Storage of haemopoietic stem cells for autologous bone marrow transplantation

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ABSTRACT

For reinfusing autologous bone marrow cells after high-dose chemotherapy and/or radiotherapy it is necessary that an effective technique for their storage is available. The traditional method uses 10% dimethyl sulphoxide as cryoprotectant, a rate-controlled computerized freezer programmed to cool the cells at a constant rate of 1 °C per minute and liquid nitrogen as the storage system. The method is time-consuming, expensive and requires technical expertise. Moreover, it is often associated with varying levels of clinical toxicity following infusion of the preserved cells. Processing the harvest to reduce the initial volume and the mature cells has been shown to be beneficial in reducing the volume of the cryoprotectant and the incidence of toxicity. An alternative, cost-effective method using a cryoprotectant mixture of 5% dimethyl sulphoxide, 6% hydroxyethyl starch and 4% albumin has been found to be effective even when the cells are stored at −80 °C without rate-controlled freezing. However, its efficacy needs to be evaluated for extended periods. The current use of purging and cell sorting methods seems to be promising.

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INTRODUCTION

Autologous bone marrow transplantation involves reinfusion of the patient's own marrow as protection from the myeloablative effects of high-dose cytotoxic chemotherapy and/or radiotherapy. The minimum storage interval between the collection and reinfusion of the marrow depends upon the time required to administer the therapeutic regimen, as well as the half-life of the drugs used. While stem cells are preserved for short periods at 4 °C, they can be stored successfully for extended periods at extremely low temperatures of −80 °C and −196 °C. For optimal cryopreservation and prevention of problems upon thawing, marrow or peripheral blood stem cells are usually processed to reduce the volume (and to eliminate mature cells), and resuspended in a cryoprotectant. Processing also involves purging the harvested marrow of leukaemia or tumour cells. Various cryoprotectants, either singly or in combination, are currently being used at different concentrations for preserving the stem cells. Although they have several beneficial effects, their use has been associated with various levels of toxicity when given indiscriminately. A combination of these cryoprotectants seems to be beneficial for optimal cryopreservation of the haemopoietic stem cells. Different techniques are available to assess the efficacy of these storage methods each with its own scope and limitations. We review here the existing knowledge on the subject and discuss its future prospects.

MECHANISM OF CRYOINJURY

When subjected to extremely low temperatures, haemopoietic cells are damaged by thermal shock during cooling, osmotic changes during freezing and thawing, and membrane-shearing forces due to intracellular ice formation when liquid water goes through the transition phase into the solid ice state. Substances which protect the cells from these factors are called 'cryoprotectants'. Cryoprotectants usually work by decreasing the ice crystal formation when liquid water goes through the transition phase into the solid ice state. Intracellular ice formation occurring with high rates of cooling may rupture the cell. Extracellular ice formation results in a progressive increase in the osmolality of the extracellular medium as water is taken up by the growing ice crystals, resulting in severe dehydration of the cell which affects its viability.

CRYOPROTECTANTS

Various cryoprotectants currently available include dimethyl sulphoxide (DMSO), hydroxyethyl starch (HES), albumin, glycerol, 1,2-propanediol and polyvinyl pyrrolidone. Of these, the use of glycerol as a cryoprotectant is avoided as it may cause lactic acidosis. Also, washing out glycerol from the stem cells is a cumbersome process and often results in loss of cell viability. A colligative cryoprotectant, i.e. one that depends on the concentration of a particular particle, must be capable of permeating the cell, otherwise it will merely add to the extracellular osmolality. DMSO is a penetrating cryoprotectant, which affords protection by its colligative properties and lowers the freezing temperature of the medium, thus reducing the proportion of water incorporated into ice and the extent of cellular dehydration.

Stem cells cryopreserved in DMSO may demonstrate a
is also effective in preventing post-thaw macroscopic fociating agent and has a proven effect on the differentiation proliferative and malignant potential. DMSO is a differen-
decrease in the proliferation of malignant cells, because
4 °C or 37°C for incubation durations of up to 1 hour. of the cell cycle progression genes
DMSO has been shown to specifically inhibit the expression of cytokine IL-6 without having any effect on genes not
enzymes=-'? and modification of the cellular cytoskeleton.P
cells up to 1 hour was also not toxic to haemopoietic
progenitor cells. At a concentration of 40% DMSO, a
direct toxicity to haemopoietic progenitors was found. Delay in the removal of DMSO after thawing of cryopreserved cells up to 1 hour was also not toxic to haemopoietic progenitor cells. Direct addition of DMSO at 1% or 10%
final concentration (volume per volume; v/v) to the culture dishes suppressed colony formation.

