

Application of a Novel High Yield Volume-Reduction Method for the Cryopreservation of 950 UC Blood Units

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Abstract

Umbilical cord blood represents an increasingly useful source of hematopoietic stem cells for Bone Marrow Transplantation (BMT), although currently, low cell numbers generally limit its use to pediatric patients. The aim of this study was to evaluate the routine use of a recently published volume reduction protocol for long term cord blood banking as was applied in a large number of cord blood samples. 950 cord blood samples with mean volume collected 78.81 ± 23.56 ml, Nucleated Cell (NC) $0.91 \pm 0.44 \times 10^9$ NC, Monocytes (MN) $0.49 \pm 0.23 \times 10^9$ MN and CD34⁺ HPCs (Haematopoietic Progenitors Stem Cells) $2.4 \pm 2.08 \times 10^6$ CD34⁺ HPCs counts, have been collected respectively. We found a significant correlation between leukocyte counts and CD34⁺ HPCs with the volume collected ($r=0.71$, $P<0.0001$). We found a highly statistically significant correlation between leukocyte cell count and CD34⁺ HPCs cell number, ($r=0.66$, $P<0.0001$). After volume reduction the respective UCB units contained $1.06 \pm 0.54 \times 10^9$ NC and $3.4 \pm 3.07 \times 10^6$ CD34⁺ HPC, $17.88 \pm 9.38 \times 10^9$ Red Blood Cells (RBC), while the viability and the volume were 92 ± 5.9 % and 6.43 ± 0.99 ml respectively.

Keywords: Stem cell; Cord blood; CD34⁺; Transplantation

Introduction

In haematopoietic reconstitution, Umbilical Cord Blood (UCB) is used as an alternative source of stem cells, especially in cases where matching of the major Human Leukocyte Antigen histocompatibility complex (HLA) for related or unrelated bone marrow or peripheral blood stem cell donor is not possible [1,2]. UCB transplantation has been associated with a reduced risk of developing severe Graft-Versus Host Disease (GVHD), even when cells from partially major histocompatibility complex mismatched donors are used [3]. Furthermore cord blood offers several advantages over bone marrow and peripheral blood as a source of stem cells. These advantages include the zero risk for the mother and infant during collection, the low frequency of transmitting infections like cytomegalovirus and the availability of immediate use. Moreover cord blood's Hematopoietic Stem Cells (HSC) have greater proliferative and colony forming capacity, and are more responsive to some growth factors. In addition since umbilical cord blood derived haematopoietic stem cells are more 'naive' than proliferative cells from bone marrow, they seem to produce fewer complications associated with some aspects of HSC transplantation [4].

As a consequence of the success of UCB transplants many cord blood banks have been established worldwide. Cord blood banks have been listed at registries of bone marrow donors to facilitate availability (Bone Marrow Donors Worldwide <http://www.bmdw.org>). Netcord Foundation (<https://office.de.netcord.org/index.html>) is another organisation that provides search mechanisms and guidance on standards for affiliated cord blood banks. There are currently more than 85,000 cryopreserved cord blood units available through Netcord for clinical use.

In the present report we describe the evaluation of the first 950 samples collected and stored during 2006 in a Cord Blood Bank in Greece using a modification of the protocol described by Yang et al. [5] that uses a triple blood collection bag. The addition of a final centrifugation step resulted in a small final volumes comparable to volumes achieved only by the Ficoll-Hypaque procedure [6]. This method allows the maintenance of sterility, reduces significantly

the volume of blood to be cryopreserved and yields a satisfactory sequestration of the red blood cells.

Materials and Methods

Collection of umbilical cord blood

950 UCB units were collected in a commercial 450 ml triple blood donation bags containing 63 ml of Citrate-Phosphate-Dextrose-Adenine anticoagulant (CPD-A) in bag 1 and 100 ml of Saline-Adenine-Glucose-Mannitol (SAG-M) in bag 2 (T2313, Compoflex, Fresenius HemoCare, Germany). In all cases, informed consent of the mother was obtained.

The donors were selected by the following criteria: negative testing for viral infections, absence of maternal fever during labor or delivery, gestation longer than 35 weeks, and delivery occurring less than 24 h after rupture of membranes. For collection, umbilical cord was clamped immediately after baby delivery and cleaned with betadine swab and 70 percent alcohol. UCB was collected from the umbilical vein by gravity in the blood collection bag that contained 63 ml of CPD-A. The units were stored at 7°C and processed within 48 hours of blood collection.

Temperature maintenance of CB during transportation from the collection hospital to the processing laboratory

Each CB was packed individually for transportation to the laboratory. The CB was placed between pre-freezing bags in order to reach the temperature of 7°C in a way to obtain the maximum number of cells for transplantation and to avoid an excessive loss of them [7].

