



## Controlled-rate versus uncontrolled-rate cryopreservation of peripheral blood progenitor cells: a prospective multicenter study

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### Abstract

**Background and Objective.** Cryopreservation of hemopoietic progenitors for transplantation has been traditionally performed by the use of a controlled-rate freezer. Several groups have reported successful cryopreservation of progenitor cells at  $-80^{\circ}\text{C}$  without a controlled-rate freezer. In an attempt to elucidate whether both methods are equally efficient, we compared controlled-rate versus uncontrolled cryopreservation of peripheral blood progenitor cells (PBPC) in a prospective, multicenter study.

**Design and Methods.** Apheresis products from patients undergoing PBPC mobilization were split into two aliquots, and cryopreserved simultaneously by both methods, in autologous plasma plus 10% dimethylsulfoxide. Controlled-rate samples were placed into a programmable freezer with a cooling rate of  $1-2^{\circ}\text{C}/\text{min}$ . Uncontrolled-rate samples were directly introduced into a  $-80^{\circ}\text{C}$  mechanical freezer. After thawing, cell counts, assays for viability, clonogenic cultures and  $\text{CD}34^{+}$  cell enumeration were performed.

**Results.** A total of 105 cases were included. No significant differences were found in viability (mean  $88.8 \pm 13\%$  in the controlled-rate group vs.  $89.7 \pm 12\%$  in the uncontrolled-rate group), nucleated cell loss ( $23.5 \pm 23\%$  vs.  $23 \pm 22\%$ ), mononuclear cell loss ( $19 \pm 23\%$  vs.  $19.1 \pm 22\%$ ), and loss of  $\text{CD}34^{+}$  cells ( $34.3 \pm 33\%$  vs.  $28.6 \pm 34\%$ ). On the other hand, recovery of granulomonocytic colony-forming units (CFU-GM) was significantly better with the controlled-rate technique, than with the non-controlled-rate method ( $104.3 \pm 95$  vs.  $86.5 \pm 80$ , respectively;  $p=0.048$ ).

**Interpretation and Conclusions.** Our results indicate that both techniques are suitable for cryopreservation of PBPC, although a better recovery of committed progenitors is achieved by the controlled-rate method. Therefore, the use of controlled-rate freezer should probably be recommended.

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Key words: cryopreservation, stem cells, transplantation, cryoprotective agents, lymphoma

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It has been widely accepted that cryopreservation of hemopoietic progenitors requires the use of controlled-rate freezing devices, in order to minimize freezing injury to stem cells.<sup>1</sup> A constant cooling rate between  $1-2^{\circ}\text{C}$  per minute, and compensation for the heat of fusion during phase transition, have been considered critical issues for optimal cryopreservation.<sup>2</sup> Other factors that may affect cell survival after freezing and thawing are the medium and cryoprotectant in which cells are suspended, the sample volume, cell concentration and plastic material of the container.<sup>3</sup> Nevertheless, programmed controlled-rate freezing apparatuses are expensive and may not be available in many centers. For this reason, several authors have developed simplified cryopreservation methods characterized by the use of  $-80^{\circ}\text{C}$  mechanical freezers, without control of cooling rate.<sup>4,5</sup>

Although both bone marrow and peripheral blood progenitor cells (PBPC) frozen with the uncontrolled-rate techniques have been used for autologous transplantation,<sup>6</sup> there is a lack of comparative studies that prospectively evaluate whether this method is as efficient as the standard controlled-rate cryopreservation.

Here we present a prospective, multicenter study comparing the recovery of different cell populations after controlled-rate versus uncontrolled-rate cryopreservation of PBPC. This is the only reported study in which the comparison has been done in parallel, and all the conditions potentially affecting cell viability have been identical in the samples frozen by both methods.

