

The Effect of Freezing on the Recovery and Expansion of Umbilical Cord Blood Hematopoietic Stem Cells

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Abstract

Objectives: Cell populations residing in waste tissues (cord blood, umbilical cord, and placenta) may be collected without any medical or ethical contraindications concerning the mother or newborn baby. Cord blood hematopoietic stem cells are routinely used for clinical transplants; however, the low cell dose of the graft limits their therapeutic efficacy as it is associated with increased delayed or failed engraftment. The cell dose can be increased, and the efficacy of cord blood transplant potentially improved, by ex vivo expansion before transplant.

Materials and Methods: Twelve umbilical cord blood samples were included. The effect of cord blood storage at -80°C on CD34⁺ cell count (mean $\pm$ standard deviation [SD]), cell viability (mean $\pm$ SD percent), and cell cycle status (percent quiescent versus dividing) was estimated. Ex vivo culture of cord blood mononuclear cells was done before storage, and after 1 week of freezing, and after 2 weeks of freezing. Ex vivo liquid culture was performed with media supplemented with stem cell factor, interleukin-3 (IL-3), and both.

Results: The count of CD34⁺ cells in pre-expansion aliquots decreased from $15.00 \pm 9.96 \times 10^6$ cells before freezing to $7.70 \pm 3.20 \times 10^6$ cells after 2 weeks of freezing ($P = .024$). Cell viability in pre-expansion aliquots decreased from $99.5\% \pm 1.0\%$ before freezing, to $52.5\% \pm 27.5\%$ after 1 week of freezing ($P = .013$) and to $32.5\% \pm 9.5\%$ after 2 weeks of freezing ($P = .001$). Mean fold of cell expansion and proportion

of quiescent versus dividing cells did not change significantly from before to after freezing, and was not significantly different for culture with stem cell factor, IL-3, or both.

Conclusion: Although freezing decreased cell count and viability, it did not impair the expansion potential of cord blood hematopoietic cells. Whether IL-3 or stem cell factor should be considered as essential components of expansion media is uncertain.

Key words: *Ex vivo expansion, Cryopreservation, viability, Stem cell factor, Interleukin-3*

Umbilical cord blood is increasingly used as an alternative stem cell source in place of bone marrow or peripheral blood stem cells (1). Advantages of cord blood transplant over those of bone marrow or mobilized peripheral blood include the ease of collection, less stringent requirement for HLA blood group matching between donors and recipients, lower incidence of graft-versus-host disease after transplant (2), presence of donors from minority groups that are poorly represented in bone marrow donor registries, and much lower incidence of cytomegalovirus-infected donors (3). However, the relatively low cell dose has limited the use of umbilical cord blood transplants mostly to pediatric patients or low-weight adults (2). Low doses of cord blood hematopoietic progenitor cell are associated with delayed neutrophil and platelet engraftment and a high incidence of graft failure (4). To obtain optimum engraftment, the most important factors are the number of nucleated cells and the absolute CD34⁺ cell content per kilogram of body weight in the recipient (5). In addition, the viability and proliferative state of the infused cells are important. Different approaches to increase cell doses are being considered, such as by using multiple units of umbilical cord blood (6) and by ex vivo expansion to

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improve the applicability and outcome of the transplant (7).

It is crucial that an optimized condition is established for the expansion of stem cells and progenitor cells that possess the ability to engraft in the bone marrow of the recipient (8). Also, to consistently achieve an adequate cell dose, umbilical cord blood processing methods must minimize cell losses. Each additional manipulation of a cellular product potentially leads to further loss of cells. In most studies, CD34⁺ cell selection was done before initiating cell culture (9), but the CD34⁺ cell selection itself is associated with a substantial loss of progenitor cells. This cell loss, which may not be significant for smaller children, may become critical in reaching a suitable dose for transplant in older children and adults (10).