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The processing of the samples was taken place with a maximum delay of 12 hours.

Volume reduction and cryopreservation

Volume reduction and cryopreservaton were performed as described before [6]. After removal of aliquots for routine tests, HES 200/0.5 (HEAS-steril 10% Fresenius Kabi, Deutschland GmbH Germany) was added to the cord blood in the collection bag (bag 1) to obtain a final concentration of 2%. In addition 26 ml of HES 200/0.5 was added to the bag that contained 100 ml of SAG-M (bag 2). The bag 1 was hanged on a stand for 45 minutes to allow RBC sedimentation. Supernatant was slowly expressed using a plasma extractor into bag 3 (empty bag) and as soon as red cells started to enter connecting tube, connecting tube was clamped temporarily.

In order to recover any remaining NC trapped between the sedimented RBC, the content of bag 2 (100 ml of SAG-M plus 26 ml of HES 200/0.5) was transferred to bag 1 that contained the RBC. Connecting tube was temporarily clamped and bag 1 was shaken gently, hanged for 45 minutes and the supernatant was then transferred to bag 3.

Bag 3 was then centrifuged at 400 g for 12 min. After completion of the centrifugation the supernatant plasma was transferred back to bag 2 using a plasma extractor and the cells were re-suspended in the about 15 ml of remaining plasma. The bag was then centrifuged again as described above. After centrifugation the bottom of the bag containing the white blood cells was clamped using a Kocher clamp and the supernatant plasma was transferred to bag 3. The residual cells (about 3 ml) were gently resuspended in 4 ml pre-cooled (7°C) cryoprotectant solution [6]. The suspension was transferred into 1.8 ml cryovials (Nalge Nunc International Rochester NY, USA). The samples were then incubated at 7°C for 10 minutes, frozen using a rate controlled freezer (Minicool Air liquide) at a rate of 1°C/min up to -85°C and then immediately placed in the liquid phase of liquid nitrogen.

Hematological cell counts and platelet parameters

The number of total NC, MNC, RBC, platelets, Mean Platelet Volume (MPV), Plateletcrit (PCT) and Platelet Distribution Width (PDW) were determined using a Coulter Ac T diff analyzer (Beckman-coulter, Maimi, FL, USA).

Flow cytometry

A commercial kit (Stem-Kit™ Immunotech Beckman Coulter, Marseille France) based on the ISHAGE guidelines as described in detail by Keeney et al. [8], was used for assessing the viability and for the enumeration of viable CD45 + CD34 + and Haematopoetic Progenitors Stem Cells (HPCs) (CD 45dim CD 34+). The samples were analyzed using in a Beckman Coulter EPICS XL/MCL flow cytometer according to the above mentioned protocol and the instructions of the kit manufacturer.

Colony-Forming Units assay (CFU)

CFU in pre-processed (whole cord blood), post processed and post thaw cell suspensions were assessed using a commercially prepared complete methylcellulose medium, Methocult GF H44/34 (Stem Cell Technologies, Vancouver, BC, Canada). Cells were plated without further separation in triplicate at a concentration of 1.25×10^4 cells per well. After incubation at 37°C for 14 days in humidified air containing 5% CO₂, Granulocyte-Macrophage (CFU-GM), Erythroid (BFU-E) and Granulocyte-Erythroid-Macrophage-Megakaryocyte (CFU-GEMM) colonies were scored by inverted microscopic examination.

Bacteriology

Samples were removed from the UCB pre and post-volume reduction for sterility control. Aerobic and anaerobic contamination were tested using the Bactec method (Becton Dickinson, Mountain View, CA, USA).

Thawing

Thawing solution contained 50 ml of 25% Human albumin UPS (Baxter, 12.5 gm/50 mL) and 36 ml anticoagulant citrate dextrose solution (formula A) that were added to 250 mL of 10% HEAS-steril.

Cryopreserved samples were rapidly warmed in a 37°C water bath (usually 1 min). Then the samples were diluted stepwise under gentle shaking at room temperature by stepwise addition of thawing solution, with an equilibrium time of 1 min between steps as described by Yang et al. [5].

Statistics

Results are expressed as mean ± Standard Deviation (SD) and range (minimum-maximum). Correlations were analyzed with the Pearson Correlation. A two sided p<0.5 was considered to be significant.

Results

Ninety-five percent of the pregnant woman informed about donation agreed to the collection, while 5.3 percent were excluded due to microbial contamination (n=21) and positive serologic testing (n=4) or low volume (lower than 40 ml) and hematopoietic stem cell number (26).

