A strategy of splitting individual high volume cord blood units into two half subunits prior to processing increases the recovery of cells and facilitates ex vivo expansion of the infused haematopoietic progenitor cells in adults

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INTRODUCTION

Since the first successful allogeneic cord blood (CB) transplantation (CBTx) performed in 1988 to treat a child with Fanconi anaemia (Gluckman et al., 1989), the development of cord blood banks (CBB) and the numbers of transplants performed have been increasing steadily. The vast majority of recipients were...
children with an average weight of 20 kg; however, more than 1000 CBTxs have already been performed in adults (Cohen & Nagler, 2004).

Although banked unrelated CB is a feasible alternative source of stem cells for transplantation in adults and expands the available unrelated donor pool, the results in adults have been hampered by the low total nucleated cells (TNCs) contained in the CB unit (CBUs; Lim et al., 1999; Laughlin et al., 2001; Wagner et al., 2002). The successful outcome depends on this parameter, with low cell numbers frequently resulting in delayed or failed engraftment. Thus, the use of CB in adults has permitted a better definition of cell dose limitations and thresholds (Gluckman et al., 1997; Lim et al., 1999; Laughlin et al., 2001, 2004; Long et al., 2003; Fruchtman et al., 2004; Rocha et al., 2004; Takahashi et al., 2004). Recently, it has been reported (Gluckman & Rocha, 2006) that a minimum cell dose of $3 \times 10^7$ TNC/kg at collection and of $2 \times 10^7$ TNC/kg at infusion must be targeted in malignant diseases where cell dose was the most important factor for clinical outcome. Patients with nonmalignant diseases must receive a higher cell dose to obtain engraftment than patients with malignant diseases; it should not be below $4.9 \times 10^7$ TNC/kg at collection and $3.5 \times 10^7$ TNC/kg at infusion (Gluckman & Rocha, 2006). In addition, all agree that the minimum cell dose should be $>4 \times 10^7$ TNC/kg at collection and $3 \times 10^7$ TNC/kg at infusion (Barker et al., 2001; Wagner et al., 2002; Dalle et al., 2004; Wall et al., 2005; Hurley et al., 2006). Currently, a TNC dose of $2.5 \times 10^7$ cells/kg is generally considered to be the threshold, with significantly higher rates of graft failure and transplant-related mortality reported in patients transplanted with single CBUs that contain lower numbers of TNC (Majhail, Brunstein & Wagner, 2006). However, only 25% of adult patients meet this cell dose requirement and hence the majority are ineligible for CB transplantation (Majhail, Brunstein & Wagner, 2006). Because of the limited inventory of suitable CBUs, most adults have access to only four of six HLA-matched grafts and there is increasing evidence to show that increasing HLA disparity might lead to poorer engraftment with CBUs (Barker et al., 2005a,b).

The current recommended numbers of TNC contained in a CBUs are much lower than the currently accepted dose for marrow or peripheral blood transplantation (Schmitz & Barrett, 2002); this makes CB especially useful for recipients weighing <40 kg. Moreover, as only 12% of the current inventory in established public CBB contains sufficient cells to deliver this dose to patients weighing >60 kg, alternative strategies to increase cell dose for adults and larger patients have to be explored (Kurtzberg, Lyerly & Sugarman, 2005). As a consequence, it is critical that all efforts are made to ensure a large scale dose in a CBUs, sufficient for a rapid and sustained engraftment (Gluckman et al., 1997; Rubinstein et al., 1998; Lim et al., 1999). In this respect, the importance of cell dose for transplantation outcomes provides the most compelling argument for focusing on the collection of larger CBUs. Besides, it’s time now for the CBBs to support the growth of adults’ CBTxs by developing strategies of banking, which will facilitate the transplantation in this category of patients.

In the present study, we describe the results obtained in the Hellenic Cord Blood Bank (HCBB) using a new strategy of CB banking, where CBUs with a collection volume $\geq 120$ ml were split into two half CBUs (a) and (b). This strategy was designed to improve the postprocessing cell recovery by maximizing the total number of cells contained in the freezing bag. This could optimize CB donor selection for adolescents and adults by providing two HLA-matched CB subunits, with high cell dose and the same HLA phenotype, derived from the same donor. To fully validate this strategy, white blood cell (WBC), mononuclear cell (MNC), and CD34$^+$ cell counts were used to demonstrate that the strategy of separation of the initial collection volume into two half CBUs caused no detrimental effect on the ultimate recovery of the nucleated and progenitor cells.