In this study, we evaluated the effect of storage and its duration on the expansion of unselected cord blood hematopoietic stem cells (HSCs), in 3 liquid culture media using different cytokine combinations, aiming to establish a clinically applicable culture system for ex vivo expansion of umbilical cord blood HSCs.

Materials and Methods

Twelve umbilical cord blood samples were obtained with oral consent of the mother and the approval of Cairo University ethical committee. Samples were collected from the umbilical vein after spontaneous delivery of the placenta following full-term vaginal delivery. The cord blood was collected in a bag containing citrate phosphate dextrose anticoagulant. Mononuclear cells were isolated by centrifugation of cord blood over Ficoll-Hypaque density gradient (density 1.077, Biochrom KG, Berlin).

The mononuclear cells from each sample were then divided into 3 aliquots. One was directly cultured, while the other 2 were stored at -80°C after adding 10% dimethyl sulfoxide as a cryoprotectant. Thawing was done after 1 week and after 2 weeks.

The mononuclear cells were suspended at 10⁵ cells/mL in Royal Park Memorial Institute culture medium (GIBCO, Sigma, St. Louis, MO, USA) supplemented with 20% fetal calf serum (GIBCO-BRL Grand Island, NY, USA), penicillin (10 000 units/mL), and streptomycin (10 mg/mL) (GIBCO-BRL Grand Island, NY, USA). Samples were cultured for 48 hours with stem cell factor, interleukin-3 (IL-3),

and both. Stem cell factor (R&D Systems GmbH, Wiesbaden-Nordenstadt, Deutschland) was used at a concentration of 20 ng/mL, and IL-3 (R&D Systems), at 10 ng/mL. All cultures were maintained at 37°C in 5% CO₂ and a humidified atmosphere.

Enumeration of CD34⁺ cells was performed according to the protocol of the International Society of Hematotherapy and Graft Engineering (11) before storage, after thawing after 1 or 2 weeks of freezing, and after 48 hours of culture with different cytokine combinations. Samples were mixed with fluorescein isothiocyanate-conjugated mouse monoclonal antibody against CD34 (Dako, Glostrup, Denmark) and with appropriate isotype-matched control monoclonal antibody. Cells were incubated with monoclonal antibody for 30 minutes at 4°C, washed once with phosphate-buffered saline, and resuspended in a small volume of phosphate-buffered saline for analysis by means of FACScan flow cytometer (Coulter Epics Elite, Miami, FL, USA). Forward and side scatter gates were established to exclude cell debris and clumps before analysis for expression of CD34.

The viability assay was performed using Trypan blue dye exclusion test. Mononuclear cells were mixed with Trypan blue dye and incubated at 37°C for 5 minutes. Two hundred cells were counted using a light microscope at low power. Cells not taking the dye were counted as viable, whereas cells taking the dye were considered nonviable.

For determining cell cycle phase distribution (i.e. quiescent versus dividing cells), mononuclear cells were permeabilized and nuclear DNA was labeled with propidium iodide (Beckman Coulter, Miami, FL, USA). Cell cycle phase distribution of nuclear DNA was determined on FACScan fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using Cell Quest software (Becton Dickinson, San Jose, CA, USA). A total of 10000 events was acquired, and analysis of flow cytometry data was performed using Mod Fit software (Verity Software House, Topsham, ME, USA). A histogram of DNA content (x-axis, propidium iodide fluorescence) versus counts (y-axis) was displayed.

Statistical analysis was performed with SPSS 13.0 (Statistical Package for the Social Sciences, version 13.0, SPSS Inc, Chicago, IL, USA). Data analysis included descriptive statistics (mean and standard deviation). One-way analysis of variance was used

Table 1. Variables studied in 12 umbilical cord blood samples.

