

Cell Recovery Sufficient for Adult Transplantation by Additional Cord Blood Collection From Placenta

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ABSTRACT

The amount of newborn blood that can be collected from a single cord donor is limited, but a significant amount remains in the placenta. We used a simplified perfusion method to collect this additional blood. Umbilical cord blood from 15 newborns was collected before placental delivery by umbilical vein puncture. After delivery, the placenta was placed on sterile gauze and 63 mL of citrate-phosphate-dextrose-adenine anticoagulant were injected into the umbilical vein that was then clamped near the placenta. The placenta was gently massaged, hung over a sterile vessel, and the umbilical cord cut sterilely near the embryonic surface. Additional blood was collected into the sterile vessel by pressuring a gauze bag around the placenta. We assessed the contribution of this second fraction to the total volume, total nucleated cell (NC), CD34⁺, hematopoietic progenitor cell, and colony forming unit count and bacterial contamination risk.

The total collected volume was 127.3 mL (range 92–170) and the NC content was $1.6 \pm 0.73 \times 10^9$. The mean second fraction contribution from 15 units to the total nucleated and mononuclear cell content was $54 \pm 9.87\%$ and $54 \pm 9.52\%$, respectively. The added percentage of CD34⁺ and hematopoietic progenitor cells was $54.3 \pm 10.35\%$ and $46.7 \pm 11.5\%$, respectively, while the additional percentages of colony forming-granulocyte macrophage and colony forming-erythroid in the second fraction were $43.2 \pm 5.5\%$ and $39.8 \pm 4.3\%$, respectively, indicating that the cells collected after placental perfusion (second fraction) had similar HPC content and in vitro hematopoietic potential. The method did not increase the risk of bacterial contamination.

THE NEWBORN BLOOD that remains in the umbilical cord after birth the placental unit contains a high concentration of stem cells. Accordingly, the remaining newborn blood can be collected to be used as a hematopoietic transplant unit or as a human stem cell source for stem cell and regenerative medicine studies.^{1–4}

Newborn blood donation from the umbilical cord remnant is a one-time-only opportunity. However, due to the spongy structure of the placental vasculature, only a part of the remaining newborn blood in the umbilical cord-placenta unit can be collected; the remaining part is discarded with placenta. The amount of collected blood may be critical in hematopoietic transplantation because the total nucleated cell (NC) dose infused is the most critical factor determining the speed of engraftment and survival.^{2–4}

It has been reported that using standard collection techniques only 25% of umbilical cord blood (UCB) units contain enough cells to provide a sufficient NC dose for

transplantation for patients weighing 50 to 70 kg.⁵ This percentage may be smaller after the inevitable loss during cryopreservation-defreezing processes.

Consequently, other researchers reported that placenta perfusion can collect the cells remaining in the placental vasculature, achieving hematopoietic grafts with NC counts sufficient for adult transplantation.^{6,7} However, the methods proposed have proved difficult to adopt in delivery rooms worldwide, mainly because of the skill needed to achieve a closed perfusion system.

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Here we have described a simplified placental perfusion method that can be easily and rapidly performed by a trained nurse just after delivery of the placenta.

MATERIALS AND METHODS

Umbilical cord blood was obtained from 15 healthy newborns after vaginal delivery. A consent form was signed by all mothers whose medical and family history obtained prior to collection did not reveal any exclusion criteria for UCB donation. The blood was taken using a two-fraction collection protocol.

First Fraction of UCB Collection

The first blood fraction was obtained with the placenta in utero, according to the procedure used in most UCB banks. The umbilical cord was clamped immediately after delivery and cleaned with iodine and a 70% alcohol swab. Umbilical cord blood was collected from the umbilical vein by gravity in a triple blood bag containing 63 mL of citrate-phosphate-dextrose-adenine anticoagulant (Fresenius).

