

Cord blood processing by a novel filtration system

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Abstract

Objectives: Availability of cord blood (CB) processing has been limited by the need for electrically aided centrifugal techniques, which often produce only low final cell product yield. Here, we describe development and characterization of a novel filter device aimed at allowing CB processing, using gentle gravity-led flow.

Materials and methods: CB was processed with a novel filter device (CellEffic CB, consisting of non-woven fabric), without any centrifugation. Cells were harvested by flushing the filter with either HES or physiological saline solution (SALINE). Differential cell counts and viability analysis, combined with Fluorescence-Activated Cell Sorting (FACS) (total nucleated cells [TNC], mononuclear cells [MNC], CD45+ CD34+ cells, hematopoietic precursor cells [HPCs]) and clonogenic assay, were employed for analysis of CB pre- and post-processing, and after freeze/thawing.

Results: Processing using the novel filter yielded high quality RBC depletion while maintaining good recovery of TNC, MNC, CD34+, HPCs and colony forming unit (CFU) output. The filter performed equally well using HES or SALINE. Gravity-led flow provided gentle cell movement and protection of the stem cell compartment. Post-thaw CFU output was maintained particularly, an important indicator for CB banking.

Conclusions: Geographical limitations of CB transplantation and banking have required a non-electrical, non-centrifugal solution. This novel filter CellEffic CB device revealed rapid yet gentle cell processing while maintaining the stem/progenitor

cell compartment required for both haematological and regenerative medicine therapies.

Introduction

By 2011, an estimated over 1.18 million cord blood units (CBUs) had been stored in private and public cord blood banks (CBBs) around the world (1). Cord blood (CB) was first reported as a potential alternative transfusional product, in 1939 in the *Lancet* (2,3). Although this never became routine, resurgence of interest in CB as a therapeutic product reappeared in the 1970s, particularly with the brother medical doctors Ende and Ende who unsuccessfully attempted transplantation with multiple CBUs (4). This attempt, while ground breaking, failed due to immunological mismatching of units, but led to many years of new research culminating in the first successful CB therapy for Fanconi's anaemia 1988 (5).

There is now significant and growing evidence for efficacy of cord blood transplantation (CBT) for haematological diseases, with CBT being increasingly chosen in some countries (6,7). Furthermore, stem cells in CB are not only able to give rise to haematopoietic cells but also to epithelial, endothelial and neural tissues (8–10). This raised interest in application of CB in regenerative medicine, both for tissue production and tissue repair (10). Today multiple clinical trials in regenerative medicine area are underway using CB as primary cell source (11). Development of neural cell populations from CB has furthermore led to pioneering uses of CB for neurological injury and disease, including traumatic brain injury, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (12–15). Applications have also been found in other clinics such as for cardiac lesions (16,17). This potential use of CB stem cells in non-blood-related conditions or for organ regeneration, also led to interest in storage of CB for autologous (same-patient) use. Today more CB is stored in private CB banking companies than in public banks (1). Nevertheless, therapeutic use of CB must still be considered

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to be in its early stages, particularly from an autologous perspective.

Increasing use of CB therapeutically, particularly allogeneic transplant, has led to application of 'minimal manipulation' rules being enforced, to ensure not only lack of infectious contamination and transfer, but also prevention of stimulation of the stem/progenitor cell compartment and unnatural change to the transplantable product. The USA Food and Drug Administration has published guidance for preparation of CBUs (FDA, 2015) (18).

The further and important issue of red cell depletion in CB processing has been highlighted following transplantation of CBUs replete with red blood cells (RBCs), and with negative clinical outcome (19,20). This transplant experience together with FDA guidance lead to recommendations that CBUs be depleted as much as is possible of both plasma and RBC content. The issue of red cells is particularly related to known complications of red cell debris and free haemoglobin, that can interfere with demarcation of interfaces between mononuclear cells and supernatant during pre- and post-thaw processing, and can contribute to viscosity and clumping. This side effect of conventional processing can lead to infusional toxicity, that is of clinical concern. This was reported to the National Marrow Donor Program (NMDP which country?) prompting detailed investigation of CB processing parameters which could influence patient safety, and an alert from the NMDP in 2009 (19).

A further and increasing issue concerns solutions used for CB processing. The European EMA made the decision that hydroxyethyl starch (HES) should no longer be used in patients with sepsis or burn injuries, or in critically ill patients (EMA, 2015) (21). Furthermore, HES was withdrawn from the market in the UK in 2013 by the MHRA (MHRA, 2015) (22). These decisions were made following reports concerning increased risk of mortality in patients with sepsis and increased risk of kidney injury requiring dialysis, in critically ill patients (23–25). This revealed an increasing shortage of HES available in general, and particularly to CB processing.

A third issue is finance. Currently, CB banking has been restricted largely to first world nations, with few second or third world countries being able to routinely store CB in public blood banks. Costs of processing include staff time, processing materials and long-term liquid nitrogen storage, which all together make the procedure prohibitive for many hospital systems. In private CB banking, additionally long-term agreements with clients (sometimes 20 years or more) can lead to increased costs particularly due to liquid nitrogen and insurance. Thus, there is current and urgent need for more standardized systems, usable in all geographical locations at

reasonable costs, ideally reducing volume to lower storage costs in liquid nitrogen, while maintaining useable high quality final products. Current automated systems, while offering standardization, often require expensive and complicated equipment which can break, and are run by software and with electrical and servicing/maintenance requirements. This, while realistic in high GDP countries with publically funded health care systems, is not suitable for many smaller places.

