Recovery of cord blood hematopoietic progenitors after successive freezing and thawing procedures

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Background and Objectives. Cord blood (CB) is a valuable source of stem cells. Most CB units are still cryopreserved in single bags in the world's CB banks. Thawing a single CB unit, dividing it into two parts, expanding the smaller one and refreezing the other would optimize *ex vivo* expansion of CB progenitors prior to transplantation: expanded and unexpanded cells could be infused together to accelerate early engraftment.

Design and Methods. The feasibility of refreezing CB samples was investigated by evaluating the effect of 3 successive cryopreservation procedures in 9 CB units. The number and viability of WBC, BFU-E, CFU-GM, CFU-MIX, LTC-IC, and the absolute CD34$^+$ cell count were assessed at time 0 and after each thawing. The percentage of CD34 cells expressing CD38, L-selectin, VLA-4, VLA-5, H-CAM, LFA-1 and CXCR4 was also evaluated.

Results. After three freezing and thawing procedures, WBC counts decreased, while lymphocytes were unchanged. Viability was 90% of basal values after the first thawing and did not change. BFU-E decreased significantly only after the third thawing. CFU-GM and CFU-MIX did not change significantly, nor did LTC-IC, CD34$^+$ cell counts and CAM and CXCR4 expression on CD34$^+$/CD38$^-$ cells.

Interpretation and Conclusions. These data show that two successive freeze-thaw procedures do not significantly affect the clonogenic potential and CAM expression of cord blood progenitors. This information could be exploited to devise new options in *ex vivo* expansion procedures and quality controls prior to transplantation.

Key words: cord blood, cryopreservation, refreezing, stem cell expansion

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Cord blood (CB) is a valuable source of stem cells for transplantation in the treatment of hematologic, oncologic, immunologic, and metabolic diseases.[1-5] Most CB units in the world's CB banks are stored in liquid nitrogen in individual bags for several years and quickly thawed just prior to transplantation. A single unit contains a limited number of hematopoietic progenitors although with high proliferative and repopulating capability.[6,7]

The main disadvantages deriving from this are the delayed neutrophil and platelet engraftment in comparison with that provided by bone marrow and peripheral blood stem cell transplantation and the difficulty in obtaining a large enough graft for adult patients or those weighing more than 30 kg.[4] *Ex vivo* expansion of CB progenitors could overcome the problem by accelerating engraftment and facilitating transplantation in adults.[8-11] Thus, two major problems in CB banking are cytokine-mediated expansion of CB progenitors and quality control of cryopreserved units.

The best method of cryopreserving CB units for expansion purposes is to divide them into two bags before freezing, thaw them individually, and then infuse expanded and unexpanded progenitors at the same time. Alternatively one can separately cryopreserve previously *ex vivo* expanded CB progenitors and then infuse them with the not expanded component at day 0.[12] However, this approach is only feasible in selected cases of sibling collections because of its high costs. In transplants from single bag-cryopreserved CB units, *ex vivo* expanded progenitors have been infused 10 days after the not expanded component,[13] although this is not the best way to accelerate neutrophil and platelet engraftment. A better approach would be to thaw the single unit and divide it into two unequal parts: expand the smaller part, refreeze the other and eventually infuse the expanded and unexpanded progenitors at the same time to accelerate early engraftment. This technique would also allow proper quality control before the crucial myeloablative treatment of the recipient. To evaluate the feasibility of refreezing CB samples, we studied the effect of three successive cryopreservation procedures on 9 CB units. For this purpose, WBC, lymphocytes, viability, CFU, LTC-IC, CD34$^+$ cell counts, cell adhesion molecules (CAMs) and CXCR4 expression on CD34$^+$ cells were evaluated before and after each procedure.