Second Fraction of UCB Collection

Immediately after its delivery, the placenta was transferred onto a sterile gauze cloth on a sterile surface and inspected for integrity. Sixty-three milliliters of citrate-phosphate-dextrose-adenine anticoagulant was then injected through the umbilical cord vein into the placenta vasculature, and umbilical cord clamped above the puncture site. The placenta was covered with a gauze cloth, massaged, and then hung on a stand. The umbilical cord was sterilized and cut near the placenta. Blood was collected in a sterile tube by pressing the placenta within the gauze cloth. To facilitate blood flow into the sterile tube, additional incisions on the fetal placental surface were also made. The sterile tube was then closed and transferred to the laboratory for further processing. In the laboratory, the placenta-derived blood was collected with a 60-mL syringe under laminar flow and transferred into a blood donation triple bag.

Volume Reduction

The UCB volume from the two fractions was estimated by subtracting the tare weight of the bag and the volume of anticoagulant from the total weight of the blood-containing bag. After removal of aliquots for routine testing, Hydroxyethyl Starch was added to the cord blood in the collection bag (bag 1) to obtain a final concentration of 2% and 26 mL of hydroxyethyl starch was added to the bag (bag 2) containing 100 mL of saline adenine glucose manitol. Bag 1 was hung on a stand for 45 minutes to allow red blood cell (RBC) sedimentation. The supernate was slowly expressed into bag 3, an empty bag, using a roller clamp to control flow to avoid turbulence. As red cells started to enter the connecting tube, it was clamped temporarily.

For the second recovery of remaining NC in sedimented RBC, 100 mL of bag 2 were transferred to bag 1 that contained RBC, and the above steps were repeated.

Bag 3 containing the RBC-depleted supernate was held upright in a centrifuge bucket using a styrofoam insert and centrifuged at 400g for 12 minutes at 10°C. After completion of the centrifugation, plasma was extracted to bag 2, and the sediment containing the nucleated cell fraction was resuspended in 15 mL of the remaining plasma and collected in a syringe equipped with a long spinal puncture needle.

Hematologic Cell Counts

The numbers of total NC, mononuclear cells, and RBC were determined using a Coulter A.T differential analyzer (Beckman-Coulter, Miami, Fla, USA).

Viability and Enumeration of CD45⁺ and CD34⁺ Cells

A method based on the ISHAGE guidelines, described in detail by Kenney et al⁸ was used to assess the viability and enumerate the CD45⁺ and CD34⁺ cells. The samples were analyzed using a flow cytometer (Beckman Coulter EPICS XL/MCL) and stem kit. A stem-kit containing CD45-FITC/CD34-PE, isotype control PE, stemcount fluorospheres (microbeads with known concentration/ μ L), concentrated ammonium chloride lysing solution and the vital dye 7-aminoactinomycin D (7-AAD) for enumeration and viability. A CD34⁺/CD45^{dim} gate was established to enumerate of CD34 HPCS (hematopoietic progenitors cells).

Colony-Forming Assay

Colony-forming assay (CFU) in preprocessed and after-process fractions were assessed using a commercially prepared complete methylocellulose medium (Methocult GF H4434; StemCell 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 microscopic examination.

Bacteriology

Samples were removed from both fractions prevolume and postvolume reduction for sterility control. Aerobic and anaerobic contaminations (fungal and bacterial contamination) were tested using the Bactec method (Becton Dickinson, Mountain View, Calif, USA).

Statistical Analysis

All statistics used the Graph Pad InStat Program (San Diego, Calif, USA). For the comparison of the numbers of NC mononucleated cells, CD34⁺ cells and HPCs cells in the two different fractions we used paired Student's *t* test and for comparisons of mean values, the nonparametric Mann-Whitney U Test. Pearson's correlation coefficient estimated relations between blood volume and NCs.

