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Hematopoietic Stem Cell Cryopreservation: A Review of Current Techniques

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ABSTRACT

Hematopoietic stem cells (HSC) can be stored for prolonged periods at cryogenic temperatures. The techniques currently used were derived from the initial report in 1949 of cryopreservation of bovine sperm in glycerol. The addition of this penetrating cryoprotectant protected the cells from the injury associated with ice formation. Current cryopreservation techniques (with minor variations) suspend cells in an aqueous solution of salts, protein, and one or more cryoprotectants. Cells are frozen at slow rates and stored generally below −120°C in mechanical freezers or nitrogen refrigerators. That these techniques are successful in maintaining HSC viability is evident from the engraftment of these cells in patients treated with marrow-lethal conditioning regimens. However, issues such as the composition of the cryoprotectant solution, cell concentration during freezing, cryoprotectant toxicity, and storage temperatures have not been adequately studied, primarily because of a lack of appropriate assays for HSC cryosurvival. HSC cryobiology will become an increasingly important subject as new HSC collection and processing techniques are developed. Improved cryosurvival of HSC using modified cryoprotectant solutions may improve engraftment kinetics and decrease the cost and morbidity of autologous transplantation.

INTRODUCTION

CRYOPRESERVATION of bone marrow or peripheral blood-derived hematopoietic stem cells (HSC) permits the administration of intensive chemo-radiotherapy to patients with dose-responsive malignancies. Although cryopreservation is not a requirement for autologous transplantation, and patients have recovered marrow function after reinfusion of marrow cells stored for several days at 4°C, a progressive loss of hematopoietic stem cells occurs during nonfrozen storage. Definition of ideal conditions (*e.g.*, temperature, initial processing of the cells, additives) for nonfrozen storage of cells may slow this loss. However, merely maintaining survival, much less growing additional HSC, is beyond the current capabilities of *in vitro* cell culture techniques. Therefore, most transplant centers cryopreserve HSC intended for autologous transplantation. Cryopreservation allows administration of multiple-day transplant conditioning regimens as well as prophylactic storage for patients to be transplanted months to years later. Although some unavoidable loss of

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HSC occurs with marrow or peripheral blood stem cell (PBSC) processing and cryopreservation, progressive loss over months of proper storage is not obvious and may not occur.

That HSC can be successfully cryopreserved is evident from the success of autologous HSC transplantation in regenerating marrow function after marrow-lethal conditioning regimens. Engraftment failure is generally not attributed to HSC cryopreservation, although some investigators correlated poor HSC cryopreservation with delayed engraftment after transplantation (Gorin, 1986; Rowley *et al,* 1989). Most cryopreservation laboratories use a variation of the technique outlined in Table 1. Cryopreservation research, however, has not been a focus of the transplantation teams, and HSC cryopreservation is one of the least understood aspects of HSC processing. This lack of understanding of cryobiology hinders the ability to adapt techniques for handling unusually small or large collections of cells or other accidental or intentional deviations from laboratory protocol. Although easily performed by a number of transplant centers, HSC cryopreservation and reinfusion are not without risk of toxicity to both the stem cell inoculum and the recipient.

It is the purpose of this commentary to review the development of our current cryobiology techniques, examining the parameters depicted in Table 1. For this review, HSC is intended to refer to both primitive hematopoietic stem cells responsible for durable engraftment as well as the more mature progenitor cells that can be cultured *in vitro,* regardless of source (*e.g.,* bone marrow, peripheral blood).

CRYOPRESERVATION THEORY

Preservation of viability after cryopreservation and thawing became possible after the discovery that glycerol could be used for the freezing of bovine sperm (Polge *et al,* 1949). Subsequent experiments demonstrated that bone marrow frozen with glycerol could be used to reconstitute the bone marrow of irradiated mice (Barnes & Loutit, 1955). Considerable exploration of the cryobiology of various mammalian and nonmammalian cells ensued. Those studies defined the mechanism of cell damage incurred during cryopreservation, and techniques to moderate that damage. The current understanding of cryobiology contends that ice crystal formation during cooling is the primary cause of cell damage (Karow & Webb, 1965a). Intracellular ice crystals may form at rapid rates of cooling, resulting in *mechanical* rupture of cell structures. At slower rates of cooling, ice crystal formation occurs in the extracellular space, resulting in increasing osmolality as free water is incorporated into the ice crystals. This loss of free water results in the concentration of extracellular solutes, such as sodium ions, that do not freely penetrate the cell membrane (or are actively excluded), extreme hyperosmolality, and *dehydration* injury. For example, the molality of Na^+ in a saline solution at −10°C is about 2.8 molal, and at −20°C about 5.6 molal (Mazur, 1970).

