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A novel high-yield volume-reduction method for the cryopreservation of UC blood units

4 G Koliakos[1], DH Alamdari[1], N Tsagias[2], K Kouzi-Koliakos[3], E Michaloudi[4] and
5 V Karagiannis[2]

6 [1]Department of Biological Chemistry, Medical School, Aristotle University Thessaloniki, [2]Third University Obstetrics and Gynaecology Clinic,
7 Ippokration General Hospital, Medical School, Aristotle University Thessaloniki, [3]Department of Histology-Embryology, Medical School, Aristotle
8 University of Thessaloniki, and [4]Biohellenika SA, Thermi, Thessaloniki, Greece

9 **Background**
10 *For the application of umbilical cord blood (UCB) units as*
11 *hematopoietic grafts, a dose of 3.7×10^7 nucleated cells (NC)/kg*
12 *body weight is required. NC can be lost during volume-reduction*
13 *processing and during thawing. A novel modification of the double-*
14 *processing protocol with the aim of minimizing NC loss is described*
15 *and evaluated.*

16 **Methods**
17 *One-hundred and fifty UCB were collected. The volume was reduced*
18 *by a centrifugation step following double-processing in the presence of*
19 *2% HES 200/0.5. Pre- and post-processing cell counts and platelet*
20 *parameters were measured with an automatic counter. The number of*
21 *viable $CD34^+$ hemopoietic stem cells was measured by flow cytometry.*
22 *In 25 of the samples, colony-forming units (CFU) were also*
23 *determined. The same samples were thawed 6 months after*
24 *cryopreservation and re-evaluated.*

25 **Results**
26 *The volume was reduced to 6 ± 1.5 mL. The recovery of NC, MNC,*
27 *$CD34^+$ hemopoietic stem cells, RBC depletion and CFU following*
double-processing was $93.6 \pm 3.2\%$, $95.8 \pm 2.2\%$, $98.4 \pm 1.5\%$, $96.8 \pm 1.1\%$ and $107.1 \pm 6.1\%$ (for 25 samples), respectively. The post-thaw recoveries of NC, MNC, $CD34^+$ hemopoietic stem cells and CFU (for 25 samples) were $78.6 \pm 5.4\%$, $90.8 \pm 4.4\%$, $96.4 \pm 2.5\%$, $89.1 \pm 4.1\%$, respectively. No post-thaw cell aggregation was observed. A significant ($P < 0.05$) post-thaw loss of platelets and signs of platelet activation was observed.

Discussion
The protocol uses non-expensive equipment and clinically approved materials and results in samples that can be used in patients with a mean weight of 32.7 kg.

Keywords
$CD34^+$ hematopoietic stem cells, cryopreservation, hydroxyethyl starch, leukocyte separation, platelets, umbilical cord blood.

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28 ## Introduction
29 Umbilical cord blood (UCB) serves as an alternative source
30 of HPC transplantation. For the establishment of large
31 long-term UCB storage banks, efficient and cost-effective
32 volume-reduction methods are required.
33 In 1994, Harris *et al.* [1] described a double Ficoll–
34 Hypaque procedure in which the final preparation was
35 virtually devoid of RBC and PMN and contained virtually
36 all colony-forming cells. The method achieved very low final storage volumes; however, it could not be widely applied because Ficoll and Hypaque are not approved for clinical use.

In 1995, Rubinstein *et al.* [2] first described the use of hydroxyethyl starch (HES 450/0.7 or Hespan), which enhances sedimentation of red cells by rouleaux formation [3], and a single centrifugation method for UCB volume reduction. This protocol has been widely accepted and is presently used by most cord blood banks world-wide

Correspondence to: **George G. Koliakos**, PO Box 17034 54210, Thessaloniki, Greece. E-mail: koliakos@med.auth.gr

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because hydroxyethyl starch provides a significantly higher recovery for nucleated cells (NC), mononuclear cells (MNC), $CD34^+$ cells and colony-forming units (CFU) compared with other materials with similar actions [4–6].