RESULTS

Increased Blood Volume and NC Content Using a Modified Placental/Cord Blood Collection Method

The technique for UCB collection used in this study comprised two separate blood harvests. We suggested that

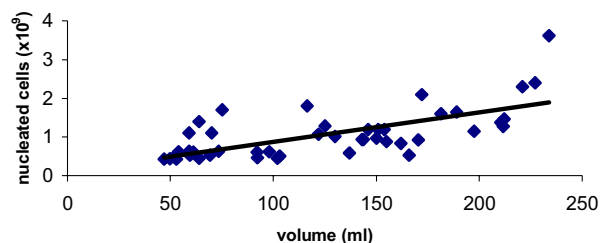


Fig 1. Correlation of umbilical cord blood volume and nucleated cells. Pearson's correlation coefficient, $r = 0.68$.

Table 1. Volume, Nucleated Cell Content, and Mononucleated Cell Content of Umbilical Cord Blood Units After the Two Fractions of Collection

	Volume* (mL)	Nucleated Cells ($\times 10^9$)	Mononuclear Cells ($\times 10^9$)
Mean	127.3	1.6	0.895
SD†	26.9	0.73	0.31
Range	92–170	0.88–1.3	0.45–1.5

*Umbilical cord blood volume (fraction 1 and fraction 2).

†Standard deviation.

a second blood fraction obtained after placental perfusion and incisions in addition to the standard umbilical venipuncture collection would result in a greater blood volume and yield of NC, CD34⁺, and HPCs. We observed a significant correlation between umbilical cord blood volume and total nucleated cell content (Fig 1).

Using this technique, we obtained an average volume of 127.3 mL (range 92–170) and a NC content of $1.6 \pm 0.73 \times 10^9$ (Table 1). Our results indicated that the additional blood collected after placental perfusion increased the total number of NCs.

Moreover to determine the benefits of the second fraction, we analyzed its contribution to the total volume and total nucleated and mononucleated cell content (Table 2).

Several reports have suggested that 2×10^7 /kg NC is the target dose for transplantation.^{9–11} With the standard venipuncture collection (fraction 1), a person of mean weight 37 kg would reach the target dose, while with the double collection the mean weight rose to 80 kg.¹² In contrast to other reports,^{6,7} the NC contribution by the second fraction was not variable ranging at 54% (Fig 2). Therefore, the two fraction collection method described here increased the number of UCB units clinically useful for adult patients.

CD34⁺, HPCs, and CFUs Analysis in First and Second UCB Fractions

To assess the hematopoietic potential of the first and second UCB fractions, we determined the number of CD34⁺, hematopoietic progenitors cells (HPCs) and CFUs in each fraction. The number of CD34⁺ and HPCs were analyzed by flow cytometry. The proportion of CD34⁺ and HPCs was $54.3 \pm 10.35\%$ and $46.7 \pm 11.5\%$, respectively. There were no significant differences in CD34⁺ and HPCs content between the two UCB fractions (Fig 3).

Moreover, we analyzed the clonogenic capacity of the two fractions. The proportion of CFU-GM and BFU-E for

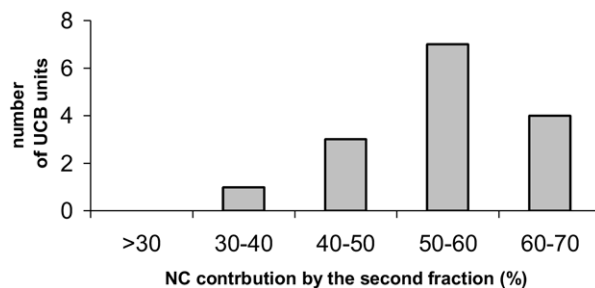


Fig 2. Contribution of the second fraction to the total NC number collected. The percentage of NCs contributed by the second fraction is presented in 10% intervals. In 11 of the 15 cases a more than 50% contribution was achieved.

the second fraction was $43.2 \pm 5.5\%$ and $39.8 \pm 4.3\%$, respectively (Fig 4).

There were no differences in colony size between the two fractions. All these results indicated that the proliferative capacity of the clonogenic progenitors was the same.

