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Cryopreservation of Hematopoietic and Non-Hematopoietic Stem Cells – A Review for the Clinician

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1. Introduction

The transplantation of stem cells can be performed in an autologous, where the recipient donates his own stem cells for later use, or in an allogeneic fashion, where the donor and the recipient are two different persons (Berz, McCormack, Winer, Colvin, & Peter J Quesenberry, 2007; A. Gratwohl et al., 2010). In some clinical settings, stem cells can be utilized within a 72 hour timeframe without the need for extensive storage (Fleming & A Hubel, 2006; Allison Hubel, Carlquist, Clay, & Jeff McCullough, 2004; Pettengell, Woll, O'Connor, Dexter, & Testa, 1994). The autologous transplantation of cellular products and the therapeutic use of umbilical cord stem cells rely heavily on the preservation of stem cells after initial collection to be utilized at a later point in time (Berz, McCormack, Winer, Colvin, & Peter J Quesenberry, 2007).

Hematopoietic stem cell transplantation has been in clinical use for decades to treat benign and malignant hematologic and non-hematologic conditions (C. J. Hunt, 2011). The principal sources for those therapeutic strategies are bone marrow, peripheral blood hematopoietic progenitor cells and stem cells derived from umbilical cord blood. Although clinicians have decades of experience with the use of hematopoietic stem cells(HSC), the interest in the clinical use of non-hematopoietic stem cells, such as embryonic(C. J. Hunt, 2011; Leeb et al., 2011), mesenchymal (Ding, Shyu, & Lin, 2011; Leclerc et al., 2011; Puglisi et al., 2011) and induced pluripotent stem cells (Leeb et al., 2011; Sohni & Verfaillie, 2011) is rapidly expanding. The therapeutic strategies utilizing such cellular products, depend heavily on the effective preservation of those cell products for clinical use at a later point in time. The need for ready availability of such products calls for storage procedures with favorable graft survival rates and a tolerable toxicity profile. While cryopreservation protocols for HSC are well developed, the field for non-hematopoietic stem cells still remains to be defined.

The cryopreservation of all stem cells follows certain principal steps; First, Cytoreduction and prefreezing processing of the freshly collected graft. Second, the cryopreservative medium is prepared and added. Third, the graft is assessed for viability and integrity. Microbial contamination is ruled out. Fourth, freezing of the stem cells is performed. Fifth, thawing of the cryopreserved graft is conducted. Last, the post thawing processing is

performed, including in certain situations washing of the stem cell concentrate. A standard algorithm for the preservation of HSC populations is depicted in figure 1

No single, standardized cryopreservation protocol has been universally used and differences in techniques continue to exist in between different centers.

We at our institution utilize a standardized NIH protocol. We collect Hematopoietic Progenitor Stem Cells in a minimally manipulated fashion as defined by the Foundation for the Accreditation of Hematopoietic Cell Therapy (FAHCT) with a minimal cell dose of at least $1.5\text{-}3.0 \times 10^6\text{-}5.0 \times 10^6$ CD34(+) cells/kg body weight, depending on the clinical indication. The specimen is then centrifuged to develop the cell rich pellet. In autologous transplants donor plasma is used for re-suspension, if available. Human serum albumin solution is an alternative. Then, a solution of heparinized Plasmalyte and 10% DMSO (Dimethylsulfoxide) is added. This usually eventuates into a cellular concentration of 200×10^6 cells in the cryopreservate. We store the bone marrow or peripheral blood stem cell product at initially -4°C . Then the sample is frozen down to the target temperature of -135° to -156°C to be placed into the vapor phase of the nitrogen tank.

2. Types of stem cells

- a. Hematopoietic stem cells (HSC) are in therapeutic use for more than 50 years (THOMAS, LOCHTE, CANNON, SAHLER, & FERREBEE, 1959). In 2006 50,417 hematopoietic stem cell transplants were performed globally, 21 516 allogeneic (43%) and 28 901 autologous (57%) (A. Gratwohl et al., 2010). The principal sources for HSCs are the bone marrow (BM), mobilized peripheral blood stem cells (PBSC) and umbilical cord stem cells (UCB)(Berz, McCormack, Winer, Colvin, & Peter J Quesenberry, 2007). Allogeneic grafts are often used shortly after collection, but autologous grafts and cord blood units are generally cryopreserved. The amount of cryopreserved cord blood units is rising and the storage time in cord blood banks often exceeds a decade (Valeri & Pivacek, 1996).
- b. Mesenchymal stem cells (MSC) were initially identified in 1976 as bone marrow stromal cells with the capability to form mesenchymal components such as fat, cartilage and bone(Friedenstein, Gorskaja, & Kulagina, 1976). Along with the large regenerative potential of damaged mesenchymal tissues, MSCs are powerful immune modulators with promising results in autoimmune diseases and GVHD(Dazzi & Krampera, 2011; McGuirk & Weiss, 2011).
- c. Human embryonic stem cells(hESC) are primitive precursor cells with an unlimited potential for self- renewal and the capability to differentiate into any cell type derived from all three germ cell layers(Murdoch et al., 2011). This pluripotent property makes hESCs powerful candidates for regenerative cellular therapies.
- d. Induced pluripotent stem cells (iPSC) are somatic cells which are transformed by genetic reprogramming into cells with pluripotent stem cell character. Initially described in 2007, such cells would be optimal candidates for autologous regenerative approaches without the ethical concerns about hESCs (Walia, Satija, Tripathi, & Gangenahalli, 2011). However, immunologic barriers have recently been identified even in autologous settings. Before a viable clinical use, such obstacles have first to be overcome (Zhao, Z.-N. Zhang, Rong, & Y. Xu, 2011).

Although most of our clinical expertise exists with HSC, efforts to develop appropriate cryopreservation protocols for non-hematopoietic stem cells are ongoing. This chapter will

primarily focus on the recent developments in the field of cryopreservation of hematopoietic stem cells, but also outline some of the similarities and differences between the cryopreservation of HSCs and non-hematopoietic stem cells.

3. Processing prior to cryostorage

3.1 Liquid phase storage

Several centers, particularly in remote rural settings rely on the performance of autologous bone marrow transplantation without a local cryopreservation expertise. Along with that, umbilical cord blood processing is only performed in highly specialized cell processing facilities, which are often geographically remote from the place of collection. In such clinical settings, the initial cell collections have to be transported to a center with the necessary expertise in a liquid form (Fleming & A Hubel, 2006; Rodrigues et al., 2008). Liquid storage, either for transport purposes or to bridge a short time span prior to definitive clinical use has been successfully used for different clinical indications (Corato et al., 2000; Pettengell, Woll, O'Connor, Dexter, & Testa, 1994; Pettengell, Woll, Thatcher, Dexter, & Testa, 1995).

Several studies have investigated the effects of temperature, total liquid phase duration and storage media with varying cell concentrations on functional outcomes. A short term storage temperature of 4° Celsius has been shown to be suitable for UB cord blood collections (Burger, A Hubel, & J McCullough, 1999; Allison Hubel, Carlquist, Clay, & Jeffrey McCullough, 2003) and non cord blood hematopoietic progenitor cells (Burger et al., 1999; Allison Hubel, Carlquist, Clay, & Jeff McCullough, 2004; Allison Hubel et al., 2003). The major rationale for the choice of 4° Celsius was the concern about crystal formation and corresponding cell death at lower temperatures (Matsumoto et al., 2002). However, the actual freezing/crystallization point of human plasma has been shown to be at -0.8° Celsius (K. B. Storey & J. M. Storey, 1990). Hence, different temperatures have been investigated for short term storage. A study from Japan established the feasibility of storage of HPC at -2 Celsius in University of Wisconsin medium without cryopreservative additive (CPA) for up to 72 hours. In this short term storage conditions, appropriate for transport purposes, the post-thaw nucleated cell count recoveries and functional assay outcomes were both above 90%. This was superior to a control, which was stored at -80° Celsius with DMSO for the same amount of time (Matsumoto et al., 2002). The results of this and similar studies suggest that storage around the freezing point is more appropriate in short term storage for periods of less than 72 hours than the usual deep freezing temperatures.

3.2 Centrifugation and resuspension

The pre-cryostorage processing entails the actual collection procedure, the removal of cell bulk, volume reduction with concentration of the stem cells and addition of the cryo-medium. The process is performed in a strictly sterile environment. It also includes the identification of the donor with labeling of the bag, general assessment of the collected specimen (such as weighing and cell enumeration) and ascertainment of a sterile specimen with microbiologic studies (S. M. Watt, Austin, & S. Armitage, 2007).

Subsequently the specimen undergoes centrifugation and resuspension of the pellet in order to achieve volume reduction and concentration of the target cell population (Laroche et al., 2005; Rebullá, 2002; Rowley, Bensinger, Gooley, & Buckner, 1994).

