

Cryopreserving Human Peripheral Blood Progenitor Cells

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Abstract: High-dose chemotherapy followed by autologous peripheral blood progenitor cell (PBPC) transplantation is used in the treatment of chemosensitive malignancies. Cryopreservation of PBPC in 10% dimethyl sulfoxide (DMSO) has been the standard procedure in most institutions. Infusion of PBPC cryopreserved with DMSO can be associated with toxic reactions such as vomiting, cardiac dysfunction, anaphylaxis and acute renal failure. The grade of toxicity experienced by patients is related to the amount of DMSO present in the PBPC. Cryopreservation with lower DMSO concentrations would be expected to reduce the toxicity. In recent studies done with PBPC cells cryopreserved with 5%, 4% and 2% DMSO, using 10% DMSO as a reference control, CD34⁺ cells were investigated for preservation of viability, apoptosis, and necrosis. Also preservation of mature colony-forming (CFU) cells, specifically mature myeloid, erythroid progenitors, CFU-megakaryocytes and long-term culture-initiating cells (LTC-ICs) were investigated, using 5% and 10% DMSO as cryoprotectant. All samples were frozen in a rate-controlled programmed freezer and stored in the vapor phase of liquid nitrogen until used. Conclusion: 5% DMSO is the optimal concentration for cryopreserving human PBPC *in vitro*. Consequently, some hospitals have started using 5% DMSO as cryoprotectant for the autologous PBPC as a standard procedure.

Keywords: Cryopreservation, DMSO, progenitor cells, CD34⁺ cells, viability, CFUs.

INTRODUCTION

High-dose chemotherapy followed by autologous peripheral blood progenitor cells (PBPC) transplantation is used in the treatment of chemosensitive malignancies [1-4]. The standard method for stem cell mobilization today is the use of G-CSF/GM-CSF (granulocyte or granulocyte-macrophage-colony stimulating factor) alone or in combination with chemotherapy [5,6]. PBPCs are usually harvested and stored in liquid N₂ until reinfusion. Storage at this low temperature will block all enzymatic pathways and metabolism in the cell [7]. Cryopreservative(s) must be added to the PBPC before freezing in order to protect the cells. The concentration of the cryoprotectant and the rate at which the cells are frozen are the main factors governing the survival of the cells. Current protocols for the human PBPC are based on freezing to -160°C in 10% (v/v) dimethyl sulfoxide (DMSO) [8] in a freezer programmed for temperature rate control. Thereafter the cells are stored in liquid N₂. DMSO has been the most favored additive for long-term storage of stem cells obtained from bone marrow or by leukapheresis since the beginning of the 1960s [9-11].

The first report of a laboratory investigation of DMSO as a cryopreservative in a mammalian system was of human and bovine erythrocytes and bovine spermatozoa [12]. DMSO is relatively freely permeable through cell membranes [13-14] and prevents intracellular formation of ice crystals and disruption of cell membranes under freezing [15]. A 5% DMSO concentration in combination with 6% hydroxyethyl starch (HES) [7,16-19] is also in use. HES coats the outside of the cell membrane and thereby prevents post-thawing clumping or gel formation [7, 13]. However, Donaldson *et al.* [19] concluded that HES did not improve

the recovery of CD34⁺ stem cells after cryopreservation in combination with 5% DMSO. Different procedures for freezing have been performed through the years, both by programmed rate-control and by uncontrolled freezing (placement of the PBPCs into a -70 to -80°C freezer).

Infusion of DMSO-preserved PBPC in patients is often associated with toxic reactions such as nausea, vomiting, cardiac dysfunction, anaphylaxis, acute renal failure, hypotension and transient hypertension. The amount of DMSO present in the graft is related to toxicity [20-22]. In addition to the negative effect of DMSO, the number of dead cells infused may also cause side effects of fever and abdominal cramp [22]. Prior to the infusion the patients may be medicated with i.v. methylprednisolone, ranitidine and/or antihistamine to prevent the side effects caused by DMSO [23]. Health personnel also experience physical symptoms such as headaches and gastrointestinal reactions both from the DMSO odor and from its metabolite dimethyl sulfide (DMS). About 45% of DMSO is secreted through urine, whereas DMS is secreted through the skin, breath, feces, and urine from patients up to 2 days after stem cell infusion. Thus, administration of DMSO can be a problem for several days after infusion [24].

