Effects of red blood cells on the final product of automated closed system in stem cells isolation and its elimination method

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ABSTRACT

Nowadays, the use of umbilical cord blood (UCB) stem cells is promoting for related and unrelated donor transplantation. A successful transplant happens when quality of isolated cells will be high. Therefore, in addition to yield of quantitative production, to provide safe and effective products is very important in cord blood banks, because these products have to create the conditions and results allowing a therapeutic exploitation. So these principles are the main aims of cord blood banks to achieve an accurate transplantation from stem cells (originating from: bone marrow, peripheral blood, cord blood). For this purpose, it requires some important technological innovations. This goal remains to be achieved for cord blood cells too. In many researches, quality of fresh samples and final products after thawing are evaluated by staining. For assessing the quality and recovery of UCB cells following laboratory manipulation in a study, for example, mononuclear cells (MNC) from fresh (<48 h old) and thawed UCB units were stained with 7-amino-actinomycin D (7-AAD) and illustrated revealing 2-3% dead cells. The frequencies of apoptotic cells in fresh and thawed sample were similar. However, UCB held for 72 h showed higher levels of cell deterioration. Finally, the utilization of an automated closed system “Sepax®” with washing after thawing is one of the best solution methods before transplantation.

INTRODUCTION

Three different sources of bone marrow (BM), peripheral blood progenitor cells (PBPCs), and umbilical cord blood (UCB) are available for hematopoietic progenitor cells (HPCs)12]. UCB was recognized as a rich source of HPCs for potential clinical use such as the pediatric setting, oncohematologic and inherited diseases5,9,10,12] and the first successful hematopoietic cell transplant in which UCB, instead of marrow that was performed in Paris, France, in 19887,13] accepted that UCB could be an alternative source of HPCs for transplantation13,9]. Stem cell therapy is currently mainly under clinical investigation for cardiac and orthopaedic purposes4,6,8]. More than 30 years ago it was demonstrated that significant numbers of primitive hematopoietic cells are found in UCB and Over the past decade, its banking and transplantation have increased significantly worldwide7]. Until 2001, more than 1200 familial and unrelated UCB transplants have been performed

KEYWORDS

Washing; Umbilical cord blood; Transplantation; Sepax®; Automated closed system.
Effects of red blood cells on the final product of automated closed

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worldwide for the treatment of a variety of malignant or hereditary blood diseases, immunologic defects, and metabolic disorders and the greater uses of UCB were resulted when banking facilities with large numbers of HLA-typed UCB samples are established to provide UCB allografts for transplantation, especially in unrelated recipients[13]. However, the experience in developing countries is still limited[7].