The volume reduction process is well documented to be associated with loss of active cells (Koliakos et al., 2007). This is of particular relevance in UCB specimens, in which a paucity

of donor cells is a concern (Koliakos et al., 2007; Laroche et al., 2005; J. C. Wang, Doedens, & Dick, 1997).

Hence, volume reduction during pre-freezing processing remains a field of ongoing research to limit the cell loss. One example is a study, by Koliakos et al, which achieved limited cell loss by a careful double processing in the presence of 2% HES(Koliakos et al., 2007).

3.3 Cell concentration

Triggered by concerns of toxicities of the cryopreservative additive to the cells, high stem cell concentrations in the cryopreserved unit were initially deemed to be detrimental. Also the osmotic shock during manipulation of the specimen could potentially be associated with untoward effects (Luciano et al., 2009). Hence the recommended cell concentrations in cryopreserved hematopoietic stem cells was suggested to be not above 2×10^7 /mL (Aird, Labopin, Gorin, & Antin, 1992; Gorin, 1986; Rowley, 1992; Rowley, Bensinger, Gooley, & Buckner, 1994; Silberstein & Jefferies, 1996). This would lead to a total storage space of 7 liters for every patient needing a routinely used cell dose (Rowley, Bensinger, Gooley, & Buckner, 1994). Early work by Law et al., established that stem cells have a high osmotic resistance (Law, Alsop, D C Dooley, & Meryman, 1983), what led to the investigation of higher cell concentrations with a smaller corresponding storage volumes.

After initial preclinical experiments, several studies established that higher cell concentrations were compatible with good functional outcomes and engraftment kinetics (Cabezudo et al., 2000; Yoshifumi Kawano et al., 2004; Rowley, Bensinger, Gooley, & Buckner, 1994; Villalón et al., 2002). Cell counts between 1 and 2×10^8 were found to be safe and feasible (Alencar et al., 2010; Cabezudo et al., 2000; Yoshifumi Kawano et al., 2004; Rowley, Bensinger, Gooley, & Buckner, 1994; Villalón et al., 2002). A more recent study from Brazil (Alencar et al., 2010) compared the effect of PBSC concentrations on cell viability, functional assays and engraftment kinetics. No significant differences were observed between cell concentrations of 1×10^8 /mL and 2×10^8 mL. Today cell concentrations of 2×10^8 /mL and above are considered safe and are used in many cryopreservation centers.

3.4 Creation and addition of the cryopreservation medium

The cryopreseved unit for storage consists of the stem cell collection, the diluent medium and the cryopreservative additive(CPA). Often the cell concentrate is added to the cryopreservative medium (diluent medium+CPA) in a 1:1 volume ratio. The optimal consistency of the cryopreservation medium is still a matter of active research and is poorly standardized (S. M. Watt, Austin, & S. Armitage, 2007; Zeisberger et al., 2010).

3.4.1 The diluent

The diluent consists most frequently of cryoprecipitated autologous or allogeneic plasma. If unavailable human albumin solution is widely used (Berz, McCormack, Winer, Colvin, & Peter J Quesenberry, 2007; Rodrigues et al., 2008; S. M. Watt, Austin, & S. Armitage, 2007; Zeisberger et al., 2010). However, particularly in non-hematologic stem cell cryopreservation protocols, fetal bovine serum(FBS) is still used as a standard diluent (Ellerström et al., 2006; Holm et al., 2010; Unger, Skottman, Blomberg, Dilber, & Hovatta, 2008; Zeisberger et al., 2010). The use of FBS, a xenobiotic mix with variable consistency, is associated with certain tangible risks. Immunologic responses to animal serum components

(Mackensen, Dräger, Schlesier, Mertelsmann, & Lindemann, 2000) or stimulated by hapten formation (Martin, Muotri, Gage, & Varki, 2005) and the transmission of known or unknown animal pathogens to the recipient (Elsaadany et al., 2011; Will et al., 1996) have been raised as concerns. Hence, current research focuses on the development of standardized, xeno-free cryopreservation practices for human stem cells (Ellerström et al., 2006; Holm et al., 2010; Zeisberger et al., 2010). Recently, Zeisberger et al. presented excellent viabilities with a xeno-free, predefined cryomedium, suitable for hematopoietic and mesenchymal stem cells.

3.4.2 The cryopreservative additive

3.4.2.1 DMSO and its Toxicities

Dimethylsulfoxide is the most widely used and was already described as cryoprotectant in 1959 (LOVELOCK & BISHOP, 1959). After having been initially synthesized by Alexander Mikhaylovich Zaitsev in 1867 (Lewis, 1994a; 1994b), DMSO has been used in the wood industry since the 19th century (Ruiz-Delgado et al., 2009). It also found medical application in a wide spectrum of musculoskeletal, autoimmune and metabolic diseases, including gonarthrosis, interstitial cystitis and amyloidosis (Albanell & Baselga, 2000; Eberhardt, Zwingers, & Hofmann, 1995; Iwasaki, Hamano, Aizawa, Kobayashi, & Kakishita, 1994; McCammon, Lentzner, Moriarty, & Schellhammer, 1998; Morassi, Massa, Mesesnel, Magris, & D'Agnolo, 1989). As a small amphiphatic molecule, DMSO penetrates also into stem cells and acts as a strong hydrogen bond disrupter and hence exerts colligative effects (Ruiz-Delgado et al., 2009; N. C. Santos, Prieto, Morna-Gomes, Betbeder, & Castanho, 1997).

The pulmonary excretion of DMSO accounts for the typical garlic like smell, noticed during stem cell infusion (Jacob & Herschler, 1983). Secondary to the wide pharmacokinetic distribution volume, including good blood brain barrier penetration, DMSO affects multiple organ systems with a wide spectrum of toxicities. Those adverse effects include CNS-, respiratory-, hemolytic-, gastrointestinal-, hepatic-, dermatologic-, cardiovascular- and renal toxicities.

The overall most frequently reported side effects are of gastrointestinal and cardiovascular nature. Nausea and abdominal cramping are observed with incidences of up to 70%. Early studies by Davis et al. (J. M. Davis, Rowley, Braine, Piantadosi, & G. W. Santos, 1990; J. Davis, Rowley, & G. W. Santos, 1990) demonstrated that the incidence of side effects rose with the volume of DMSO and amount of cell lysis products infused.

Vasovagal reactions with hypotension and bradycardia are observed with a high incidence. In a multinational survey study performed by Windrum et al., data from 97 EBMT transplant centers were included. DMSO related toxicities other than nausea and vomiting were observed in about one out of 50 transplants with a mean incidence of 2.2% in all administered units. Cardiovascular side effects were most frequently observed, witnessed in 27% of the participating centers (Windrum, Morris, Drake, D Niederwieser, & Ruutu, 2005). The vagolytic effects of DMSO have been documented in physiologic experiments and the hypotension can be attributed to DMSO induced histamine release (J. M. Davis, Rowley, Braine, Piantadosi, & G. W. Santos, 1990; KLIGMAN, 1965). However, confounding is the fact, that stem cell concentrates are usually infused after thawing to a slushy state at a temperature of 4°-8°C. Hence, a substantial amount of the frequently observed bradycardia and hypotension could be calorically induced. Along with this, recipients of stem cell concentrates are usually premedicated with IV glucocorticosteroids, hydration, mannitol and anti-histamines (J. M. Davis, Rowley, Braine, Piantadosi, & G. W. Santos, 1990; J. Davis,

Rowley, & G. W. Santos, 1990). Several of the cardiovascular side effects, including the frequently observed hypertensive episodes, could hence be multifactorial, like the small vessel smooth muscle constriction by DMSO, hydration in combination with mannitol and the influence of glucocorticosteroids in the presence of antihistamine premedication. Hypotensive attacks are more frequently observed in the absence of antihistamine premedication (English et al., 1989; O'Donnell, Burnett, Sheehan, Tansey, & G. A. McDonald, 1981). Other cardiovascular toxicities, which are potentially related to DMSO are electrocardiographic abnormalities (J. M. Davis, Rowley, Braine, Piantadosi, & G. W. Santos, 1990), pulmonary edema and rarely observed case fatalities, such as cardiac arrest (Baum, Weissman, Tsukamoto, Buckle, & Peault, 1992; PEGG & KEMP, 1960; Ruiz-Delgado et al., 2009).

Respiratory side effects are also frequently observed with stem cell infusions and can often be attributed to DMSO toxicity. Mild bronchospasm and subclinical reductions of pulmonary capacity are frequently observed, but severe respiratory depressions with the need for pulmonary resuscitation have been reported (Benekli et al., 2000; Miniero, Vai, Giacchino, Giubellino, & Madon, n d).