### Materials and Methods

#### Study design

This is a prospective multicenter study in which individual experiments were performed in parallel, by simultaneous cryopreservation of each sample by the two methods being tested. Seven centers participated in the trial. The study subjects were patients with hematologic malignancies and solid tumors included

in a program of autologous peripheral blood progenitor cell (PBPC) transplantation. PBPC were obtained by repeated aphereses after mobilization treatment with hemopoietic growth factors either alone or in combination with chemotherapy, according to each center's protocol. Informed consent was obtained according to local ethics committee regulations. Aphereses were performed using either COBE Spectra (COBE Iberica, Barcelona, Spain) or Fenwall CS3000 (Baxter Biotech, Barcelona, Spain) cell processors.

### **Cryopreservation**

Apheresis products were split into two identical freezing bags, either teflon-captan (Gambro DF700, Gambro GmbH, Hechingen, Germany) or ethylene-vinyl-acetate (Cryocyte, Baxter Biotech, Barcelona, Spain). Cells were suspended in pre-cooled autologous plasma and bags were placed on ice. An equal volume of autologous plasma plus 20% dimethylsulfoxide (DMSO) (Sigma, Madrid, Spain) was slowly added to each bag, to bring a final 10% DMSO concentration, a final volume of 150 mL, and equal cell concentration, not exceeding 100 million cells per mL. If the total number of cells exceeded the capacity of the two bags at such concentration, additional bags were prepared in the same way. One of the study bags was cryopreserved by the controlled-rate method and the other one by the uncontrolled-rate method, simultaneously. For controlled-rate freezing, the bag was placed in a metal canister, introduced into a programmed freezer (Cryoson, or CM25, Carbuos Metálicos, Madrid Spain), and cooled as previously described.<sup>7</sup> Briefly, there was a  $-1^{\circ}\text{C}/\text{min}$  cooling rate from  $4^{\circ}\text{C}$  to  $-6^{\circ}\text{C}$ , then a rapid decrease of temperature to induce nucleation, followed by a constant decrease of  $-1^{\circ}\text{C}/\text{min}$  down to  $-40^{\circ}\text{C}$ . The second phase was a decrease of  $-2^{\circ}\text{C}/\text{min}$  down to  $-60^{\circ}\text{C}$ , and finally a rapid  $-12^{\circ}\text{C}/\text{min}$  rate down to  $-120^{\circ}\text{C}$ . After completing the program, the bag was transferred to a liquid nitrogen tank for long term storage. For the uncontrolled-rate cryopreservation, the bag inside its metal canister, was introduced in a pre-cooled methanol bath at  $4^{\circ}\text{C}$ , and placed directly into a  $-80^{\circ}\text{C}$  mechanical freezer, as previously described.<sup>8</sup> After 16-24h this bag was also transferred to a liquid nitrogen tank for long term storage. On the day of reinfusion, both bags were thawed by immersion in a  $37^{\circ}\text{C}$  water bag and infused to the patient without further manipulation.

### **Biological controls**

Samples for cell counts, viability, CD34 analysis and functional assays were drawn directly from the bags, not from pilot tubes, before freezing and after thawing. Cell viability was assessed by trypan blue dye exclusion. CD34<sup>+</sup> cells were analyzed by flow cytometry using the HPCA2 (Beckton Dickinson, Mountain View, CA, USA) monoclonal antibody. Assays for granulomonocytic colony forming units (CFU-GM)

were performed using semisolid cultures, according to each center's policy. Overall, three methods were used, as follows: 43% of the experiments were performed in agar, as described elsewhere.<sup>9</sup> Feeder layers prepared from normal peripheral blood mononuclear cells were used as a source of colony stimulating factors. Twenty-eight percent of the cases were plated in methylcellulose plus 10% leukocyte conditioned medium (Methocult, Stem Cell Technologies, Vancouver, Canada), and 31% were done with the Stem Sell CFU Kit (Baxter Biotech, Barcelona, Spain), following the manufacturer's indications. In all cases an identical CFU-GM assay was used for both controlled-rate and uncontrolled-rate samples within each paired experiment. Cultures were plated in triplicate and incubated for 14 days at  $37^{\circ}\text{C}$ , with 5%  $\text{CO}_2$ , in a humidified atmosphere. Colonies (more than 40 cells) were counted under an inverted microscope.

