Cryopreservation Techniques and Freezing Solutions

6

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6.1 Introduction

Hematopoietic progenitor cell (HPC) products remain viable and capable of engraftment in a transplant recipient when stored in the "liquid state" at either refrigerated or room temperatures for approximately 72 h following collection. Cryopreservation enables long-term storage of HPC products and is used extensively for autologous products collected prior to high-dose chemotherapy (HDT) that are intended for use after treatment to rescue hematopoiesis. Allogeneic products may also be cryopreserved if treatment is delayed due to the donor's or recipient's medical condition, the donor's cells are collected prior to their anticipated use because of donor availability, or more donor cells are obtained than needed and remaining cells are stored for future use. Similarly, allogeneic cord blood products are cryopreserved, prospectively banked, and made available through donor registries. In order for cryopreservation to be an effective tool, when thawed and infused, HPC must be able to regain their ability to proliferate, engraft, and provide the same functional capability as prior to cryopreservation. Pre-cryopreservation storage conditions and manipulation procedures, cryoprotectant formulation, freezing rate, long-term storage temperatures, length of time in storage, and thawing conditions all have the potential to affect the quality and engraftment of thawed HPC products.

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6.2 Pre-cryopreservation Transport, Storage, and Processing Considerations

The temperature(s) at which HPC products are stored and transported prior to processing, manufacturing, and cryopreservation can influence the viability, sterility, potency, efficacy, and overall quality of the products. However, "standard" temperatures have not been established. Storage and transport temperature(s) vary by collection and manufacturing facility, and some facilities even use different temperatures for products collected from different sources (e.g., apheresis, marrow, and cord blood) (Antonenas et al. 2006; Hahn et al. 2014; Lazarus et al. 2009; Fry et al. 2013; Kao et al. 2011). These temperatures range from refrigeration (2–10 °C) to ambient or room temperatures (up to 20 °C). Even the National Marrow Donor Program (NMDP, Be the Match®), which transports more than 6000 HPC products annually from collection centers to transplant centers throughout the world, *does not* have a standard transport temperature but rather leaves storage and transport conditions to the discretion of the transplant center.

Minimal manipulations of HPC products, such as plasma and red blood cell reduction, are often performed prior to cryopreservation. Plasma reduction rarely results in significant cell loss; however, red blood cell reduction may result in loss of as many as 30% of total nucleated cells (TNCs). Removal of donor red blood cells and/or plasma effectively reduces the risks of hemolysis associated with donor and recipient ABO/Rh incompatibility. The volume of cryoprotectant used is proportional to the volume of the product. Removing red blood cells and/or plasma reduces the volume of the product and also serves to reduce the volume of cryoprotectant in the cryopreserved product, thus reducing the risks of infusion adverse reactions attributable to the concentration of cryoprotectant administered to the patient. From a practical standpoint, the reduced total volume also conserves reagents, cryopreservation bags, and valuable freezer storage space. More than minimal manipulation such as $CD34^+$ cell enrichment and $CD3^+$ cell reduction with monoclonal antibodies and ex vivo expansion cultures may make HPC more susceptible to damage during cryopreservation, and thawing and cryopreservation protocols may or may not need to be modified to improve recovery of these much needed cells (Gorin 1986; Reich-Slotky et al. 2016) (Table 6.1).

6.3 Cellular Composition and Concentration

HPC, Marrow and *HPC, Apheresis* differs significantly in cell content and volume. HPC, Marrow contains a large proportion of mature granulocytes and red blood cells which lyse when cryopreserved and thawed. The lysed cells and the toxic materials released can cause serious infusion complications including renal failure and, in rare instances, death (Rowley 1992). Historically, *HPC, Marrow* products were processed and cryopreserved prior to the advent of *HPC, Apheresis* collections. Based on experience with *HPC, Marrow*, cell concentrations up to 1×10^8 nucleated cells (NCs)/mL in a cryoprotectant containing 10% dimethylsulfoxide (DMSO, volume/volume) were suggested for cryopreserved products (Rowley 1992; Cabezudo et al. 2000;