CD34 antigen expression is used as a surrogate marker for hematopoietic stem cells and enumeration of CD34⁺ cells has been used to quantify progenitor and stem cell content [25]. Assays by flow cytometry for measuring the viability of CD34⁺ cells, apoptosis and cell death of the harvested PBPCs have improved in the last 10 to 15 years [19, 26-28], thereby confirming the effectiveness of the different cryopreserving methods. To evaluate the functional capacity of the cell subsets, the colony assay is a functional tool to assess the ability of the hematopoietic cells to divide and differentiate. Functional tests for progenitors provide important complementary information for CD34⁺ cell enumeration. Measuring the formation of the different

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colony forming units (CFU) of the myeloid, erythropoietic, megakaryocyte lineage and the long-term culture initiating cells before and after freezing are appropriate methods for testing the cryopreservation of PBPCs [29, 30].

CRYOPRESERVATION

Cryoprotectors

Freezing of cells at low temperatures preserves their metabolic pathways, but will damage the cell membranes because rapid freezing of cells dissolved in physiological salt water results in formation of ice crystals within the cells (too short time for the water to diffuse out of the cells). Ice crystals may disrupt the membrane barriers and lyse the cells and their subcellular organelles [16, 31]. Further, slow freezing results in extra-cellular ice crystal formation and osmotic dehydration injury with an osmotic gradient across the membrane. Addition of different cryoprotectants to the cells is a necessity. DMSO has been the most used cryoprotective agent for human peripheral blood stem cells, either alone or in combination with HES and human serum albumin (HSA). DMSO is a clear, colorless, organic liquid that freezes at 18.5°C and has a strong affinity for water [32]. Since DMSO is highly soluble in water and readily crosses cell membranes, the addition of DMSO to a cell suspension results in a high DMSO osmolality both outside and inside the cell, offsetting the salt gradient established across the membrane [16]. In a controlled-rate freezing setting, virtually all the free water existing within the cell will diffuse out of the cell, thereby preventing intracellular ice crystal formation [16]. Another often used cryoprotectant solution is a combination of 5% DMSO, 6% HES and 4% HSA [33]. Starch macromolecules will not penetrate the cell membrane, but will form a shell around the cells and prevent dehydration. This method is often used when a controlled rate freezer is not available. HES is a biodegradable cryoprotectant, which is eliminated via the kidneys; within 20 hours the HES concentration was almost completely eliminated in urine [34]. Glycerol is a cryoprotectant that penetrates into the cells, but it is slower than DMSO. It also requires a washing procedure to remove it before reinfusion [16]. The effectiveness of cryoprotectants generally increases with their concentration, but this can also have deleterious osmotic and toxic effects on the cell [19]. Further, the protein content of cryopreservation solutions appears to be important; plasma proteins exert cryoprotectant effects, possibly by modifying the viscosity of the cryoprotective solution. All cryopreservation solutions in use for PBPCs contain plasma proteins added as part of the cryopreservation or during cell processing. Empirically either plasma or albumin have been proven to be sufficient [35].

Cell Collection, Cell Concentration and Freezing Procedures

PBPC are usually collected after mobilization with disease-specific chemotherapy followed by treatment with G-CSF [36-38]. The progenitor cells are harvested by the use of leukapheresis enrichment of the mononuclear cells relative to the granulocytes. In addition the red cell collection is omitted. For many years 2.5 - 3 times the blood volume of