Design and Methods

CB collection and processing

Collection and cryopreservation procedures were performed according to an ISO 9002 quality system validation program of the Italian Cord Blood Banks Network.[15] Nine CB units were obtained by umbilical vein catheterization after delivery of full-term newborns. Informed consent was given by the mothers. CB was collected into PVC MacoPharma (Rho, Milan, Italy) bags containing 21 mL CPD.[15,16] Total volumes (including anticoagulant) ranged from 75 to 120 mL (mean 86±15 mL). The units were transferred with no prior cell separation into 200 mL Haemofreeze DF700 bags (NPBI, Emmer-Compascuum, The Netherlands) and, after the addition of sterile saline solution plus 5% human albumin, 10% DMSO and 2000 I.U. heparin to obtain 200 mL final volume, cryopreserved by a standard method (–1 C°/min programmed cooling rate, stored in liquid nitrogen). CB units were thawed at 37°C, gently mixed in melting ice and quickly refrozen within 15 min. A 5 mL sample was drawn from an inserted injection coupler, slowly mixed with 5 mL of sterile washing solution (5% Dextran40, 2.5% human albumin in 0.9%NaCl) and centrifuged once (400 g/10 min). The supernatant was removed and the sedimented cells were slowly resuspended in fresh washing solution added to restore the 5 mL volume, then tested.[17] This procedure was repeated three times with an interval of one month or more between freezing and thawing. Clonogenic tests and flow cytometry analysis were performed on the basal sample (whole blood) and after the first, second and third thawing. WBC, lymphocytes, $CD34^+$ and CFU counts were expressed per microliter. To compare whole blood basal values with values after thawing, a correction factor was applied because the units had been diluted to 200 mL final volume before cryopreservation. After thawing WBC, lymphocytes and CFU counts were multiplied by 200 and divided by basal volume. WBC viability was evaluated by the ethidium bromide and acridine orange method (Fluoro-Quench, ONE LAMBDA, Canoga Park, CA, USA).

Cell cultures

The colony assays for BFU-E, CFU-MIX and CFU-GM were performed by plating 2×10^4 MNCs in 1 mL of methylcellulose medium with recombinant cytokines Methocult GF H4434 (StemCell Technologies, Vancouver, Canada). LTC-IC were assessed using bulk cultures (StemCell Technologies). Briefly, 6×10^5 CB MNCs were cultured in 2 cm^2 wells in the presence of MyeloCult H5100 medium with 10^{-6} M hydrocortisone onto a previously established feeder layer of irradiated (8000 cGy) M2-10B4 cells. Cultures were performed at 37°C in a humidified incubator with 5% CO_2 in air. One half of the medium was removed weekly and replaced with fresh medium. After five weeks, non-adherent and adherent cells (after trypsinization) were seeded in 35 mm Petri dishes in Methocult GF H4435 (StemCell Technologies). After 14 days colonies were scored with an inverted microscope. LTC-IC were calculated according to Hogge *et al.*[18]

Antibodies

The following monoclonal antibodies were utilized: anti-CD34 FITC (8G12, Becton-Dickinson, San José, CA, USA); anti-CD38 TC (HIT 2, Caltag, South San Francisco, CA, USA); anti-CD13 PE (L138, Becton-Dickinson); anti-CD62L PE (L-selectin, SK11, Becton-Dickinson); anti-CD49d PE (VLA-4, 44H6, Serotec, Kidlington, Oxford, England); anti-CD49e PE (VLA-5, SAM-1, Serotec); anti-CD44 PE (H-CAM, F 10-44-2, Serotec); anti-CD11a PE (LFA-1, B-B 15, Serotec); anti-CXCR4 PE (PharMingen, San Diego, CA, USA).

Flow cytometry

The percentage of cells expressing CD34, CD38 and/or CAM and/or CXCR4 was assessed by three-color flow cytometry analysis as follows: 5×10^5 nucleated cells were incubated for 20 min at 4°C with anti-CD34, anti-CD38, and anti-CAM or anti-CXCR4 MoAb. Isotype control was performed. After incubation and red cell lysis by ammonium chloride, cells were analyzed with a flow cytometer XL2 Epics Coulter equipped with an argon laser. Almost two hundred $CD34^+$ cells were analyzed for each sample. A three-color analysis for CD34, CD38 and CD13 was also performed in six further samples. Absolute $CD34^+$, $CD34^+/38^-$, $CD34^+/38^-/13^-$ counts were assessed by a two-platform method, utilizing a Sysmex K 4500 Counter (Sysmex Corporation, Kobe, Japan) and a standard Milan protocol. As a control, absolute $CD34^+$ cell counts were assessed in each sample by a two-platform ISHAGE-derived protocol. The coefficient of variation between the Milan and ISHAGE counts was never more than 5%. In the first three CB units, leukocytes were counted with a Sismex K 4500 Counter in triplicate after each thawing. The results were highly reproducible.

Statistical analysis

Statistics were obtained with Student's t test.

