cell survival in the cold: the basis for biopreservation of tissues and organs. In: Baust JG, Baust JM, editors. *Advances in biopreservation*. Boca Raton: CRC Press; 2007. p. 15–62.
- [94] Baust JM, Van B, Baust JG. Cell viability improves following inhibition of cryopreservation-induced apoptosis. *In Vitro Cell Dev Biol Anim* 2000;36(4):262–70.
- [95] Nicoud IB, Clarke DM, Taber G, Stolowski KM, Roberge SE, Song MK, et al. Cryopreservation of umbilical cord blood with a novel freezing solution that mimics intracellular ionic composition. *Transfusion* 2012;52(9):2055–62.
- [96] Cosentino LM, Corwin W, Baust JM, Diaz-Mayoral N, Cooley H, Shao W, et al. Preliminary report: evaluation of storage conditions and cryococktails during peripheral blood mononuclear cell cryopreservation. *Cell Preserv Technol* 2007;5(4):189–204.