the patient has been processed for 1 - 4 days in a row to achieve appropriate amounts of CD34⁺ cells for reinfusion. Lately, large-volume apheresis (4 -5 times the patients blood volume) has been processed in some hospitals. This results in an increased CD34⁺ collection [38, 39], achieving at least 2 - 2.5 x 10⁶ CD34⁺ cells/kg in one day (about 4 hours) which is enough cells for engraftment after reinfusion. Before freezing, the cell count is usually adjusted with autologous citrated plasma to a mean concentration of 100 - 200 x 10⁶/ml [27, 28, 38]. Thereafter cells are placed on ice before DMSO is infused slowly (because of heat exposure) into the freezing bag containing the PBPC. A small sample is drawn from the collection bag, divided into small cryotubes and frozen together with the bag for control and/or research. To ensure uniform distribution of temperature the collection bag is compressed between two flat metal plates [15] before freezing to -160°C in a programmed freezing machine. Thereafter the bag and samples are transferred to a liquid N₂ container and stored. This programmed freezing method, which preserves the cells for years, is supposed to be the best way to handle PBPC cells [11]. However, several reports conclude it is safe and acceptable to use a combination of DMSO and HES in a -80°C freezer, which is less expensive compared to the programmed freezing technique [1, 7].

Recovery and hematopoietic potential depend on both the cooling rate and the concentration of the cryoprotectant [40]. Cryopreservation is associated with loss of adhesion molecules from the cell surface [41, 42]. The percentages of apoptotic and necrotic CD34⁺ cell in the autografts show a wide variation between patients [27, 43].

The PBPC product comprises a heterogeneous cell population, only a very small proportion of which is hematopoietic stem cells required for engraftment after transplantation. PBSC transplantation is characterized by the need to collect, process, and infuse large quantities of cells. Unfortunately, ex vivo manipulations that are optimal for hematopoietic cells may be damaging to these other cells [35]. Freezing techniques differ for stem cells and erythrocytes [31] and the granulocytes are poorly cryopreserved with any current technique available [35]. The mature blood cells may affect the cryopreserved hematopoietic stem cells in different ways such as clumping caused by platelets and/or granulocytes both before and after thawing. Second, damaged mature cells may increase the infusion-related toxicity [11].

Optimal Cell Concentration and Storage Temperatures

Although most institutions cryopreserve PBPCs at a concentration of around 100 - 200 x 10⁶ cells/ml [28, 29], cryopreservation of nucleated cells in 10% DMSO as high as 3.7 ± 1.9 x 10⁸ (range 0.4 - 8.0) cells/ml has been reported by Rowely *et al.* [44]. They claimed that this high cell concentration was not deleterious to the hematopoietic stem cells in the cell preparation. The nucleated cell recovery was about 75% after thawing. The infusion of these products was generally well tolerated, and it was concluded that both PBPC and BM may be cryopreserved at very high concentrations without loss of engraftment. The observations were confirmed by Cabezudo *et al.* [45]. They cryopreserved

the PBPC in 10% DMSO at cell concentration of 332×10^6 /ml (range 171 -582) and claimed that this had no adverse effects on the function of the hematopoietic progenitor cells (HPC) after thawing compared to standard concentration at 91×10^6 cells/ml (range 45-92). Even cell concentrations as high as 1000×10^6 cells/ml in 5% DMSO and 6% HES in PBPC has been cryopreserved and reported to give viability of 75% of recovered cells [46]. A significant decrease in viability of the thawed PBPC was not observed until a cell concentration of 2000×10^6 cells/ml. Platelets and MNC were removed from the PBPCs at a high efficiency, and a -70°C freezer was used for storage until reinfusion. However, these results contrasted greatly with that of *Liseth et al.* [47] who found a decrease in recovery of CD34^+ cells in the samples containing 300×10^6 cells/ml when the 10% DMSO was reduced to 5% DMSO, and the cells were kept in a liquid N_2 storage container.

For long-time cryopreservation of PBPC or BM it is important that stem cells are frozen in a programmed freezer and stored at -140°C or lower until reinfusion. Stem cells stored up to 11 years still capable of engrafting have been reported [48].