However, about 20% of the collected units [7] do not meet the recovery standard criteria post-processing and are therefore discarded [8]. An additional problem for the use of this protocol is that HES 450/0.7 is no longer approved for human use in several countries because of reported side-effects and therefore is no longer available. The lower molecular weight hydroxyethyl starch 200/0.5 (HES 200/0.5, HEAS-steril or Pentaspan) that replaced HES 450/0.7 for clinical use is, however, equally effective as HES 450/0.7 in inducing RBC sedimentation [9]. Yang *et al.* [8] reported a modification of the protocol proposed by Rubinstein *et al.* [2] using HES 200/0.5, and a double-processing method that significantly improved the average cell recovery and RBC depletion.

We present our experience using a novel modification of the double-extraction protocol described by Yang *et al.* [8], which utilizes a commercial triple blood collection bag and, by adding a final centrifugation step, can result in a small final volume comparable with the volumes achieved with the Ficoll–Hypaque procedure. Because small cryopreservation volumes may lead to cell aggregation [10], we also evaluated post-thaw recovery and cell aggregation.

Methods

Collection of UCB

One-hundred and fifty UCB units were collected in commercial 450-mL triple blood donation bags, containing 63 mL citrate-phosphate-dextrose-adenine anticoagulant (CPD-A) in bag 1 and 100 mL 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 study was approved by the local ethics committee.

The donors were selected with 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, the UC was clamped immediately after baby delivery and cleaned with a betadine swab and 70% 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 6–8°C and processed within 3–48 h of blood collection. Specimens (pre-processed) that showed bacterial or fungal contamination following a 7-day culture (about 1% of cases) were not included in the study because these were defined as 'not appropriate for use'; however, the post-processing yield of these units was not different from the rest of the units.

Volume reduction and cryopreservation

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 HES 200/0.5 was added to the bag that contained 100 mL SAG-M (bag 2). Bag 1 was hung on a stand for 45 min to allow RBC sedimentation. The supernatant was slowly expressed using a plasma extractor into bag 3 (an empty bag) and, as soon as red cells started to enter connecting tube, the connecting tube was clamped temporarily.

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

Bag 3 was centrifuged at 400 *g* for 12 min. After completion of centrifugation, the supernatant plasma was transferred back to bag 2 using a plasma extractor and the cells were resuspended 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 WBC was clamped using a Kocher clamp and the supernatant plasma transferred to bag 3. The residual cells (about 3 mL) were gently resuspended in 3 mL pre-cooled (7°C) cryoprotectant solution that contained 20% DMSO and 4% HEAS-steril in CPD-A, and was slowly added at rate of 1 mL/min with continuous gentle stirring. The suspension was then harvested using an Abbocath™ catheter and transferred into 1.8-mL cryovials (Nalge Nunc International, Rochester, NY, USA) covered with cryoflex™ (Nalge Nunc International). A small volume was taken for cell counting, flow cytometry and CFU cell culture assay. The samples were then incubated at 7°C for 10 min, frozen using a rate-controlled freezer (Minicool Air liquid) at a rate of 1°C/min up to −85°C, and then immediately placed in the liquid phase of liquid nitrogen.

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Hematologic cell counts and platelet parameters

The numbers 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, Miami, 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.* [11], was used for assessing viability using 7-AAD and for enumeration of viable $CD45^+$ $CD34^+$ and hematopoetic stem cells ($CD45^{dim}$ $CD34^+$). 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.

CFU assay

CFU in pre-processed (whole cord blood), post-processed and post-thaw cell suspensions was assessed using a commercially prepared complete methylcellulose medium, Methocult GF H44/34 (StemCell Technologies, Vancouver, Canada). Cells were plated without further separation in triplicate at a concentration of 1.25×10^4 cells/well. After incubation at 37°C for 14 days in humidified air containing 5% CO_2, 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

The thawing solution contained 50 mL 25% human albumin UPS (12.5 g/50 mL; Baxter) and 36 mL anticoagulant citrate dextrose solution (formula A), which were added to 250 mL 10% HEAS-steril.

Cryopreserved samples were rapidly warmed in a 37°C water bath (usually 1 min). Then the samples were diluted stepwise as described by Yang *et al.* [12], under gentle shaking at room temperature, by stepwise addition of 20% (1/5 dilution), 33% (1/3 dilution) and 25% (1/4 dilution) proportion by volume thawing solution with an equilibrium time of 1 min between steps.