Results

After the three procedures, WBC counts decreased significantly from a baseline of 9.6±1.4 to $6.7\pm1.3\times10^3/\mu L$ ($p<0.001$), whereas lymphocyte counts were unchanged (basal count = $3.4\pm0.6 \times10^3/\mu L$, third thawing = $3.4\pm1.0\times10^3/\mu L$). The

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mean basal percentage of viable cells was 96.6±1.7% and did not show significant changes from the first to the third thawing (first, second and third thawing: 89.1±3.6%, 90.6±2.2%, and 92.4±1.9%, respectively). BFU-E decreased significantly from 2.9±1.4/µL to 2.0±0.9/µL after the third thawing (p=0.01), whereas there was no significant difference between the first and second thawing. CFU-GM and CFU-MIX did not change significantly. LTC-IC were unchanged. The absolute count of $CD34^+$ cells was virtually constant (23±14.4/µL basal value; first, second and third thawing: 25.2±15.9/µL, 23.6± 16.3/µL, and 25.7±13.5/µL, respectively). The absolute count of $CD34^+/38^-$ cells increased significantly from the second to the third thawing (basal count: 2±1.2/µL; first, second and third thawing: 2.5±1.7/µL, 2.8±1.5/µL, 3.8±1.4/µL, respectively), though in six further experiments the absolute count of $CD34^+/38^-/13^-$ cells did not change significantly (1.2±0.9/µL basal value; first, second and third thawing: 1.1±1.4/µL, 0.9±1.3/µL, 0.8±0.7/µL).

The percentage of CXCR4 and CAM-positive $CD34^+/38^-$ cells did not change significantly, though CD11a-positive cells increased from the first to the second thawing (respectively, 8.3±2.6% and 10.5±1.1%, p=0.04). CXCR4, CD49d, CD11a, and CD62L expression on $CD34^+/CD38^+$ cells decreased significantly from basal values to the third thawing (but not between the first and second) (82.8±3.9%, 86.9±5.8%, 85.0±6.4%, 82.4±5.8% versus 70.4±6.8%, 77.8±2.8%, 71.1±7.2%, 70.8±5.7%, p<0.01). The mean fluorescence intensity for CD11a, CD62L, CD49d and CXCR4 decreased significantly in $CD34^+/38^-$ cells after three procedures but did not change from the first to the second thawing, except for CD11a, which decreased from 3.7±1.1 to 3.1±0.8 (p=0.03). The results are summarized in Tables 1-3 and Figure 1.

Table 1.

	WBC ×10³/µL	Lymph ×10³/µL	BFU-E /µL	CFU-GM /µL	CFU-MIX /µL	LTC-IC /10⁵MNC	CD34⁺ /µL
Basal	9.6±1.4	3.4±0.6	2.9±1.4	1.4±0.8	1.5±1.4	1.0±0.6	23.0±14.4
Thaw 1	8.3±1.4	3.0±0.8	2.4±0.9	1.1±0.6	1.2±0.9	0.9±0.6	25.2±15.9
Thaw 2	7.7±1.5	3.2±0.8	2.3±1.0	1.2±0.6	0.8±0.4	1.1±0.3	23.6±16.3
Thaw 3	6.7±1.3	3.4±1.0	2.0±0.9	0.9±0.4	0.7±0.5	1.2±0.7	25.7±13.5

Number of WBC, lymphocytes (Lymph), BFU-E, CFU-GM, CFU-MIX, LTC-IC, $CD34^+$ cells before (Basal) and after three successive cryopreservation procedures (Thaw1, Thaw2, Thaw3) in 9 CB units. Basal = whole blood.

Discussion

Hematopoietic progenitors were evaluated in our study by clonogenic assays and flow cytometry. Assays of colony-forming cells detect the more differentiated progenitors and the LTC-IC assay detects early progenitors. Flow cytometry evaluation of CD34 and CD38 expression gives information about $CD34^+/CD38^-$ cells, a very immature population highly enriched in SCID repopulating cells.[19] After three freeze and thaw procedures, the more differentiated hematopoietic progenitors decreased, though significantly only for BFU-E. LTC-IC were unchanged.

The absolute count of $CD34^+$ cells did not change significantly, while $CD34^+/38^-$ counts unexpectedly showed a significant increase after the third thawing. To investigate this phenomenon, we performed further experiments. These demonstrated that after three freeze-thaw cycles $CD34^+/CD38^-/CD13^-$ cell counts were unchanged. We thus suppose that successive freeze-thaw cycles can reduce the surface expression of CD38 antigen. A reduction of CD38 expression of $CD34^+$ cells without a

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Table 2.