### **Statistical methods**

The primary endpoint was recovery of CFU-GM after thawing. Secondary endpoints were loss of nucleated cells, mononuclear cells and CD34<sup>+</sup> cells. Statistical analysis was performed with NCSS software (Hintze, Kaysville, UT, USA).<sup>10</sup> An  $\alpha$  value of 0.05 was used throughout the study. Variables showing normal distribution were analyzed by two-tailed paired t-tests. Variables showing other than normal distribution were analyzed by the nonparametric Wilcoxon test for matched pairs. Pearson's correlation was used to evaluate the influence of cell concentration and storage time on the recovery of CFU-GM.

### **Results**

PBPC from a total of 105 patients were cryopreserved by both methods. Of these, 101 had their cells thawed and reinfused, and were, therefore, evaluable for post-thawing analysis. Four cases were not thawed because of removal from the transplant program, due to either tumor relapse or clinical complications. Patients' diagnoses included 47 solid tumors, 29 multiple myeloma, 18 lymphoma, 5 acute leukemia and 1 chronic myeloid leukemia. The apheresis machine was the COBE Spectra in 69% of the cases and the Fenwall CS3000 in 31%. Fifty-eight percent of the freezing bags were Gambro and 42% were Cryocyte. Data of different cell populations per bag prior to cryopreservation are shown in Table 1. Although cell concentration was intended not to exceed 100 million per mL, up to 188 million/mL was achieved in one case due to the need for volume reduction. Median time of PBPC storage in liquid nitrogen prior to reinfusion was 33 days, the range was from 7 to 561 days. After thawing, median cell viability was 95% (range 45-100) in the uncontrolled-rate group and 95% (range 45-100) in the controlled-rate group. Loss of nucleated and mononuclear cells after thawing was also similar in both samples, as shown in Figure 1. There was no significant difference in the mean loss of CD34<sup>+</sup> cells,

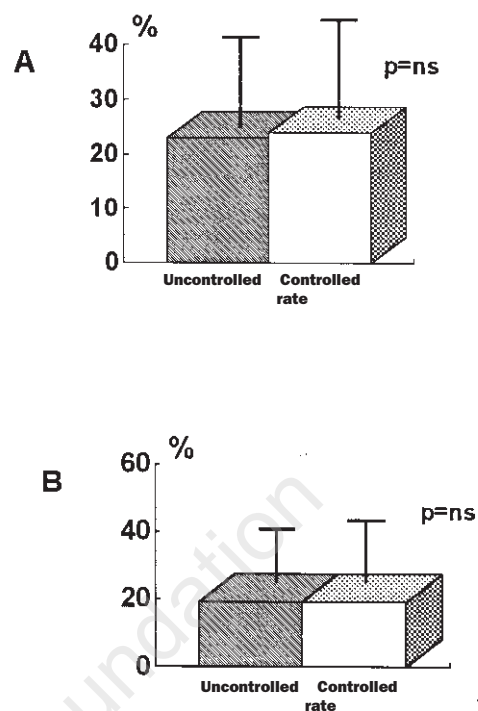
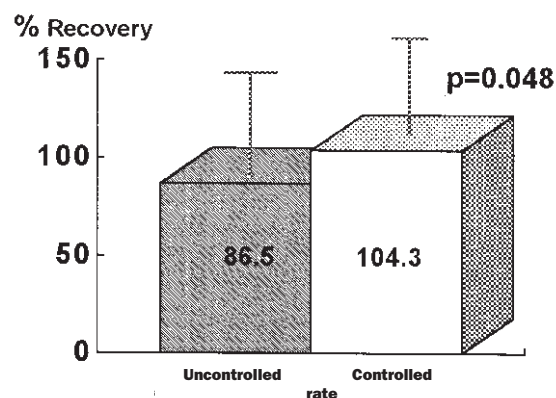
**Table 1. Cell content per bag before freezing.**