		CD34 ⁺ Cell Count ($\times 10^6$)	Viability (%)	Quiescent Cells (%)	Dividing Cells (%)
Before freezing	Pre-expansion	15.00 $\pm$ 9.96	99.5 $\pm$ 1.0	95.1 $\pm$ 3.8	4.6 $\pm$ 3.6
	SCF	22.25 $\pm$ 9.90	98.0 $\pm$ 2.8	96.1 $\pm$ 3.5	4.0 $\pm$ 3.0
	SCF + IL-3	24.20 $\pm$ 7.00	98.0 $\pm$ 1.2	95.2 $\pm$ 3.9	4.5 $\pm$ 3.5
	IL-3	25.15 $\pm$ 12.60	98.0 $\pm$ 0.5	95.6 $\pm$ 2.6	3.9 $\pm$ 2.1
After 1 week of freezing	Pre-expansion	16.30 $\pm$ 5.30	52.5 $\pm$ 27.5	95.5 $\pm$ 5.3	5.1 $\pm$ 2.7
	SCF	38.95 $\pm$ 22.20	45.0 $\pm$ 20.4	87.4 $\pm$ 9.8	7.5 $\pm$ 7.1
	SCF + IL-3	40.90 $\pm$ 24.10	43.8 $\pm$ 22.1	83.1 $\pm$ 16.3	11.7 $\pm$ 10.6
	IL-3	44.75 $\pm$ 25.20	45.0 $\pm$ 21.2	81.5 $\pm$ 16.9	14.6 $\pm$ 14.3
After 2 week of freezing	Pre-expansion	7.70 $\pm$ 3.20	32.5 $\pm$ 9.5	95.9 $\pm$ 1.9	4.7 $\pm$ 5.2
	SCF	43.25 $\pm$ 10.70	17.5 $\pm$ 13.2	83.4 $\pm$ 5.3	11.7 $\pm$ 11.1
	SCF + IL-3	50.90 $\pm$ 18.70	16.3 $\pm$ 11.1	78.6 $\pm$ 3.0	17.3 $\pm$ 16.1
	IL-3	52.50 $\pm$ 17.10	16.3 $\pm$ 9.5	73.1 $\pm$ 2.4	18.6 $\pm$ 17.0

Data are presented as mean and standard deviation.

Abbreviations: IL-3, interleukin-3; SCF, stem cell factor.

in testing for statistically significant differences for the comparisons between the culture media. Scheffé posthoc test was used for pair-wise comparisons between the means when analysis of variance rendered a statistically significant result. Paired *t* test was used to study the effect of time on different variables in each culture. Pearson correlation coefficient was used to determine significant associations between fold expansion and other variables. The statistical significance level was set as $P < .05$.

Results

Twelve umbilical cord blood units were included in this study. Mean values of all studied variables (CD34⁺ cell count, viability, and proportions of quiescent and dividing cells) at each testing point are summarized in Table 1. The CD34⁺ cell count was decreased from $15.00 \pm 9.96 \times 10^6$ cells before freezing to $7.70 \pm 3.20 \times 10^6$ cells after 2 weeks of freezing in the pre-expansion aliquots ($P = .024$). In addition, viability was decreased from $99.5 \pm 1.0\%$ before freezing to $52.5 \pm 27.5\%$ after 1 week of freezing ($P = .013$) and to $32.5 \pm 9.5\%$ after 2 weeks of freezing ($P = .001$) in the pre-expansion aliquots.

Cultures showed the same patterns in viability as for cell counts. In liquid culture using stem cell factor, viability was lower in samples after 1 week of freezing ($P = .013$) and after 2 weeks of freezing ($P = .002$) than before freezing. In liquid culture using IL-3, viability was lower after 1 week of freezing ($P = .015$) and after 2 weeks of freezing ($P < .001$) than before freezing. In liquid culture using both stem cell factor and IL-3, viability was lower after 1 week of freezing ($P = .016$) and after 2 weeks of freezing

($P = .001$) than before freezing. The mean fold expansion of CD34⁺ cells did not change significantly through the periods; before freezing to 1 week and before freezing to 2 weeks in the 3 sets of culture media. (With stem cell factor alone, $P = .747$ and $.085$, with both stem cell factor and IL-3, $P = .995$ and $.125$ and with IL-3 alone, $P = .916$ and $.146$ respectively) (Table 2).