Estimation of the post-thaw aggregation

For the quantification of post-thaw aggregation, the number of nucleated cells was counted before and after passage through a 70-μm nylon mesh (cell strainer BD Falcon™; BD Biosciences, Bedford, MA, USA) designed to obtain single-cell leukocyte suspensions [13–15].

Results

As presented in Table 1, using the described protocol for 150 samples, the volume was reduced to 6 ± 1.5 mL; most RBC were depleted, whereas nucleated cell viability and sterility remained intact. Furthermore, as presented in Table 2, a high post-process recovery was achieved.

Post-thaw recovery, as estimated in 25 of the 150 samples, was about 80% for NC, whereas the recovery of MNC, $CD34^+$ hemopoietic stem cells and CFU reached 90% (Table 3). The numbers of nucleated cells, stem cells and other hematologic parameters (such as platelet parameters and RBC parameters) were the same before and after filtration; accordingly, no aggregation was detected.

A significant increase in PCT was observed during pre-cryopreservation processing, indicating co-purification of platelets with the NC. However, platelet count and PCT decreased post-thaw ($P < 0.05$). This decrease was mirrored by a significant post-thaw increase ($P < 0.05$) of MPV and PDW, indicating platelet activation (Table 4).

Discussion

We have described and evaluated a novel modification of the double-processing method proposed by Yang *et al.* [8].

Table 1. Pre- and post-process characteristics of 150 UCB units

	Pre-process	Post-process
Volume (mL)*	75.4 ± 8.4	6.0 ± 1.5
RBC depletion (%)*	NA	96.8 ± 1.1 (93.4–98.9)
Viability (%) nucleated cells*	91.4 ± 2.8 (89.7–99.3)	90.7 ± 1.2 (87.1–98.7)
Sterility test	Negative	Negative

NA, not applicable.
**Mean ± SD (range).*

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Table 2. Mean ± SD of pre- and post-process cell count ($\times 10^6$), and mean ± SD (median/range) of the percentage yield for 150 UCB units

Cell type	Pre-process	Post-process	% Yield
NC	1294 ± 185	1211 ± 168	93.6 ± 2.3 (94.2/87.9 – 100.1)
MNC	568 ± 84	544 ± 71	95.8 ± 3.1 (96/87.1 – 103.6)
CD34$^+$ HPC	5.7 ± 1.4	5.6 ± 1.7	98.4 ± 1.5 (95.2/87.9 – 102.2)

126 The modification comprises gravity sedimentation instead
127 of centrifugation and the use of a commercial triple blood
128 bag. Centrifugations are used in the final steps in order to
129 reduce further the volume of NC preparation. The data
130 indicate that this method for UCB volume reduction offers
131 almost 100% RBC depletion as well as almost 100% NC
132 and CD34 HPC recovery. This obviously exceeds the
133 standard 60% NC recovery from most cord blood banks
134 [6] and can be compared favorably with recently reported
135 data of 80.3 ± 7.7% for NC and 86 ± 11.6% for automatic
136 AQ5 devices [16]. Considering the optimum NC dose for
137 hematopoietic cell transplantation that is 3.7×10^7/kg of
138 patient weight, cord blood samples with a mean volume
139 of 70 are sufficient for patients with a mean body weight of
140 about 20 kg. Using the proposed double-processing tech-
141 nique, patients with a mean weight of about 33 kg can be
142 treated (a 65% increase). The viability of NC and CD34$^+$
143 and the number of CFU did not differ significantly before
144 and after processing. The method is safe because only
145 clinically approved materials are used, and it is aseptic
146 because it is closed. In addition, the materials used are
147 widely commercially available, used in routine clinical
148 procedures and therefore can be purchased at affordable
149 prices. Accordingly, the method may become widely
150 applicable in public and commercial UCB banks world-wide. The low post-processing volume achieved permits low post-thaw amounts of DMSO and free hemoglobin [17] and the preservation of samples in small cryovials that can be stored at constant temperature within the liquid phase of nitrogen. It should be noted that storage in the liquid phase of nitrogen is the only cryopreservation method with documented cell viability for 15 years [18]. It has been also reported that hematopoetic stem cells can be satisfactorily stored at high cell concentrations [19].