**MATERIALS AND METHODS**

**Cord blood collection**

Donors were recruited for CB donation at the first presentation in two hospitals selected on the basis of number of obstetric deliveries and the enthusiasm of staff. Written informed consent was obtained from the mother before delivery and CB was collected in utero (before placental delivery) by trained CBB staff. Briefly, after the spontaneous vaginal delivery of full-term newborns, the HCBB staff harvested the CB by clamping immediately the umbilical cord, cleaned
with 70% alcohol and an iodine swab. CB was collected from the umbilical vein by gravity in a closed sterile double overwrap single CB collection bag (Maco Pharma Cord Blood Collection Bag, MSC 1201 DU; Mouvaux, France) containing 21 ml of citrate–phosphate–dextrose (CPD) anticoagulant in the collection bag and an additional 8 ml of CPD in a satellite bag for flushing the tubes after finishing the collection. The collected CBUs were stored at 4°C and those with a collection volume of 120 ml or above were split within 24 h of collection. Exclusion criteria were congenital malformations and known hereditary or blood-transmissible diseases affecting the foetus or mother.

Groups of cord blood units

Cord blood units with collection volumes ≥120 ml were handled in the routine processing laboratory and they were categorised into three groups: CBUs with collection volume 120–139 ml, CBUs with volume 140–159 ml and CBUs with collection volume ≥160 ml. Once a week, all CBUs with a collection volume ≥120 ml were separated before volume reduction in two half CBUs; (a) and (b) referring with the same code number (i.e. CB0450a and CB0450b). For each CB subunit, all processing stages were performed in a closed system of three bags as described below.

Automated separation procedure

For the automated separation procedure, the Sepax Cell Processing System (Biosafe, Geneva, Switzerland) was used [umbilical cord blood (UCB)-HES protocol], with the CS-490 cell separation kit (a closed system of three bags: buffy coat collection bag, plasma and red cells collection bags), which was connected with the CB collection bag with a sterile docking system (Haemonetics TCD). The Sepax is a cell centrifugation device for processing blood volumes from 20 to 200 ml in one-step procedure. It consists of a centrifuge and a pneumatic system with vacuum or pressure capability to fill or empty the disposable separation chamber. Moreover, an optical line sensor and a system of three rotary pins control the blood flow and the position of the three-way valves that are part of the separation kit, thus controlling the direction of blood flow in the separation kit.

After removal of samples for routine testing and QC a volume of 20% of CBUs volume of hydroxyethylstarch (6% HES in 0.9 NaCl; Fresenius Kabi, Bad Homburg, Germany) was added to CB collection bag about 20 min prior to starting the process. HES allows faster red cell sedimentation and contributes to maintain cell viability after cryopreservation (Reboredo et al., 2000). CBUs were reduced to a standard volume of 26 ml prior of freezing, removing excess plasma and red cells to leave the required cells in the buffy coat component. The UCB-HES protocol allowed a volume reduction of CB in 25–30 min.

Haematological cell counts

A full blood differential cell count was performed on all samples pre- and postvolume reduction using an automated blood cell counter (Abbot Cell-DYN 4000, Abbott Diagnostics, Santa Clara, CA, USA). WBC, mononuclear cells (MNC), red blood cells (RBC) and platelets (PLTs) concentration was converted into absolute cell numbers using the corresponding volumes, and the % recovery of each blood cell type postvolume reduction was calculated.

