

Freeze it and forget it: the value of having a robust formulation and cryopreservation strategy for cell therapy manufacturing

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Introduction

Cell-based therapies promise to transform medicine and human health as they offer solutions for treatment of diseases where pharmaceutical drugs have failed. The recent commercial success of products such as Kymriah™ and Yescarta™ cell therapies has generated tremendous excitement and acceleration of new product development in this area. Currently, over 500 cell therapies are in various phases of clinical development and over 1,000 clinical studies are underway globally. The ultimate success of these therapies is dependent on their seamless transition from bench to bedside without compromising safety, quality, and efficacy.

Manufacturing processes for cell therapies are complex and pose unique challenges in comparison with traditional pharmaceutical drug manufacturing, as cell therapies are living pharmaceuticals. While significant strides have been made in optimizing the manufacturing process for cell therapies, post-manufacturing steps such as formulation, final fill, and cryopreservation have been often overlooked. In order to achieve clinical and commercial success, the final cryopreserved product needs to have an extended shelf life, be conducive to long-term storage, and withstand global transportation. Failure to deliver optimally cryopreserved product to the patient's bedside can mean depriving the patient of their only viable treatment option.

It is important to note that cryopreservation is useful only if it achieves the goals of arresting biological degradation, conserving cellular attributes, maintaining product sterility, and attaining optimal viability post-thaw. Development of a standardized cryopreservation strategy is challenging due to factors such as diversity of cell types, nature of therapy, and the size of the dose. It is therefore vital to utilize the fundamental principles of cryobiology to design cryopreservation processes that overcome the complexity and protect final product integrity.

The purpose of this review is to discuss challenges related to cryopreservation of the final cell-based therapy product along with formulation and final fill, summarize the lessons learned from recent commercial successes, and review the best practices that will ensure product safety.

Cryopreservation

Cryopreservation is defined as the process of lowering the temperature of biological systems including cells in order to preserve their structural and functional integrity. The goal of an optimal cryopreservation strategy is to lower the temperature below -130°C without intracellular ice formation during the transition from aqueous phase to ice phase. Successful cryopreservation will ensure that cells achieve glass transition temperature (i.e., when liquid begins to behave as a solid), arrest molecular transport, and remain in the state of "suspended animation" without compromising the quantity and quality of cells [1]. Once cryopreserved, cells can be stored in a stable state at or near the temperature of liquid nitrogen (-196°C). Cryopreservation is preceded by sequential unit operations that include cell harvesting and formulation (addition of cryoprotectant (CP) and ancillary materials and/or excipients). All of these steps are critical, and even minor execution errors can result in an unstable and suboptimal final product [2]. Following arrival at the site of treatment, the final product may simply be thawed and administered or may undergo washing (removal of DMSO and animal origin products) and reformulation.

Benefits of cryopreservation

Cryopreservation has benefits for both patient-specific autologous therapies and “off-the-shelf” allogeneic therapies. Cell-based therapies are often the last resort for relapsed or refractory patients, and it is the flexibility of cryopreserved cells that enables these patients to have access to therapies on demand. In many instances, the final product may have a limited shelf life or may need to be administered in the form of multiple doses over a specific period of time. Cryopreservation of the product not only makes this possible, but also permits short-term storage of the final product to allow time for extended microbiological testing and the preparation of documentation for product release.

Delivering fresh product for small-scale autologous therapies is feasible, and is routinely carried out at many point-of-care facilities. However, cryopreservation reduces the complexity of cold-chain management for late-stage clinical or commercial products that require the manufacture and shipment of thousands of doses to hospitals and clinics all over the globe. Kymriah and Yescarta therapies are examples of cryopreserved cell therapies that have experienced global success; the stage is set for future treatments.

Manufacture of cell-based therapies requires isolation, manipulation, and expansion of cells prior to harvesting and cryopreservation. *Ex vivo* culturing of cells for extended periods can result in reduced proliferative potential, senescence, genetic drift, and epigenetic changes [2]. Such changes can be detrimental and ultimately reduce the potency and efficacy of the final cellular product. Cryopreservation obviates the need to maintain cells in culture for long periods of time, thus protecting them from such undesirable changes. Optimal cryopreservation allows the maintenance of desired cell phenotypes in near-perfect condition for long-term storage and during transportation to the final destination. It offers flexibility in managing cold-chain logistics, as manufacturing of a cryopreserved product can be completed at any point during a seven-day work week. The product can be stored until it is ready to be shipped at a time that is most convenient for the patient and the clinical staff. It also allows for a better overall strategy for modeling, and accurate forecasting of manufacturing capacity that will ultimately save time and reduce costs.