We therefore intended to create a system which could potentially be used globally. Our first paper describing the system discussed processing potential of the device and its mechanical properties and structure of the filter (26). Here, we have developed the device further for use in the CB industry, with the ability to use it with and/or without HES, and compare its performance to a centrifugal, well-established, automated device SEPAX. CelleEffic CB is a manual system, which requires no electricity, software or maintenance and our primary aim was to process CB to an extremely high-quality, available not only for blood-related transplantation but also for the wider regenerative medicine community.

Materials and methods

Inclusion criteria

Samples were collected only after obtaining informed, written parental consent, from Natecia Hospital, Lyon, France. The protocol followed was reviewed and approved for research-only work. A negative viral profile and infection status was mandatory. CBUs were processed only if time between collection and cryopreservation was <48 h and the sample was of ≥ 35 ml. CelleEffic CB can process 35–100 ml CB including anticoagulant, using larger volume of CB results in slightly lower recovery ratio of TNC (data not shown).

Collection of CBUs

Samples were collected from caesarean section births, post-partum, after the placenta had been delivered. The umbilical cord was clamped (as directed by the surgeon performing the procedure), in two places: close to the placenta and close to the baby. The placenta was then applied to a specific cone-shaped collection vessel with the cord depending from the underside. The collection bag contained citrate phosphate dextrose adenine (CPD-A) anticoagulant. The appropriate intravenous needle attached to the collection bag, punctured the lower end of the cord allowing the blood sample to drain into the bag. Blood was sampled from the umbilical cord vein only, not from the placenta. Once sample collection was complete, the

unit was transported to the laboratory and stored at room temperature until processing was initiated.

CellEffic CB device

CellEffic CB consists of a filter and two bags as shown in Fig. 1. Multilayered non-woven polyester fibres are used as filter. Kits were designed comprised of plastics such as polyvinyl chloride and polycarbonate, without any metal parts, in order to be easy to store and, after being used, to be disposed off (Table 4). For use the device was taken from a sterile stored blister pack and primed with SALINE, followed by infusion of the CB. CB samples were introduced into the filter under the influence of gravity only. Cells trapped on the filter were collected into the cell harvesting bag by reverse wash of either HES solution (6% HES (w/v) hydroxyethyl starch) or SALINE (0.9% NaCl), applied with a kit syringe, as harvesting solution. Cells that flowed through the filter into the waste solution bag were also collected for subsequent analysis.

SEPAX (Centrifugal-based automated device)

This technique allows separation in an automated system, which is controlled by computer software. SEPAX

isolates haematopoietic stem cell-rich buffy coat of MNCs from 35–290 ml CB to a final volume of 10–50 ml. Each unit was always separated with a single use kit, inserted into the apparatus. CB was added to the device where it filled the central rotating chamber. While filling, each sample was spun at a speed of up to $1900 \times g$, and components were directed to individual blood bags. In this study, SEPAX 2 apparatus with UCB mode and CS490 disposable kits were used. As optimal CB volume for SEPAX, over 100 ml of CB was processed.

Cord blood processing

In total, 26 CB samples were processed by CellEffic CB and 13 CB samples were harvested for each harvest solution (SALINE or HES). Eight CB samples among 13 processed-CB samples were used for freeze/thawing processes for each harvest solution. For SEPAX, nine CB samples in total were processed.

Enumeration by differential cell count

Cell counts were performed using CellDyn4000 Analyser, a mechanical method. A sample of 1 ml was applied to the device at each of three points during processing. WBC count and differential cell count, that is percentage of each type of WBC, were determined in each unit of blood.

Flow cytometry

Flow cytometric analysis was carried out using Becton Dickinson (BD) FACS Caliber apparatus. Samples were prepared as follows: 100 μ l blood was added to each tube and followed by 50 μ l antibody cocktail (antibodies, fluorochromes and supplier details are shown in Table 1). Tubes were then incubated at room temperature, in the dark for 20 min. Cells were then lysed and washed on the BD FACS Lyse/Wash Assistant, after which samples were run on the flow cytometer. BD and BD Pharmingen antibodies and reagents were sourced from Pont-de-Claix (France).

Clonogenic assays

Clonogenic assays to evaluate content of stem and progenitor cell compartments were conducted based on our previously described method (27).

Freeze/thaw analysis

To evaluate effects of control-rate freezing parameters and thawing effects on unit content, freezing and thaw-

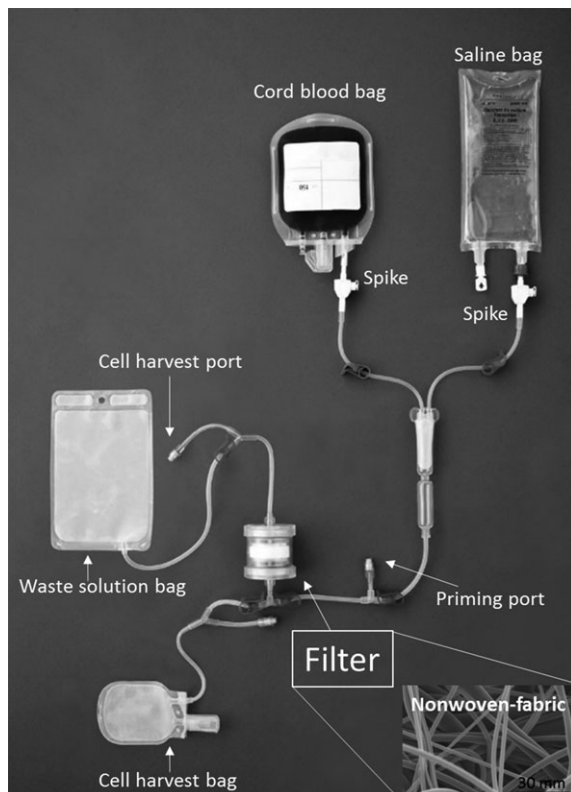


Fig. 1. Configuration of CellEffic CB and view inside the filter.