Other, less frequently observed toxicities are anaphylaxis, renal failure, seizures, acute hepatotoxicity and hemolysis.

3.4.2.2 Alternatives to standard DMSO

The above described toxicities led to efforts to reduce the patients' exposure to DMSO. Principally five different approaches are possible. First, the reduction of DMSO concentration in the stem cell concentrate, second creating a stem cell product with a higher cell concentration and corresponding smaller volumes and a lower cumulative exposure to DMSO, third a prolonged infusion with a less intense exposure to DMSO, fourth DMSO depletion in post-thaw processing and last, using alternative CPAs, either alone or in combination with DMSO.

A DMSO concentration of 10% in the stem cell concentrate is still considered standard in most centers around the world. However, several investigators examined the effects of lower DMSO concentrations on recovered cell counts, viability and colony forming capacity. DMSO concentration as low as 2% were used (Bakken, O Bruserud, & J F Abrahamsen, 2003; Balint et al., 1999; Galmés et al., 1999; Halle et al., 2001; Syme et al., 2004; Zeisberger et al., 2010). Overall, 5% DMSO concentrations delivered comparable results to the standard 10% concentration, whilst DMSO concentrations of less or equal to 2% revealed inferior cell integrity, at least in some reports (Zeisberger et al., 2010). In a recent retrospective report from Norway, 103 consecutive patients underwent autologous PBSC transplants after high dose chemotherapy for lymphoma or myeloma (Akkök et al., 2008). The stem cell concentrates were preserved with 10% DMSO in the initial period and with 5% DMSO in the later period. Clinical outcomes, such as transfusion requirements and neutrophil/platelet recovery were essentially the same.

In recent years, the disaccharides trehalose and sucrose have been evaluated as cryopreservative additives (Buchanan et al., 2004; Rodrigues et al., 2008; Scheinkönig, Kappicht, Kolb, & Schleuning, 2004; E J Woods et al., 2000; X. B. Zhang et al., 2003). The exact mode of action of those small molecules remains elusive, but it has been well demonstrated that the integrity of membrane layers and proteins during cryopreservation remains well preserved in the presence of those molecules (J H Crowe et al., 2001; John H Crowe, 2007). Along with a favorable toxicity profile of those compounds, certain

pharmacokinetic properties may be of benefit. Trehalose is not able to permeate into the interior of the cells. This facilitates removal during post-thaw washing (Rodrigues et al., 2008). Sucrose/trehalose/DMSO combinations with DMSO concentrations as low as 2.5% have been shown to be comparable with standard 10% DMSO as cryo-additive. Cell count recovery, viability and clonogenicity were similar in several studies (Rodrigues et al., 2008). Similarly encouraging results have been noticed in the presence of catalase type natural bio-oxidants in the cryopreservation medium (Motta, Gomes, L F Bouzas, Paraguassú-Braga, & Porto, 2010; Sasnoor, Kale, & Limaye, 2003), although the exact role of the natural bio-oxidant is still not known.

Another group of substances which are excellent candidates as cryo-preservative additives are hydrophilic macromolecules. They also have the pharmacokinetic benefit of being restricted to the extracellular space and some follow a first order elimination kinetic. Albumin, modified gelatin, hydroxyethyl starch(HES), polyvinylpyrrolidone and polyethylene oxide are members of this group of substances. HES has been most widely studied, particularly to reduce the DMSO concentration (Clapisson et al., 2004; Jeffrey McCullough et al., 2010). A combination of 5% DMSO and 6%HES has been shown to be associated with successful long term storage of PBSC(Jeffrey McCullough et al., 2010). In a blinded randomized phase III, patients underwent high dose chemotherapy with autologous PBSC support, either preserved with a standard solution containing 10% dimethylsulfoxide (DMSO, v/v) or 5% DMSO in combination with 6% hydroxyethylstarch (HES, w/v). One hundred and forty eight patients received PBSC frozen with 10% DMSO and 146 received cells frozen in 5% DMSO/6% HES. Whilst platelet recovery and the median amount of blood products transfused did not differ in between the two groups, the patients obtaining cell concentrates, cryopreserved with the DMSO/HES combination obtained neutrophil recovery in average one day earlier and needed one day less antibiotic administration($p=0.04$)(Rowley et al., 2003).

Unfortunately hematopoietic and other stem cells, particularly human embryonic stem cells, undergo apoptotic transformation during the cryopreservation process (Sangeetha, Kale, & Limaye, 2010; Stroh et al., 2002). This led to the study of caspase inhibitors as cryopreservatives. The initial encouraging results on cell cultures (Heng, Clement, & T. Cao, 2007; Stroh et al., 2002), were recently supported by in vivo experiments(Sangeetha, Kale, & Limaye, 2010).

Other cryo-preservative additives, such as α tocopherol(Neunert et al., 2009; E J Woods et al., 2000), are under investigation and may be of future interest.

3.5 The freezing process

3.5.1 Freezing rate

The optimal method of freezing the cell concentrate to the target storage temperature still remains a matter of debate. Only few high quality studies are available to guide the clinician and laboratorian in the choice of the optimal technique. The controlled rate freezing(CRF) procedure still remains the defined standard in many countries (S. M. Watt, Austin, & S. Armitage, 2007). The principal rationale for CRF as choice is the limited cell damage during the freezing process (Donaldson et al., 1996; Douay, 1985; Yang et al., 2001), particularly at the eutectic transition point. At the eutectic transition point the liquid phase transits into a solid phase and fusion heat is released. Prolonged cell exposure at this point is regarded

detrimental to the survival of cells (Douay, 1985). The principal time trajectory during controlled rate freezing involves initially slow freezing, at a rate of -1° to -2° Celsius per minute, then very rapidly around the eutectic point, to be then further cooled at a steady, preset rate to a target temperature to be finally placed into nitrogen for durable storage. Several modifications of this technique have been described in the literature (Berz, McCormack, Winer, Colvin, & Peter J Quesenberry, 2007; Donaldson et al., 1996; Gorin, 1986; Perez-Oteyza et al., 1998; Valeri & Pivacek, 1996; S. M. Watt, Austin, & S. Armitage, 2007). Briefly, one typical algorithm for controlled rate freezing using the KRYO10 freezer encompasses, pre-cooling of the specimen to 6° Celsius and placement into a cryo-cassette. Then the cooling process proceeds at a rate of -1° to -2° Celsius/minute to a temperature of -5° C, followed by rapid cooling around the eutectic point to avoid damage by fusion heat release. Then the cell concentrate continues to be cooled at a rate of -1 Celsius /minute to a temperature of -40 Celsius followed by a rate of -5 Celsius /minute to a target of -135 to -160 Celsius. Finally, the product is placed into permanent nitrogen storage.

Unfortunately, this technique requires sophisticated, costly equipment and a certain personal expertise, which are not available at every center (Almici et al., 2003; Perez-Oteyza et al., 1998).

Several single armed studies have reported excellent viability and hematopoietic recovery rates with uncontrolled freezing procedures in bone marrow (Clark, Pati, & D. McCarthy, 1991; Stiff, Murgo, Zaroulis, DeRisi, & Clarkson, 1983) and peripheral blood progenitor specimens (Almici et al., 2003; Cilloni et al., 1999; Halle et al., 2001).

Few studies directly compared outcomes of controlled rate to uncontrolled rate freezing processes.

A preclinical study, using murine bone marrow samples compared two controlled rate freezing protocols with one uncontrolled rate protocol. Nuclear cell count, viability and several functional assays (MRA, CFU-S and CFU-GM) were assessed after thawing and washing. Although comparable cell counts and viability assays were achieved, superior functional assays (particularly CFU-S and CFU-GM) were achieved with the controlled rate freezing procedure (Balint et al., 1999).

In a clinical study from Japan, two different freezing protocols were compared in the cryopreservation of peripheral blood stem cells (PBSC) (Y Takaue et al., 1994). The PBSC were cryopreserved by either controlled rate or uncontrolled rate freezing methods. No differences were observed in the granulocyte/platelet engraftment times and transfusion requirements were similar in both groups (Y Takaue et al., 1994).

The interpretation of this study is somewhat complicated by the fact that the cells in the uncontrolled rate freezing arm also differed in the cryopreservative additive from the standard arm. In addition, only twelve patients obtained the cells, preserved by uncontrolled rate freezing and the study design was not randomized, using matched historical controls as reference.