The efficacy of HES as a cryoprotectant for human red blood cells was demonstrated in 1967. This compound offers the advantage of being non-toxic if administered intra-
venously. Also being a plasma substitute, HES, in combina-
with human red blood cells, can restore the blood volume to normal and improve oxygen transport when there is major blood loss.6 Macromolecular cryoprotectants such as HES remain outside the cell and appear to provide protection by undergoing a reversible 'glass transition',11 forming a viscous shell around the cell and thereby prevent-
ing water shifts and progressive cellular dehydration. This is also effective in preventing post-thaw macroscopic clumping or gel formation after long term preservation.25,26

The combination of DMSO, an intracellular cryoprotec-
tant, and HES, an extracellular cryoprotectant, has been previously reported to reduce post-thaw granulocyte lysis and clumping.11 A mixture of 5% DMSO, 6% HES and 4% albumin possesses a sponge-like property of absorbing free liquid water thereby decreasing the water available for icicle formation during the transition phase. Although albumin is not a cryoprotectant, it may help in stabilizing frozen-thawed cells by maintaining their viability.13

FACTORS AFFECTING STORAGE VIABILITY, THAWING AND ASSESSMENT OF VIABILITY
The removal of contaminating red cells and the reduction of initial volume are important prerequisites for correct cryopreservation. These can also decrease the amount of cryoprotectant and products of lysed cells transfused into patients at the time of bone marrow infusion. The success of engraftment of the stored stem cells depends on the dose and the functional integrity of the cells infused, which, in turn, depend on the quality of the cryopreservation pro-
cedure and post-thaw handling. Most methods involve direct infusion of the cells immediately after thawing usually by keeping the marrow-containing bag in a water bath main-
tained at temperatures ranging from 37-40 °C to simplify the procedure and reduce cell loss. However, Schaeffer et al.30 reported that the rapid infusion of thawed undiluted human marrow can cause severe osmotic pressure due to the high osmotic pressure of the cryoprotectant, leading to a reduction in stem cell survival. Such potential losses in vivo are difficult to assess. Moreover, the greatest loss of stem cells occurs in the process of freezing the marrow and is therefore not related to the duration of storage. Efficient granulocyte–macrophage colony forming unit (CFU-GM) and BFU-E recovery relies on numerous factors: the use of a cryoprotective agent,14 a slow initial freezing rate,15 a slow freezing rate beyond this point13 and the use of non-
lipophilic, non-plastic bags or ampoules of polypropylene for storage.34,35

One of the mechanisms which causes injury in preserved tissues is the formation of oxygen-free radicals. The cell is protected from oxidative stress by many defence mechanisms. One of these involves glutathione and glutathione-dependent enzymes. During tissue preservation by simple cold storage the loss of glutathione may sensitize the tissue to free radical damage after transplantation. Methods to suppress the loss of glutathione during the preservation of tissues may be an important factor in suppressing oxygen-
free radical injury. Addition of antioxidants into the preservation solution improves the cryoprotection of the human bone marrow cells. Antioxidant drugs—seleno-
methionine, methionine, tocopherol and penicillin/Fe++—increase the tolerance of the stem cells to freezing and thawing and elevate the number of surviving granulocyte–
macrophage colony forming cells (GM-CFC) up to two-fold. In this way more immature cells can be protected.7,36

STORAGE TEMPERATURES
Storage at 4 °C
Several researchers utilize stem cells without freezing, usually storing them at 4 °C in a refrigerator because the process is simple and inexpensive.1-3 Such a procedure is used when the half-life of the drug is short such as intra-
venous melphalan, where the stored bone marrow needs to be reinfused within 24-48 hours.1 One interesting fact is that in refrigerated marrow the platelet count recovers faster. In vitro studies have demonstrated that bone marrow cells can be preserved from 48 hours to 9 days in the liquid state without loss of colony forming units (CFUs).37 Further, recent in vivo studies have shown that marrow stored at 4 °C from 8-54 hours can provide haemopoietic reconstitut-
ion in patients receiving myeloablative therapy.38 Storage of whole unfractionated marrow is superior to storage of buffy coat cells in tissue culture medium and 20% foetal calf serum.39 Delforge et al.2 have shown that unfractionated bone marrow cells may be stored at 4 °C for 4 days with 97% recovery of CFUs. Therefore, such procedures need expert handling and selective usage. However, the quality
and quantity of the stem cells stored at such temperatures for a longer period cannot be consistently predicted.