In 1999, it is showed that successful in engraftment and overall survival correlated with nucleated cell (NC) dose and CD34+ cell content[13,5], then, they are the important paramounts to ensure a high recovery of HPCs and a minimal loss of function when UCB is processed[13]. Total nucleated cell (TNC) lead to the necessity of saving as much as possible the total number of cells contained in the UCB unit not only in the prestorage manipulation step but also in the postthawing phase, immediately before the reinfusion to the patient[9]. The freezing process requires the addition of cryoprotectants to prevent cell injury from low temperature effects[11]. In this regard dimethyl sulfoxide (DMSO), the most commonly used penetrating cryoprotectant, is added up to 10 percent (vol/vol) to reduce intracellular ice formation and solution effects during freezing and ameliorate the transfusion-related side effects[11]. If thawed UCB transfusion is usually transfused without manipulation, the cells, DMSO, and lysis products enter into the patient’s blood. It is well documented that during transfusion and/or in the following hours several complications can occur ranging from minor (nausea and vomiting, cough, flushing, rash, chest tightness, chills, abdominal pain, and hypotension) to severe (renal failure and cardiovascular as well as neurologic complications). These transfusion-related complications are mainly attributed to DMSO content but also to the transfused cell debris and released intracellular products. Washing out DMSO in thawed UCB is performed by use of defferent approaches[11]. Therefore, the commonly practiced methods of reducing the number of RBCs and plasma volume in either closed or open systems, employ manual or semi-automated centrifugation with or without the addition of exogenous media, such as ficoll, percoll, hydroxyethyl starch (HES), dextran, poligeline, and gelatin on the large-scale banking of UCB, in particular the standardization of collection and storage procedures[13]. The volume reduction protocol of the closed centrifuge allows for the highest concentration, and therefore, is a promising candidate for instant stem cell therapy[8]. Also, many of published results show that the direct transfusion of thawed HPCs associate to transfusion-related side effects that are thought to be dose-dependent on the infused dimethyl sulfoxide (DMSO)[11] and some patients may sometimes develop DMSO-related life-threatening complications as acute renal failure or cardiac and pulmonary arrest[9]. As it is mentioned, Washing after thaw, increase cell losses[5]. So, to minimize the complications, cryopreservation of grafts highly concentrated with nucleated cells (NCs), the use of less DMSO in the preservation solution, mixture of cryoprotectants, fractionated transfusion, and CD34+ cell selection techniques have been applied[11]. Then, washing PT was justified by the in vitro inhibitory effect of dimethyl sulfoxide (DMSO) on progenitor cell viability and clonogenicity, better control over the thawing conditions, and reduction in infusional toxicity related to DMSO[5,1]. Various washing methods have been proposed to abolish or significantly reduce the DMSO-related complications, diminishing the cryoprotectant content after thawing that the most of these methods need a long manipulation time and are not free from a significant cell loss with a potential negative impact on the graft take[9]. To date, Current standard postthaw (PT) processing includes a wash step to remove dimethyl sulfoxide (DMSO), lysed red cells, and stroma[5]. Recently, the new automated closed separation device (Sepax® S-100 Biosafe SA) has showed interesting results[9] that it is used in most of cord blood banks (CBB) in worldwide[10]. The use of this device and its kits are safe and efficient in terms of recovery and viability of nucleated and progenitor cells[11]. The application of Sepax® (Biosafe SA) allowed RBC depletion before cryopreservation and washing of the thawed UCB units before infusion while getting good recoveries[5,10] and moreover, cord blood banks should participate in the improvement and/or development of new technology in order to minimize risks during the manipulation of UCB grafts[10]. The rationale behind RBC depletion is only to reduce the total volume of the product being frozen to maximize storing capacity[5].

According to performed researches in many of CBBs, Sepax® S-100 showes acceptable recovery
efficiencies for total nucleated cells (TNC), mononuclear cells (MNCs) and CD34+ cells, short processing times and clinical safety, without microbial contamination, in a closed kit and using a traceable protocol\[^{10}\]. In these researches, cryopreserved CB units are thawed, and cryoprotectant is usually removed before clinical use.\[^{10}\] To achieve this, several techniques have been proposed, aiming at maintaining cell viability and functionality\[^{10}\].

At present, the more widespread technique used is that introduced by Rubinstein \textit{et al.}, based on a manual two-step washing method which reduces dimethylsulphoxide (DMSO) content and minimizes osmotic shock to the cells. Although this technique is widely used in CB transplantation, is manual approach results in unpredictable cell losses, and cell clumping is not infrequent. Some authors have also proposed direct infusion without washing as an alternative, but clinical outcomes are limited and published biological data suggest advantages of washed cells in terms of clonogenic activity and viability\[^{10}\]. Also, direct infusion of the thawed cells at the bedside could produce complications related to DMSO toxicity as well as lysed cells and microaggregate exposure after infusion\[^{10,11}\]. To minimize these risks and to facilitate interlaboratory reproducibility of the washing results, we propose an automated washing procedure using the Sepax\textsuperscript{®} S-100 device, which shows a good recovery of nucleated and progenitor cells. The washing process is performed automatically and uses an initial 1 : 1 dilution with a hyperosmolar buffer containing dextran and albumin, and may be one step towards full CB banking automation. Furthermore, dissemination of this good manufacturing practice (GMP)-based technique could improve reproducibility of results among different laboratories, favouring standardization of the washing procedure in transplant centres\[^{10}\].

Final product cryopreservation of UCB In the transplantation, the time interval between collection of UCB final product and transplantation is several days up to weeks. To keep the prolong time of the separated product, it is cooled slowly at a controlled rate and stored at -196°C in the vapor phase of liquid nitrogen. Cooling the cells slowly avoids intracellular ice building, which can cause rupture of the cell membrane, however, it may result in dehydration of the cells by formation of extracellular ice and to prevent this, a cryoprotectant, such as DMSO, is added. It is used the most widely and thus, cryopreserved UCB product can be stored for years\[^{12}\]. The presence of the cryoprotectant and changes resulting from the freezing and thawing process necessitate special precautions during and after the infusion of the HPC product into the patient.