In a prospective, randomized controlled multicenter study from Spain, apheresis products from 105 patients, who obtained a peripheral stem cell transplant for various malignancies, were split into two bags. One was processed with controlled rate, the other one with uncontrolled rate freezing. Nucleated cell counts, viability and a committed functional assay (CFU-GM) were assessed after thawing. No difference in the loss of cell counts and viability were observed, but again the CFU-GM assay performed superior in the controlled rate freezing arm (Perez-Oteyza et al., 1998). Although the strength of the study was that each

sample functioned as its own control, i.e. all other the outcome influencing parameters were kept identical in both arms, the study was not powered to detect differences in the patients' clinical outcomes, such as engraftment kinetics and survival. It remains hence unclear, if the differences in the functional assay outcome hold clinical relevance.

In conclusion, we feel that the controlled rate and the uncontrolled rate freezing procedure are both viable techniques for the preservation of stem cells. At our institution, we perform controlled rate freezing only for bone marrow. Future studies, sufficiently powered to examine relevant clinical outcomes will be needed to determine if the more elaborate controlled rate approach is truly superior.

3.5.2 Storage temperature

The minimal requirements for a long term storage temperature are technical feasibility and a successful clinical outcome. The storage temperature for hematopoietic stem cells varies between different centers. Temperatures range from -196°C to -80°C (Aird, Labopin, Gorin, & Antin, 1992; Cilloni et al., 1999; Galmés et al., 1999; Halle et al., 2001; Rubinstein et al., 1995; Son, Heo, Park, H. H. Kim, & K. S. Lee, 2010; Valeri & Pivacek, 1996). The initially used storage temperatures of -196°C , reflecting the liquid phase nitrogen storage, have largely been replaced by temperatures of -156°C to -135°C , reflecting the vapor phase storage (Berz, McCormack, Winer, Colvin, & Peter J Quesenberry, 2007) in BM-, PB- as well as umbilical cord derived stem cells (Aird, Labopin, Gorin, & Antin, 1992; Cilloni et al., 1999; Galmés et al., 1999; Halle et al., 2001; Rubinstein et al., 1995; Son, Heo, Park, H. H. Kim, & K. S. Lee, 2010; Valeri & Pivacek, 1996). This shift in culture was mainly induced by the observation that infectious pathogens can survive and be propagated in the liquid nitrogen phase (Bielanski & Vajta, 2009). This fact received a large deal of public attention, when in the mid-1990s six recipients of BM- and PBSC- transplants developed icteric hepatitis B. Follow up examinations revealed that leakage from one auto-donor's stem cell product induced viral contamination of five patients' units, which were stored in the same tank (Tedder et al., 1995).

A recent study by McCullough et al. compared 5 different protocols for five year PBSC storage. Along with other variables, liquid phase nitrogen storage (-196°C) was also compared to a mechanical freezer temperature at -135°C . No significant outcome differences were observed between both temperatures (Jeffrey McCullough et al., 2010).

For human embryonic stem cells, which are stored after vitrification, the storage temperature is a more crucial subject. Even slight temperature variation can lead to devitrification of the metastable cryopreservate with crystal formation and corresponding cell damage (Baicu, M. J. Taylor, Z. Chen, & Rabin, 2008; Wusteman, Robinson, & D. Pegg, 2004). Techniques have been developed to minimize those potentially detrimental temperature fluctuations (C. J. Hunt, 2011; Rowley & Byrne, 1992).

3.5.3 Infectious considerations

The contamination of stem cell products with infectious pathogens can occur at several points during the stem cell processing-The marrow harvest, cord blood collection or apheresis, the transport of the product to the cryopreservation facility, the pre-thaw processing, the thawing and washing process as well as the infusion of the final product are all processing points with the potential for microbial contamination. Infectious

contaminations during those processes are observed with varying degrees, generally 0-4.5% for PBSC and up to 26% for bone marrow, even if strictly aseptic protocols are followed (Attarian, Bensinger, Buckner, D. L. McDonald, & Rowley, 1996; Espinosa, Fox, Creger, & Lazarus, 1996; Jestice et al., 1996; Kamble et al., 2005; Larrea et al., 2004; Lowder & Whelton, 2003; Majado et al., 2007; D. J. Padley et al., 2003; D. Padley, Koontz, Trigg, Gingrich, & Strauss, 1996; Schwella et al., 1994; Webb et al., 1996). Along with this, bacteria, fungi and viruses are able to survive in the liquid nitrogen storage phase and cross-contaminations between different units, stored in the same container have been observed (Bielanski, 2005; Bielanski, Bergeron, Lau, & Devenish, 2003; Bielanski & Vajta, 2009).

If microbial contaminations in other blood products, such as red blood cells, platelet concentrates or plasma products are detected, they are usually discarded. However, in stem cell concentrates this decision is complicated by the fact, that the total amount of stem cells is limited. Several reports described case fatalities when stem cell recipients received microbially contaminated products ("Current good tissue practice for human cell, tissue, and cellular and tissue-based product establishments; inspection and enforcement. Final rule," 2004; "Eligibility determination for donors of human cells, tissues, and cellular and tissue-based products. Final rule," 2004; Kamble et al., 2005). The FDA recommends caution with the use of contaminated products and estimated that seven deaths per year could be prevented by the elimination of contaminated stem cell units.

The predominant cultured bacterial subspecies are part of the skin flora and other commensal organisms, with the remainder being mainly enteric organisms. However, other opportunistic and non-opportunistic organisms like *Stenotrophomonas maltophilia* and MRSA have been identified (Kamble et al., 2005; Larrea et al., 2004; Patah et al., 2007; Vanneaux et al., 2007).

The clinical relevance of microbial contamination of stem cell products has been questioned by several authors. Two recent studies examined cultures of stem cell collections at different time points of the cryopreservation process. All patients were routinely prophylaxed with fluoroquinolones, acyclovir and fluconazole. Further, organism specific antimicrobial therapy was added when positive cultures were detected prior to the infusion (Majado et al., 2007; Patah et al., 2007).

A study from the MD Anderson cancer center reported the experience with 3078 autologous or allogeneic hematopoietic stem cell infusions over a six year time span. Cultures were taken at apheresis or marrow harvest, infusion for fresh products (mainly allogeneic) and thawing/infusion of the cellular products. The overall rate of positive cultures in this study was relative low at 1.2% and coagulase negative Staphylococci were the most frequently detected organisms. None of the 21.6% of the patients who died in the post-transplant period died from a cause, ascribed to the contaminated stem cell product (Patah et al., 2007). Another study from Spain summarized the experience with 152 patients receiving a total of 617 bags of autologous, cryopreserved PBSC. Cultures were taken pre-freezing and at the end of the infusion from the post-thaw specimen. Overall, 31 were found to be contaminated (5%) and skin commensals were again the most frequently identified species. No increased mortality or other severe clinical sequelae were observed in the patients obtaining the contaminated stem cell infusions. The length of hospitalization in the acute transplant period was longer in this group of patients. However, this could not be attributed to infections with the contaminant, suggesting other patient specific factors in this patient group (Majado et al., 2007).

Other studies with a similar methodology were performed and confirmed all the following results (Kamble et al., 2005; Larrea et al., 2004; Vanneaux et al., 2007): First, skin commensals are the most prevalent cause of microbial contaminations; Second, the relatively increased incidence of positive cultures after bone marrow harvest when compared to peripheral blood apheresis products seems to be decreasing, possibly reflecting the implementation of recent asepsis guidelines. Third, the rate of positive blood cultures is the highest directly after stem cell collection and lowest after cryopreservation. This eludes to a certain antimicrobial effect of the cryopreservation procedure and possibly the cryopreservative DMSO.

Cultured organism	Overall incidence of positive cultures[%]	Fraction of positive cultures[%]
Staph. epidermidis and other coagulase negative Staphylococcus (CNS)	3-11.7	53.1-87.2
Propionibacterium acni	0.6-2.2	0.1-27.2
Staphylococcus aureus	0-1.6	0-2.3
Bacillus cereus and other Bacillus spec.	0.06-0.35	0-0.8
Pseudomonas spec.(aeruginosa, putida and fluoresces)	0-0.32	0-0.8
Corynebacterium spec.	0-0.3	0-6.5
Stenotrophomonas maltophilia	0-0.3	0-5.9
Aspergillus fumigatus	0-0.3	0-7.6
Mixed cultures	0.1-15.6	0-0.82

Table 1. The table above describes the growth of different organisms when cultures were assessed in after thawing, before infusion of the unit. The studies, used to extract the table are referenced in the paragraph above. The middle column reflects the overall incidence of positive cultures by organisms over different studies, the right column lists the fraction those organisms represented. All values are expressed in percentages.

However, bacteria and other organisms can survive cryopreservation and have to be considered when antibiotic coverage after infusion is administered. Fourth, the clinical impact of infusion with contaminated stem cell products seems to be manageable, when handled by expert hands. Table 1 summarizes the results of the referenced studies.