Table 1. Antibody cocktails used for flow cytometric analysis

| Marker | Fluorescence | Channel | Provider | ref |
|---------------------------------------|--------------|---------|-----------------------------|-------------|
| CD7 | FITC | 1 | BD 100tests | 555360 |
| CD3 | PE | 2 | BD 100tests | 555340 |
| CD14 | APC-H7 | 6 | BD 100tests | 560180 |
| CD19 | APC | 5 | BD 100tests | 555415 |
| CD33 | PE-Cy7 | 4 | BD 100tests | 333952 |
| CD34 | PE-Cy7 | 4 | BD 50 tests | 560710 |
| CD44 | APC-H7 | 6 | BD 100tests | 560532 |
| CD45 | V500 | 8 | BD 100tests | 560777 |
| CD73 | PE-Cy7 | 4 | BD Pharmingen™ | 561258 |
| CD90 | FITC | 1 | BD 100tests | 555595 |
| CD133 | PE | 2 | Miltenyi biotec 100tests | 130-090-853 |
| CD235a | APC | 5 | BD0,1 mg | 551336 |
| BD Pharmingen 7-AAD Staining Solution | | | | 559925 |

ing were conducted, based on our previously described method (27).

Sterility (microbiology)

Microbiological status of CS samples pre- and post-processing was assessed with BacT/Alert (Biomerieux, Lyon, France), to monitor growth of aerobic and anaerobic organisms, by automatic measurement of CO₂ production, with a colorimetric sensor. Prior to incubation and analysis, samples were inoculated into BacT/ALERT FA FAN Aerobic bottles (Cat N° #259791) and BacT/ALERT FN FAN Anaerobic (Cat N° #259793) respectively.

Statistical analysis

Significance of differences between groups was determined using Student's *t*-test; *P* value of <0.05 was considered statistically significant.

Results

For this study, a total of 26 CB samples was processed. Of those, 13 were harvested by HES and 13 were harvested by SALINE. Performance was compared between HES and SALINE used as harvest solution. Details of differential cell count data are shown in Table 2. For SEPAX, details of differential cell count data are also shown in Table 2.

Viable TNC and MNC recovery

TNC recovery was measured using values from differential cell count combined with flow cytometry analysis of CD45+ cells and their viability measured by the uptake

Table 2. Average differential cell counts for CellEffic CB (each harvest solution) and SEPAX. Mean ± SD are shown

| Cell type | CellEffic CB (HES) | | | CellEffic CB (SALINE) | | | Sepax2 | | |
|-------------------------------|--------------------|---------------|--------------|-----------------------|---------------|--------------|--------------|---------------|--------------|
| | Pre (n = 13) | Post (n = 13) | Thaw (n = 8) | Pre (n = 13) | Post (n = 13) | Thaw (n = 8) | Pre (n = 9) | Post (n = 9) | Thaw (n = 9) |
| WBC ($\times 10^6$ /ml) | 4.52 ± 2.57 | 11.41 ± 6.30 | 3.32 ± 1.12 | 5.65 ± 2.36 | 12.13 ± 6.38 | 4.35 ± 2.81 | 8.12 ± 2.54 | 25.98 ± 8.73 | 8.63 ± 3.26 |
| RBC ($\times 10^9$ /ml) | 2.27 ± 0.71 | 1.58 ± 0.71 | 0.14 ± 0.05 | 2.07 ± 0.64 | 1.51 ± 0.44 | 0.12 ± 0.05 | 3.11 ± 0.61 | 4.81 ± 0.69 | 0.40 ± 0.14 |
| Platelet ($\times 10^6$ /ml) | 114.9 ± 53.4 | 198.0 ± 129.9 | 25.9 ± 20.8 | 101.6 ± 49.1 | 196.0 ± 89.5 | 25.9 ± 17.0 | 139.7 ± 44.4 | 539.1 ± 296.4 | 44.7 ± 30.3 |
| %Lymphocyte | 31.2 ± 8.4 | 31.6 ± 7.8 | 58.5 ± 9.7 | 30.8 ± 5.0 | 32.0 ± 4.5 | 59.0 ± 8.9 | 39.6 ± 7.6 | 43.9 ± 6.0 | 51.6 ± 8.7 |
| %Monocyte | 9.2 ± 3.6 | 9.1 ± 4.1 | 24.1 ± 4.5 | 8.7 ± 2.4 | 8.5 ± 1.6 | 23.6 ± 4.6 | 13.1 ± 9.9 | 9.8 ± 1.3 | 24.9 ± 4.1 |
| %Granulocyte | 51.0 ± 13.9 | 53.2 ± 12.5 | 6.5 ± 6.6 | 55.6 ± 6.4 | 54.6 ± 5.4 | 7.6 ± 4.0 | 45.2 ± 15.1 | 43.1 ± 7.1 | 19.9 ± 6.1 |
| %Hematocrit | 23.1 ± 6.7 | 16.1 ± 7.0 | 1.2 ± 0.4 | 21.1 ± 6.4 | 15.0 ± 4.2 | 1.1 ± 0.4 | 27.7 ± 5.2 | 44.6 ± 6.6 | 3.7 ± 1.5 |
| Hemoglobin (g/dl) | 7.45 ± 2.04 | 6.00 ± 1.83 | 0.54 ± 0.21 | 7.41 ± 2.26 | 5.49 ± 1.48 | 0.55 ± 0.18 | 9.76 ± 2.53 | 14.50 ± 8.36 | 1.24 ± 0.45 |