DMSO

DMSO is a polar compound with cryoprotectant properties that increases tolerance of cells to freezing at slow cooling rates to withstand osmotic stress. DMSO decreases amount of water taken into ice crystals at any given temperature and penetrates the cell membrane, allowing a high concentration within and external to the cell because it decreases the degree of cellular dehydration and osmotic stress. RBCs undergo lysis when the product is thawed. Thus, the thawed final product contains granulocyte debris (e.g., membrane fragments and enzymes), RBC stroma, and free Hb. These contaminants may cause side effects when infused into the recipient. DMSO is converted to dimethylsulfone (DMSO\textsubscript{2}) and dimethylsulfide (DMS\textsubscript{2}) and eliminated by urinary excretion\[^{12}\].

Sedation, headache, nausea, and dizziness are the most common symptoms after cutaneous application. DMSO is also known to induce histamine release, causing hypotension or anaphylactic infusion reactions. Intravascular hemolysis, hyperosmolality, and increased serum transaminase levels have been reported. The LD\textsubscript{50} values reported infusion of DMSO are 2.5 g per kg for dogs and greater than 11 g per kg for monkeys\[^{12}\].

DOSE OF DMSO

The dose of DMSO given during the infusion of thawed BM or PBPC varies. Some studies report doses between approximately 0.2 and 0.7 g per kg of the recipient, and some report absolute doses between 15 and 92 mL of DMSO, which may equal up to approximately 1 mL per kg in a normal size adult\[^{12}\]. Most commonly, HPCs are cryopreserved in 10 percent DMSO\[^{12,11}\]. Nevertheless, some studies show satisfactory results after using 5 percent DMSO as cryoprotectant and storage for several months\[^{12}\].
INFUSION OF UCB

Cord blood is cryopreserved in 10 percent DMSO, but is usually washed after thawing to remove DMSO and free Hb\textsuperscript{1,2,4}. The volume of the separated UCB with Sepax® Biosafe SA and thawed is 20 mL. Therefore, there is a lower rate of side effects, so the volume of amount of DMSO being infused must be related to the patient’s size. Regarding the infusion of UCB, recommendations for thawed products and ABO-mismatched products apply\textsuperscript{4,11}. Because of the small volume of the products, it is necessary that a method be applied to minimize cell loss. Therefore, the most method to solve this problem is the use of Sepax® S-100 that it is not only fast but also effective. The most method for thawing of UCB products frozen is rapid thawing in a 37°C-waterbath\textsuperscript{13}.

AUTOMATIC WASHING

The UCB bags stored in liquid nitrogen are thawed by immersion in a preheated 37 °C waterbath. Once thawed, the UCB bags were weighed, samples are taken for laboratory analysis and the bag is then connected to the prototype kit designed for UCB cell washing (Biosafe, Eysins, Switzerland) and processed using the Sepax® S-100 (Biosafe). Briefly, the UCB bag is connected to the washing kit (Figure 1) in a sterile flow hood. This kit has two additional bags: one for the final product and a second for the waste product. The final product remains in equilibrium for 5 min while agitates. The washing is then started\textsuperscript{10}.

Mean ±SD recoveries are calculated for all the analysed cell subsets by using the formula\textsuperscript{10}:

\[
\% \text{Recovery} = \left[ \frac{\text{absolute cell count postwash}}{\text{absolute cell count post thaw}} \right] \times 100
\]

COMPARISON RESULTS OF AUTOMATIC AND MANUAL WASHING PROCESSES

In a performed research by Rodríguez et al., the automatic procedure results in a recovery of 93% CD34+ cells and 89% TNC; no significant differences were observed between methods. As shown in the results of TABLE 1, no considerable discrepancies were observed in terms of cell recovery when manual and automated washing methods were compared. In done experiences, the manual method takes 70 min (range: 65–90 min) to perform, and only RBC recovery is significantly different. The automated washing using the Sepax® S-100 protocol takes 28 min (range: 26–30 min) to perform and has resulted, as for the manual washing procedure, in an absence of microbiological contamination during the process after the washing procedures. TABLE 2 showes, respectively, the cell content and the relevant recovery data of UCB units before and after the automatic washing process. As it is showed, after washing more than 50% of the thawed RBC and platelets were eliminated.\textsuperscript{10} After UCB automated washing procedures, the recovery of TNCs, CD34+ cells and MNCs was 89% (range: 83–95), 93% (range: 87–104) and 94% (range: 87–104), respectively\textsuperscript{10}. Non-specific measured cell losses were, on average, 2 ± 0.5%.\textsuperscript{10} For fresh units (prefreeze), the recovery of TNC was 86% (range: 74–88) for the automatic washing procedure and 89% (range: 84–104) for the manual washing procedure.