Table 2. Mean fold expansion of CD34⁺ cells in culture media with the three cytokine combinations

	SCF	SCF + IL-3	IL-3
Before freezing	2.01 $\pm$ 1.13	2.57 $\pm$ 1.95	2.64 $\pm$ 1.97
After 1 week	2.41 $\pm$ 1.28	2.56 $\pm$ 1.11	2.81 $\pm$ 1.53
After 2 weeks	6.64 $\pm$ 3.34	7.38 $\pm$ 2.86	8.11 $\pm$ 4.49

Data are presented as mean and standard deviation.

Abbreviations: IL-3, interleukin-3; SCF, stem cell factor.

The mean percentage of quiescent cells decreased from before freezing to 1 week of freezing for cultures with stem cell factor alone ($P = .002$) and with both stem cell factor and IL-3 ($P = .003$), although not for cultures with IL-3 alone ($P = .121$). The mean percentage of quiescent cells did not change significantly from before freezing to 2 weeks after freezing with the 3 cytokine combinations ($P = .245$ for stem cell factor alone, $P = .304$ for both stem cell factor and IL-3, $P = .272$ for IL-3 alone; Table 3).

The mean percentage of dividing cells did not change significantly from before freezing to 1 week of freezing and from before freezing to 2 weeks of freezing with the 3 cytokine combinations (with stem cell factor alone, $P = .485$ and $.317$, with both stem cell factor and IL-3, $P = .427$ and $.333$ and with IL-3 alone, $P = .269$ and $.283$ respectively; Table 3).

The correlation between fold expansion and the mean percentage of dividing cells showed a positive relationship with the 3 cytokine combinations and

Table 3. Mean percentage of quiescent cells and dividing cells with the three cytokine combinations

		Before Freezing	After 1 Week of Freezing	After 2 Weeks of Freezing
Quiescent cells (%)	SCF	96.1 ± 3.5	87.4 ± 9.8	83.4 ± 5.3
	SCF + IL-3	95.2 ± 3.9	83.1 ± 16.3	78.6 ± 3.0
	IL-3	95.6 ± 2.6	81.5 ± 16.9	73.1 ± 2.4
Dividing cells (%)	SCF	4.0 ± 3.0	7.5 ± 7.0	11.7 ± 11.1
	SCF + IL-3	4.5 ± 3.5	11.7 ± 10.6	17.3 ± 16.1
	IL-3	3.9 ± 2.1	14.6 ± 14.3	18.6 ± 17.0

Data are presented as mean and standard deviation.

Abbreviations: IL-3, interleukin-3; SCF, stem cell factor.

during the different periods of freezing, with no statistically significant difference between the 3 cytokine combinations (Figures 1-3).

Discussion

Over the last few years, many studies have been carried out with the aim of identifying conditions that support the self-renewal and expansion of human umbilical cord blood HSCs in ex vivo culture