of 7-AAD (Fig. 2a). Average CD45+ cell recoveries for HES and SALINE harvesting were 76.1% (SD \pm 8.7) and 73.4% (SD \pm 5.9) respectively. There was no statistical difference between HES and SALINE harvesting ($P > 0.05$). As a result of analysis for SEPAX, average CD45+ cell recoveries was 76.6% (SD \pm 16.1). There was no statistical difference between Cellefficient CB (HES or SALINE harvesting) and SEPAX ($P > 0.05$). Recovery of CD45+ cells was further analysed, excluding granulocytes, using data collected from flow cytometric analysis. Cells excluded were CD14- with high granularity, which was measured using side scatter separation (Fig. 2b). After exclusion of granulocytes, CD45+ minus granulocyte (MNC) recovery for HES and SALINE harvesting were 79.3% (SD \pm 9.5) and 79.3% (SD \pm 11.1) (Table 4), respectively and there was no statistical difference between HES and SALINE harvesting ($P > 0.05$). As a result of analysis for SEPAX, average CD45+ minus granulocyte (MNC) recoveries was 79.8% (SD \pm 10.9). There was no statistical difference between Cellefficient CB (HES or SALINE harvesting) and SEPAX ($P > 0.05$).

Recovery of CD45+/CD34+ cells to examine haematological stem and progenitor cell compartments

Next, we examined recovery of CD45+/CD34+ cells. Recovery of CD45+/CD34+ cells was measured using the nucleate cell count, from the differential cell count, combined with flow cytometry, analysis of CD34+ cells and their viability measured by uptake of 7-AAD (Fig. 3). HES harvesting yielded average recovery of 75.2% (SD \pm 12.2), compared to 80.2% (SD \pm 15.2) from SALINE harvesting (Table 4) and there was no statistical difference between HES and SALINE harvesting

($P > 0.05$). As a result of analysis for SEPAX, average CD45+/CD34+ recovery was 68.6% (SD \pm 17.2). Although Cellefficient CB has higher CD45+/CD34+ cell recovery than SEPAX, there was no statistical difference between Cellefficient CB (HES or SALINE harvesting) and SEPAX ($P > 0.05$).

Recovery of stem cells useful for regenerative medicine purposes, and immune cells

More detailed flow cytometric analysis was conducted with Cellefficient CB for checking stem cells for regenerative medicine, and immune cells. Recovery of other cells was measured using flow cytometric analysis combined with differential cell counting. We focused on three different populations of haematopoietic stem cells as previously described (early stage (CD34-/CD133+), mid stage (CD34+/CD133+) and late stage (CD34+/CD133-)),

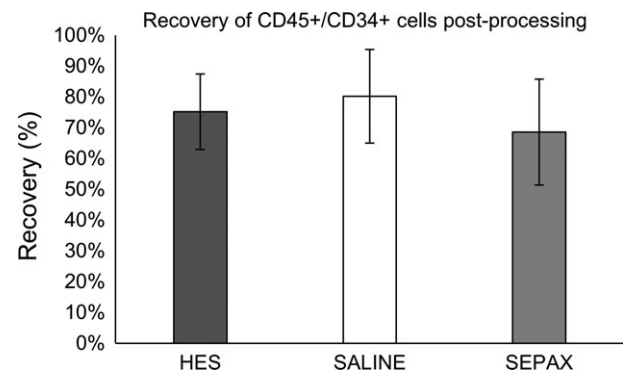


Fig. 3. Hematopoietic stem cell (CD45+/CD34+ cells) recovery by Cellefficient CB. High recovery was achieved with both harvest solutions. Mean \pm SD are shown. $n = 13$ for each harvest solution of Cellefficient CB, $n = 9$ for SEPAX.

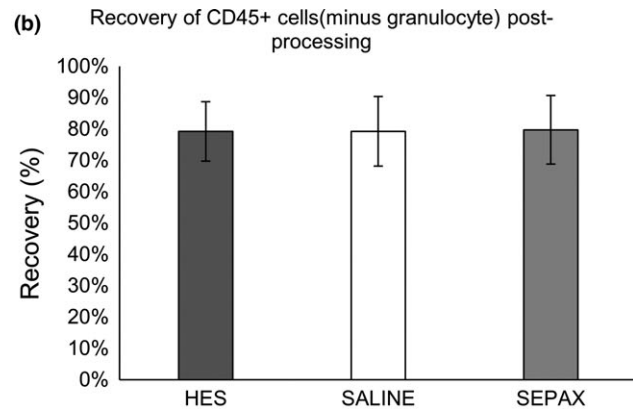
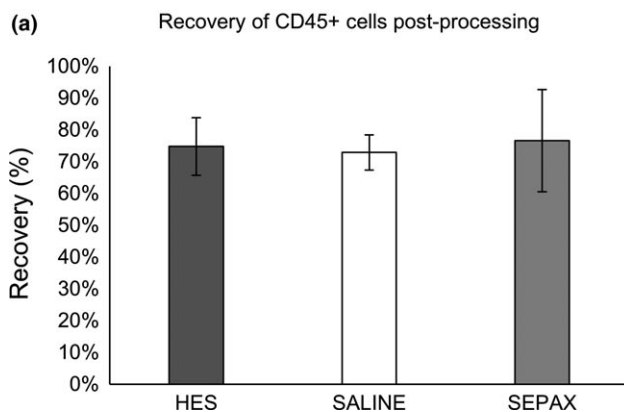