METHOD

In this consideration the effect of red blood cells on final products and their quality is evaluated. The automatic method is compared with the manual washing procedure. For this purpose, viability and mean recovery of nucleated cells (TNC) post-thaw are evaluated.

RESULTS

Umbilical cord blood (UCB) products are thawed using an automatic washing method intended to reduce DMSO toxicity and remove RBC. The results show that the automatic washing method has no significant differences on final products. So mean TNC recovery depends on thawing method.

DISCUSSION

It is proved that the automatic washing method is as effective as the manual method on viability, but this procedure is faster and easier for the operators. Safety of final products is very important that the automatic
The method is safe and suitable for washing of thawed final products. So an accurate washing method after thawing is necessary.

In Table 3, Phenotype % of Collected, Red Cell-depleted and CD34+ cell-enriched CB by both of two protocols Ficoll and HES are compared.

### Table 1: Recoveries comparison of results from automatic and manual washing processes

<table>
<thead>
<tr>
<th></th>
<th>Automatic Washing</th>
<th>Manual Washing</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC*10^9</td>
<td>89±4</td>
<td>83-95</td>
<td>94±5</td>
</tr>
<tr>
<td>CD34+*10^6</td>
<td>94±4</td>
<td>87-104</td>
<td>92±3</td>
</tr>
<tr>
<td>RBC*10^10</td>
<td>50±11</td>
<td>32-70</td>
<td>39±4</td>
</tr>
</tbody>
</table>

NS = not significant

### Table 2: Comparison of UCB units content before and after the automatic washing process

<table>
<thead>
<tr>
<th></th>
<th>Pre-wash</th>
<th>Post-wash</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC*10^9</td>
<td>1.28±0.26</td>
<td>1.14±0.22</td>
<td>0.90-1.52</td>
</tr>
<tr>
<td>CD34+*10^6</td>
<td>1.73±0.76</td>
<td>1.61±0.69</td>
<td>0.65-2.5</td>
</tr>
</tbody>
</table>

### Table 3: Phenotype % of collected, red cell-depleted and CD34+ cell-enriched CB

<table>
<thead>
<tr>
<th></th>
<th>Collected</th>
<th>Depleted</th>
<th>Isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD45+/CD34+</td>
<td>CD34+/CD38+</td>
<td>CD45+/CD34+</td>
</tr>
<tr>
<td>Ficoll</td>
<td>2.58</td>
<td>0.17</td>
<td>3.06</td>
</tr>
<tr>
<td>HES</td>
<td>2.43</td>
<td>0.15</td>
<td>1.26</td>
</tr>
</tbody>
</table>

### Abbreviations

HPCs = hematopoietic progenitor cells; UCB = umbilical cord blood; CBB = cord blood banks; TNCs = total nucleated cells; PBPCs = peripheral blood progenitor cells; PT = postthaw; HES = hydroxyethyl starch; 7-AAD = 7-aminoactinomycin; RBC(s) = nucle-
Effects of red blood cells on the final product of automated closed

CONCLUSION

As is known, a major concern in UCB banking is the limited storage space available in nitrogen tanks. Therefore, it is imperative to reduce UCB volume before cryopreservation not only for maximizes storage space, but also decreases the toxicity resulting from the infusion of larger volumes of DMSO and hemolyzed products. It may also lessen transfusion reactions associated with incompatible RBCs in major ABO-mismatched UCB allografts. CD34+ and TNCs is relatively low in UCB and those are a crucial point for the transplant outcome. So, cell recovery after DMSO removal is an important aspect on which to be focused. The most important inconveniences are the efficiency in DMSO removal, the debris derived from the damaged cells, and the risk of microbial contamination. To date, they have been compared the different automated devices and manual methods in the thawed UCB setting to evaluate the possibility of efficiently removing DMSO, minimizing at the same time the cell loss.