Flow cytometry

To assess CB countenance in CD34+ cells relative to the WBC and absolute numbers as well as their viability, in every step of the processing, three-colour flow cytometry was used. Briefly, the samples taken just before and after volume reduction were dual labelled with anti-CD45 FITC (fluorescein isothiocyanate) and anti-CD34 (phycoerythrin) conjugated antibodies. Viability of CD34+ cells was assessed using the membrane integrity stain 7-amino-actinomycin D (7-ADD). Analysis was carried out on a Beckman Coulter Epics XL flow cytometer using the EXPO32 ADC software. In total, 75,000 CD45+ events were acquired. Sequential gating of the CD45+ cell population was used to identify the CD34+ cells subpopulation according to the dual platform International Society of Hematotherapy and Graft Engineering guidelines (Gratama et al., 1998). Using this method, an absolute CD34+ cell counts could be determined for each sample by the addition to the analyte of a known volume of Flow-Count fluorospheres (Stem-Kit Reagents; Beckman Coulter, Miami, FL, USA).
Statistics

Descriptive statistics are presented for CB variables (WBC, MNC and CD34+ cell counts). Computer software (Minitab Statistical Software, release 13.1; Minitab Inc., State College, PA, USA) was used to perform the statistical analysis. The Kolmogorov–Smirnov test was employed to investigate the normality of the variables distribution. The correlation between the cell counts of CBUs before and after volume reduction were analysed by means of Spearman correlation coefficient (rho, \(\rho\)). For categorized variables, the groups were compared by the Student’s 2-sample \(t\)-test (equal variance hypothesis was controlled using the Fisher or the Levene’s test according to the variables distribution). A \(P \leq 0.05\) was considered significant.

RESULTS

Nonsplit and split cord blood units: volume reduction

In CBUs with collection volume \(\geq 120\) ml, the results were analysed as two groups: the initial 106 nonsplit CBUs and the following 128 two half CBUs \(a\) and \(b\) that were handled in the routine processing laboratory using the SEPAX volume reduction method.

The preprocessing volume of the initial 106 nonsplit CBUs ranged from 120.2 to 202.5 ml (mean: 140.4 \(\pm\) 19.55); postprocessing the reduced mean volume of the CBUs was 25.31 \(\pm\) 0.89 ml with a range from 27.2 to 21.3 ml. The results of the first 128 split CBUs processed under routine conditions, for inclusion into the unrelated bank, showed before splitting a preprocessing mean volume of 146.487 \(\pm\) 20.48 ml. The preprocessing volume of the initial two half CBUs \(a\) and \(b\) ranged from 40.2 to 96.1 ml (mean; 74.29 \(\pm\) 10.60) and from 39.2 to 95 ml (mean: 72.18 \(\pm\) 10.50), respectively; postprocessing the reduced mean volume of the two half CBUs \(a\) and \(b\) showed very similar results (25.75 and 25.52 ml respectively).

Cell counts

The data for the pre- and postprocess WBCs, MNCs and CD34+ cell counts in nonsplit and in split CBUs are presented as absolute numbers in Table 1. All the units were categorized, according to their collection volume, in three groups: 120–139, 140–159 and \(\geq 160\) ml.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Preprocess [mean (\pm) SD (range)]</th>
<th>Postprocess [mean (\pm) SD (range)]</th>
<th>Recovery (%), (P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCs Nonsplit</td>
<td>947 ± 265 (464–1845)</td>
<td>1323 ± 366 (629–1992)</td>
<td>67.08, 0.000</td>
</tr>
<tr>
<td>Split</td>
<td>955 ± 270 (496–1557)</td>
<td>1214 ± 344 (609–1915)</td>
<td>80.67, 0.000</td>
</tr>
<tr>
<td>MNCs Nonsplit</td>
<td>418 ± 124 (77–770)</td>
<td>591 ± 229 (198–1247)</td>
<td>74.22, 0.007</td>
</tr>
<tr>
<td>Split</td>
<td>437 ± 146 (145–884)</td>
<td>538 ± 159 (299–984)</td>
<td>82.79, 0.000</td>
</tr>
<tr>
<td>CD34+ Nonsplit</td>
<td>2.782 ± 1.927 (0.30–9.50)</td>
<td>3.685 ± 2.547 (0.80–10.60)</td>
<td>77.11, 0.001</td>
</tr>
<tr>
<td>Split</td>
<td>2.259 ± 1.522 (0.30–7.10)</td>
<td>3.424 ± 1.831 (0.80–9.10)</td>
<td>86.45, 0.000</td>
</tr>
</tbody>
</table>