Fig. 2. Nucleated cell recovery by Cellefficient CB. (a) Recovery of CD45+ cells (TNC), (b) Recovery of CD45+ cells (minus granulocyte) (MNC). No statistically significant differences were found. Recoveries were similarly high with both harvest solutions. Mean \pm SD are shown. $n = 13$ for each harvest solution of Cellefficient CB, $n = 9$ for SEPAX.

haematopoietic stem cell subsets (CD45+/CD34+/CD38- and CD45+/CD34+/CD90+), multipotent lineage-negative stem cells (LinNEG cells (CD45-/CD235a-/CD7-/CD33-)), circulating mesenchymal stem cells (CD45-/CD73+/CD44+/CD90+) and immune cells (T cells (CD45+/CD3+), B cells (CD45+/CD19+)) (28). As shown in Table 3, and summarized in Table 4, Celleffic CB yielded high recovery.

Depletion of RBC and haemoglobin

We measured RBC depletion using cell differential counting. After processing, average RBC depletion for Celleffic CB was 73.5% (SD \pm 11.8) for HES harvesting and 70.9% (SD \pm 11.4) for SALINE harvesting (Table. 4) with no statistical difference between HES and SALINE ($P > 0.05$) (Fig. 4). As a result of analysis for SEPAX, average RBC depletion was 56.9% (SD \pm 25.5). Although Celleffic CB had higher RBC depletion than SEPAX, there was no statistical difference between Celleffic CB (HES or SALINE harvesting) and SEPAX ($P > 0.05$).

We also measured haemoglobin. Haemoglobin level post-processing was 6.0 g/dL (SD \pm 1.8) for HES harvesting and 5.5 g/dl (SD \pm 1.5) for SALINE harvesting (Fig. 5). Haemoglobin level post-thaw was 0.5 g/dl (SD \pm 0.2) for HES harvesting and 0.6 g/dl (SD \pm 0.2) for SALINE harvesting; there was no statistical difference between HES and SALINE ($P > 0.05$). As a result of analysis for SEPAX, haemoglobin level post-processing and post-thawing were 14.5 g/dl (SD \pm 8.4) and 1.2 g/dl (SD \pm 0.5) respectively (Fig. 5). As there was statistical

Table 3. Average cell recovery of each cells for Celleffic CB. Mean \pm SD are shown

| Cell type | Celleffic CB Post-processing |
|---|------------------------------|
| %Recovery of CD34-/CD133+ cells (Early stage hematopoietic stem cells) | 75.8 \pm 11.8 |
| %Recovery of CD34+/CD133+ cells (Mid stage hematopoietic stem cells) | 84.2 \pm 14.9 |
| %Recovery of CD34+/CD133- cells (Late stage hematopoietic stem cells) | 78.2 \pm 14.6 |
| %Recovery of CD45+/CD34+/CD38- cells (Hematopoietic stem cells subset) | 76.6 \pm 18.3 |
| %Recovery of CD45+/CD34+/CD90+ cells (Hematopoietic stem cells subset) | 84.4 \pm 46.9 |
| %Recovery of CD45-/CD235a-/CD7-/CD33- cells (Lineage negative stem cells) | 61.6 \pm 31.8 |
| %Recovery of CD45-/CD73+/CD44+/CD90+ cells (Circulating mesenchymal stem cells) | 76.5 \pm 28.7 |
| %Recovery of CD45+/CD3+ cells (T cells) | 64.1 \pm 15.7 |
| %Recovery of CD45+/CD19+ cells (B cells) | 71.1 \pm 11.3 |

difference between Celleffic CB (HES or SALINE harvesting) and SEPAX at pre-processing, statistical analysis on post-processing and post-thawing was not conducted.

Ability for colony forming units

As CFU number varies with normal CB variability, we put CFU/10cells at pre-processing to 100. As a result, post-processing number ratios were 90 (SD \pm 26) and 97(SD \pm 26) for HES harvesting and SALINE harvesting respectively (Fig. 6a); there was no statistical difference between pre-processing and post-processing

Table 4. Summary of Celleffic CB evaluation

| Item | Results |
|-------------------|---|
| RBC content | <ul style="list-style-type: none"> Red Blood cell removal was 74% (HES) and 71% (SALINE) |
| Stem cell content | <ul style="list-style-type: none"> CD45+ cell recovery was 76% (HES) and 73% (SALINE) MNC (CD45+ cell minus granulocytes) recoveries were 79% (HES) and 79% (SALINE) CD45+/CD34+ cell recoveries were 75% (HES) and 80% (SALINE). Other sub-populations and stem cell recoveries were high. |
| Final volume | <ul style="list-style-type: none"> The volumes of post-processing were 21.0 (HES) and 21.7 (SALINE) while originating from 35–94 ml pre-processing unit volumes. |
| Freeze/thaw | <ul style="list-style-type: none"> There were no differences in CFU numbers between post-processing and freeze/thaw in both harvest solutions. |
| Ease of use | <ul style="list-style-type: none"> Disposable kits were composed of only plastic without metal. Compacted package. No special training is needed. |
| Availability | <ul style="list-style-type: none"> No electricity/No machine/No software/No maintenance |