70% of all UCB samples had a volume range between 50-100 ml. Figure 1 shows the validated cord blood collection volume. The mean volume was 78.81 ± 23.56 ml corresponding to a NC $0.91 \pm 0.44 \times 10^9$ and to $2.4 \times 10^6 \pm 2.08 \times 10^6$ CD34+ HPC containing sufficient cells numbers for a pediatric transplantation.

The number and viability of nucleated cells and the number of mononuclear cells, CD34+ HPCs cells, immediately after collection are indicated in Table 1.

Figure 2 represents our statistical results. There is extremely significant correlation between leucocytes count and sample volume ($r=0.71$, $P<0.001$) and between cord blood volume and CD34+ ($r=0.45$, $P<0.001$). In addition a statistically significant correlation between

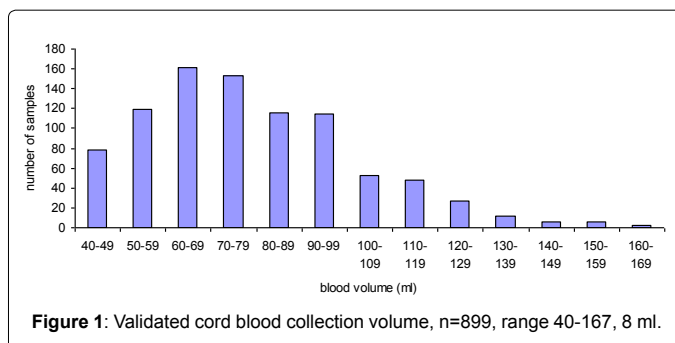


Figure 1: Validated cord blood collection volume, n=899, range 40-167, 8 ml.

Characteristic	Mean ± SD	Range
Sample size (ml)	78.81 ± 23.56	40-167.8
Number of nucleated cells (10 ⁹)	0.91 ± 0.44	0.18-3.52
Viability (%)	96.1 ± 4.14	72-99.7

Table 1: Characteristics of cord blood samples (N=899) at collection.

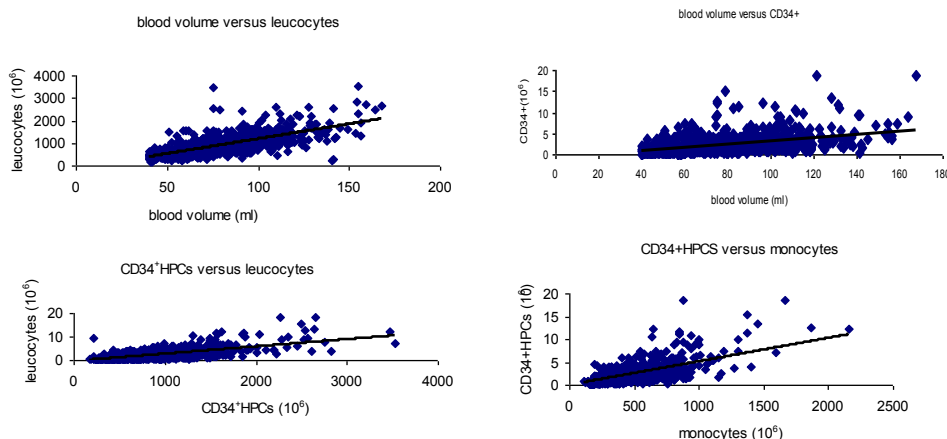


Figure 2: Positive relationship between UC blood volume and viable leucocytes. Positive relationship between UC blood volume and viable CD34⁺HPCs ($r = 0.45$, $P < 0.0001$). Positive relationship between viable leucocytes and viable CD34⁺HPCs ($r = 0.66$, $P < 0.0001$). Positive relationship between viable monocytes and viable CD34⁺HPCs ($r = 0.68$, $P < 0.0001$).

	Volume (ml)	Viability (%)	Nucleated cells (10 ⁹)	CD34 ⁺ HPCs cells (10 ⁶)	Red Blood Cells (10 ⁹)
Mean	6.41	92	1.06	3.4	17.88
SD	0.99	5.9	0.54	3.07	9.35
Range	3.6-11.2	58.5-99.5	0.17-4.9	0.18-28.56	5.5-81.1
Median	6.2	93.8	0.95	2.52	16

Table 2: Characteristics of cord blood samples (N=899) after processing.

leucocytes cell count and CD34⁺ HPCs cell number was also found, ($r = 0.66$, $P < 0.001$) and between monocytes cell count and HPCs cell number, ($r = 0.68$, $P < 0.0001$).

Taken together, these data suggest that measured volume appears to be a good predictor of both total leucocyte and CD34⁺ HPC cell counts. Furthermore there is a statistically significant correlation between leucocyte cell count and CD34⁺ HPCs cell number, and between monocyte cell count and HPCs cell number.