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Preprocessing, in the nonsplit CBUs, the initial WBC count in the three collection volume groups ranged from $464 \times 10^6$ to $3035 \times 10^6$ (mean: $947 \times 10^6$, $1323 \times 10^6$ and $1746 \times 10^6$ respectively) whilst, in the split CBUs, the WBC count ranged from $955 \times 10^6$, $1214 \times 10^6$ and $1708 \times 10^6$ respectively). These preprocessing WBC counts were not statistically significant ($P > 0.05$). On the contrary, postprocessing, the mean WBC counts ($767 \times 10^6$, $988 \times 10^6$ and $1311 \times 10^6$ per collection volume range) of the split CBUs were significantly higher ($P \leq 0.05$) than those of the corresponding nonsplit CBUs (mean: $616 \times 10^6$, $765 \times 10^6$ and $910 \times 10^6$ respectively). The mean initial MNC counts in the nonsplit CBUs ($418 \times 10^6$, $591 \times 10^6$, $870 \times 10^6$ per collection volume range) and in the split CBUs ($437 \times 10^6$, $538 \times 10^6$ and $742 \times 10^6$ per collection volume range) were not statistically significant ($P > 0.05$). Postprocessing and for the 120–139 ml collection volume range, the mean MNC count in the split CBUs was significantly higher ($P \leq 0.05$) than that of the corresponding nonsplit CBUs ($352 \times 10^6$ and $304 \times 10^6$ respectively). Finally, the preprocessing CD34+ counts in the nonsplit CBUs (mean: of $2.782 \times 10^6$, $3.685 \times 10^6$, $5.377 \times 10^6$ CD34+ cells per collection volume range) and in the split CBUs (mean: of $2.259 \times 10^6$, $3.424 \times 10^6$ and $4.304 \times 10^6$ per collection volume range) were not statistically significant ($P > 0.05$). Postprocessing and for the 120–139 ml collection volume range, the mean CD34+ counts ($3.194 \times 10^6$ and $3.592 \times 10^6$ respectively) in the split CBUs were significantly higher ($P \leq 0.05$) than those of the corresponding in the nonsplit CBUs ($2.206 \times 10^6$ and $3.320 \times 10^6$ respectively).

Linear regression analysis of the WBCs cell content pre- and postprocessing gave regression coefficients of $1.3063$ and $1.1765$, as well as intercepts of $256.69$ and $74.411$, in the nonsplit CBUs and in the split CB units respectively (Figure 1).

**Cell recovery**

Cell recovery was assessed by estimating the WBC, MNC and CD34+ cell content postprocessing in the nonsplit and split CBUs with collection volumes of 120–139, 140–159 and ≥160 ml. The percentage cell recovery data after processing is presented in Table 1. The mean WBC recoveries (80.67%, 81.71% and 77.67% per collection volume range) of the split CBUs were significantly higher ($P \leq 0.05$) than the corresponding of the nonsplit CBUs (mean: 67.08%, 61% and 51.04%). Similarly, the mean MNC recoveries (80.57%, 82.79%, 76.31% per collection volume range) of the split CBUs were significantly higher ($P \leq 0.05$) than those of the nonsplit CBUs (mean: 74.22%, 65.32%, 52.92%). The same pattern was observed when comparing the CD34+ recoveries of the nonsplit CBUs (mean: 77.10%, 67.64% and 61.12% per collection volume range) with those of the split CBUs (mean: 86.45%, 90.08% and 85.72%), the latter being significantly higher ($P \leq 0.05$) than the former.

**Cell dose and weight of the potential recipients**

Before freezing, the weight of the potential CBU recipient was estimated according to the WBC dose of the volume reduced nonsplit and split CBUs with collection volumes 120–139, 140–159 and ≥160 ml. Briefly, the mean weights of the potential recipients for the split CBUs were 30.69, 39.54 and 52.46 kg per collection volume range, which were significantly higher...
(P ≤ 0.05) than those of the corresponding nonsplit CBU (mean: 24.66, 30.62 and 36.41 kg respectively).