	34⁺/38⁻ 44⁺ %	34⁺/38⁺ 44⁺ %	34⁺/38⁻ 11a⁺ %	34⁺/38⁺ 11a⁺ %	34⁺/38⁻ 62L⁺ %	34⁺/38⁺ 62L⁺ %	34⁺/38⁻ 49e⁺%	34⁺/38⁺ 49e⁺ %	34⁺/38⁻ 49d⁺ %	34⁺/38⁺ 49d⁺ %	34⁺/38⁻ CXCR⁺ %	34⁺/38⁺ CXCR4⁺ %
Basal	2.7±2.4	87.2±5.4	7.0±3.1	85.0±6.4	7.6±3.2	82.4±5.8	8.2±2.9	87.2±6.2	6.7±2.0	86.9±5.8	7.3±2.7	82.8±3.9
Thaw 1	6.1±3.4	85.7±3.7	8.3±2.6	83.0±7.0	8.6±4.2	76.5±8.5	8.9±3.0	85.7±5.5	8.3±2.2	86.2±6.0	8.1±1.3	79.0±7.0
Thaw 2	3.8±1.9	85.8±3.2	10.5±1.1	81.0±5.1	9.6±2.1	75.8±7.5	8.8±2.5	86.0±3.8	8.4±3.0	82.8±5.2	9.8±2.5	78.2±6.6
Thaw 3	2.9±2.2	80.7±4.5	10.0±4.3	71.1±7.2	6.7±1.9	70.8±5.7	11.5±3.7	78.9±4.2	7.3±3.1	77.8±2.8	8.9±2.8	70.4±6.8

Percentage of CAM and CXCR4-positive cells in $CD34^+/38^-$ and $CD34^+/38^+$ populations before and after three successive cryopreservation procedures (Thaw1, Thaw2, Thaw3) in 9 CB units.

Table 3.

	$34^+/38^-$ 44^+	$34^+/38^+$ 44^+	$34^+/38^-$ $11a^+$	$34^+/38^+$ $11a^+$	$34^+/38^-$ $62L^+$	$34^+/38^+$ $62L^+$	$34^+/38^-$ $49e^+$	$34^+/38^+$ $49e^+$	$34^+/38^-$ $49d^+$	$34^+/38^+$ $49d^+$	$34^+/38^-$ $CXCR4^+$	$34^+/38^+$ $CXCR4^+$
Basal	2.0±1.6	7.8±4.2	4.6±2.0	6.5±7.4	3.8±1.5	5.1±2.5	4.4±1.9	3.6±1.8	2.7±0.9	8.3±4.9	3.6±1.2	8.7±5.2
Thaw1	3.0±1.3	6.8±2.8	3.7±1.1	6.0±8.5	2.7±0.8	2.9±1.1	3.7±1.1	3.4±1.5	3.2±1.0	3.1±1.1	2.5±0.6	6.3±3.9
Thaw2	2.1±0.4	9.7±6.3	3.1±0.8	3.7±2.6	2.3±0.7	2.3±0.5	3.5±1.6	3.1±1.5	2.8±0.9	3.3±0.8	2.3±0.6	7.6±7.9
Thaw3	1.5±1.0	6.3±3.1	1.9±0.8	2.8±1.3	1.7±0.5	2.1±0.9	3.4±1.8	2.7±0.9	1.6±0.3	3.3±2.4	1.8±0.4	5.7±7.8

Mean fluorescence intensity of CAM and CXCR4 expression in CD34+/38− and CD34+/38+ populations before and after three successive cyopreservation procedures (Thaw1, Thaw2, Thaw3) in 9 CB units.

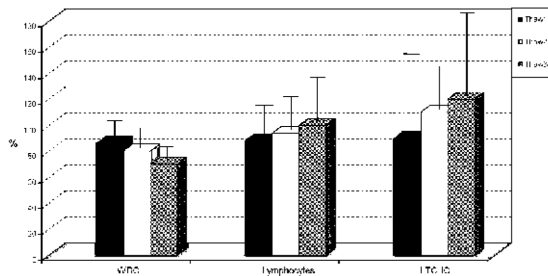

Figure 1. Recovery of CB WBC, lymphocytes, LTC-IC after 3 cryopreservation procedures (expressed as percentage of basal values)

concomitant increase of repopulating capacity has been observed in expansion experiments.[20] Our study on CD34+/ CD38−/ CD13− cells matches the LTC-IC results and confirms that more immature progenitors are unchanged after successive cryopreservation procedures.