3.5.4 Durability

The durability of a stem cell graft, is defined as the timespan a stem cell graft can be preserved and still exert the desired clinical effect when utilized. Although the time span between collection and infusion into the recipient for most allogeneic and autologous hematologic transplants is relatively short, for cord blood it can be much longer than a decade. The maximal possible cryopreservation time span is still unknown.

Several of the above listed functional substitute assays have been used to determine the functional integrity of cryopreserved stem cell grafts. For bone marrow, peripheral blood as well as cord blood stem cells, it has been shown that clonogenic assays, such as the BFU-E and CFU-GM are compromised fairly early during the course of cryopreservation. In

contrast, the recovery of nucleated cells (NC) and CD34+ cells remains relatively well preserved (Attarian, Feng, Buckner, MacLeod, & Rowley, 1996; Hal E Broxmeyer et al., 2003; Buchanan et al., 2004; Cilloni et al., 1999; E J Woods et al., 2000; Xiao & Douglas C Dooley, 2003; Yang et al., 2001; X. B. Zhang et al., 2003). In addition, the engraftment capacity in the immunodeficient NOD/SCID mice remains relatively well preserved for long periods of time (Bock, Orlic, Dunbar, H E Broxmeyer, & D M Bodine, 1995; Vormoor et al., 1994). The durability of cord blood stem cells beyond 15 years of cryopreservation has been shown by several authors with different functional substitute assays (Hal E Broxmeyer et al., 2003; Kobylka, Ivanyi, & Breur-Vriesendorp, 1998; Mugishima et al., 1999). Kobylka et al. and Mugishima et al. proved the durability after up to 15 years of storage with flow cytometric and clonogenic assays, whilst Broxmeyer et al. also demonstrated hematopoietic reconstitution of sublethally irradiated NOD/SCID mice (Hal E Broxmeyer et al., 2003).

The clinical experience with long term cryopreserved stem cell grafts remains somehow limited. Anecdotal reports confirmed successful trilineage engraftment with BM derived stem cell grafts, which were stored for 7 years (Walter et al., 1999). A systematic review evaluating the combined experience of the Brigham and Women's Hospital and the EBMT Group (Aird, Labopin, Gorin, & Antin, 1992) noticed that HSC can be effectively cryopreserved for up to 11 years. A retrospective study from Seattle revealed full trilineage recovery in patients receiving HSC, stored for up to 7.8 years without consistent detrimental effects (Attarian, Feng, Buckner, MacLeod, & Rowley, 1996). It also remains unclear if any of the currently used cryopreservation protocols reveals superior results when used for clinical applications in humans. A recent report by McCullough et al. compared four different protocols for cryopreservation of PBSC. After five years of storage the relative integrity of PBSCs was preserved, regardless which of the cryopreservation protocols was used (Jeffrey McCullough et al., 2010).

3.5.5 Storage containers

Containers for the long term storage of stem cell concentrates need to fulfill at least closure integrity, sample stability over long periods of time and easy accessibility as minimal requirements (Erik J Woods & Thirumala, 2011). With the increasing financial and environmental strain on health care systems, even in developed nations, and the increasing amount of umbilical cord stem cells banked, additional points of relevance are a low cost, potential for reuse, environmental sustainability and economic use of available long term storage space. Container devices prior to the actual long term cryopreservation need to allow for convenient collection of the stem cells and storage in the liquid phase at supra-freezing temperatures for up to 72 hours. In addition, the processing of the stem cell collection often entails centrifugation and hence the requirement for the container to be resistant to significant gravitational forces. To date, most institutions use several containers or bags prior to the actual cryopreservation bag. In addition, to prevent spillage and cross contamination of concentrates stored in the same storage nitrogen tank, the ISCT recommends over-rap bags on their web site. Historically most stem cell storage institutions arose from blood banks with long standing expertise with bags as storage containers (Khuu et al., 2002; Thirumala, Goebel, & Erik J Woods, 2009; Erik J Woods & Thirumala, 2011). Hence, freezing bags are the most frequently used long term storage containers for stem cells and ethylene vinyl acetate (EVA) based products represent the majority in the market (Thirumala, Goebel, & Erik J Woods, 2009).

A 2002 publication reported breakage of EVA based bags, storing PBSC and lymphocyte concentrates, with a microbial contamination rate of 42% (Khuu et al., 2002). Along with that, a recent publication described a breakage rate of 3.5% over a 6.5 year storage period on umbilical cord concentrates (Thyagarajan, Michael Berger, Sumstad, & McKenna, 2008). Similar concerns were raised in the past with different plastic materials (Valeri & Pivacek, 1996).

The historical experience with catastrophic viral cross infection (Tedder et al., 1995) as well as such recent reports triggered increasing concerns about EVA based products. EVA experiences a glass transition below -15°C , what renders it brittle and potentially fragile below this temperature (Kempe MD, Jorgensen GJ, Terwilliger KM, McMahan TJ, Kennedy CE, n d). Those concerns, along with the desire to produce reusable materials, have prompted the exploration of other materials as base for cryocontainers (Eakins MN, n d).

Trade name	Material
<ul style="list-style-type: none"> • Cryocyte/Baxter® • CellFlex/Maco Pharma® • Cell Freeze™ Charter Medical • Pall Medical® Freezing Bag 791-05 • Cryostore EVA/Origen Biomedical Inc. • Thermogenesis® Corp./Freezing bag 80346-0 • Origen Cryostore® • EVA CryoMACS® 	EVA* based
<ul style="list-style-type: none"> • KryoSure® • American Fluoroseal® • Fresenius Hemocare/Hemofreeze® • Origen Biomedical Inc./Permalife Bag 	FEP# FEP/Teflon Teflon/Kaplon FEP/Polyimide

Table 2. The table above lists several commercially available bags for cryopreservation of stem cells. Please note, that some of them may not be currently available or FDA approved.

A recent publication by Woods et al described a cyclic olefin co-polymer based container system with favorable cryo-physical properties. The small size of those containers at 2mL and 5mL would be prohibitive for large scale HSC storage but was successfully assessed for suitability of dental pulp derived MSC(Erik J Woods & Thirumala, 2011).

Another report from the Czech republic demonstrated success cryopreservation in a reusable stainless steel container specifically designed for PBSC(Měricka et al., 1991). Table 2 describes some of the products used for cryopreservation of stem cells around the world.

3.6 Thawing and post-thaw processing

At our institution the thawing of the cryopreserved unit is performed by the technician at the bedside. The unit is then handed over to the treating physician or nurse for infusion into the patient. Depending on the regulatory environment, in certain countries the unit is retrieved from the storage tank by the technician to be handed over for transport to the patient and thawing by the physician or supporting clinical staff. Regardless of the logistic specifics, the unit is thawed to a slushy state and then slowly infused into the recipient

under close clinical observation. Several elements of the post thaw process are still a matter of debate and we will here discuss relevant points on washing and the functional post-thaw assessment of stem cells.

3.6.1 Washing of stem cells

Since increased toxicities of DMSO have been observed with increasing amounts, attempts to develop protocols to deplete the DMSO content in the infused stem cell product have been undertaken. Several protocols to reduce the infused DMSO content have been published (Lemarie et al., 2005; Syme et al., 2004). Some of those procedures achieve 2 log reductions in the infused DMSO concentration. Theoretical concerns have been raised that extensive washing can negatively impact on engraftment kinetics. Unfortunately, only few prospective studies have examined the influence of post-thaw washing on toxicities and hematopoietic reconstitution (Akkök, Holte, Tangen, Ostenstad, & Oystein Bruserud, 2009; Lemarie et al., 2005; Syme et al., 2004).

In a recent study by Akkök et al., 53 patients obtained high dose chemotherapy with PBSC transplant for multiple myeloma, non Hodgkins lymphoma, amyloidosis or POEMS syndrome. The maximal liquid storage time prior to cryopreservation was less than 24 hours. The cryomedium contained AB allogeneic plasma and controlled rate freezing was routinely performed. The patients either received the graft either directly post-thaw (n=34) or cells, DMSO depleted cells by virtue of a one-step washing procedure(n=19). Only patients with a CD34+ count of at least 2.5×10^6 as backup were allowed for depletion. The washing solution consisted of an ACD-saline mixture. Directly after thawing, the graft was mixed with the washing solution and centrifuged at 850Xg for 6 minutes at 22°C. The supernatant was discarded and the pellet re-suspended with ACD-saline solution to a total volume of maximal 150mL. The total ex-vivo handling time after removal from liquid nitrogen storage was 50-60 minutes longer for the depleted grafts(Akkök et al., 2009). A statistically significant 23.1% loss of CD34+ cells was observed. However, the extracellular DMSO concentration and number of neutrophils were both reduced in the washed stem cell product. Overall less infusion related adverse events were observed in patients receiving the washed autografts (16% vs. 36%, $p=0.024$). The neutrophil recovery and length of neutropenic fever episodes were comparable in both arms, but the platelet recovery was delayed by two days (14 vs 12 days) in the group obtaining a washed graft. This was associated with a statistically not significant increase in clinically meaningful hemorrhage events. The authors concluded that in certain patient populations, a simple one-step washing procedure can reduce DMSO related adverse infusion reactions with a tolerable compromise of platelet engraftment kinetics.