Table 6.1 Pre-cryopreservation storage and processing considerations

Condition	Risks
Liquid storage temperature	Refrigeration temperatures may reduce viability Room temperature may increase the risk of microbial contamination
Plasma reduction	Reduces hemolysis risk due to minor ABO/Rh incompatibility Reduces concentration of DMSO required, thereby reducing risks of infusion adverse reactions Minimal cell loss
RBC reduction	Reduces red cell lysis during thawing, thereby reducing infusion adverse events Reduces hemolysis risk due to donor and recipient major ABO/Rh incompatibility Cell loss may be significant (≥30% total nucleated cells)
Cell concentration	*HPC, Marrow* has lower cell counts than HPC, Apheresis Large dilutions can result in large volumes of DMSO which may cause adverse reactions when infused
Cell enrichment (or reduction), e.g., $CD34^+$ cell enrichment (or $CD3^+$ cell reduction	Reduced cell viability and recovery Cells may be more sensitive to DMSO
Cell types to be recovered	HPC may be more resistant to cryopreservation than lymphocytes
Ex vivo culture/expansion	Increased apoptosis Culture media may improve viability due to amino acids, protein, and antioxidants

Rowley et al. 1994). However, *HPC, Apheresis* can contain as many as fivefold more cells making dilution to similar cell concentrations impractical since these dilutions would result in very large volumes that are difficult to store and infuse and contain large volumes of DMSO which can be toxic (Gorin 1986). Recovery of nucleated and $CD34^+$ cells, clonogenic assays, and engraftment have been used to compare *HPC, Apheresis* products cryopreserved at low and high cell concentrations (Cabezudo et al. 2000; Rowley et al. 1994; Lecchi et al. 2016; Martin-Henao et al. 2005). In these studies, *HPC, Apheresis* was cryopreserved at concentrations up to approximately fourfold the recommended *HPC, Marrow* concentrations. In all instances, the concentration of cells at the time of cryopreservation did not significantly affect the post-thaw recovery of HPC or the time to engraftment of these cells.

6.4 Cryoprotectants

Cryoprotectants are added to HPC products prior to cryopreservation in order to limit cell lysis during the cooling/freezing process (Gorin 1986). DMSO, a small molecule that diffuses into cells, is the most commonly used cryoprotectant.

Intracellular DMSO changes the osmotic balance of cells and interferes with ice crystal formation, thereby reducing cell lysis and preserving cell viability (Rowley 1992; Lecchi et al. 2016; Karow and Webb 1965). Because cryopreservation in DMSO decreased the proliferative potential of bone marrow progenitor cell colonies in some studies, it has been assumed that DMSO is toxic to HPC and that exposure to DMSO in the non-cryopreserved state (Douay et al. 1982) and post-thaw (Rowley 1992) should be limited. However, colony-forming progenitor cells were resistant to toxic effects of up to 20% DMSO for up to 1 h (Rowley and Anderson 1993) and 8–10% DMSO for at least 2 h (Branch et al. 1994). DMSO is generally diluted in an isotonic solution containing a source of protein such as human serum albumin or autologous plasma (Rowley 1992; Smagur et al. 2015). Concentrations ranging from 3.5% to 10% DMSO maintain HPC viability and have been reported to have similar engraftment outcomes when products are thawed and infused (Rowley 1992; Smagur et al. 2015; Lovelock and Bishop 1959; Veeraputhiran et al. 2010; Fry et al. 2015; Stiff et al. 1987).

DMSO does not protect red blood cells and granulocytes in HPC products from lysis during freezing and thawing. Lysis of these cells, and the toxic materials that they release (hemoglobin and nucleoprotein and lysosomal enzymes, respectively), has been implicated in infusion adverse reactions. Hydroxyethyl starch (HES) is a high-molecular-weight extracellular cryoprotectant that reduces post-thaw granulocyte lysis and clumping (Stiff et al. 1987). Addition of 6% HES to DMSO enables cryopreservation at a lower DMSO concentration (5%) and lowers granulocyte lysis, both of which reduce the occurrence of infusion adverse reactions without compromising viability and engraftment of HPC (Stiff et al. 1987). Dextran 40, another high-molecular-weight extracellular cryoprotectant, has been used extensively for cryopreservation of HPC, Cord Blood. Addition of dextran 40 to the cryoprotectant reduces DMSO toxicity during the cooling process (Rubinstein et al. 1995).

Because DMSO can be toxic to cells and may cause infusion-related adverse events, a non-DMSO-containing cryoprotectant may be advantageous. HPC cryopreserved in DMSO and the low-molecular-weight carbohydrate Pentaisomaltose (PIM) were compared (Svalgaard et al. 2016). Post-thaw, cells were analyzed for $CD34^+$ cell recovery and viability, and $CD34^+$ cell subsets by flow cytometry; and in vitro colony assays were also performed. All analyses indicated that PIM was comparable to DMSO as a cryoprotectant and therefore may have the potential to be used as a less toxic cryoprotectant. Preclinical, in vivo animal studies will be needed to confirm these findings prior to clinical studies.