**Storage at liquid nitrogen temperatures**

The traditional method is to use DMSO at a molality of 1.6 (10% v/v) as a cryoprotectant which is added to the source of stem cells (bone marrow and/or peripheral blood) enriched with mononuclear cells, then introduced into a computer freezer programmed to cool at a constant rate of 1 °C per minute and subsequently stored at −196 °C (liquid nitrogen). This method of cryopreservation often causes agglutination of the stem cells upon thawing and in some instances may require DNAse therapy to disrupt the clumps that form during DMSO washout before infusion.

Other agents which can prevent leucoagglutination are dextran 110, HES and collagenase. After thawing, the grafts are generally reinfused without washing because of the potential risk of reducing the number of progenitor cells. However, despite proven clinical effectiveness adverse effects have often been associated with the infusion of untreated cryopreserved bone marrow cells and peripheral blood stem cells.

Clinical toxicity includes nausea, abdominal cramps, flushing, decreased forced vital capacity and transient hypertension. Such toxicity may be due to the infusion of large amounts of cell suspensions (and therefore large doses of DMSO) or, in certain procedures, due to products of cell lysis, which are less in highly purified density gradient separated grafts. Some investigators have used 2% human serum albumin or even a COBE cell processor to wash the grafts without major loss of repopulating cells.

Although this type of cryopreservation is most effective and widely used currently for long term storage of the stem cells, it is time-consuming, expensive and requires technical expertise. For a developing country like India, adopting this method would be difficult because of the expensive equipment involved. The large liquid nitrogen containers require large laboratory spaces, thus pressurizing the laboratory administration. These limitations led researchers to explore other rapid, economical and simpler methods such as storage at −80 °C without rate-controlled freezing and yet yield results similar to those after liquid nitrogen storage.

**Storage at −80 °C**

Many institutions involved in the cryopreservation of stem cells have found encouraging results using a mixture of 5% DMSO, 6% HES and 4% human serum albumin for cryoprotection and storing the bone marrow in a −80 °C deep freezer by simple immersion. This method is inexpensive, rapid, requires minimal manipulation and is effective as cells largely retain their biological activity. It would, therefore, ideally suit Indian conditions. Using this method several investigators have emphasized that storage at liquid nitrogen temperatures with rate-controlled freezing was not essential for successful cryopreservation and further demonstrated that immersion at −80 °C did not cause agglutination upon thawing therefore requiring no DNAse therapy.

Gorin et al. reported that recovery rates of CFU-GM from cryopreserved human marrow cells were 50% or more when post-plateau cooling rates were less than 5 °C/minute, 45% when they ranged from 5–10 °C/minute and 22% when they exceeded 10 °C/minute. Simple placement of cells in a −80 °C freezer provides a cooling rate of approximately 3 °C/minute and the post-plateau lasts for less than 4 minutes, thereby rendering it effective even when rate control was circumvented. The nucleated cell recovery after thawing in this procedure ranged from 50–96%.

This method of stem cell preservation holds good for a few months, with the maximum period of successful storage reported to be 18 months. Gulati et al. have found better cell viability (90%) and growth patterns while storing the marrow at −120 °C in a newer generation electrical freezer for up to seven months.

Malinin et al. reported that cryopreservation of human bone marrow cells at −196 °C produced better recoveries than those at −79 °C. On the other hand, other reports propose that both these methods give more or less similar results. It remains to be seen whether such a comparison holds good for longer periods of storage.

**EVALUATION OF THE STORAGE TECHNIQUES**

All these techniques can be assessed by the following methods prior to and after the storage. However, each has its limitations.

**Trypan blue dye exclusion technique**

Trypan blue is one of several rapid staining techniques recommended for use in dye exclusion procedures for viable cell counting. The percentage of dye excluding viable cells can be calculated. Staining also facilitates the visualization of cell morphology. However, dye exclusion assays are not specific as they measure damage caused to both mature and stem cells. With freezing there are large losses of differentiated cells compared to the more immature cells, thereby making this method a poor representative of the actual number of living cells.