TABLE 1. SEATTLE HSC CRYOPRESERVATION TECHNIQUE

Cell processing:	Buffy-coat or light-density cells
Cell concentration:	$>2 \times 10^7$ cells/ml final concentration
Storage container:	Blood storage bag tolerant of cryogenic temperatures
Sample volume:	Dependent upon bag size, generally 40–60 ml per bag
Cryoprotectant:	10% (vol/vol) dimethylsulfoxide final concentration
Protein:	20% autologous plasma
Electrolytes:	Tissue culture medium
Rate of cooling:	1°C/minute to −40°C with compensation for heat of fusion, then −10°C/minute to −80°C
Storage temperature:	Below −135°C in nitrogen vapor-phase refrigerators or mechanical freezers
Rate of thawing:	Rapidly (>100°C/minute) in a 37°C water bath
Post-thaw manipulation:	Addition of ACD (20% vol/vol of product volume), then infusion immediately after thawing without further processing; filtration through blood administration set (170 μ) if clumped after thawing

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Intracellular ice formation may be limited by cooling cells slowly. At slow rates, it is probable that ice nucleation will first occur in the larger volume outside the cell. Progressive dehydration of the cell will result if the rate of cooling is slow enough to allow water to shift to the extracellular space and be incorporated into the growing ice crystals. The permeability of the cell to water appears to define the optimal cooling rate for any particular cell (Mazur, 1966). Dehydration with concentration of intracellular solutes protects the cells from intracellular ice formation and growth.

Glycerol and dimethylsulfoxide (DMSO) are colligative cryoprotectants that prevent dehydration injury by moderating the increased concentration of nonpenetrating extracellular solutes such as sodium ions during ice formation, and by decreasing the amount of water absorbed by (in equilibrium with) the ice crystals at a defined temperature. Colligative refers to properties dependent upon the number of particles (solute), not the composition of the particles. Freezing is the crystallization of water, and the freezing point is the temperature at which ice crystals can be sustained in equilibrium with water. The freezing point of water is depressed by the addition of solute. For any particular mixture of solute(s) and water, there will be a defined temperature at which ice crystals can initially form. Unlike pure water, ice crystal formation and growth in aqueous solutions occurs over a temperature range. Growing ice crystals absorb free water and exclude solute particles. The incorporation of water into ice results in concentration of the solute and further depression of the freezing temperature of the remaining water, thereby preventing additional ice formation unless further cooling occurs. Thus, temperature (and pressure) defines the equilibrium between ice and the nonfrozen solution. With further cooling, a temperature is eventually reached at which the solute itself crystallizes (eutectic point). The molality of a solution in equilibrium with ice is determined by the temperature of the solution (and not the initial concentration of the solute). In our example above, a saline solution at −20°C has a Na^+ molality of about 5.6 molal. For a two-component system such as DMSO and saline, both solutes will contribute to the molality of the unfrozen solution. The molality (before freezing) of 10% DMSO (vol/vol) in solution is about 1.6 molal, about 10 times the molality of Na^+ in the medium. At −20°C, this molal ratio between DMSO and Na^+ will be maintained. Therefore, the molality of the Na^+ will be about 0.56 molal, or a little less than four times the molality of Na^+ in a solution without ice. The addition of a penetrating cryoprotectant to an aqueous saline solution, therefore, reduces the osmotic stress across the cell membrane at −20°C from about 36 times to less than four times that of an ice-free solution. According to this theory, colligative cryoprotectants must be capable of penetrating the cell to avoid merely contributing to the molality of the extracellular medium (and must be nontoxic to the cells at the concentration required for cryopreservation).

The tolerance of cells to freezing, therefore, depends on the ability of the cells to withstand osmotic stress. The osmotic tolerance of granulocytes is much less than that for lymphocytes or mature hematopoietic progenitors, probably accounting at least in part for the difference in survival after cryopreservation (Dooley & Takahashi, 1981; Law *et al,* 1983). With DMSO concentrations less than 10%, the degree of dehydration caused by concentration of the nonpenetrating solutes during freezing will be greater because nonpenetrating solutes will contribute proportionately more to the molality of the nonfrozen solution in the extracellular medium. With greater concentrations of DMSO,the osmotic stress will be less. The optimal concentration for a colligative cryoprotectant, therefore, depends upon the osmotic tolerance of the cell to be frozen, the toxicity to the cell of high concentrations of the cryoprotectant itself (Karow & Webb, 1965b; Fahy, 1986; Arakawa *et al,* 1990), and the presence of other cryoprotectants.