Challenges in cryopreservation

General considerations

The challenges of cryopreservation are influenced by many factors, including cell size, morphology, permeability of the cell membrane, and composition of organelles, as well as the composition and density of the cell culture medium and the CP [3]. Rather than using cookie-cutter legacy protocols adapted from the pharmaceutical industry, cell therapy cryopreservation protocols need to be tailored specifically for each type of final cellular product. Suboptimal cryopreservation can result in loss of viability, insufficient cell number per dose, and dose-to-dose variability that may affect the overall efficacy of the therapy.

Cryopreservation process parameters

A major challenge in cryopreservation is ensuring that cells not only survive the freezing process, but also maintain safety and potency profiles post-thaw. Optimizing the process is critical for avoiding osmotic shock and membrane damage, which may lead to post-thaw cell death. Improper use of freezing parameters can lead to artificial selection of subpopulations with phenotypic characteristics that are different from the population of interest. Studies have shown that immediate post-thaw viability measured by membrane integrity tests such as trypan blue dye exclusion or fluorescent cell imaging is not an accurate measure of cryopreservation process quality [4]. These studies highlight the need for implementing assays other than membrane integrity in early stages to determine and improve product profiles. Post-thaw assessment of cell viability and cell number should be carried out beyond the 24-hour period [4]. Long-term testing by thawing cells at multiple intervals will be helpful in evaluating the robustness and stability of the cryopreservation. Designing cryopreservation strategies to effectively overcome these challenges is paramount to ensure cell quality and potency.

Cryopreservation-induced stress

Cryopreservation-induced stresses represent a major hurdle in cold-chain management and contribute to significant loss of cell viability and cellular function. These stresses can be attributed to various factors, including CP toxicity, intra- and extracellular ice crystallization, altered intracellular pH, osmotic imbalance, and suboptimal rates of cooling and post-thaw warming.

Cryopreservation-induced stress can result in two types of cell death: apoptosis and necrosis. Post-cryopreservation apoptosis and necrosis are normally observed 6 to 24 hours into post-thaw culture [5,6]. Necrosis is characterized by swelling and disintegration of cellular organelles, resulting in rupture of the cell membrane from chemical or mechanical stress. Necrosis is fast acting, caused by external stressors, and results in massively significant cell loss. In contrast, apoptosis is physiologically programmed cell death that affects single cells or small populations of cells. It is characterized by cell shrinking, formation of apoptotic blebs, and eventual cell rupture [5,6].

In 2001, Baust et al. [7] presented the concept of cryopreservation-induced delayed-onset cell death (DOCD). DOCD is associated with overall cryopreservation failure and may not be obvious through one time-point analysis of viable cells during the first few hours post-thaw. Cryopreservation-induced DOCD appears to be due to a combination of necrotic and apoptotic events following thawing of cryopreserved samples and is characterized by a significant decrease in viability 12–24 hours post-thaw. DOCD results from permanent damage to cells when the level of oxidative stress is beyond the cells' ability to sustain or repair [7]. Choosing the right kind of CP and freezing medium formulation that minimizes DOCD and improves cell survival will define the success of the cryopreservation.

Choice of CP

Classification

CPs preserve cells and tissues from cryopreservation injuries by minimizing both physical and chemical damage. CPs are an integral part of cryopreservation as they promote cell survival and maintain structural integrity of the cells. Effective CPs have a low molecular weight, are nontoxic, and do not influence the behavior of post-thaw cells. CPs can be divided into two main classes:

- Cell membrane-permeating or intracellular agents can penetrate the cell membrane and prevent the formation of ice crystals that could result in rupture. Examples include DMSO, glycerol, ethylene glycol, and propylene glycol.
- Nonmembrane-permeating or extracellular agents have a high molecular weight and do not penetrate the cell membrane. They act to improve the osmotic imbalance that occurs during freezing. Examples include sucrose, trehalose, dextrose, methylcellulose, and polyvinylpyrrolidone (PVP).