Thawing and Reinfusion

Thawing is simply the reverse of freezing, with similar risks of cellular damage. Empirical observations reveal that rapid thawing yields the least loss of progenitor cells [40]. The best procedure for thawing the PBPC is by immediate immersion of the cryopreserved bag from N_2 vapor into a 37°C water bath [23, 49], gently shaking until thawed, and then the cells should be infused into the patient as soon as possible. Rapid processing is important in order to avoid cell clumping and minimize the time the cells are exposed to DMSO at 37°C since direct chemical toxicity of DMSO to the hematopoietic progenitors has been suggested [50]. However, short-term exposure of the cells for up to 1 hour in 10% DMSO concentration [14] and up to 2 hours [15] in 8% DMSO, at temperatures above the freezing point, resulted in no significant loss of hematopoietic progenitor cells. This was assessed by *in vitro* colony formation of colony-forming units (CFU-GM), CFU-GEMM and burst-forming units (BFU-E) in methyl cellulose cultures.

Although purified stem cells [51] thawed at 20°C showed no significant difference from those thawed at 37°C , thawing at 37°C is well established. The progenitor cells are seldom purified and contain a heterogeneous cell population, which may cause clumping after thawing. As mentioned granulocytes are poorly cryopreserved in medium that seem optimal for stem cells, and will release DNA and lysosomal enzymes after freezing and thawing which causes clumping and loss of cell viability [52].

Detection of CD34^+ Cells

Since the early 1990s flow cytometry has been in use to detect CD34^+ stem cells. A method based on the ISHAGE guidelines, described in detail by Keeney *et al.* (1998) [26] is commonly in use. In short, the PBPC is stained immediately after thawing and analyzed according to the single platform, flow cytometric absolute CD34^+ cell count

method [26, 53]. A stem cell CD34^+ HPC enumeration kit containing CD45-FITC/CD34-PE , isotype control PE, stem-count fluorospheres (microbeads with known concentration/ μl) and a concentrated ammonium chloride lysing solution are used together with the vital dye 7-amino actinomycin D (7-AAD) [28, 54]. Both the total and the absolute number of viable CD34^+ cells, and the apoptotic and necrotic cells can be counted and calculated using this method when the volume of the PBPC concentrate in ml is known. This method is used routinely to validate the PBPC [19, 27]. A correlation between the initial of total CD34^+ cells and the viable CD34^+ cells after thawing has been shown, also a significant correlation between the transplanted viable CD34^+ dose and the time until both platelet and neutrophil engraftment has been found [54].

Dye Exclusion Test

The dye exclusion test with Trypan Blue may offer several advantages [10] since it is an extremely quick and easy method that can be performed in any laboratory when performed immediately after thawing. Cells are considered viable if they are able to exclude Trypan Blue; nonviable cells appear stained dark blue and to be crenate [15]. Unfortunately it is not possible to differentiate between the cells.

Different Cryopreservation and Assay Methods for Human Progenitor Cells

After 25 years of stem cell autotransplantations, new and better methods for cryopreservation and freezing techniques are still being developed. Simultaneously, methods in the laboratory using flow cytometer for detection of stem cells have been improved and expanded since in the late 1980s, such as counting of total CD34^+ cells, viable CD34^+ cells, apoptotic and necrotic cells [26]. Various procedures for identifying different types of primitive hematopoietic cells have been described for their ability to produce clonogenic progenitors (colony-forming units (CFUs)). Colony-forming-erythroid (CFU-E), CFU-granulocyte, CFU-erythroid, macrophage, megakaryocyte (-GEMM), CFU-granulocyte, macrophage (GM), CFU-megakaryocyte (CFU-Mk) and the long-term culture-initiating cells (LTC-ICs) [25] can all be evaluated in the PBPCs. These culture assays are all time-consuming and take from 2 to 5 weeks to investigate. However, they are very useful and can give a good prediction of how suitable a new cryopreservative method can be compared to the one in use, since samples from both methods may be investigated in parallel before and after cryopreservation. Microscopic enumeration of hematopoietic colonies is inherently subjective; therefore it may be important that the same technologist perform all the assay counting throughout a study [29].