**'In vitro' semi-solid methyl cellulose/agar colony assays**

Engraftment of the donor bone marrow is associated with haemopoietic precursors at different stages of maturation. Early recovery is facilitated by transplanting committed progenitor cells whereas sustained engraftment is the result of reinfusion of pluripotent stem cells. Because the marrow repopulating stem cell has not yet been identified, the committed progenitor CFU-GM is the most commonly used indirect indicator of marrow repopulating ability. Clinical evidence indicates that rapid and sustained haemopoietic reconstitution can be achieved when a CFU-GM dose of 15–50×10⁶/kg body-weight is given to the patient. Although the success and speed of engraftment have been related to the recovery of CFU-GM after thawing of unpurged marrow, this correlation of in vitro colony assays with engraftment has not always been observed. Moreover, quantification of the stem cells using clonogenic assays can only yield results after a delay of several days, thereby limiting its application.

However, it is well known that haemopoietic stem cells have the ability to form colonies in in vitro semi-solid cultures. In rodents, the assay of bone marrow stem cells (colony forming units-spleen; CFU-S) capable of repopulating the marrow and forming colonies in the spleen of lethally irradiated animals is probably the best method of monitoring the viability of the stored marrow. It correlates well with
the results of CFUc in agar though the latter technique measures a more mature precursor cell that is already committed to differentiation along the granulocyte-macrophage lineages. The agar culture technique can be applied to man and the measurement of CFUc is probably the best available index of the viability, proliferative capacity and lineage characteristics of stored human bone marrow stem cells.37

Ideally, the percentage of CFUc recovered from each specimen after storage reconstitution should take into account the possible loss of nucleated cells during storage and subsequent manipulation. In a given sample the nucleated cell recovery may be calculated as follows:22

\[
\text{No. of cells after reconstitution} = \frac{\text{No. of cells before freezing}}{\text{CFUc recovery}} \times 100
\]

The CFUc recovery may then be calculated as:

\[
\text{CFUc recovery} = \frac{\text{CFUc} \times 10^6 \text{nucleated cells after reconstitution}}{\text{CFUc} \times 10^6 \text{nucleated cell recovery cells before freezing}}
\]

Haemopoietic progenitor cells, monoclonal antibodies and the repopulating ability of stored bone marrow

The discovery of monoclonal antibodies has revolutionized the field of diagnostic pathology in pinpointing the specific cellular types. Several types of antibodies have been raised to the CD34 cluster by the International Workshop on Leukocyte Differentiation.59 The CD34 antigen is expressed on the CD34+ progenitor cell populations based on the expression of the CD45RA and CD71 antigens. CD34+ CD45RA+ CD71- are myeloid progenitors, CD34+, CD45RA- CD71- are erythroid progenitors and CD34+ CD45RA- CD71- are the most primitive multipotent stem cells.66-68 A strong positive correlation between the number of CFC-GM colonies and CD34+ cells has been demonstrated.59,70

The estimation of transplanted CD34+/CD33- and CD34+/CD33+ cells would be a more practical way to anticipate the marrow repopulating ability of bone marrow or peripheral blood derived grafts. However, enumeration of the stem cell fraction utilizing these monoclonals would not be beneficial to know the true repopulating potential of the infused bone marrow cells, unless it is combined with the in vitro progenitor cell assays.

FUTURE PROSPECTS

Several authors believe that leukaemic cells do not cryopreserve as well as normal haematopoietic cells. They have further observed that leukaemic cells undergo preferential cytolysis over normal haematopoietic stem cells when subjected to cryopreservation—a phenomenon termed ‘cryopreservation trauma’.23,46,71,72 This was one explanation of why the cryopreserved bone marrow (unpurged) from patients with leukaemia in remission may have a purging effect, and patients thus have a higher rate of relapse-free survival.73,74

Effective eradication of the malignant cells by high-dose chemotherapy and/or radiotherapy together with successful engraftment by the infused (cryopreserved) cells is the main principle behind autologous bone marrow or peripheral blood stem cell transplants. Various purging methods are currently being developed to separate the malignant clones based on in vitro models utilizing cell lines, fresh cancer cells and drugs like 4-hydroperoxycyclophosphamide and VP-16.46,75-77 Several centres have developed monoclonal antibodies specific to lymphoma, leukaemia, small cell lung cancer and neuroblastoma which are currently being used to purge the cancer cells.46,78,79 Using the recent counterflow elutriation/centrifugation and FACS methods, researchers have been able to isolate different fractions of stem cells based on their antigen expression, size, age and proliferating stage.80,81

Therefore, the future lies in expanding the scope of purging methods using specific monoclonal antibodies to more diseases, counterflow elutriation and FACS to fractionate the stem cells and to expand such cells ex vivo82 for more successful bone marrow transplantation procedures.

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REFERENCES