Viability of the Two Fractions

The vital dye 7-aminoactinomycin D (7-AAD) was used to determine viability. The viability in the first and second fractions (mean value \pm SD) was $96.36 \pm 1.823\%$ and $93.53 \pm 3.046\%$, respectively (Fig 5).

Bacterial Contamination of UCB Collected by the Two Fractions

Only one unit was positive for aerobic and anaerobic bacteria in both fractions, indicating that the second fraction collection did not increase the risk of bacterial contamination.

DISCUSSION

For UCB transplantation, cell dose is the most critical determinant of outcome. Currently, four to six HLA antigen matched grafts are considered acceptable. While the minimum acceptable UCB graft cell dose is yet to be unanimously agreed upon, a minimum threshold of 1.5×10^7 nucleated cells/kg or 1.7×10^5 CD34⁺ cells/kg has been suggested.^{12,13} Among 0 to 2 HLA-antigen mismatched grafts, current data suggest that for the same cell dose, survival is superior with better-matched grafts; although, the negative effect of HLA mismatch can be at least partially overcome by a higher cell dose.^{12,14} Hence, higher

Table 2. Volume and Number of Nucleated Cells and Mononuclear Cells in Umbilical Cord Blood by Umbilical Venipuncture (Fraction 1) and Placental Perfusion and Incisions After Delivery (Fraction 2)

	Fraction 1	Fraction 2	Whole UCB Units	Fraction 2/Whole UCB Units (%)
Volume (mL)	60.64 ± 8.8	64.7 ± 18.8	$127.3 \pm 26.9^*$	66 ± 4.5
Nucleated cells (10^9)	0.754 ± 0.41	0.86 ± 0.38	$1.6 \pm 0.7^*$	54 ± 9.87
Mononuclear cells (10^9)	0.41 ± 0.18	0.48 ± 0.184	$0.895 \pm 0.31^*$	54 ± 9.52

Results (mean \pm SD) show volume and NC and MNC content of the first and the second fraction as well in the whole UCB unit after adding the two fractions. The last column shows the percentage contribution of the second fraction provided for the whole unit concerning the volume the NCs and the MNCs content.

*Significantly different ($P < .001$, paired-samples *T* test).

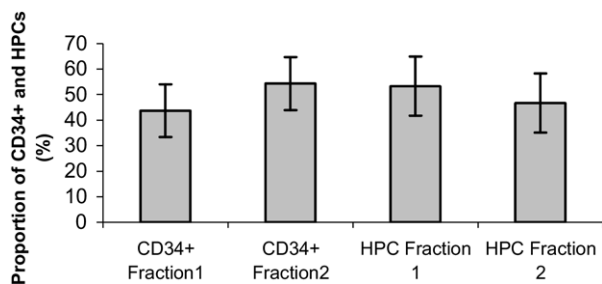


Fig 3. Comparative analysis of CD34⁺ and hematopoietic progenitor stem cells (mean ± SD) in first and second fraction. The proportion of CD34⁺ cells in the fraction obtained after placental perfusion and incisions (fraction 2) was 54.3 ± 10.35% and 46.7 ± 11.5%, respectively. Differences between both UCB fractions are not significant (paired samples *T* test).

cell doses are even more important with HLA-mismatched grafts.

In addition to placenta perfusion after delivery^{6,7} several other strategies have been proposed that could increase the nucleated/CD34⁺ cell dose, including ex vivo expansion of UCB hematopoietic stem cells¹⁵⁻¹⁷ and multiunit UCB transplantation,¹⁸ because the cell dose provides the most important parameter for using umbilical cord blood, even in adults. However, these methods are expensive and can increase the risk to the patient.

In the present study we evaluated a simplified placenta perfusion technique that can be easily performed by a nurse in the operating room. A blood fraction obtained by umbilical venipuncture, similar to the procedure used in most banks, was followed immediately upon placental delivery by perfusion with citrate-phosphate-dextrose-adenine anticoagulant. Our data indicated that the proposed method increased the blood volume and resulted in an increase in the total number of NCs and in hematopoietic potential with no significant risk of bacterial contamination.