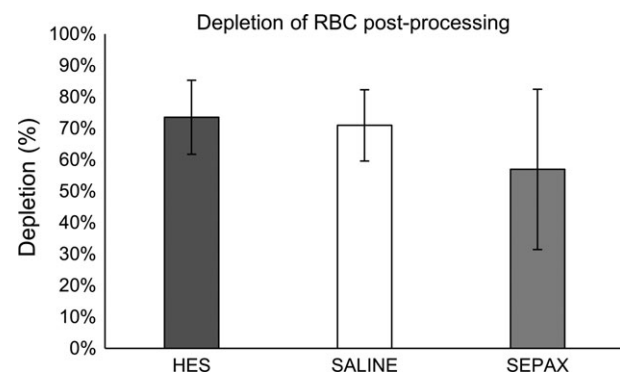


Fig. 4. Red blood cell depletion of Celleffic CB. Celleffic CB depleted RBC effectively with HES as well as with SALINE as harvest solution. Mean \pm SD are shown. $n = 13$ for each harvest solution of Celleffic CB, $n = 9$ for SEPAX.

($P > 0.05$). Post-thaw CFU ratio for HES harvesting was 114 (SD \pm 36) compared to 92 (SD \pm 32) SALINE harvesting respectively but there was no statistical difference between HES and SALINE ($P > 0.05$). Furthermore, there were no statistical differences between post-processing and post-thawing ($P > 0.05$) (Table 4). As a result of analysis for SEPAX, average CFU ratio was 83 (SD \pm 8) at post-processing and there was no statistical difference between Celleffice CB (HES or SALINE harvesting) and SEPAX ($P > 0.05$). Furthermore CFU ratio at post-thaw was analysed with SEPAX and average CFU ratio was 19 (SD \pm 17). There was a statistical difference between Celleffice CB (HES or SALINE harvesting) and SEPAX ($P < 0.05$).

Next, we examined TNC viability measured by uptake of 7-AAD. TNC viability ratios post-processing to pre-processing were 98.3% (SD \pm 2.9) and 99.4% (SD \pm 2.3) and TNC viability ratios post-thaw to pre-processing were 87.8% (SD \pm 7.1) and 85.24% (SD \pm 12.45) for HES harvesting and SALINE harvesting respectively (SD \pm 12.1) (Fig. 6b). There were no statistical differences between HES and SALINE

harvesting ($P > 0.05$). As a result of analysis of SEPAX, TNC viability ratio post-processing to pre-processing was 99.6% (SD \pm 4.7) and TNC viability ratio post-thaw to pre-processing was 64.8% (SD \pm 15.4) (Fig. 6b). Although there was no statistical difference in TNC viability ratio at post-processing between Celleffice CB (HES or SALINE harvesting) and SEPAX ($P > 0.05$), there was statistical difference in TNC viability ratio at post-thaw between Celleffice CB (HES or SALINE harvesting) and SEPAX ($P < 0.05$).

Microbial contamination

Results of sterility testing confirmed that both HES harvesting and SALINE harvesting caused no incidence of infection during processing and freeze/thawing (data not shown).

Volume reduction during processing

For this study, 35–94 ml CB was processed with Celleffice CB; volume post-processing ranged from 19 ml to

Fig. 5. Hemoglobin level. Mean \pm SD are shown. Pre-processing and post-processing ($n = 13$ for each harvest solution of Celleffice CB, $n = 9$ for SEPAX), Post-thaw ($n = 8$ for each harvest solution of Celleffice CB, $n = 9$ for SEPAX).

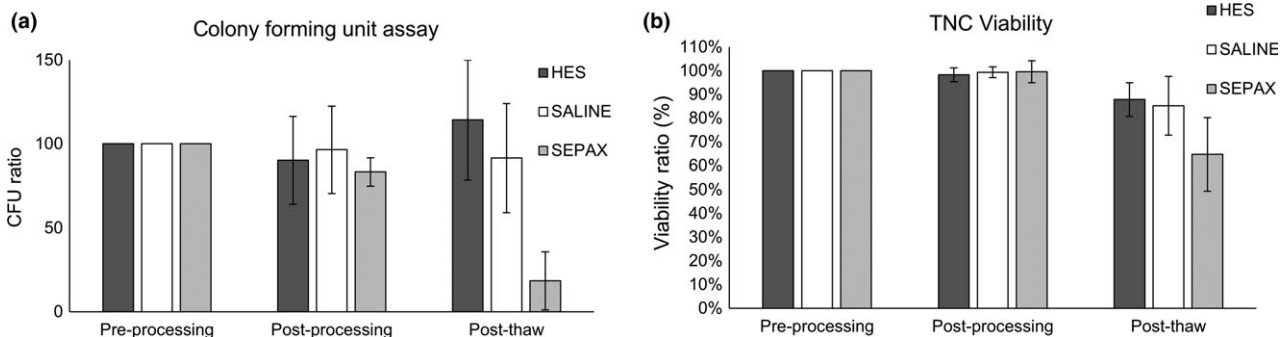
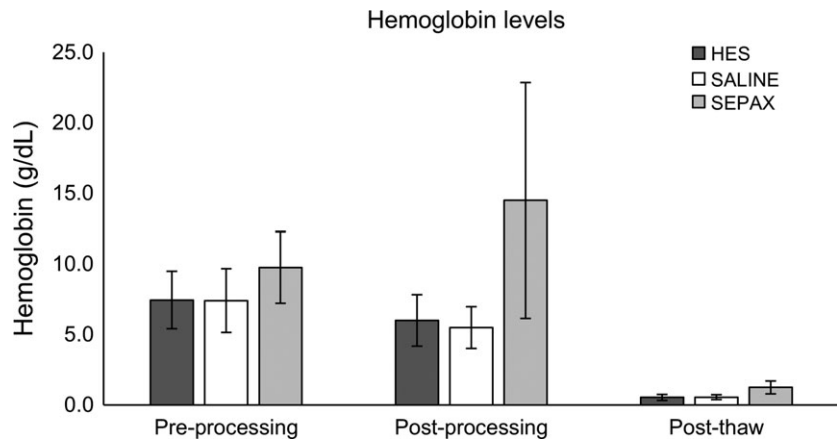