Through the years many different combinations of cryoprotectants have been tried for hematopoietic stem cells. Most autologous transplant programs use 10% DMSO as cryoprotectant for human PBSC [11], although some institutions use HES in combination with DMSO, since it has been shown that HES reduce post-thaw granulocyte lysis and clumping [55].

Stiff *et al.* (1983) [1] reported a procedure for cryopreservation of bone marrow (BM) in which 10% DMSO was replaced with 5% DMSO + 6% HES in an attempt to increase the viable cell yield and reduce the clumping of cells after thawing, which was observed when using DMSO alone. In this study the unfractionated cells were simply placed in a -80°C freezer and then later into a liquid nitrogen container for 6 months. Samples were then thawed and assayed for Trypan Blue exclusion and CFU-recovery. The cells in the DMSO/HES mixture had higher recovery values than the ones in 10% DMSO alone compared to the pre-freeze values. Additional samples were kept at only -80°C and then thawed 12 to 16 months later. Thawing of the DMSO/HES mixture gave an excellent CFU-C recovery and eliminated the clumping. Stiff *et al.* concluded that there was no need for a rate-controlled freezer and liquid nitrogen temperatures for storage of BM cryopreserved in 5% DMSO + 6% HES up to one year. Unfractionated BM from 60 patients was later cryopreserved in the DMSO/HES mixture as above. The patients underwent chemotherapy, and the cryopreserved BM was reinfused [1]. Stiff *et al.* concluded that BM cells can successfully be cryopreserved in the DMSO/HES mixture rapidly and inexpensively, without rate-controlled freezing or storage at liquid nitrogen temperatures. Later this method has also been applied to PBPCs [7, 46, 56].

Cryopreservation of hematopoietic progenitor cells with 5% DMSO instead of 10% at -80°C without rate controlled freezing was reported by Galmés *et al.* [57] after first having successfully frozen PBPCs with 10% DMSO in a mechanical freezer [58]. They also investigated the effects on mononuclear cell (MNC) recovery and viability when using various DMSO concentrations up to 10%. The recovery (measuring the CFU-GM and CFU-E) of the MNC was 124% when using 1% and 3% DMSO, 96.6% using 5% DMSO and 90% when using 10% DMSO as the sole cryoprotectant. However, the best viability of the MNCs was 91.6% using 5% DMSO, and the viability was reduced only to 90.6% when the DMSO was increased to 10% [57]. Further, Galmés *et al.* found a recovery of the MNC at 96%, CFU-GM at 82%, BFU-E at 70% and CD34⁺ at 129%, and a viability of the MNCs at 77% after storage of the cells at -80°C for 1 year in 5% DMSO. They concluded it was possible to store the hematopoietic progenitor cells (HPCs) in 5% DMSO as the sole cryoprotectant at -80°C . Later Galmés *et al.* [59] reported that cryopreserving HPCs at -80°C in 5% or 10% DMSO resulted in 80% viability of MNCs over 1 to 12 months. After 18 months freezing, however, the viability decreased to 60%, as measured by Trypan Blue exclusion. CFU-GM and BFU-E, measured by clonogenic assay kits showed decreased recovery over 1 - 12 months (around 40%, range 0-100%), and then the recovery dropped dramatically to zero. The colony recovery was not significant between 5% and 10% DMSO samples, although there was a trend to higher recovery for the 5% compared to the 10% DMSO throughout the study. Galmés *et al.* [59] concluded that HPC can be cryopreserved at -80°C with 5% DMSO for no longer than 6 months.

Sputtek *et al.* [17] claim that cooling rates between 1 to 5 K/min (-80°C mechanical freezer) is not critical with 5% DMSO in combination with 6% HES. However, Rowely *et al.* [60] cryopreserved PBPCs in a controlled-rate freezer and

stored the grafts in the vapor phase of N₂ either in 10% DMSO or 5% DMSO + 6% HES as cryoprotectants. Patients who received PBPC products frozen in DMSO/HES achieved a statistically significant faster WBC recovery $>1.0 \times 10^9/\text{l}$ (a median of one day faster) than patients who received products frozen in DMSO alone. The time required to achieve neutrophil counts of $>0.5 \times 10^9/\text{l}$ and $>1.0 \times 10^9/\text{l}$ were similarly faster for recipients of cells frozen in the combination solution. In contrast, median times for recovery of platelet counts $>20 \times 10^9/\text{l}$ were equivalent for each group (10 days) [60].