Although post-thaw washing is not a routinely performed technique at our center for PBSC and BM derived stem cell concentrates, it holds a higher relevance for UCB products. Umbilical cord blood transplantation is gaining increasing popularity for a variety of malignant and non-malignant diseases. To date, more than 20,000 UCB transplants have been performed on children and adults, and more than 400,000 UCB units are available in more than 50 public CB banks (Solves, Mirabet, & Roig, 2010). The way cord blood units are collected from the donor, renders them rich in hypertonic cryopreservative, red cells, plasma and cell debris. This demands additional processing, not routinely applied in PBSC and BM derived stem cells. This processing is partially performed before and partially after the cryopreservation. Initially, after collection, the collected unit contains a large amount of

red blood cell bulk and plasma. Volume reduction is essential to reduce the total amount of required storage space. The standard New York Blood Center protocol implies a two-step procedure. Here, 6% HES is added in a 1:5 volume ratio to the collected, anticoagulated UCB. This increases the erythrocyte sedimentation rate. The mixture is then centrifuged at 50Xg for 5 minutes at 10°C. The leukocyte rich supernatant is expressed and again centrifuged at 400Xg for 10 minutes. The pellet is resuspended in supernatant plasma to a total volume of 20mL, which then is used to create the finally cryopreserved unit. The post-thaw washing procedure entails the addition of an equal volume 2.5% albumin, 40 dextran solution and centrifugation at 400Xg for 10 minutes. The sedimented cells are then re-dispensed in fresh albumin/dextran solution prior to infusion (Rubinstein et al., 1995).

Although the washing UCB units has been shown to increase the viability of remaining cells (Rubinstein et al., 1995), concerns of cell loss in a product which intrinsically suffers from a paucity of cells have been raised (Laroche et al., 2005). Hence, alternative washing procedures, excluding the post-thaw centrifugation step have been proposed. Here, the deleterious osmotic shift is prevented by introduction of a dextran/albumin solution under laboratory conditions, but no centrifugation is subsequently performed. In a recent report from the Memorial Sloan Kettering Cancer Center (MSKCC), such a technique was applied in 54 consecutive patients, obtaining double cord blood transplants (Barker et al., 2009). The sustained donor engraftment rate was excellent (94%) and the amount of DMSO infused compared favorably with autologous transplants. The infusion reactions were manageable with the most prominent concern being the episodes of renal compromise, probably induced by residual cell debris.

3.6.2 Functional substitute assays

The ultimate functional stem cell assay is the engraftment with subsequent reconstitution of the desired physiologic function, such as cell count recovery in hematopoietic stem cells in the myeloablated human host.

However, for obvious reasons we are relying on functional substitute assays to estimate the functional integrity of the stem cell graft. Such substitute assays consists of cell counting experiments enumerating cell populations with a high stem cell potential, viability tests, quantifying certain biologic functions, clonogenic assays, reflecting the potential of cell population to give rise to other subspecialized cell types and direct engraftment experiments in previously myelo-ablated or immune-deficient mammals (see table 3).

The most frequently used cell counting assays are the total nucleated cell count and the count of CD34 positive cells (Kurtz, Seetharaman, N. Greco, & Moroff, 2007; Yang et al., 2001). Increasing numbers of both have been shown to correlate positively with the hematopoietic recovery potential in HSC transplantation (Kurtz, Seetharaman, N. Greco, & Moroff, 2007; Yang et al., 2001). Another flow-cytometry marker, which has recently been found to correlate positively with the enumeration of viable stem cell populations is aldehyde dehydrogenase (ALDH+). The particular benefit of this marker is, that ALDH positive cells show a very low or absent apoptotic potential (D. A. Hess et al., 2004; Kurtz, Seetharaman, N. Greco, & Moroff, 2007). Trypan blue-, 7-aminoactinomycinD (7-AAD) and propidium iodide-exclusion have been shown to be particularly strongly expressed in immature, viable cell populations, such as stem cells (Kurtz, Seetharaman, N. Greco, & Moroff, 2007; K. Liu et al., 2003; M. Solomon, Wofford, C. Johnson, Regan, & Creer, 2010; Ware, Nelson, & Blau, 2005; E J Woods et al., 2000; Xiao & Douglas C Dooley, 2003). They

function as true viability assays and are diminished or absent in apoptotic cell populations. The simple and fast feasibility of such tests qualifies them as excellent functional substitute assays, especially before freezing and after thawing of stem cell concentrates.

Clonogenic assays such as the CFU-Sd12, CFU-GM, CFU-GEMM, BFU-E, LTC-IC and direct engraftment assays performed on NOD/SCID mice are more sophisticated functional substitute assays. Figure two displays images of clonogenic experiments. However, such assays are expensive, time intense, require specific personal expertise and laboratory equipment and are poorly standardized. Hence, they are not routinely used in standard qualitative assessments of stem cell grafts.

Biologic function	Assay type	Corresponding references
Cell enumeration assays	Nucleated Cell counts	(Donnenberg et al., 2002; Shlebak et al., 1999; E J Woods et al., 2000)
	Flow cytometry for CD34+cells	(Donnenberg et al., 2002; Sasnoor et al., 2003)
Viability/apoptosis tests	Trypan blue	(K. Liu et al., 2003; Ware, Nelson, & Blau, 2005)
	7-AminoactinomycinD	(Xiao & Douglas C Dooley, 2003; Yang et al., 2001)
	Propidium Iodide	(K. W. Johnson et al., 2007)
	SYTO16 assay	(Sparrow & Tippett, 2005)
	Flow cytometry for ALDH+ reactivity	(D. A. Hess et al., 2004; Kurtz, Seetharaman, N. Greco, & Moroff, 2007)
Clonogenic assays	CFU-sd12, CFU-GM, CFU-GEMM	(Balint et al., 1999; Kobylka et al., 1998; Perseghin et al., 1997; Shlebak et al., 1999)
	BFU-E	(Balint et al., 1999; Kobylka et al., 1998; Perseghin et al., 1997)
	LTC-IC	(Barker & Wagner, 2003; Ito et al., 2010)
Direct engraftment experiments	Engraftment in NOD/SCID mice	(Halle et al., 2001; Matsumoto et al., 2002; Perez-Oteyza et al., 1998; Valeri & Pivacek, 1996)

Table 3. The table above outlines different methods to assess a stem cell graft. The right column displays the biologic function assessed, the middle column the actually used assay and the right column references for review of the corresponding subject. long-term culture-initiating cells (LTC-IC), CFU-GM-colony forming unit granulocyte/macrophage; -GEMM-colony forming unit granulocyte/erythrocyte/macrophage/megakaryocyte; BFU-burst forming unit-erythrocyte, CFU-Sd12-colony forming unit spleen on day 12.

No final consensus is reached as to the optimal assessment of a donor graft. Cell counting assays, either with or without the enumeration of CD34 positive cells are still the most frequently used tests. Several problems have recently been outlined with the use of those assays. First, a substantial inter-laboratory variability in CD34 counts can be observed (Dzik, Sniecinski, & Fischer, 1999; Moroff et al., 2006). Second, although the total NC did not significantly change in several studies, the number of CD34+ cells can be significantly reduced after thawing (Kurtz, Seetharaman, N. Greco, & Moroff, 2007). Third, a significant number of CD34 bright cells are not functional when tested on viability assays. In a recent study by Pranke only about 1.8% of the bright CD34+ cells were alive, whereas a small part (19.0%) was actively undergoing apoptosis and most of them (79.2%) were dead, when judged by the 7-AAD exclusion assay (Pranke et al., 2006). Last, when novel enzymatic, such as the ALDH (N. J. Greco, W. R. Lee, Kurtz, Seetharaman, & Moroff, 2003), or apoptotic, such as SYTO16 (Sparrow & Tippett, 2005), markers are used, a more sophisticated assessment of the clonogenic/regenerative potential of the collected cell population may be possible. In a recent study by Kurtz et al., three different methods to enumerate CD34+ cells were assessed on pre-freeze and post-thaw samples, along with the viability marker 7-AAD and the apoptotic marker SYTO16. Although only minor changes in NC and CD34+ counts were observed, the functional assays were significantly impacted by the freezing/thawing process (Kurtz, Seetharaman, N. Greco, & Moroff, 2007).