While intracellular CPs are most commonly used in cell-based therapies, there is growing interest in the use of a combination of CPs to reduce toxicity while maintaining structural and functional integrity.

DMSO as an intracellular CP

Dimethyl sulfoxide (Me₂SO, DMSO) has been proven to be the choice CP in pharmaceutical manufacturing as it offers enhanced penetration, provides long-term stability, and maintains safety and potency of the cells in final formulation [8]. DMSO has been used both as an ancillary agent and as an excipient in final formulations.

When used as an excipient, the toxicity associated with DMSO requires that it be used at very low concentrations. Intake of DMSO at <50 mg/day is acceptable, and intravenous administration of up to 1 g/kg/day is common practice in transplantation therapies [9,10]. DMSO is used in hematopoietic progenitor cell (HPC) cord blood and commercial chimeric antigen receptor (CAR) T cell formulations. For example, the two commercial CAR T cell therapy products use DMSO in the final formulation. T cells for Kymriah therapy are formulated in glucose, NaCl, human serum albumin (HSA), dextran 40 for injection (10 mg/mL), DMSO (82.5 mg/mL), Na-gluconate, Na-acetate, KCl, MgCl₂, Na-N-acetyltryptophanate, Na-caprylate, aluminum, and water for injection, and have a shelf life of 9 months at below –120°C in the vapor phase of liquid nitrogen. T cells for Yescarta therapy are formulated in 68 mL solution composed of CryoStor™ CS10 medium (proprietary formulation containing 10% DMSO, dextran 40, sodium, potassium, calcium, magnesium, phosphate, HEPES, lactobionate, sucrose, mannitol, glucose, adenosine, and glutathione), NaCl, and HSA, and have a shelf life of 1 year at below –150°C in the vapor phase of liquid nitrogen [11]. In the case of both these products, cells are thawed and directly infused into patients. The advantages of using DMSO as an excipient at low concentration are the elimination of the post-thaw washing step at the receiving site, minimization of labor and specialized training, and reduction in chances for errors and contamination.

If DMSO is used as an ancillary material, it needs to be removed through cell washes. This can be accomplished by traditional methods of centrifugation or newer approaches such as filtration by spinning membrane, stepwise dilution and centrifugation using a rotating syringe, diffusion-based DMSO extraction in microfluidic channels, or controlled dilution and filtration through a hollow-fiber dialyzer [12,13]. Recent advancements have been made in the choice of non-DMSO agents using a combination of osmolytes like sugar, sugar alcohol, amino acids, and proteins that show promise by improving post-thaw recovery [14,15].

Extracellular CPs

The use of extracellular CPs has been investigated in research settings, but their use in cell-based therapies has been hampered due to their inability to achieve optimal cryoprotection and consequent post-thaw recovery with low cell viability. In research studies, the use of PVP in cryopreservation of human adipose tissue-derived adult stem cells resulted in recovery of cells that was comparable to DMSO with animal serum [16]. In another study, successful outcomes were achieved by using methylcellulose either alone or in conjunction with low concentrations of DMSO along with HSA [17]. Further studies are warranted to evaluate the use of extracellular CPs for cell-based therapies.

Addition of CP and cooling

The rate of cooling during cryopreservation has a dramatic impact on cell viability in the final product. Cooling rates control the formation and size of both intracellular and extracellular ice crystals and can impact solution effects during the freezing process. While rapid cooling maximizes intracellular ice formation and minimizes solute concentration effects, slow cooling has opposite effects. Currently, slow cooling is the most frequently used method of cryopreservation for a variety of cell types [6]. While rapid cooling methodologies minimize intracellular and extracellular ice crystal formation, they require a much higher concentration of cryoprotectant resulting in toxicity-induced cell loss and addition of a washing and reformulation step at the clinical site [6].