Although cryopreserved HPC products have been used clinically for decades, successful cryopreservation and subsequent clinical use of other cellular therapy products have been more challenging and may require modification of procedures and cryopreservative formulations. Aliquots of allogeneic *HPC, Apheresis* products may be cryopreserved as a source of donor lymphocytes for infusion if indicated to treat relapse or speed immune recovery posttransplant making stability and recovery of white blood cells critical (Stroncek et al. 2011). The post-thaw recovery and viability of HPC and lymphocytes cryopreserved under standard conditions were compared (Fisher et al. 2014; Berens et al. 2016). In one study (Fisher

et al. 2014), $CD34^+$ cell recovery from related and unrelated donors was similar; however, $CD3^+$ cell recovery was lower in products from unrelated donors. Since all unrelated donors had undergone mobilization with G-CSF while many related donors had not, products from mobilized related and unrelated donors were also compared. Recovery of $CD3^+$ cells was found to be similar in both groups, indicating that donor mobilization with G-CSF may have a negative effect on recovery of $CD3^+$ cells post-cryopreservation. Another study (Berens et al. 2016) demonstrated that recovery of cryopreserved $CD3^+$ cells and $CD4^+$ and $CD8^+$ subsets was decreased compared to recovery of $CD34^+$ cells. In this study, there was no correlation between lymphocyte recovery and donor mobilization with G-CSF, and the investigators concluded that resistance to freezing and thawing is cell specific and independent of other factors. Ex vivo expanded HPC, Cord Blood survives poorly when cryopreserved using standard procedures. Addition of serum-free culture medium containing carbohydrates and antioxidants improved the recovery of viable HPC as evidenced by decreased apoptosis, increased numbers of in vitro progenitor cell assays, and engraftment in primary and secondary immunodeficient NSG mice (Duchez et al. 2016).

6.5 Cooling Process: Controlled Versus Passive Rate Freezing

HPC must be cooled slowly in order to preserve post-thaw viability and function. Many transplant centers use controlled rate freezers to cryopreserve these products (Rowley 1992). The programmed freeze cycle consists of *three phases*. The first is the equilibrium phase during which the product is cooled to the same temperature as the freezing chamber. This ensures that the product's temperature can be monitored accurately throughout the process to provide freezing curve uniformity for all products. A constant cooling rate is achieved during the second phase. The optimal cooling rate for HPC products is 1–2 °C/min. Extracellular ice formation which is an exothermic reaction occurs during this phase, and the program must compensate for the heat that is released. This is usually accomplished by an "ice seeding" procedure whereby the product is cooled very quickly and then warmed slightly before continuing the constant cooling rate. Extracellular ice formation decreases the available water in the extracellular space, thereby increasing the extracellular concentration and efflux of water from the cells can occur. Decreasing the temperature at which extracellular ice formation occurs protects cellular viability. In the final phase, cooling continues at a constant rate until the desired temperature is achieved (Hubel 2009).

Controlled rate freezing (CRF) requires costly equipment and cooling program development. Once the cooling cycle is started, controlled rate freezers cannot be opened to add additional products. As a result, processing labs must either have multiple freezers or coordinate processing of multiple products so that all can be placed in the freezer at the same time assuring minimal exposure time (usually <15 min) to DMSO prior to cooling process. As a less expensive, rapid, easy, and

very reproducible alternative, "dump" or "passive" freezing has been developed and is used extensively (Stiff et al. 1987; Halle et al. 2001; Detry et al. 2014). Products in metal freezing cassettes are placed horizontally on shelves in a −80 °C mechanical freezer. The freezing rate can be monitored easily by placing the probe of an electronic temperature monitor inside the cassette, against the cryopreservation bag. The metal cassette can be wrapped in disposable absorbent pads or Styrofoam insulation to adjust the cooling rate to the desired 1–2 °C/min. Cell viability, recovery, and engraftment are comparable to CRF.