Guidelines for umbilical cord blood banking have recommended the same procedures as for PBPC and BM. However, to optimize the methods for cord blood, Donaldson *et al.* [19] investigated different methods for cryopreservation of these progenitor cells. The recovery of CD34⁺ cells when cryopreserving in 4% HES in increasing DMSO concentrations of 0, 1, 2, and 5% DMSO in the medium resulted in a far better recovery at 5% DMSO compared to the lower concentrations and an even better recovery when omitting HES in 5% DMSO. Increasing to 7.5% DMSO alone gave higher recovery, while adding 4% HES reduced the recovery of CD34⁺ cells. The cooling rate was $-1^{\circ}\text{C}/\text{min}$ throughout the experiments which also gave the maximal recovery of cells compared to -5 or $-10^{\circ}\text{C}/\text{min}$. Donaldson *et al.* [19] included DNase to the thawing media immediately after thawing the cells. This was performed to disperse the clumps that formed during thawing. Their conclusions were that HES did not improve the recovery of CD34⁺ cells after cryopreservation with 5% DMSO, and that the use of HES to supplement the cryoprotective effect of DMSO was found to be of little value. Concentrations of 5 or 10% DMSO were similarly effective, and a concentration above 5% would be recommended as safe, because survival of CD34⁺ cells fell markedly when DMSO concentration fell below 5%.

Lakota and Fuchsberger [61] mobilized and harvested stem cells for further processing in Earle's medium containing 10%, 5% or 2.2% DMSO, 0.6% HES and 5% autologous serum (inactivated at 56°C for 30 min). The cells were frozen in a programmed freezer and stored in the liquid phase of N₂. They report that for all 20 patients (6 to 7 in each group) engraftment was rapid, but they did not investigate the recovery of the CFU-GM or the CD34⁺ cell survival.

Later Halle *et al.* [18] reported a prospective evaluation of 109 autologous PBPC transplantations after uncontrolled freezing and storage at -80°C of the apheresis products. The cryopreservative solution contained in a final concentration 1% human serum albumin, 2.5% HES and 3.5% DMSO. They found the procedure successful for engraftment when comparing to the standard procedure with 10% DMSO and controlled-rate freezing. With *in vitro* assays, the median recoveries of nucleated cells were about 61%, for CD34⁺ cells 80%, for the CFU-GM 36% and for BFU-E 33%. However, the parameters showed a great variation. The median length of storage was 7 weeks (range 1 - 98). They claimed that the hematopoietic reconstitution did not differ in patients undergoing myeloablative or nonmyeloablative conditioning regimens before transplantation.

Another group, Curcoy *et al.* [62], reported successful engraftment in children when cryopreserving PBPCs at high cell concentration $>200 \times 10^6/\text{ml}$ in 5% DMSO. The PBPCs were frozen in a methanol bath in a mechanical freezer at -80°C , median time for transplantation was 10 days. These good results may be caused by the short time between cryopreservation and transplantation.