The above mentioned concerns notwithstanding, Yang et al demonstrated that viable CD34+ cell counts are reliable indicators for successful clinical hematopoietic recovery on pre-freeze and post-thaw samples (Yang et al., 2005). This observation reflects most likely the dogma of stem cell biology, that principally only one cell with stem cell potential is necessary to regenerate the entire hematopoietic system of a myeloablated host. Although, the theoretical definition of a stem cell is relatively clear, it still remains unclear how to define its immunophenotype and although the CD34+ cell population certainly harbors cells with stem cell potential, those represent certainly only a small fraction (K. W. Johnson, M. Dooner, & P J Quesenberry, 2007; Peter J Quesenberry, M. S. Dooner, & Aliotta, 2010). Table 3 depicts different methods to assess the functionality of a stem cell graft. At our center, we routinely perform NC-, CD34+- count as well as the trypan blue viability assay before freeze and after thaw with excellent observed engraftment correlations.

3.7 Cryopreservation of Non-Hematologic Stem Cells

The cryopreservation for the different hematologic stem cell populations are well defined with rather subtle evolving refinements. In contrast, the cryopreservative technology for non-hematologic stem cells is still evolving. The highest level of sophistication to date has been reached with the MSCs. A comprehensive discussion of cryopreservation for non-hematopoietic stem cells would be beyond the scope of this chapter. Detailed reviews have been recently published (Hanna & Allison Hubel, 2009; C. J. Hunt, 2011). We will briefly outline some of the parallels and differences between non-hematologic and hematologic stem cell cryopreservation in the following paragraph.

- a. Mesenchymal stem cells(MSC). No consensus has been reached as to the optimal cryopreservation protocol of MSC, but most of the published efforts were guided by cryopreservation approaches for HSCs (G. Liu et al., 2008; Thirumala, Gimble, & Devireddy, 2010; Thirumala, Goebel, & Erik J Woods, 2009; Erik J Woods et al., 2009). Several particular concerns were identified. First, MSCs seem to lose their viability very

rapidly post-thaw, what can most likely be attributed to the rapid development of apoptotic processes. When Pal et al. investigated the behavior of MSC post thawing, a rapid decrease in viability from >80% at 2 hours to <40% at 8 hours was identified, even when the cells were maintained at 4°C (Pal, Hanwate, & Totey, 2008). Protocols utilizing anti-apoptotic agents, such as ROCK-inhibitors, have shown promise in preclinical experiments (Heng, 2009). Second, the reliability of standard viability assays for MSCs has been questioned. Trypan blue, which is still in wide application in the clinical use of HSCs in the US, may not as accurately as 7-AAD, the preferred HSC-viability-assay in Germany, reflect the viability of MSCs (D. E. Pegg, 1989). Third, MSCs may play a role in the treatment of acute and chronic processes of the heart muscle. DMSO as cryopreservative additive(CPA) with the associated cardio-toxicity may not be the best choice for these clinical scenarios (Zenhäusern, Tobler, Leoncini, O. M. Hess, & P. Ferrari, 2000). Fourth, the alternative CPAs for MSCs are still poorly defined. Trehalose, glycerol and proline did have good efficacy in HSC but do not work in MSC (Grein et al., 2010; Y. Liu et al., n d). Last, most published protocols to date encompass the use of xenobiotic components, such as fetal calf serum(FCS). This is associated with the corresponding immunologic as well as infectious concerns. Recent developments with xeno-free cryopreservation protocols seem to be viable options for MSCs (Zeisberger et al., 2010).

- b. Human embryonic stem cells(hESC). Initial attempts to preserve hESC with protocols analogous to the slow controlled rate freezing HSC protocols were associated with recovery rates below 30% and high post-thaw differentiation (Ha et al., 2005; Reubinoff, Pera, Vajta, & Trounson, 2001; C.-quan Zhou, Q.-yun Mai, T. Li, & Zhuang, 2004). Hence, alternative methods for cryopreservation of hESC were explored. The vitrification technique is for long in use for cryopreservation of embryos and oocytes in veterinary and human application (Yan et al., 2011). Vitrification represents the direct transformation of a substance into a glass like state without the formation of ice crystals (M. Ojovan, n d). This can be reached by very high cooling rates and high concentrations of cryopreservative. In Reubinoff's original open pulled straw (OPS) protocol 20% DMSO, 20% ethylene glycol and 0.5 mol/l sucrose were used as CPA(Reubinoff et al., 2001). The specimen consisting of a 20µL solution in an open straw, was then plunged directly into liquid nitrogen, achieving a cooling rate of approximately -75°C/second. This technique results in high post-thaw viabilities and low post-thaw differentiation rates. However, several practical problems exist with the OPS method. First, the volume preserved in one pulled open straw is with 20µL very small and hence logistically prohibitive for a large scale clinical use. Second, the open character of the system with storage in liquid nitrogen is associated with infectious concerns. Third, the glass like state achieved by this vitrification method is metastable and requires consistent temperatures below -135°Celsius, what is often a problem in conventional freezing tanks, which imply internal temperature gradients (C. J. Hunt, 2011; Rowley & Byrne, 1992). Fourth, most formulations use animal proteins in the solution, what corresponds to infectious and allergic concerns. Fifth, substitute assays, to measure the functionality of hESC for extended periods of time are still poorly defined (C. J. Hunt, 2011). Last, the method requires technical and personal sophistication, which is not necessarily everywhere available.

Closed straw as well as potentially scalable techniques have been recently published(T. Li, Q. Mai, J. Gao, & C. Zhou, 2010; Richards, Fong, S. Tan, Chan, & Bongso, 2004). The reason for the difficulties with the preservation of hESC with traditional slow cooling protocols

may be the cooperative growth behavior and the inter-cellular interaction via gap junctions. Hence, ice crystal niduses formed within a hESC colony can easily cause mechanical damage throughout the entire cell cluster. The awareness of this idiosyncrasy in the growth behavior of hESC has led to a series of publications, trying to modify the different cryobiological parameters, such as cooling rate, ice nucleation, cryoprotectant concentration, osmotic effects associated with the introduction and removal of the cryoprotectant and matrix systems allowing for the seeding of cell clusters, maintaining continuous cell-to-cell contact in slow cooling protocols (Baran & Ware, 2007; J. Y. Lee et al., 2010; Yang Li, J.-C. Tan, & L.-S. Li, 2010; Valbuena et al., 2008). The recovery success in those experiments was promising.

c. Most of the principals applying to hESC also appear to apply to iPS and so far cryopreservation approaches similar to hESCs have been explored (C. J. Hunt, 2011; Nishigaki et al., 2011).