Fig. 6. Freeze/thaw analysis. (a) Clonogenic assay and (b) TNC viability. Mean \pm SD are shown, Pre-processing and post-processing ($n = 13$ for each harvest solution of Celleffice CB, $n = 9$ for SEPAX), Post-thaw ($n = 8$ for each harvest solution of Celleffice CB, $n = 9$ for SEPAX).

22 ml for HES harvesting and 21 ml to 23 ml for SALINE harvesting (Table 4). For SEPAX, 50–142 ml of CB was processed and volumes post-processing ranged from 24 to 25 ml.

Discussion

CBUs stored globally increase each year. However, early CBUs stored were either unprocessed units with added DMSO cryoprotectant, or minimally reduced units. Due to large numbers of red cells in these early units, it is likely that many of them can never be used clinically. Indeed, there have been incidences where entire collections of larger stored units had to be discarded. The urgent need for volume-reduced and red cell reduced units led to many different systems being designed, both automated and manual. However, the majority of these use one form or other of centrifugal force, relying on haemoglobin molecular weight.

Centrifugal force is well known to have detrimental effects on cells if applied incorrectly. In addition, we believe that stem and progenitor cells following mitosis are more vulnerable to centrifugal effects. Nevertheless, requirement for human CB in haematological transplantation and regenerative medicine increases both in paediatric and adult settings (6,11,29). Increasing collections of high quality CBUs globally are needed to fulfil future needs, even if regenerative medicine is still considered to be in its early stages. Estimates that current CB storage is sufficient, have been historically flawed being based on only haematological transplantation requirements and current stocks of CBUs (including larger units already noted as not being ideal). There is therefore, a requirement for an optimal system for CB processing which fulfils needs of cost, global reach and quality of the final product.

Such an optimal system would ideally reduce RBCs efficiently, as RBC-replete CB has been associated with negative clinical outcome (19,20). Our results demonstrated that the CelleEffic CB device produced highly effective reduction in RBCs and in free haemoglobin. CelleEffic CB achieved RBC depletion of $70.9 \pm 11\%$ and $73.5 \pm 11.8\%$ for SALINE and HES harvesting respectively. SEPAX achieved lower RBC depletion, namely $56.9 \pm 25.5\%$. In this study, SEPAX apparatus was used with the UCB protocol (no HES). RBC depletion of $47.5 \pm 9.1\%$ has also been reported for SEPAX operating without HES (30), making it considerably lower than when using SEPAX with HES ($88.28 \pm 5.62\%$) (31). Taken together, these data indicate that CelleEffic CB is superior in RBC depletion than SEPAX when operating without HES while being inferior when using HES. However,

current trends are in the direction of omitting HES in processing CB and for this condition, CelleEffic CB is superior to SEPAX.

Many public CBB have historically decided to choose final CBUs for storage or for transplantation based on final TNC or MNC content (32,33). While this does not in itself specifically describe content of CBU in terms of stem cells, it has become standard practice. Noteworthy, CelleEffic CB processing maintained excellent post-processing TNC and MNC levels, both with HES ($76.1 \pm 8.7\%$ and $79.3 \pm 9.5\%$, respectively) and with SALINE ($73.4 \pm 5.9\%$ and $79.3 \pm 11.1\%$ respectively) as cell harvest solution. TNC recovery is in the range of those reported for SEPAX system with HES ($76.76 \pm 7.51\%$) as well as AXP without HES ($78.81 \pm 7.25\%$) (31). Furthermore, it is of importance that we did not pre-screen the CBU for highest TNC count or highest volume, to avoid any bypass in the data which is similar to PrepaCyte data described previously by us (27). However, novel scoring system analysing and evaluating multiple markers at different times in order to optimize selection of CBU for transplantation are underway (34). Importantly, these markers, such as CFU and CD34 next to TNC and MNC were also highly recovered by CelleEffic CB processing. To prove that our system, CelleEffic CB, is at least equally efficient in these parameters as a well-established, automated system, we chose to compare CelleEffic CB data with SEPAX. We found similar TNC recovery ratios with all methods (CelleEffic CB using HES harvesting recovery was $76.1 \pm 8.7\%$, CelleEffic CB using SALINE harvesting was $73.4 \pm 5.9\%$, SEPAX was $76.6 \pm 16.1\%$). There was also no statistically significant difference in MNC recovery ratios when comparing all methods (CelleEffic CB with HES recovery was $79.3 \pm 9.5\%$, CelleEffic CB with SALINE was $79.3 \pm 11.1\%$, SEPAX was $79.8 \pm 10.9\%$). Furthermore, CD34+ cell recovery ratios were similar between all methods compared to slightly but not statistically significant better recovery for CelleEffic CB, with both harvesting solutions (the recovery for CelleEffic CB with HES was $75.2 \pm 12.2\%$, CelleEffic CB with SALINE was $80.2 \pm 15.2\%$, SEPAX was $68.8 \pm 17.2\%$). Last but not least CFU analysis was performed. No differences were found between all methods analysed post-processing (for CelleEffic CB with HES, CFU ratio was found to be 90 ± 26 , CelleEffic CB with SALINE was 97 ± 26 , SEPAX was 83 ± 8). However post-thaw, CelleEffic CB was found to be superior to SEPAX (for CelleEffic CB with HES, CFU ratio was $114 \pm 36\%$, CelleEffic CB with SALINE was $92 \pm 32\%$, SEPAX was $19 \pm 17\%$), whose tendency to post-thaw CFU reduction was similar to our previous data (27).