Colligative properties do not explain the cryopreservation achieved by freezing cells in solutions of macromolecules such as hydroxyethyl starch (HES). Solutions of high-molecular-weight, polymeric cryoprotectants contain relatively few particles and, moreover, do not freely penetrate the cell. These cryoprotectants may protect the cell by forming a viscous, glassy shell that retards the movement of water, thereby preventing progressive dehydration as water is incorporated into the extracellular ice crystals (Takahashi *et al,* 1988). Solutions of some compounds, when present in sufficiently high concentrations, will solidify to an amorphous glass without first forming ice crystals, a process termed "vitrification" (Fahy *et al,* 1984). The "glass-transition temperature" (T_g) depends upon both the structure and concentration of the solute. Pure water forms a glass at about −139°C (Grout, 1991). The addition of cryoprotectants such as DMSO, glycerol, or HES raises the T_g (Luyet, 1960). At very high concentrations of cryoprotectants, (6.3 *M* for DMSO [Fahy *et al,* 1984]), the T_g is higher (warmer) than the temperature at which ice crystals can form, thereby preventing crystallization during cooling and its resulting mechanical and osmotic stresses. The

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practical difficulty with achieving vitrification arises from the necessity to use very high concentrations of cryoprotectants. Vitrification has been used for the cryopreservation of murine embryos, for example, using a solution of 20% DMSO, 15.5% acetamide, 10% propylene glycol, and 6% polyethylene glycol (Rall & Fahy, 1985). Prolonged exposure to this solution was toxic, however, with complete loss of viability after 30 min at 4°C. With lower concentrations of cryoprotectants, the crystallization temperature is higher than the vitrification temperature and ice crystals will form during cooling. Yet, glass transition may occur even in the presence of ice crystallization. With the formation of ice crystals and resulting loss in free water, the concentration of the cryoprotectant in the nonfrozen water will increase. With decreasing temperature causing increasing cryoprotectant concentration, a point will be reached at which the cryoprotectant forms a glass. At this "limiting glass transition temperature" (T'_g), the solution suddenly becomes viscous, retarding, if not stopping, the flow of water through the extracellular matrix. This has been proposed as the mechanism for cryoprotection afforded by the extracellular cryoprotectants (Takahashi *et al,* 1988). In one study of peripheral blood monocyte cryopreservation using extracellular cryoprotectants, a limiting glass-transition temperature of −20°C was optimal, and this was achieved using a 20% solution of HES (Takahashi *et al,* 1988). Substances forming glasses at higher or lower temperatures were less effective. This model of cryopreservation requires adequate dehydration to occur to concentrate intracellular solutes and decrease the probability of intracellular ice formation, but glass formation at an appropriate temperature (please recall that temperature defines the osmolality of the unfrozen aqueous solution) to prevent excessive dehydration of the cell.

Cells are not frozen in simple, two-component solutions, but rather in complex solutions containing salts, sugars, penetrating cryoprotectants with or without extracellular cryoprotectants, and plasma proteins (Table 2). Phase transition temperatures (such as T'_g) for these solutions have not been determined. The improved cryosurvival observed with increasing protein concentration (Ragab *et al,* 1977) and combined penetrating and extracellular cryoprotectants (Stiff *et al,* 1983; Conscience & Fischer, 1985) may be explained at least in part by these theories on ice and glass formation and their effects on the cell. Moreover, the existence of glass formation may explain the relationship between storage temperature and survival of HSC over time. Below the T'_g temperature, ice crystal growth cannot occur and the cells are protected from mechanical disruption by growing ice crystals. The optimal storage temperature is below the T'_g for the cryoprotectant solution used.

TABLE 2. PUBLISHED CRYOPRESERVATION TECHNIQUES

	Final concentration						
Author	*Cell (per ml)*	*DMSO (%)*	*Protein (%)*	*Medium*	*Other*	*Pre-chill cells*	*End-freeze temperature*
Weiner & Gross	$0.2–1 \times 10^8$	10	25, FFP	HBSS	Heparin	Yes	−120°C
Soken	4×10^7	10	10, AP	TC199	—	Yes	−90°C
Janssen & Lee	NS	10	5, PPF	TC199	—	Yes	−90°C
Meagher, Herzig & Herzig	$<10^8$	10	5, AP	RPMI-1640	—	Yes	−90°C
Hill, Robertson & Dickson	$<10^8$	10	0.5, HSA	MEM	—	Yes	−100°C
Hollinsworth	NS	10	NS, AP	TC199	—	Yes	−90°C
Dyson, Haylock & To	$2–4 \times 10^7$	10	20, AP	HBSS	—	Yes	−80°C
Yao	NS	10	NS, AP	TC199	Heparin	Yes	NS
Bouzgarou *et al.*	4×10^7	10	NS, AP	HBSS	—	Yes	−140°C
Oldenburg & Stiff	NS	5	3.6, HSA	Normosol-R[R]	6% HES	Yes	NA
Rosina & Jiang	$<10^8$	5	5, HSA	Plasma-Lyte[R]	6% HES	Yes	NA

All techniques were recently published in a manual of marrow processing techniques (Areman, Deeg & Sacher, 1992). Only technical details without supporting clinical data documenting cryopreservation efficacy were provided. Abbreviations used are: FFP = fresh frozen plasma, AP = autologous plasma, PPF = plasma protein fraction, HSA = human serum albumin, NS = not specified, NA = not applicable.