The cryopreservation medium is usually added to the cell suspension in steps or at a controlled rate to prevent cell losses resulting from osmotic stress. It is common to prechill the cryopreservation medium and to keep the cell suspension and the admixture chilled using cold packs, a frozen blanket, or a chilled work surface to prevent heat-related cell damage during addition of DMSO. After adding cryopreservation medium, the cell suspension is transferred to the precooled chamber of a controlled-rate freezer. During the freezing process, product temperatures can be recorded using a probe that helps generate a freeze curve. Regardless of the cooling strategy, there is an unmet need to develop optimized and standardized cryopreservation procedures that preserve the potency and viability of the final cell-based product.

Formulation

Close attention needs to be given to formulation and final fill steps as they precede the cryopreservation step. Optimal formulation is critical for the success of the final cellular product that is stable, safe, efficacious, and meets regulatory requirements. Formulation is the process of combining cells, buffers, proteins, ancillary materials, and CPs and is carried out immediately after the cells are harvested at the end of the manufacturing process. Formulation is a temperature-dependent and time-sensitive step since the harvested cells during this step are held in suboptimal environmental conditions and without nutrition. Appropriate formulation is needed to stabilize the cells so they can withstand stress factors such as temperature excursions, pH changes, and mechanical stress caused by handling, storage, shipment, and bedside preparation.

Formulation and final finish steps for cell-based therapies are unique, which reflects the cell therapy industry saying that “the process is the product”. Unlike conventional pharmaceuticals, there is not a significant difference between drug substance (DS) and drug product (DP) for cell-based therapies. DS in cell-based therapies is composed of the manipulated or nonmanipulated cells that hold the therapeutic potential, and excipients. DP is drug substance that is diluted to final dose and filled into final containers that are ready to be delivered to the patients as therapy.

Formulation and final fill strategies involve selection of the appropriate CP and other excipients and the final containers. The selection of excipients plays a key role in the maintenance of critical quality attributes (CQAs) of the final product. HSA is one of the most popular excipients in cell therapy because it is the most ubiquitous protein in blood and is known to create an optimal microenvironment for sustained cell viability. It acts as a scavenger of toxins and other reactive oxygen species, maintains pH, provides insulation, and maintains cell viability during cryopreservation [18]. Additional components of the final formulation include dextran, which serves as an osmotically neutral volume expander and as parenteral nutrition, sodium chloride as a normal saline diluent, and stabilizers such as sodium caprylate and N-acetyltryptophanate that protect proteins such as HSA from oxidative stress [11].

Choice of containers for final fill

Choosing the right containers for the final product is of great consequence to the success of the overall therapy because they provide physical protection and are responsible for the stability over the entire lifecycle of the final product. The container needs to offer standardization and reproducibility for storage and shipment. It also needs to have features such as ease of use, stability at below-freezing temperatures, the absence of leachables and extractables, resistance to CPs such as DMSO, and optimal labeling surface [19]. The types of final containers most commonly used for cell-based therapies are screw-cap cryovials, bags, and plastic or glass vials.

Screw-cap cryovials have been extensively used to store many cell-based products, and especially for banking of GMP-grade master cell banks. While screw-cap vials are convenient, cost-effective, have a long-standing cryopreservation record, and work well for analytical and stability testing, they pose several challenges from a regulatory perspective for large-scale commercial manufacturing: They involve open steps for product filling that need to be carried out in a biosafety cabinet (BSC) and are labor intensive, subject to human error, and more prone to contamination. They are also limited in volume per dose, have a limited labeling surface, and require extensive manipulation at the receiving site prior to delivery into patients.

Use of bags is preferred by manufacturers of cell-based therapies as they offer the option of using completely closed systems with transfer bags and tubings, a large selection of sizes with customizable options, optimal

labeling surfaces, sampling ports, and minimal bedside manipulation. Use of bags, however, requires investment in specialty instruments such as welders and sealers, specialized training for operators, and carefully planned processes for air removal and packaging to ensure that the bags do not develop cracks and cause leakage of product after thawing. Though multiple bags can be filled using kits or automated systems, scale-up is challenging and lot sizes for a single manufacturing run are typically capped at 150–200 product bags [3].