Previous experiences have displayed a difference of effectiveness of dextran in the depletion of RBCs in UCB and marrow that it may be attributed to the greater proportion of trapped RBCs in the dead space near the infusion ports of the transfer bags associated with UCB of smaller volume. The relative abundance of RBCs may entrap MNCs during the process of density-gradient separation in the presedimentation UCB. Of course, these agents have an episodes of allergic reactions and anaphylactic shock (0.085% to HES and 0.15% to poligeline) after infusion. The by-products of postsedimentation RBCS and plasma supernatant can be saved for blood groupings, microbiology screening, and molecular studies so that nothing is wasted during the processing of UCB. This also eliminates cell losses that occurred in various UCB samples before processing.

In regard to previous researches again and comparing the manual and the automated washing methods, it was observed no significant differences in cell recoveries among methods. With Sepax it was obtained acceptable recoveries for all the cell subsets of clinical interest. The data of CD45+ cells, resulted in a wider range of values than those of TNCs, possibly because of the higher variability. Direct infusion after thawing may have been caused by infusion-related complications because of cryoprotectant and lysed cells such as slight nausea, vomiting and hypotension, severe renal failure and cardiovascular complications. Also, thawed products for direct infusion contain DMSO are still cold when infused and have a high osmolality and contain cell debris and some free Hb. ABO-mismatched products might be infused because of the importance of HLA matching. Adding the cryoprotectant, changes resulting from freezing and thawing, and transfusing blood products across the ABO barrier require special precautions before, during, and after the infusion. Therefore, Graft washing after thawing almost eliminates DMSO and cell lysis products and could prevent those undesirable effects mainly in paediatric patients who are more susceptible to such transplant-related toxicities owing to the greater ratio of the DMSO dose infused per kg and because they are currently the main UCB transplant recipients. Additionally, the washing process after thawing could be considered as a standard technique in CBB if current experimental applications, such as combined transplantation of two CB units from different donors, becomes a successful therapy in clinical trials, because of the associated additional toxicity when two CB units are transplanted. Although the viability of cells in the presence of high osmolarity solutions is time dependent and decreases rapidly, the direct infusion at the bedside, just after thawing, results in safe engraftment, possibly because of the rapid dilution of the high osmolarity solution in the bloodstream. Routine washing of UCB units after thawing could thus facilitate the infusion settings. In addition, other applications could benefit from the automated washing of UCB, such as the preparation of cells for expansion, culture or further manipulations. In summary, the evaluation of an automated process for UCB washing after thawing with a hyperosmolar buffer demonstrates that cell recoveries and progenitor cell viability are consistently high enough to consider such a technique as safe and suitable for clinical use with an important reduction in the DMSO content.

The SEPAX system could achieve the highest amount of separation with the volume reduction protocol that separates the NC cell fraction. There is no
evidence whether the injection of a mixed cell population of nuclear cells influences performance\cite{2-4}. Overall, Clinical use of UCB units follows an international approach, where units from CBB around the world are shipped to wherever they are required. So, the automation of UCB processing could help to standardize the wide range of laboratory techniques used by different CBB and transplant units, and could facilitate safe and reproducible procedures.

The results are quite similar between an automatic washing method and the manual approach in terms of viability and progenitor cells recovery, unless a processing procedure using sedimentation and washing in a automated closed system not only can reduce the RBC bulk, probable risks, the volume as well as recover HPCs in UCB, safe and suitable for the routine washing of thawed CB grafts in the clinic, but also is simple and fast for the operators to perform and efficient, reliable with a good cell recovery and a very high efficiency in DMSO removal.

So, The SEPAX® system (SEPAX®, Biosafe) that is a centrifuge which is a closed and fully automated system that is typically used to separate umbilical cord blood fractions under sterile conditions enables to help the physician to apply stem cell therapies in medical centres that do not have scientific laboratories. In addition, this system could lower costs and reduces the risk of infection\cite{4}. It is proved that the automatic washing method is as effective as the manual method on viability, but this procedure is faster and easier for the operators. Safety of final products is very important that the automatic method is safe and suitable for washing of thawed final products. So an accurate washing method after thawing is necessary.

Finally, With the Sepax S-100 device UCB can be processed and washed in a simple way, time saving and with little claim to the laboratory’s staff. The closed system reduces contamination risk during separation. The main advantage is the possibility of separating even small collection volumes and the fixable volume of the final product, which enables a more standardized freezing procedure and a constant quality of the blood products.

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