|  | Median | Range    |
|--|--------|----------|
| Total nucleated cells ( $\times 10^9$ )        | 12.1   | 3.1-28.3 |
| Mononuclear cells ( $\times 10^9$ )            | 8.8    | 1.6-25.5 |
| Cell concentration ( $\times 10^6/\text{mL}$ ) | 81.6   | 21-188   |
| CFU-GM ( $\times 10^6$ )                       | 1.6    | 0-32.6   |
| CD34 <sup>+</sup> cells ( $\times 10^6$ )      | 40.0   | 3.5-522  |

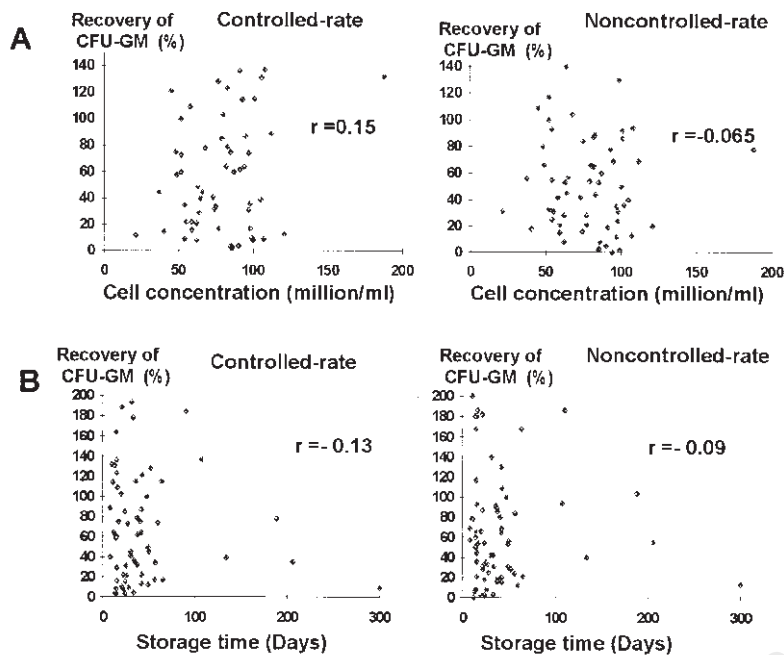
being  $34.3 \pm 33\%$  in the uncontrolled-rate sample and  $28.6 \pm 34\%$  in the controlled-rate sample ( $p = \text{ns}$ ). On the other hand, the recovery of CFU-GM after thawing was significantly better with the controlled-rate technique than with the uncontrolled-rate method ( $p = 0.048$ ), as shown in Figure 2. The recovery of CFU-GM, was not influenced by either the cell concentration in the bag or the duration of the storage in liquid nitrogen. This lack of correlation was seen in both controlled-rate and uncontrolled-rate samples (Figure 3).

## Discussion

We compared the efficiency of controlled-rate, versus uncontrolled-rate cryopreservation of PBPC in a prospective manner. Controlled-rate freezing requires the use of somewhat sophisticated equipment, not available in many centers. In 1983, Stiff *et al.* reported preliminary experiments demonstrating the feasibility of freezing bone marrow cells by direct introduction of the sample into a  $-80^\circ\text{C}$  mechanical freezer.<sup>5</sup> They used a mixture of 6% hydroxy-ethyl-starch (HES) and 5% DMSO as cryoprotectant, and obtained a recovery of 105% CFU-GM, after thawing. In 1987, the same group reported a series of 60 patients with solid tumors transplanted with bone marrow frozen by this uncontrolled-rate technique.<sup>11</sup> Mean viability after thawing was 82%, and CFU-GM recovery 81%. Engraftment was achieved in 68 out of 72 transplants. Nevertheless, their patients receiving regimens that were considered as non myeloablative, since they included neither total body irradiation nor full dose busulfan. Obviously, uncontrolled-rate cryopreservation has the advantages of being a simpler technique, of costing less and of only needing a mechanical freezer, which is a common device available in most blood banks. For these reasons several groups have attempted similar approaches with only minor modifications.<sup>12-14</sup> Clark *et al.* reported 62% CFU-GM recovery from bone marrow frozen by the uncontrolled-rate method, using DMSO as the sole cryoprotectant, and complete engraftment was achieved after transplantation.<sup>4</sup> The choice of cryoprotectant may have important implications. Makino *et al.* found relevant differences in the freezing curves obtained after uncontrolled-rate cryopreservation depending upon the use of either DMSO alone or a combination of HES plus DMSO.<sup>15</sup> In our study 10% DMSO was used, and the