Through the past few years a number of investigations have been carried out by Abrahamsen *et al.* [28, 30] and Bakken *et al.* [29] examining the difference between cryopreservation of autologous progenitor cells in 5 and 10% DMSO. The PBPCs from patients with different malignant diseases were collected on an apheresis device. Mostly large-volume apheresis procedures were processed, collecting PBPCs from 4 to 5 times the patient blood volume [38]. The cells were concentrated or diluted in autologous plasma to a mean of $100 - 200 \times 10^6$ cells/ml. Then two small test samples from the cell suspension were prepared by adding DMSO slowly to a final of 5 and 10% and mixed carefully on ice. The samples were distributed in 0.5 ml aliquots into cryotubes, thereafter into a controlled programmed freezer together with the PBPC concentrate containing 10% DMSO. The PBPC and the test tubes were frozen to -160°C and then stored in a liquid N_2 container until use. The 5 and 10% DMSO test tubes were always handled strictly in parallel, throughout the pre-freeze, thawing and staining procedure [28] and also when preparing for the different colony-forming culture assays [29, 30] and Trypan Blue exclusion test. The samples were thawed in a 37°C water bath until the ice crystals just disappeared, then immediately diluted in appropriate medium to be analyzed. Abrahamsen *et al.* [28] found that cryopreserving human progenitor cells in 5% rather than 10% DMSO resulted in significant less apoptosis and necrosis in CD34^+ cells, using the annexin V method for analysis of apoptosis [27, 63]. They also [27] reported that the absolute number of total and viable CD34^+ cells were higher in 18 and equal in one of all the samples cryopreserved with 5% as opposed to 10% DMSO. The absolute count method (single platform, flow cytometric absolute CD34^+ cell counts based on the ISHAGE guidelines method) [26] was used in these investigations. Recently the same research group cryopreserved PBPC test samples in parallel with 2, 4, 5 and 10% DMSO from 20 patients [47]. The samples were stored from 6 to 8 weeks before flow cytometer measurements of absolute and percentage of total and viable CD34^+ cells were performed. Thus, the conclusion is that 4% and 5% DMSO samples were almost identical, while 2% DMSO gave a lower CD34^+ cell survival. They concluded that 5% DMSO might be the optimal dose when used as the sole cryoprotectant for PBPCs and the cells were frozen to -160°C in a controlled programmed freezer. Since results with 4% were almost identical to those of 5%, cryopreserving PBPC with 5% DMSO should give a sufficient margin concerning minor variances in DMSO concentration that might occur when adding DMSO [47].

Simultaneously with these flow cytometer investigations [28] colony-forming cell assays also were performed [29] testing for CFU-E, CFU-GM, CFU-GEMM, CFU-Mk, CFU-Mk-total in both 5 and 10% DMSO samples. Comparison of 27 samples from patients with malignant diseases were carried out after at least 3 months and after 1

year of cryopreservation, and showed a significantly higher colony formation for the CFU-E and CFU-GEMM in 5% DMSO compared to 10%. For CFU-GM and for CFU-Mk no significant difference was demonstrated after 3 months or after 1 year in the 5 and 10% DMSO samples. There also was a significant correlation between the CFU-total and CFU-Mk-total, indicating that the CFU-total might be used in an evaluation of megakaryocytic progenitors, as the megakaryocytic cell culture assay is time consuming and not suitable for routine analysis. In parallel to these colony assays viability testing with Trypan Blue exclusion test showed that cells cryopreserved with 5% DMSO had significantly higher viability than the cells cryopreserved in 10% DMSO. Also correlation between the number of clonogenic cells in the PBSC autographs after 7 days of *in vitro* culture and the total numbers of colony subsets in conventional 14 days colony assays were investigated [64]. The total colony number after 7 days of culture correlated significantly with (i) the CD34^+ cell number, (ii) the total colony number as well as the numbers of erythroid, non-erythroid and mixed colonies in a conventional assay using 14 days of culture and (iii) the number of megakaryocytic colonies. This simple analysis may be used in combination with e.g. estimation of stem cell viability and CD34^+ cell subset analysis.

Further characterizations of the primitive progenitors responsible for long-term post-transplant reconstitution (LTC-CFC) was investigated using test samples from PBPCs as above [30]. Samples from 15 patients with malignant diseases had been cryopreserved in 5 and 10% DMSO and stored in liquid nitrogen for at least 14 months before the LTC-CFC assay was performed. The 5% DMSO cryopreservation again resulted in the highest colony scores compared to the 10% DMSO. Results also showed that the CD34^+ cells can be used as a marker for the number of primitive progenitor cells (in the 5% cryopreservation) in the graft [30]. The LTC-CFC assay is considered to reflect growth characteristics of immature hematopoietic progenitors responsible for long-term post-transplant PBPCs reconstitution.