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Warmer temperatures may be used, but at risk of cell damage, a risk that is dependent upon the temperature of storage and the stability (viscosity) of the solution at that temperature.

This simplified review of the effects of freezing and the cryoprotectant properties of penetrating and polymeric cryoprotectants does not completely explain the processes involved during freezing, and more detailed reviews of the freezing of aqueous solutions and mammalian cells have been published (Karow & Pegg, 1981; Fuller & Grout, 1991). In addition to mechanical and dehydration injuries, cooling itself may be damaging to the cell (Fuller, 1991). Also, colligative effects alone are not sufficient to explain the cryoprotectant properties of DMSO or glycerol. Other freely penetrating chemicals such as urea and $DMSO_2$ do not function as cryoprotectants for mammalian cells (Vos & Kaalen, 1965; McGann & Walterson, 1987), and some chemicals such as ethanol and guanidine may actually function as cryosensitizers (Kruuv *et al,* 1990). Obviously, the chemical structure of the cryoprotectant is important in the cryosurvival of mammalian cells, and a molecular interaction between the cryoprotectant and protein or lipid molecules appears necessary for optimal cryopreservation (Carpenter & Crowe, 1988; Crowe *et al,* 1987, 1990; Anchordoguy *et al,* 1991). The damaging effect of freezing on membrane proteins or structure compared to the survival of the cell is illustrated by the ability of HSC to proliferate *in vitro* when stimulated by peripheral blood feeder layers but lack of response to GCT-conditioned medium (Gilmore, 1983). Similar "resistance" to recombinant growth factors by thawed bone marrow progenitors, suggests a loss of cytokine receptors despite the survival of the cell (Rowley & Hattenburg, 1990). The ideal cryoprotectant solution would also protect the various cell organelles and molecular species from sublethal damage.

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PROCESSING OF BONE MARROW FOR CRYOPRESERVATION

One of the most important concepts of clinical HSC cryopreservation that must be realized is the heterogeneity of marrow and PBSC populations. HSC cryopreservation involves not only the cryobiology of the HSC, but also of the mature blood cells contained in the harvested product. The minimum number of cells required for marrow reconstitution after autologous transplantation is not known. Generally, $1–3 \times 10^8$ nucleated marrow cells per kilogram of patient weight are harvested. Less certain is the minimum amount of cells required for PBSC transplantation; under certain conditions, the quantity collected may far exceed the number harvested for marrow transplantation. Regardless, hematopoietic stem cells comprise a very small portion ($<1\%$) of either the marrow or peripheral blood product. Bone marrow consists of HSC, mature blood cells, and noncellular material such as fat. Cryopreservation techniques that are optimal for HSC may destroy mature blood cells. The standard technique for red cell cryopreservation uses glycerol (Sputteck & Korber, 1991); granulocytes cannot be cryopreserved successfully. The presence of mature blood cells affects HSC cryopreservation in at least three ways. First, the large proportion of mature blood cells collected may hinder the laboratory processing if clumping before or after thawing is induced by damaged granulocytes or platelets. We observed the loss of about 50% of cells when clumping occurred, and infusion through a standard blood administration set may be required to prevent pulmonary embolism. Second, damaged cells may also predispose toward infusion-related toxicity. The infusion of marrow that was frozen without depletion of mature blood cells was associated with acute renal failure in 3 of 33 patients in one study (Smith *et al,* 1987); this presumably resulted from massive hemolysis of red cells. Other investigators correlated the quantity of cells cryopreserved and infused to the post-infusion toxicity observed. In one study, infusion of cryopreserved buffy-coat cells incurred greater toxicity than infusion of light-density cells, which were essentially depleted of mature blood cells (Davis *et al,* 1990). Kessinger *et al* reported a high incidence of infusion-related toxicity for recipients of large quantities of peripheral blood stem cell products (Kessinger *et al,* 1990). Third, if the cell concentration affects HSC cryosurvival, then the presence of large numbers of mature blood cells requires that the cells be frozen in large volumes. It is possible that the toxicity reported by Kessinger *et al* and Davis *et al* may relate to the quantity of DMSO infused, and not specifically from lysis of mature blood cells.

Therefore, cryopreservation of HSC can be facilitated by the prefreeze depletion of mature blood elements. Collection of buffy-coat cells is the minimum processing required for cryopreservation of bone marrow. A number of apheresis or cell-washing devices are capable of processing the large quantities of cells harvested.

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