**Figure 1. Loss of total nucleated (A) and mononuclear (B) cells after thawing.****Figure 2. Recovery of granulomonocytic colony-forming units (CFU-GM), after thawing.**

recovery of CFU-GM in the uncontrolled-rate arm, was 86%, which compares favorably with results reported by other authors.<sup>4,12</sup> Nevertheless, in our hands, the controlled-rate technique gave a significantly better CFU-GM recovery. Although there is only one reported study in which such superiority has been found, several hypotheses may explain our findings.<sup>16</sup> First of all,



**Figure 3.** Absence of correlation between either (A) cell concentration in the freezing bag and recovery of CFU-GM after thawing, or (B) duration of frozen storage and recovery of CFU-GM.  $r$  = correlation coefficient.

there are few comparative studies, and none of these have been performed in a prospective fashion. Rosenfeld *et al.* found no differences in a study including 33 patients receiving uncontrolled-rate frozen peripheral blood compared to a control group of 17 patients transplanted with controlled-rate frozen bone marrow.<sup>17</sup> In their study, the cryoprotectant was HES plus DMSO in the first group, and DMSO alone in the second group, which makes the comparison less reproducible. Second, in our study, all the conditions that may have an influence on cell survival after freezing were exactly the same within each paired experiment. These include the plastic material of the bag, the sample volume, cell concentration, cryoprotectant and suspension medium.<sup>18-21</sup> Moreover, our CFU-GM assays were plated on the same day, with the same method and counted by the same person in each paired analysis, which makes the comparison more accurate. Another important issue is the fact that post-thawing samples were drawn directly from the freezing bags. In most of the previous reports samples were taken from pilot tubes, which are very different in size, shape and plastic thickness, compared to the original bag of marrow. As reported by Douay *et al.*, these differences make the results obtained from samples from ampoules less reliable than those from samples taken directly from the bags.<sup>22</sup>

The higher recovery of CFU-GM found with the controlled-rate method means only an *in vitro* superiority. Whether such a difference has any clinical implications is still to be determined. Evaluation of engraft-

ment was beyond the scope of our study, since both the controlled-rate and the uncontrolled-rate frozen bags were reinfused to the patient. Large randomized studies would be needed to compare hematologic reconstitution.<sup>23,24</sup> According to other authors, it seems that uncontrolled-rate cryopreservation could be sufficient to guarantee hematologic reconstitution in patients receiving conditioning regimens.<sup>14,17</sup> On the other hand, on the basis of our results, the controlled-rate technique should be recommended, since a better recovery of committed hemopoietic progenitors is achieved.

#### Contributions and Acknowledgments

JPO formulated the design of the study, carried out statistical analysis and wrote the manuscript. JG designed the controlled-rate cryopreservation program. RB, MC, VH, AA and MT were responsible for cryopreservation techniques and measurements at their own centers. PR performed clonogenic cultures and data handling. JO was responsible for patient management. JLN was involved in laboratory data supervision and reviewed the manuscript. The order in which the names appear has been based upon the number of patients included in the study by each participating center.

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#### Disclosures

Conflict of interest: none.

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**Manuscript processing**

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