**WBC counts in RBC bags after volume reduction**

In order to evaluate the degree of cell loss in the CBUs, WBCs were also counted in the RBC bags to be discarded after volume reduction. Thus, in the split CBUs, the absolute WBC counts in the RBC bags with collection volume ranges from 120 to 139 and ≥160 ml were significantly higher (P ≤ 0.05) in the nonsplit CBUs (mean: 257 × 10^6 and 438 × 10^6 per collection volume range), than those in the RBC bags of the split CBUs (mean: 62 × 10^6 and 163 × 10^6 respectively). The absolute WBC counts in the RBC bags of both split and nonsplit CBUs, for the 140–159 ml collection volume range, were not significantly different (P ≥ 0.05).

**DISCUSSION**

Practical issues faced by CBBs and how those issues affect the clinical use of CB in haemopoietic stem cell transplantation are the topic questions. Thus, the critical importance of the cell dose in transplant outcome has motivated CBBs to define progressively higher cut-offs for unit acceptance as well as to develop new techniques aimed at improving processing protocols for volume reduction of stored units, both to minimize cell losses and to make the unrelated CB banking more feasible (Rodriguez et al., 2004). In addition, it’s time now for the CBBs to support the growth of adults’ CBTx by developing strategies of banking, which will facilitate the transplantation in this category of patients. To our knowledge, this is the first time that a strategy of CB banking, which mainly attempts to bank CBUs for transplantation in adults, is described. Thus, the current study was designed to maximize the total number of cells contained in the CB freezing bag, in order to facilitate the possibility for ex vivo expansion of the infused haematopoietic stem cells in adults.

To maximize the cell quantity in a single CBUs, the *in utero* method of collection was used and the collection volumes ranged from 120 to ≥160 ml. The collection of high volume CBUs, using the *in utero* method, is in accordance with other studies (Surbek et al., 1998; Reboredo et al., 2000), which have demonstrated a significantly larger volume of CB collected, probably because placenta is being compressed by the uterus, allowing more blood to be collected (Lasky et al., 2002). In addition, a study (Wong et al., 2001) provided strong evidence that the collection of CB before the delivery of the placenta should be the preferred procedure, bearing in mind there is a finite amount of stem cells available in these circumstances.

In this study, the WBC content in CBUs was taken into account instead of the TNC, since there is evidence (Stevens et al., 2002) that WBC content is presumed to be a more accurate measurement than the TNC count. In the three collection volume groups (120–139, 140–159 and ≥160 ml), recoveries of WBCs, MNCs and CD34+ cells obtained in the nonsplit high volume CBUs, implicated cell losses of 32.9–48.97% (WBC), 25.78–47% (MNCs) and 22.89–38.88% (CD34+ cells). As a consequence of these cell losses, we decided to split the collected high volume CBUs into two low volume CB subunits, to test whether it would be possible to improve the cell recoveries. Indeed, splitting individual high volume CBUs into two half low volume CBUs prior to processing and storage provided a better recovery of cells than storing them as single high volume units. Thus, in the low volume two half CB subunits a and b of our study, the mean recovery with the HES method was 80% (WBCs) and 79.89% (MNCs). These results are comparable with the cell recoveries (77% for WBCs and 75% for MNCs) reported in a recent study (Takahashi et al., 2006), using the HES method. In contrary, in the nonsplit high volume CBUs of our study, the obtained recoveries were reduced (59.7% for WBCs and 62.82% for MNCs). The reason for the reduced recoveries in the nonsplit high volume CBUs, seems to be that automatic devices have been developed to process low volume CB collections, which are too low to be processed in other blood cell separators (Zingsem et al., 2003). Furthermore, a recent multi-institutional study (Takahashi et al., 2006) evaluated the performance of four methods used for volume reduction of CB units: the HES method with two centrifugations, the top and bottom (T&B) isolation ofuffy coat following one centrifugation, and two filter systems for processing CB. While cell recovery with the T&B method did not appear to be influenced by CB volume, for the other three methods relatively high volumes appeared to be associated with lower percentage cell recoveries. Overall, these results indi-
cate that the cell processing system used in this study needs further improvements for increasing the specific recoveries in the nonsplit high volume CBUs. This is also proposed in another study (Zingsem et al., 2003), where the performance of this device was compared to the well-established method described by Rubin

stein et al. (1995).