The percentage of viable cells after the first thawing was 89.1±3.6, according to previous reports; it did not change significantly from the first to the third thawing.

The recovery of hematopoietic progenitors was better than expected from previous *in vitro* studies. This could be because of the short thawing time at low temperature before refreezing, and the washing method employed to minimize osmotic damage to the hematopoietic progenitors. A previous study[21] demonstrated the resistance of CD34+/38− cells to a single freeze-thaw procedure, while an example of resistance of relatively undifferentiated cells to refreezing was observed in human blastocysts that led to a successful pregnancy after two freeze-thaw procedures.[22] Homing of stem cells to the bone marrow microenvironment is important for the outcome of a transplant. CAMs and chemokines are involved in this process.[23] Among CAMs, VLA-4, VLA-5 and LFA-1a integrins, CD 44 and L-selectin play a critical role,[24–30] while, among chemokines, stromal-derived factor-1 (SDF-1) and its receptor CXCR4 are necessary for the engraftment of human CD34+ cells in the NOD/SCID model.[31] Previous studies have demonstrated a high expression of CAMs in CB CD34+ cells, especially in the more immature CD34+/CD38− population.[32,33] In the present study L-selectin, VLA-4, VLA-5, H-CAM and CXCR4 expression on CD34+/CD38− cells did not change after two freeze-thaw cycles, while LFA-1 expression actually increased between the first and second thawing. The mean fluorescence intensity (an indirect measure of the number of cell surface antigens) for CD11a, CD62L, CD49d and CXCR4 in CD34+/CD38− cells was unchanged between the first and the second thawing, (except for CD11a). Evaluation of CAM expression on CD34+/CD38− cells after the third thawing was partially impaired by the above described loss of CD38 expression. With regard to the more mature progenitors, CD34+/CD38+, we observed a decrease of CAM expression only after the third thawing. Previous studies demonstrated a significant reduction of L-selectin expression in CB CD34+ cells after a single freeze-thaw procedure, restored after overnight incubation with serum.[34,35] Once again, we think our refreezing and washing protocol may explain the better results. Good resistance of immature progenitors to two freeze-thaw procedures indicates that single bag-cryopreserved CB units can be thawed to obtain a sample for quality controls and divided into two parts; the smaller part can be expanded, the other refrozen, and finally expanded

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and unexpanded products can be infused at the same time. The refrozen product would provide a suitable number of early progenitors, while early and more differentiated progenitors would be supplied by the expanded product.

In conclusion, the present study shows that two freeze-thaw procedures do not significantly affect the clonogenic potential and CAM expression of cord blood progenitors. If corroborated by animal studies, these results could be exploited to devise new options in *ex vivo* expansion procedures and quality controls prior to transplantation.

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Pre-publication Report & Outcomes of Peer Review

Contributions

EI conceived the study. EI, PS and FT designed the study and interpreted the data. NC, AD, LF, FT and RA analyzed and interpreted the data. FT and NC drafted the article. EI, PS, LCdM, and LP interpreted the data and revised the article. All the authors approved the final version of the paper. Primary responsibility for the paper is FT. Tables and Figure are responsibility by NC and AD. AD is also a fellow of Comitato Gigi Ghirotti. We thank Karin Judkins for the English revision, Professor Giuseppe Basso for discussion of the flow cytometry results and Donatella Mele for editorial assistance.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Paolo Rebulla, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Dr. Rebulla and the Editors. Manuscript received March 28, 2002; accepted September 24, 2002. In the following paragraphs, Dr. Rebulla summarizes the peer-review process and its outcomes.

What is already known on this topic

Hematopoietic stem cells from cryopreserved cord blood are able to reconstitute hematopoiesis in myeloablated recipients. Nonetheless, the number of cells is frequently insufficient for large body mass patients. *Ex vivo* expansion of cord blood stem cells from a portion of the cord blood unit could overcome this limitation. As cord blood units have been frequently cryopreserved in a single bag, *ex vivo* expansion might require multiple freeze/thaw procedures. It is therefore necessary to investigate whether cord blood hematopoietic progenitors can tolerate multiple freeze/thaw procedures.

What this study adds

The study shows that cord blood tolerates 2 freeze/thaw procedures without significant loss of its lymphocyte and long-term culture initiating cell content.

Caveats

In vivo studies are needed to document full preservation of cell function.

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