Using the standard collection technique, only 25% of UCB units contain enough cells to fulfill the target dose for transplantation for patients weighing 50 to 70 kg.⁵ By using

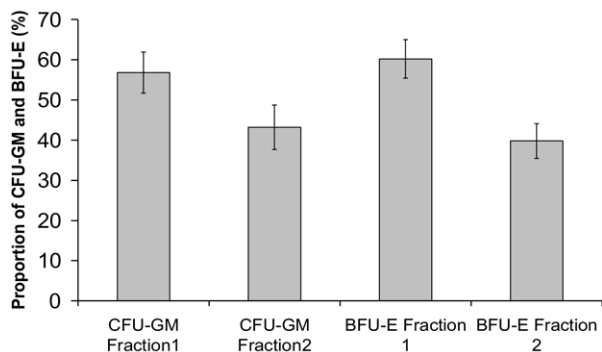


Fig 4. Comparative analysis of CFU-GM and BFU-E (mean ± SD) in first and second fraction. The proportion of CFU-GM and BFU-E for the second fraction was 43.2 ± 5.5 and 39.8 ± 4.3, respectively.

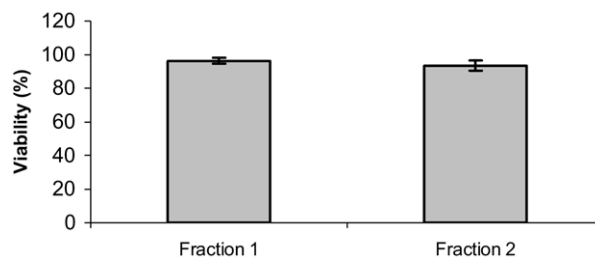


Fig 5. Viability of the nucleated cells in first and second fraction (mean ± SD). The viability in the first fraction and in the second fraction was 96.36 ± 1.823% and 93.53 ± 3.046%, respectively. Differences between both UCB fractions are not significant (paired samples *T* test).

a double fraction collection method, the mean patient weight rises to 80 kg. Similar results were presented by Bornstein et al,⁶ who perfused placenta by flushing the placental vessels with 0.9% buffer saline and without pressuring placenta tissue. The reported contribution from the placental perfusion concerning the volume and NCs content was 32% and 15%, respectively. In our study, the contribution was higher, 66% and 44%, respectively. Our results can probably be attributed to the increased amount of anticoagulant that was used and to the mild pressure that was applied to the placental surface.

Other researchers used a syringe-assisted saline “flush and drain” technique followed by catheterization and placental perfusion to achieve results similar to ours but with a higher risk of bacteria contamination (19%).⁶ With our method, only one unit was positive on aerobic and anaerobic bacterial culture in both fractions, indicating that the collection of the second fraction did not increase the risk of bacterial contamination. Moreover, the viability of NCs in the second fraction did not differ from the first fraction.

Although most clinical studies have focused on the total number of leukocytes infused, rather than the CD34⁺ cell content, the latter perhaps representing a better surrogate marker of the engraftment potential of the sample. In their study, Gluckman et al estimated the threshold dose of leukocytes to contain approximately 150,000 to 200,000 CD34⁺ cells.¹³ In another study,¹⁹ the progenitor cell another content of the umbilical cord, as measured by the colony-forming cell (CFC) content proved to be a better indicator of the speed of engraftment, than the total leukocyte cell number.

There were no differences in colony size between fractions, suggesting that UCB clonogenic progenitors present in the first and second fractions have the same proliferative capacity. Cells obtained by the standard umbilical venipuncture (first fraction) and those after placental perfusion (second fraction) had similar HPC contents and in vitro hematopoietic potential.

In conclusion, the data suggested that our method of umbilical cord blood collection resulted in a higher blood volume and NC, CD34⁺, and HPC yield. Adding the placental perfusion in the venipuncture collection provided

a cell dose sufficient for large pediatric patients as well as for most heavier adult patients requiring hematopoietic transplantation.

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