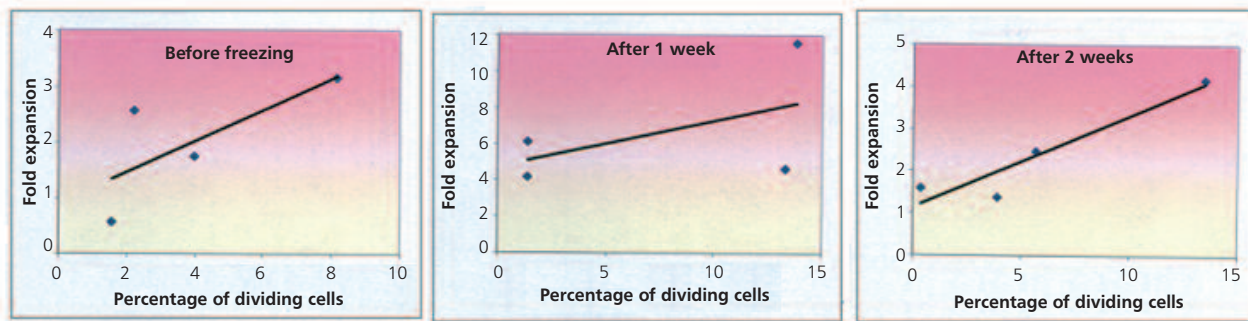


Figure 1. Correlation between fold expansion and the mean percentage of dividing cells in culture medium containing stem cell factor alone ($P = .270$, $r = .730$ before freezing, $P = .471$, $r = .529$ after 1 week of freezing, $P = .056$, $r = .944$ after 2 weeks of freezing).

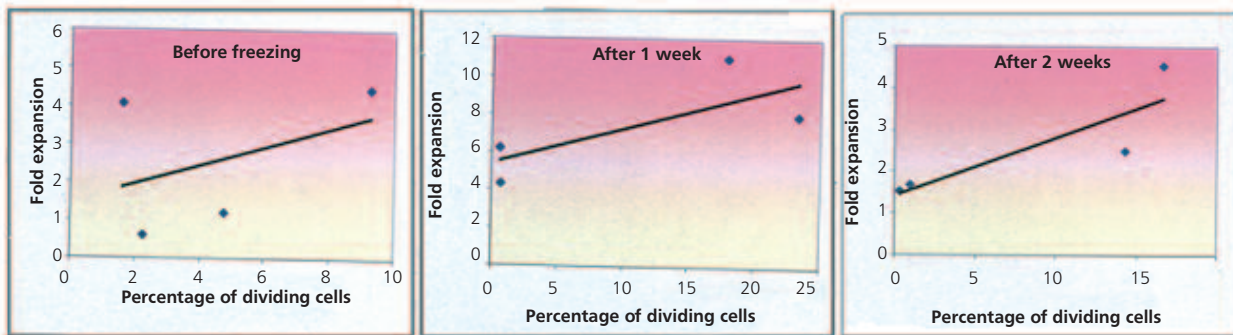


Figure 2. Correlation between fold expansion and the mean percentage of dividing cells in culture medium containing both stem cell factor and IL-3 ($P = .579$, $r = .421$ before freezing, $P = .242$, $r = .758$ after 1 week of freezing, $P = .138$, $r = .862$ after 2 weeks of freezing). **Abbreviation:** IL-3, interleukin-3.

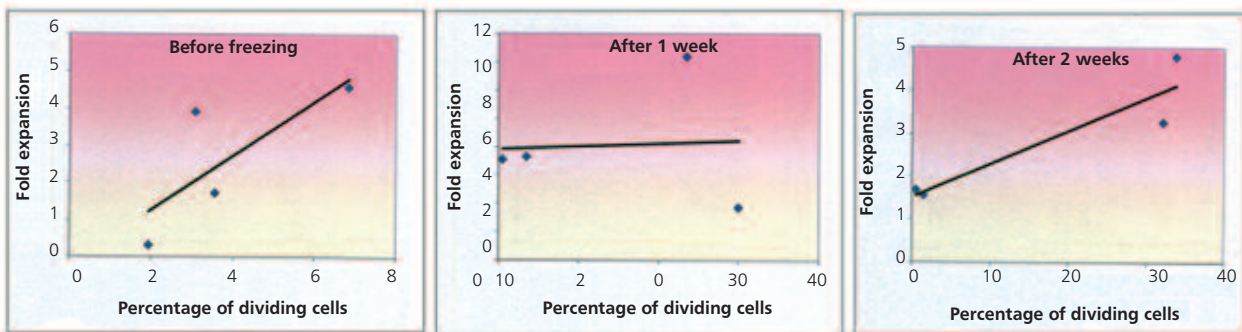


Figure 3. Correlation between fold expansion and the mean percentage of dividing cells in culture medium containing IL-3 alone ($P = .232$, $r = .768$ after 1 week of freezing, $P = .076$, $r = .924$ after 2 weeks of freezing, $P = .138$, $r = .862$ after 2 weeks of freezing). **Abbreviation:** IL-3, interleukin-3.