As shown in Table 2 after volume reduction the respective UCB units contained $1.06 \pm 0.54 \times 10^9$ NC and $3.4 \pm 3.07 \times 10^6$ CD34⁺ HPCs, $17.88 \pm 9.35 \times 10^9$ RBC while the viability and the volume were $92 \pm 5.9\%$ and 6.41 ± 0.99 ml respectively.

Discussion

UCB is increasingly used as an alternative source of hematopoietic stem cells in related and unrelated allogeneic transplantation in children and adolescents. Many cord blood banks have been established worldwide to quickly provide high quality UCB units. Transplant success is mainly related to the quality of the UCB unit with regard to the cellular dose relative to the recipient's size: total nucleated cell and Progenitor Hematopoietic Stem Cell number (HPCs), and donor recipient HLA match. However most cord blood units do not contain enough stem cells for transplantation in adults [9].

As a result of this, cord blood banking procedures should be optimized to improve the quality and quantity of cord blood units from the time to delivery as well to processing and cryopreservation time, especially in terms the amount of progenitor cells.

Our aim was to assess the collection of UCB units as well as volume reduction while maintaining sufficient progenitor cells to enable engraftment and reduce the risk of microbial contamination during processing. Moreover we studied the correlation between cell number

and volume or the correlation between leucocyte cell count and CD34⁺ HPCs cell number and between monocyte cell count and HPCs cell number.

Cell dose is the most critical factor of outcome for UCB transplantation, and currently, 0 to 2 antigen HLA-mismatched grafts are considered sufficient. A minimum of 3.7×10^7 nucleated cells/kg or 1.7×10^5 CD34⁺ cells/kg has been suggested as the minimum cells dose in UCB transplantation [4,10]. It has been referred that, survival is superior with better-matched grafts for the same cell dose and that a higher cell dose can overcome the HLA mismatch [10,11].

We have collected, processed and cryopreserved 950 samples. Cell viability, leucocyte number, blood volume and CD34⁺ HPCs numbers were analyzed for 899 samples. Our analyses demonstrated a wide variation between individual samples, although significant trends could be identified. Key question in UCB banking and haematopoietic stem cell transplantation include how many cells are required for a successful transplant and which cell type is the best measure.

Our center received UC blood samples from local and outlying areas. As a result samples were collected by many different doctors with some variability in collection technique and volume. It is important to identify parameters that may affect the final yield of nucleated cells and CD34⁺ HPCs and to set guidelines that will result in a greater yield of cells avoiding the banking of samples with insufficient cells.

According to other cord blood banks the mean volume collected, nucleated cell count, monocyte and CD34⁺ HPCs counts were 78.81 ± 23.56 ml, $0.91 \pm 0.44 \times 10^9$, $0.49 \pm 0.23 \times 10^9$, and $2.4 \pm 2.08 \times 10^6$. Moreover the leucocyte counts and CD34⁺ HPCs significantly correlated with the volume collected. We found a highly statistically significant correlation between leucocyte cell count and CD34⁺ HPCs cell number, ($r = 0.66$, $P < 0.0001$). The same correlation was found between monocyte cell count and HPCs cell number ($r = 0.68$,

$P < 0.0001$). This result indicates that leukocyte count either monocyte count can be used like predictor for total cell CD34⁺ HPCs counts.

Several centers have used separation methods for UCB prior to cryopreservation, and good recoveries have been obtained following HES sedimentation [9], 3% gelatin sedimentation [12], or Ficoll density gradients [13]. In comparison with others methods HES sedimentation does not require any laboratory preparation since it is commercially available and it can be used in a closed system, thus impeding possible microbial contamination during handling [14].

Here we describe a simple effective system for UCB processing in a triple bag which removes $93.8 \pm 8.4\%$ of RBC and allows the storage of UCB units in small volumes thus reducing the cost of large-scale UCB banking. After volume reduction the respective UCB units contained $1.06 \pm 0.54 \times 10^9$ NC and $3.4 \pm 3.07 \times 10^6$ CD34⁺ HPC, $17.88 \pm 9.35 \times 10^9$ RBC while the viability and the volume were $92 \pm 5.9\%$ and 6.41 ± 0.99 ml respectively.

Double extraction techniques using HES in a closed bag system can result in high recovery rates and significant depletion of RBC, allowing storage volumes comparable to techniques that use density gradient centrifugation while maintaining the quantity and quality of the progenitors cells. Moreover we found that the blood volume is a good predictor for the NC and CD34⁺ HPC count and consists an important factor for successful transplantation.

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