One of perhaps the most essential issues in the world of CB banking has been final storage volume. Long-term and increasingly large stores of CB in liquid nitrogen have significantly increased long-term storage costs involved. Historically, units were stored unprocessed with added DMSO. However, many of the public and private banks who chose this format, now regret it as tank space is a severely limiting factor with annual elevating costs. The CelleEffic CB device was successful in reducing overall volume of final units to a level (19–22 ml for HES and 21–23 ml for SALINE) in line with any of the currently available automated systems (24.06 ± 1.30 ml for SEPAX and 24.61 ± 3.64 ml for AXP) (31).

More than final volume and more than the issue of red cell depletion is content remaining in the unit of the stem and progenitor cell compartment. Without the early haematological stem cell compartment, the unit is not suitable for blood-related transplantation. Without the early lineage-negative populations the unit is not suitable for regenerative medicine solutions. These lineage-negative cells are known to be able to differentiate into bone, muscle, endothelial, epithelial and neural cells (35–37). It was important to see whether CelleEffic CB maintained both these compartments effectively and at high percentages post-processing. Flow cytometric analysis of the post-processed product highlighted existence of early, mid and late haematopoietic stem cells, multipotent lineage-negative stem cells and circulating mesenchymal stem cells. Additionally, using porcine bone marrow in a different study, we have shown that porcine bone marrow-derived MSC-like cells, having plastic adhesion and proliferation potency, can be isolated by CelleEffic CB strengthening our assumption that CelleEffic CB can indeed isolate MSC-like cells. Analysis of early, mid and late progenitors in the haematological hierarchy was demonstrated to be higher with the CelleEffic CB device than other processing systems currently available (data in preparation and in comparison to 27,28). Importantly, for demonstration of biological activity after processing the cells with the filter via sieving effect and adhesiveness, CD34⁺ cell differentiation was carried out and shown to be preserved *in vitro* (26). Also lymphoid and myeloid engraftment potential of Lin⁻CD34⁺ cells was retained *in vivo* (26). This allows the conclusion to be drawn that processing by the filter [consisting of non-woven fabric described by Shima *et al.* (26)] – without any chemical or antibody coating – does not influence biological activities of the isolated cells. Furthermore, to be more flexible, the filter can in principle be manufactured in different sizes to allow processing of also other volumes than the described range.

A particularly important further advantage of CelleEffic CB to us, is that there are no geographical

boundaries to use of the device as it does not require electricity, nor does it require refrigeration to store before processing. As it is a manual system requiring no electricity, no centrifugation, no additional equipment, no computer interface and therefore no software, it is easy and reproducible to use. This also addresses the challenge of reducing costs of the whole process (including harvesting, processing and storage) without compromising either quality or safety levels, as requested by Petrini (38). No servicing and no replacement parts are required. Unlike some automated systems, there are no complicated parts to dispose of or to incinerate, such as metal rotors, making disposal easy in the normal hospital environment. Significant lack of CBT and banking in many African, Asian, South American and even poorer European countries, is a problem for open and fair development of transplantation using CB. We believe that CelleEffic CB allows CB development in all geographical regions.

Finally, in a previous publication, we used mouse CB to prove bone marrow reconstitution potential of cells collected with our device (26). We demonstrated the same engraftment potential based on lymphoid and myeloid cells and repopulation of bone marrow with human CD45⁺ cells, between cells processed with our device and a conventionally used method. Repopulation of mouse bone marrow with human haematopoietic cells is considered to be the experimental gold standard for validation of biomarkers for haematopoiesis (32). Taken together with the extensive data we present here (summary in Table), one might expect that the CelleEffic CB device will be perfectly suited for routine preparation of CBUs for transplantation as well as for regenerative medicine.

In conclusion, we have developed a filter-based device, CelleEffic CB, for processing CB, which does not require centrifugation and thus omits the need for electricity, maintenance, and software, as well as reducing stress on the cells. The device not only addresses guidelines and recommendations by authorities in reducing RBC, and avoiding use of HES when desired, but also efficiently recovers TNC, MNC, CD34⁺ cells as well as HPC and other stem cells, based on differential cell counting in combination with cytometric analysis and CFU counts. Thus, CelleEffic CB is an attractive system for processing CB in private as well as public CBB or for immediate use.

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Competing interest

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