for their important applications in transplant, stem cell marking, and gene therapy (12). In this study, we aimed to evaluate the effect of storage on stem cell count, proliferation, response to cytokines, cell cycle state, and viability to provide information for the use of preserved umbilical cord blood. Three cytokine combinations were studied, using stem cell factor alone, IL-3 alone, and both stem cell factor and IL-3. Stem cell factor acts as a potent mitogen with direct action on early hematopoietic progenitor cells (13), forming the basis for hematopoietic progenitor cells *ex vivo* expansion protocols (14). Interleukin-3 enhances the amplification of early and committed progenitor cells without impairing the long-term engraftment of stem cells (15).

Regarding the effect of freezing, our results showed that the number of CD34⁺ cells and viability assessed by Trypan blue dye exclusion test were significantly lower in samples frozen for 2 weeks than in non-frozen samples in the pre-expansion aliquots. In addition, we detected significantly decreased cell viability in samples frozen for 1 week and for 2 weeks after liquid culture using the 3 cytokine combinations. Other investigators reported significantly decreased cell viability after cryopreservation (10, 16, 17) and attributed this to the effect of thawing and washing to remove the cryoprotectant. Laroche and colleagues (10) stated that thawing and washing result in loss of cells approaching 20% when compared with pre-freeze counts, with the wash step responsible for nearly half of this cell loss.

During *ex vivo* culture, we detected mean fold expansion of 6.64 ± 3.34 with stem cell factor alone, 7.38 ± 2.86 with both stem cell factor and IL-3, and 8.11 ± 4.49 with IL-3 alone after 2 days culture of the samples frozen for 2 weeks. In a similar study, Moezzi and colleagues (17) used stem cell factor, IL-3, and thrombopoietin and reported levels of expansion of (4.2-4.7 fold) after 7 days of culture of samples cryopreserved for 1 month.

There were no statistically significant differences in fold expansion between the 3 cytokine combinations before freezing and after 1 week and 2 weeks of freezing. These results agree with the work of Lazzari and colleagues (18), who reported similar expansion from both fresh and cryopreserved cord blood samples. Moreover, we detected positive correlations between fold expansion and the mean percentage of dividing cells with the different

cytokine combinations and during the different periods of freezing. From these data, we can say that although preservation procedures could decrease the count and viability of cord blood HSCs, freezing does not impair their *ex vivo* expansion potential, and this supports the data mentioned before (17).

The mean percentage of quiescent cells decreased from before freezing to 1 week of freezing in liquid cultures containing either stem cell factor alone or the combination of stem cell factor and IL-3. This decrease was not significant in the culture medium containing IL-3 alone. Stem cell factor is generally accepted as promoting the growth of primitive hematopoietic cells and thus routinely included in all cultures (15). In contrast, the role of IL-3 in *ex vivo* expansion protocols has been a matter of controversy. Sanchez-Garcia and colleagues (19) found that the percentages of cells in the G0 phase were significantly reduced when CD34⁺ cells were expanded with early acting cytokines (like stem cell factor) together with IL-3. This may be because stem cell factor initiates the proliferation of G0 CD34⁺ human cells, and then IL-3 sustains maximal proliferation of stem cell factor-prestimulated G0 CD34⁺ cells (20). Sequential cytokine exposure may be critical for optimal numerical stem cell expansion with concomitant maintenance of their functional properties (15).

In conclusion, our results suggest that freezing does not impair the expansion potential of cord blood hematopoietic cells; however, it results in a significant loss of cell viability. Further studies on larger number of samples are recommended to determine the cytokines that can be considered as essential components of the expansion medium. In the future, umbilical cord blood harvesting may become a familiar aspect of the delivery room.

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