The data of the present study indicate that an excess of CPD-A in the collection bag has no effect on the viability and recovery of nucleated cells. Moreover, it has been reported that an excess of CPD-A in the collection container decreases the risk of cell clumping during processing, cryopreservation and post-thaw recovery [10]. CPD-A also serves as a basis for the cryoprotecting solution because, during cryopreservation and thawing, a number of granulocytes may rupture, releasing molecules as calcium cations that cause aggregation, leading to loss of cell viability [20]. In addition, it has been reported that RBC lysis can cause platelet activation and aggregation [21].

It is known that a substantial number of megakaryocytes, which may release a high number of platelets during processing, circulate in UCB [22]. In an attempt to test the AQ7 above hypothesis we also measured platelet parameters such as platelet count, PCT, MPV and PDW before and after processing, as well as post-thaw. Our results indicate that, using the proposed protocol, platelets are co-purified with NC. Purification and addition of DMSO does not seem to activate platelets under the conditions used in the present study. On the other hand, our results, in accordance with the results of other researchers, indicate that thawing causes a decrease of total platelet count and PCT [23]. The observed concomitant increase of MPV may be attributed to post-thaw platelet activation, as this parameter has been

Table 3. Mean ± SD of cell and CFU count ($\times 10^6$), and mean ± SD (median/range) of the percentage post-thaw yield for 25 samples evaluated pre- and post-process, after adding DMSO and post-thaw

Cell type	Pre-process	Post-process	After DMSO	Post-thaw	% Yield
NC*	1014 ± 135	989 ± 121	957 ± 117	879 ± 139	78 ± 5.4 (79/1/64.5 – 94.2)
MNC*	486 ± 112	479 ± 98	469 ± 105	428 ± 89	90.8 ± 4.4 (90.2/77.2 – 102.3)
CD34$^+$ HPC*	4.7 ± 1.4	4.6 ± 1.5	4.6 ± 1.2	4.4 ± 1.3	96 ± 2.5 (96.8/89.8 – 104.2)
Total CFU*	2.7 ± 0.4	2.9 ± 0.7	2.7 ± 0.5	2.3 ± 0.4	89.1 ± 4.1 (88.5/75.1 – 101.4)

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Table 4. Platelet parameters for 25 samples evaluated pre- and post-process, after adding DMSO and post-thaw

	Pre-process	Post-process	After DMSO	Post-thaw
Platelet count ($\times 10^3$/μL)	144.6 ± 41	2324.2 ± 678.3	2014.7 ± 734.5	1309.3 ± 504.2
Total platelets($\times 10^9$)	10.9 ± 1.6	6.97 ± 2.1	12.1 ± 4.1	7.85 ± 2.7
MPV (fL)	6.7 ± 0.6	7.0 ± 0.5	7.5 ± 0.7	8.1 ± 0.4
PCT (%)	0.125 ± 0.06	1.68 ± 0.921	1.50 ± 0.782	1.02 ± 0.423
PDW (%)	16.8 ± 0.83	16.4 ± 0.75	16.8 ± 0.9	17.4 ± 0.51

used widely in the clinical setting as a platelet activation index [24–26].

The use of CPD-A anti-coagulant in the cryopreservation solution removes Ca^{2+} by chelating and inhibits aggregation of activated platelets [27]. Therefore the use of CPD-A in the cryopreservation solution may protect against post-thaw clumping. Mannitol and adenine, in the SAG-M bag, may also have a positive effect on the post-thaw step. It has been reported that mannitol restricts cell swelling to within previously defined tolerable limits [28] and reduces the toxicity of DMSO after infusion of stem cells [29]. In addition, adenine has an antioxidant activity that can minimize the adverse effect of oxidative stress on cells during post-thaw processing [30,31].

In conclusion, the method described offers a high post-process and post-thaw yield of hematopoetic stem cells, in combination with a small storage volume, does not require specific and expensive equipment, uses clinically approved materials and therefore can be easily applied world-wide for the processing and storage of UCB units. The main drawback of the proposed method is the prolongation of processing time. However, this delay is compensated for by the viability and yield achieved and the considerable reduction in storage volume. It should be also noted that up to six samples can be processed and centrifuged together, minimizing the processing time per sample.

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