This decreased 5% DMSO concentration in PBPCs for transfusion to patients has been in use in our institution since 2003 with good results (continuous evaluation is ongoing). The survival of CD34^+ cells however is dependant on the concentration of the cells and the percentage of the protecting DMSO in the medium. Liseth *et al.* [47] found that increasing the cell concentration to 300×10^6 cells/ml in 5% DMSO decreased the survival of CD34^+ cells, while the survival was constant in the 10% DMSO medium. They concluded that as long as the cells are not concentrated to higher levels than $200 - 300 \times 10^6$ cells/ml and rate-controlled freezing is used, cryopreservation of human PBPC autografts with 5% DMSO was satisfactory and should be further tested in clinical studies.

Extensive purification of PBPC by isolation of CD34^+ is possible, but this is costly and time consuming, and will in addition often result in loss of about half of the stem cells [3, 65, 66]. The advantage in purification of CD34^+ is a reduction of volume, thereby a reduced amount of DMSO to be added to the product. In addition unwanted cells may be reduced or omitted. Beaujean *et al.* [3] reported that purified

CD34⁺ cells had been cryopreserved in 7.5% DMSO under controlled-rate freezing and stored in the vapor phase of nitrogen. The post-freezing recovery from these investigations was about 90% for CD34⁺ cells, 59% for CFU-GM and 53% for BFU-E of progenitor cells. After reinfusion of the grafts all patients showed successful neutrophil engraftment (median 11 days), and platelets counts above 50 x 10⁹/l median, 13 days (range 9 to 125 days). The engraftment correlated negatively with the CD34⁺ cell number infused.

Recently Rodríguez *et al.* [67] investigated the elimination of DMSO from thawed PBPC in an automated cell processing device. The median recovery of viable total nucleated cells was 89% (range 46-131), the viable CD34⁺ cells was 103 % (range 62-126) and the CFU colonies was 91% (range 46-131). They concluded that washing out the DMSO was safe and efficient in terms of recovery and viability of nucleated and progenitor cells. But the procedure was time-consuming compared to direct infusion after thawing. Additionally they observed a rapid reduction of viable CD34⁺ cells and CFU-GM, with less than 50% recovery in the control group containing 10% DMSO compared to 70% in the washed group.

CONCLUSIONS

Hematopoietic stem cell transplantation has become increasingly important in the treatment of hematologic malignancies over the last 20 years. A major advantage of using auto-transplantation with PBPC instead of auto-BM transplantation is a more rapid hematopoietic reconstitution experienced [37], and especially a shorter time for platelet count stabilization [68]. However, long lasting thrombocytopenia remains a problem for a minority of patients, even when using PBPC grafts [4].

The counting of CD34⁺ cells plays a critical role in identifying the optimum product for transplantation, and this technology will continue to have a major role in defining and evaluating the most suitable product for transplantation. For safe engraftment more than 2.0 x 10⁶ CD34⁺ cells/kg patient weight are recommended. It has been shown that the amount of CD34⁺ cells transfused has significant effects on the time of engraftment [49].

During the last 10 years improvements in counting stem cells by flow cytometer and evaluating functional capacity of stem cell subsets, using different culture assays, have been important for evaluation of cryopreserving methods. To find the optimal cryopreservation for stem cells, a great number of different colony-forming assays and flow cytometer methods have to be performed, both before freezing and after thawing the cells in a 37°C water bath. The cell concentration, the cryoprotectant(s), the freezing procedure, the storage temperature and the time of storage expected for the PBPC, all have to be taken into account when cryopreserving PBPC.

To our knowledge, and under current level of understanding the method of recommendation for cryopreserving PBPC autografts should be with 5% DMSO with a cell concentration up to 250 x 10⁶/ml. The product should be frozen to -160°C in a controlled programmed

freezing machine and then kept frozen in a liquid nitrogen container until used.

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