4. Summary and conclusions

4.1 Summary

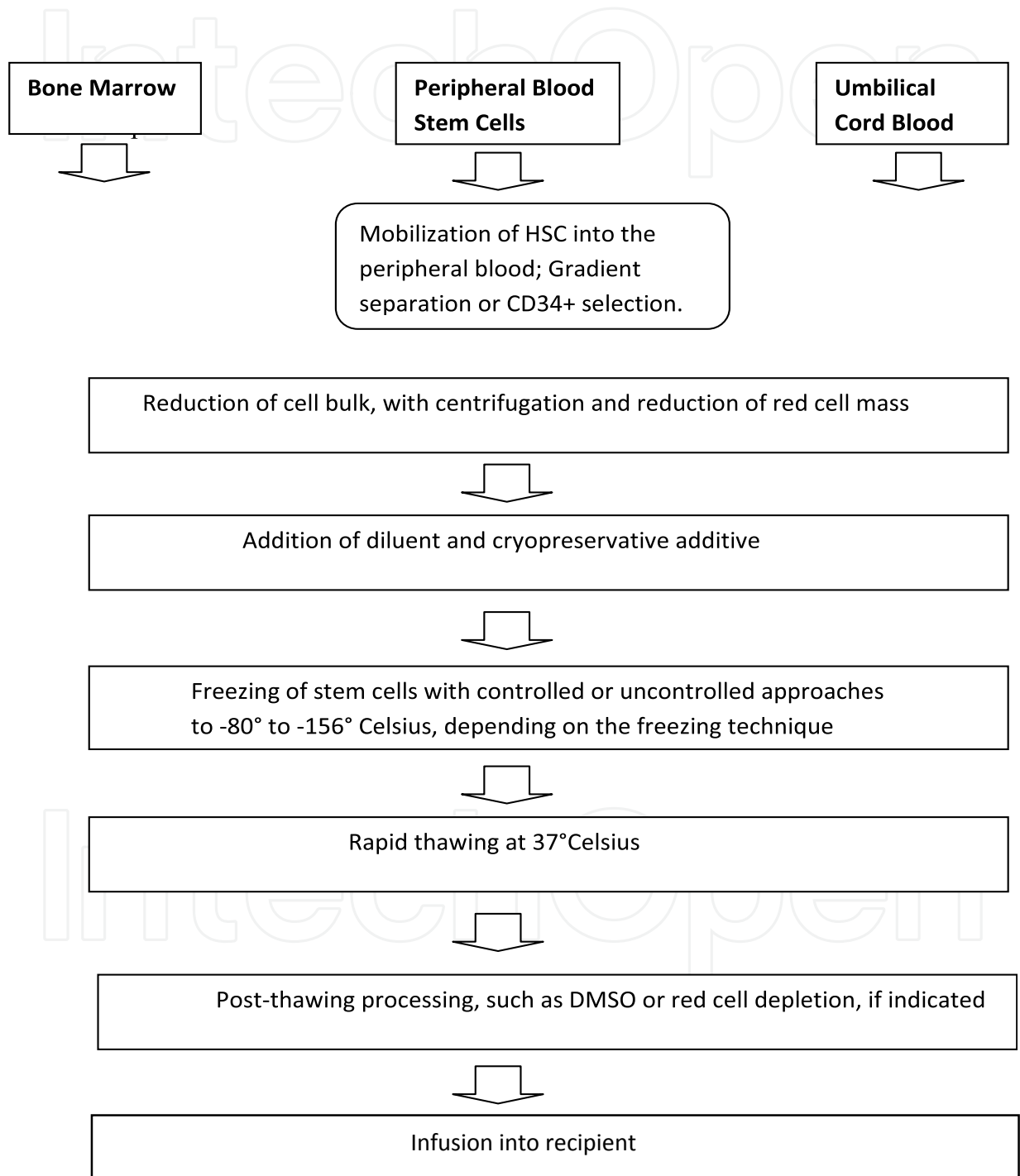
The cryopreservation of stem cells is a crucial component of their therapeutic use in hematologic disorders and regenerative medicine. Although protocols for the preservation of HSC are well defined, the standardization still remains poor. Protocols for the preservation of non-HSCs have been developed and will experience further clinical validation if hESC, MSC and iPSC will find wider clinical application in the future. Liquid phase storage is safe and effective for short term intervals. It is probably superior to cryopreservation at -80° to -156°C for period less than 72hrs, because it avoids the unnecessary physical and mechanical damage by more elaborate procedures. Volume and cell bulk reduction, although associated with a certain loss of cells, is necessary to accommodate the increasing logistical demands in the cellular therapy sector. Cell concentrations of $2 \times 10^8/\text{mL}$ or even higher are safe, effective and necessary to limit the spatial needs, particularly considering the rising amount of cryopreserved UCB units. DMSO at concentrations of 5-10% is widely used and alternative cryoprotectants have been developed. Future studies are needed to define the optimal CPA with the most favorable side effect profile. For most stem cell products, controlled rate freezing is still considered standard in most countries, but uncontrolled rate freezing approaches are safe and associated with good clinical outcomes. The optimal long term storage temperature remains to be defined but the vapor phase of nitrogen tanks is suitable for most cellular products. In conclusion, future clinical and translational studies will be needed to define and standardize the optimal cryopreservation techniques with optimal clinical outcomes and minimal clinical, environmental and financial adverse effects.

4.2 Future directives

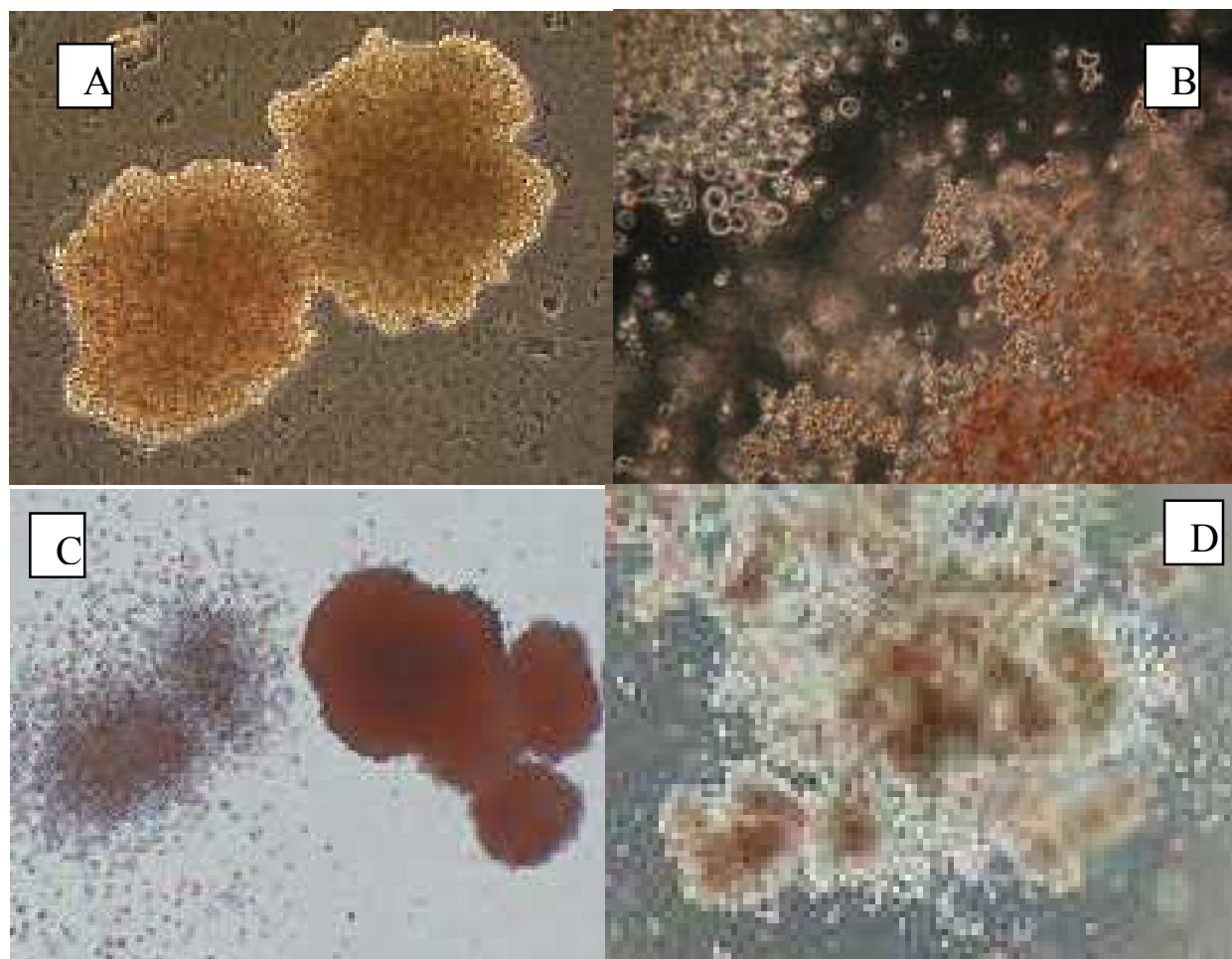
Efforts in the future should focus on clinical and basic science studies to further the understanding of the cryobiology of target cell population, to better explain the damage caused to the cells by the cryopreservation process. This will help with many issues, including the identification of optimal cryobiologic parameters, such as cell concentration, pre-freezing cell processing, freezing rate, storage temperature and others. In addition with a widening use of stem cells in regenerative medicine, less toxic cryoprotectants are in demand. Standardization and the implementation of good manufacturing practices remain a global issue. The existing and increasing financial strain on public health systems around

the world calls clearly for a global standardization with protocols, feasible also in less affluent parts of the world. Finally, an educational exchange across borders to ascertain optimal skill sets in laboratorians and clinicians, dealing with stem cell cryopreservation and utilization, has to be encouraged.

4.3 Appendix



Graphic 1.



The images above display: A-CFU-Sd12 (colony forming unit spleen on day 12); B- CFU-GM(colony forming unit granulocyte/macrophage); C- LTC-IC(long-term culture-initiating cells); D- GEMM-(colony forming unit granulocyte/erythrocyte/macrophage/megakaryocyte)

Graphic 2.

5. References

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This book documents the increased number of stem cell-related research, clinical applications, and views for the future. The book covers a wide range of issues in cell-based therapy and regenerative medicine, and includes clinical and preclinical chapters from the respected authors involved with stem cell studies and research from around the world. It complements and extends the basics of stem cell physiology, hematopoietic stem cells, issues related to clinical problems, tissue typing, cryopreservation, dendritic cells, mesenchymal cells, neuroscience, endovascular cells and other tissues. In addition, tissue engineering that employs novel methods with stem cells is explored. Clearly, the continued use of biomedical engineering will depend heavily on stem cells, and this book is well positioned to provide comprehensive coverage of these developments.

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