6.6 Long-Term Storage Temperatures and Hematopoietic Progenitor Cell Stability

Cryopreserved HPC products may remain in storage for prolonged periods of time. In order to provide adequate HPC for successful hematopoietic cell transplantation, the stability and potency of these products must be maintained during storage. Most clinical facilities store HPC products in the liquid or vapor phases of liquid nitrogen (−195 °C or −150 to −125 °C, respectively), and successful storage at −80 °C in mechanical freezers has also been reported (Halle et al. 2001; Detry et al. 2014). It has not been determined if viability and engraftment potential is increased by storage in the liquid phase of nitrogen versus the vapor phase. Accreditation standards organizations such as NetCord-FACT/JACIE (Foundation for the Accreditation of Cellular Therapy and International NetCord Foundation 2016) require *HPC, Cord Blood* to be stored at temperatures ≤−150 °C because of the assumption that a lower temperature will better maintain the stability and viability of the products over extended periods of storage experienced by public cord blood banks. Because temperature gradients can occur in liquid nitrogen vapor resulting in temperatures ≥−150 °C toward the top of vessel, many cord blood banks have chosen to store products in the liquid phase. A major disadvantage to storage in the liquid phase is the potential transmission of infectious disease and microbial contamination between products if cryopreservation bags in which products are stored do not remain intact (Tedder et al. 1995; Fountain et al. 1997). Storage in liquid nitrogen vapor phase eliminates this risk. Storage at −80 °C in mechanical freezers may not be sufficient for storage longer than approximately 2 years and may result in loss of products due to mechanical failure (Halle et al. 2001; Detry et al. 2014). Assays used to evaluate post-thaw product functionality include comparison of pre-cryopreservation and post-thaw viable total nucleated and $CD34^+$ cells by flow cytometry, in vitro progenitor cell colony growth and replating efficiencies, engraftment in animal models such as immunodeficient mice, and derivation of induced pluripotent stem cells that can differentiate into all three germ layers (Broxmeyer et al. 2003, 2011; Vosganian et al. 2012; Winter et al. 2014). Ultimately, engraftment in human subjects is the most important indicator of product stability. Cryopreserved *HPC, Apheresis* and *HPC, Cord Blood* have been evaluated and remain viable and function for at least 15 and 23.5 years, respectively (Broxmeyer et al. 2003, 2011; Vosganian et al. 2012; Winter et al. 2014).

6.7 Potential Infusion-Related Adverse Events from Thawed Products

Cryopreserved products are generally thawed quickly, often at the patient's bedside, and infused immediately to limit exposure of HPC to DMSO at warm temperatures. Procedures for thawing, washing, and preparing products for infusion are discussed in detail in Chaps. 7 and 11 of this book.

Adverse events can occur during infusion of thawed products. These reactions are generally mild and have been attributed to reactions to DMSO (Zambelli et al. 1998; Davis et al. 1990; Stroncek et al. 1991; Donmez et al. 2007), lysis of red blood cells and the resultant release of hemoglobin (Smith et al. 1987), and release of nucleoprotein and lysosomal enzymes by lysis of granulocytes (Davis et al. 1990) during the freezing and thawing process. Reported mild to moderate infusion-related adverse events include nausea and vomiting, headache, hypotension, bradycardia, tachycardia, chest tightness, fever and chills, and abdominal cramps. Less frequently, renal failure and severe cardiac and neurologic symptoms have been reported. Severe cardiac side effects require the infusion to be stopped and have been attributed to high DMSO and cell concentrations in the product (Donmez et al. 2007). In order to limit DMSO-related toxicity, infusion of DMSO should be limited to <1 g DMSO/kg of recipient body weight (Rowley et al. 1994).

6.8 Expert Point of View

HPC products have been cryopreserved by transplant centers for many years. Despite the universality of cryopreservation, there is no standard or even consensus with regard to the final cell or DMSO concentrations or other additives used (Table 6.2). However, it is generally agreed that addition of DMSO together with a slow cooling/freezing rate limits intracellular ice crystal formation and cell lysis, thereby preserving cell viability. Similarly, successful long-term storage has been reported at temperatures ranging from −196 to −80 °C. Rapid bedside thawing and immediate infusion to limit post-thaw exposure of cells to DMSO have been adopted by most centers. Fortunately, cryopreserved HPC are very robust and resilient and have been shown to be stable regardless of the technical variations reported and can be stored and remain viable, capable of engraftment, and available for patients for at least 20 years.

Table 6.2 Variable cryopreservation conditions

Cryoprotectants	3.5–10% DMSO in isotonic solution (saline, normosol, or culture media); ±serum (autologous or human serum albumin); ±hespan; ±dextran 40.
Cooling	Controlled rate versus passive; 1–2 °C/min
Temperature	*Liquid* phase of nitrogen (−195 °C), *vapor* phase of nitrogen (≤−150 °C), mechanical freezers (−80 °C)
Stability	Up to 23.5 years for CBU and 15 years for HPC, Apheresis

6.9 Future Directions

Manufacture of gene and immunotherapy products involves more than minimal manipulation and long-term ex vivo cultures. These processes may render cells more fragile and sensitive to current cryopreservation procedures. In order to make these products available to most patients, methods will have to be modified to protect the viability and functionality of complex cellular therapy products.

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