The decreased CD34 + cell loss (9–14.28%) in the split CBUs, compared with the nonsplit CBU (22.89–38.88%), is of particular interest, as several studies have demonstrated the importance of graft CD34 + cell dose in determining the outcome after unrelated donor CBTx (Wagner et al., 2002). Measurement of CD34 + cells in the transplant is a good surrogate marker of stem cells because it measures immature haematopoietic progenitors. Indeed, several authors have shown that the number of CD34 + cells in the graft correlates with engraftment (a number of >2 × 10^6 CD34 + cells/kg is optimal). Unfortunately, this measurement cannot be used for comparative studies between centres because of the absence of standardization of the counting method (Barker et al., 2001; Wagner et al., 2002). Overall, even in recipients of grafts disparate in two HLA antigens, the data suggest that higher CD34 + cell dose can partially reduce the negative impact of HLA disparity on survival (Wagner et al., 2002). Thus, it has been suggested that graft selection could be based principally on CD34 + cell dose when multiple CBUs exist with an HLA disparity of two or less (Wagner et al., 2002; Barker et al., 2005a,b). At the University of Minnesota CD34 + cell dose is not routinely used for CBUs selection, unless two units of equal HLA match that have a TNC dose within 0.3 × 10^7 cells/kg of each other are available. The unit with larger CD34 + cell dose is then selected for CBTx (Majhail, Brunstein & Wagner, 2006).

A study from the New York Blood Center concluded that raising the TNC dose by approximately 3 × 10^7 cells/kg may offset the negative effect of a HLA mismatch (Migliaccio et al., 2000). In addition, it has been shown (Gluckman et al., 2004) that the higher the number of cells and the lower the number of HLA disparities, the higher the probability of engraftment. Therefore, a variety of approaches are being studied to overcome the negative impact of low cell dose on engraftment following single CB transplantation in adults. These include ex vivo expansion of progenitors with various cocktails of cytokines, use of intra-bone marrow injection of CB to minimize nonspecific loss of circulating haematopoietic stem cells, use of nonmyeloablative conditioning regimens (Barker et al., 2003; Koh & Chao, 2004); and infusion of multiple CBUs. The method of unit selection for multiple CB transplantation is complex and there are no currently available data that specifically address the optimal selection. At the University of Minnesota each unit is required to have a cryopreserved TNC dose of at least 1.5 × 10^7 cells/kg, such that the total graft dose is 3.0 × 10^7 cells/kg or more (Majhail, Brunstein & Wagner, 2006). On the other hand, the split of the initial collected CBUs into two half CBUs provides the attractive option of ex vivo expansion of the one half CBUs, in order to increase the number of the infused haematopoietic progenitor cells. Several groups have reported that ex vivo expansion can be performed on a CBUs as a whole prior to transplantation, or on a subunit of a CBUs that is recombined with its ‘unmanipulated’ subunit at the time of transplantation, or transplanted a period of time after the unmanipulated subunit. Indeed, the combination of ex vivo – expanded and unmanipulated CB subunits might prove to be a beneficial strategy (Pecora, Stiff & Jennis, 2000; Shpall, Quinones & Giller, 2002). Shpall, Quinones & Giller (2002) expanded a portion (40–60%) of the CBUs, while the M.D. Anderson Cancer Center is currently testing CB expansion in a phase II study, comparing two unmanipulated CBUs vs. one unmanipulated and one expanded CBU (Ballen, 2005). In addition, McNiece, Kubegov and Kerzic (2000) developed a two-step 14-day CB expansion protocol, which they demonstrated yields more effective ex vivo expansion than the single-step 10-day protocol mentioned by Shpall et al. (2002).

In conclusion, the split of the initial high volume CB collections into two half CBUs is a feasible and safe strategy for the CBBs aiming to facilitate the CBTx in adults. This strategy improves the recovery of the cells and is an attractive option for ex vivo expansion, in order to increase the number of the infused haematopoietic progenitor cells, especially for adults who are typically not eligible for single CBTx because of limitations of cell dose. Improving the engraftment rate by increasing cell dose and optimizing HLA match is the key to expanding the use of cord blood (Gluckman & Rocha, 2006).
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