The use of “ready-to-use” containers such as vials made of cyclic olefin copolymer and a pierceable septum that acts as a sterile barrier offer the advantages and flexibility of a closed system and scale-up for commercial needs [19]. However, they are expensive, require specialized training, and may require filling operations to be conducted inside the BSC unless a substantial financial investment is made in purchasing large and complex multifunctional automated systems. It is possible to initially choose manual systems for filling vials or bags and then transition to automated filling systems for scale-up during commercial manufacturing.

Lessons learned and future directions

Use of fresh vs. cryopreserved final product

While some point-of-care facilities for early stage clinical trials continue to deliver noncryopreserved or “fresh” final product to the patient’s bedside, this is not a sustainable option. As the field of cell-based therapies matures, delivery of cryopreserved final product that is standardized, scalable, reproducible, in compliance with global regulatory agencies, and has a maximized shelf life for an on-demand distribution will prove to be the best option.

Post-thaw viability of cells

Kymriah CAR T cell therapy has been used in approximately 1,800 patients, but 10% of the time, the product suffered from out-of-specification issues or from manufacturing failures. In these cases, the cell viability reached 70% but failed to reach 80%, which is the commercial product specification. However, an independent study reported that doses with 70% or less viability achieved complete response and satisfied the product release specification of the minimum absolute number of viable T cells expressing the CAR [20]. This decrease in viability is an issue that is being addressed and may require enhancements in manufacturing and cryopreservation strategies.

Post-thaw functional recovery of cells

Success of the cryopreservation process is typically measured by parameters such as post-thaw recovery, viability, and stability, but true functional recovery of the cells is often ignored. As more therapies move from the clinical to commercial space, it is important to assess the phenotypic alterations caused by cryopreservation. While occurrence of cryopreservation-induced DOCD is well accepted, its underlying molecular mechanism is not sufficiently understood. Studies focusing on cryopreservation-induced biomolecular events and proteomic alterations as well as mitochondrial-associated apoptotic changes will help in understanding of intrinsic cellular behavior. A well-thought-out cryopreservation strategy that integrates cryobiology, cellular and molecular biology, biophysics, and engineering is necessary to predict and standardize dose adjustments due to post-thaw cell losses.

Use of DMSO-free CPs

There is a need to design a new class of CPs since many scientific studies have shown that DMSO can adversely affect genomic and proteomic profiles of the cells and cause damage to cellular structures including mitochondria, the nucleus, and the cell membrane. Even at a low dose, DMSO can also cause a variety of adverse reactions in patients. In formulations where the amount of DMSO exceeds ICH and FDA guidelines, removal of DMSO can be accomplished by using a specialized instrument (e.g., CytoMate™ Cell Washer, Sepax™ S-100 Cell Separation System, COBE™ 2991 Cell Processor, Lovo™ Cell Processing System, or Gibco™ CTS™ Rotea™ Counterflow Centrifugation System). However, methods and devices for DMSO removal with minimum cell loss and damage remains an unmet need. Inclusion of carrier medium that mimics the intracellular ionic balance, minimizes the gradient of ions across the cell membrane, and aids in the management of ice and chemo-osmotic perturbations is being actively explored.

DMSO-free CPs are a favorable option due to their lower risk profile, better tolerance by patients, better compatibility with bags and weldable tubings, and due to the possibility of eliminating washing steps prior to patient infusion. Trehalose (nonreducing disaccharide of glucose) is one such CP as it possesses an exceptional ability to stabilize and preserve cells and cellular structures during freezing. Studies have shown that the low penetration issue with trehalose can be overcome by addition of P2X7 (ATP-activated receptor that opens transmembrane pores of the cells) [15]. More studies that focus on improving membrane penetration of DMSO-free CPs will promote the use of nontoxic CPs for cell-based therapies.

Conclusions

Long-term success of clinical and commercial outcomes in cell-based therapies depends upon a phase-appropriate and risk-based approach. This involves:

- Introducing cryopreservation of final product as early in clinical development as possible
- Developing cold-chain logistics strategies that enable cryopreservation of cells in a manner that avoids excessive stresses and preserves the biological function of manufactured cell products
- Designing novel cryopreservation procedures to incorporate DMSO-free, serum-free, or even xeno-free CPs, devising techniques and instruments for easy and safe formulation and filling, and delivering the final product without compromising cell quantity, quality, and potency

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