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EVALUATION OF A HYDROXYETHYL STARCH SOLUTION FOR DIMETHYL SULFOXIDE REMOVAL FROM MOBILIZED PERIPHERAL BLOOD USING AN AUTOMATED SYSTEM

Carla Cristina Pedrosa de Lira de Morais[1], BSc MSc. ; Juliana Dias[2], BSc PhD.
Luciglei Nazareth Baiense[1], BSc. ; Pedro Felipe Couto Vieira[1], BSc MSc.
Flávio Henrique Paraguassú-Braga[1], BSc MSc PhD. ; Luis Fernando da Silva Bouzas[1], MD MSc PhD.

1 Cell Processing Center/Umbilical and Placental Cord Blood Bank, Bone Marrow Transplant Center, National Cancer Institute.
2 Centre for Cell, Gene and Tissue Therapeutics, Royal Free Hospital London NHS Foundation Trust, London, UK. Research Department of Haematology, University College London, London, UK.

Corresponding Author: Carla Cristina Pedrosa de Lira de Morais - cliramorais@gmail.com - ORCID: 0000-0003-0553-7272

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ABSTRACT

Background and objectives: This study evaluates the efficacy of synthetic colloid hydroxyethyl starch for use as a washing solution to remove DMSO from hematopoietic stem cells cryopreserved grafts in comparison to a crystalloid based solution. **Materials and methods:** We evaluated samples of cryopreserved mobilized peripheral blood (MPB) from 6 (six) patients that had not been used for transplant. For comparison, we used two equal bags of the same collection procedure, allowing the analysis of two different solutions simultaneously. Washing solutions were used: a crystalloid solution (solution 1, sodium chloride 0.9%) and a colloidal solution (solution 2, hydroxyethyl starch 6%), both added with human albumin 2.5%. The washes were performed using the SEPAX2™ (Biosafe) system automated methodology, using the CS-600.1 kit (Biosafe), according to the washing protocol established by the manufacturer. **Results:** The washing solution containing HES showed a statistically significant increase in the recovery of CNT and CD34+/CD45+ cells (p = 0.0313, both), in addition to a greater number of CFU-GM colonies (without statistical significance) when compared to the 0.9% sodium chloride solution. Furthermore, the wash solution containing HES also prevented significant clumping, contrary to what was observed in the wash with 0.9% sodium chloride solution. Conclusion: This work shows that the colloidal washing solution containing hydroxyethyl starch is a good option for DMSO removal procedures in samples of cryopreserved mobilized peripheral blood, maintaining the CD34+ cells viability and functionality and reducing the cell clumping.

Keywords: Cryopreservation. Dimethyl Sulfoxide. Mobilized Blood. Stem cell processing. Hematopoietic Stem Cells. SEPAX 2™.

INTRODUCTION

The first successful bone marrow (BM) transplant, performed in 1957 by Edward Thomas, led to the worldwide use of hematopoietic stem and progenitor cells (HPC) for the treatment of patients with hematologic and non-hematologic diseases, by promoting recovery of the hematopoietic activity after receiving high-dose chemotherapy[1]. In some cases, cryopreservation of the graft is necessary and an essential step for the clinical and therapeutic approach of HPC transplantation.

There are standardized cryopreservation protocols for HPC from different sources that guarantee the viability of these cells after thawing. Successful cryopreservation mainly encompasses the rate of temperature decay of 1 to 3 °C/min during freezing and the combination of cryoprotective agents such

as Dimethyl Sulfoxide (DMSO) (5%) and hydroxyethyl starch (HES - 6%) that prevent formation of ice crystals and preserve the survival of mature granulocytes after thawing, thus avoiding gel formation and macroscopic agglutination caused by cell lysis after thawing, releasing nucleoproteins and lysosomal enzymes, observed when using only DMSO as a cryoprotective agent[2].

Despite the cryoprotective action of DMSO, its use is associated with some adverse events during infusion, due to its dose-dependent toxicity[3,4]. These adverse events are related to allergic reactions, gastrointestinal, renal, cardiovascular, neurological and liver toxicity[5-9]. Based on this, Junior et al recommend that the maximum daily dose of infused DMSO be adjusted to 1 g per kg of body weight[5]. However, these toxic effects can be reduced by removing the DMSO by washing the product after thawing[10]. Over the past two decades, different methods and technologies for DMSO removal have been developed, including the use of different washing solutions, to minimize problems such as cell agglutination, loss of HPC and risk of bacterial contamination[10-17].

In general, the washing solutions that are used to remove DMSO consist of saline or cell culture medium plus osmotic active formulation, such as albumin and/or acid citrate dextrose, or non-permeable macromolecules such as dextran. These agents are not toxic to the cells and provide a hyperosmotic extracellular environment that buffers the hypertonic intracellular compartment created by DMSO, preventing osmotic damage to the cells[6, 10, 13, 14, 16, 18]. However, the increasingly frequent worldwide shortage of critical reagents, such as the qualified dextran, represents a major technical challenge for Cell Therapy Facilities[19], especially when DMSO removal is critical. Thus, this study evaluates the use of synthetic colloid hydroxyethyl starch as a constituent of the wash solution by comparing its performance with a saline-based solution, after DMSO removal using an automated system.

MATERIAL AND METHODS

Samples

Twelve mobilized peripheral blood (MPB) samples from six patients, collected between 2001 and 2014, were used after discarded with the authorization of the medical direction of the Bone Marrow Transplantation Center following the patient's death. The collection of MPB from the patients was done by apheresis 4-5 (four to five) days after administration of Granulocyte colony-stimulating factor human (G-CSF). Samples were selected according to the number of bags available for each patient from the same collection day. Those with a minimum of 2 (two) identical bags were selected for this study, allowing direct comparison of the two methodologies. Each frozen bag contains a concentration of $<3x10^8$ TNC/mL in volume of 100 mL with cryoprotectant solution composed of DMSO 5%, HES 6% and human albumin 2.5% and stored at -80 °C.

Washing solutions

Two washing solutions were evaluated in this study. Solution 1 was a crystalloid-based solution, consisting of sodium chloride 0.9%. Solution 2, was a colloidal solution, containing hydroxyethyl starch 6% (130/0,4) (Voluven™, Fresenius Kabi). Human albumin (Alburex™, CSL Behring) was added to both solutions, to a final concentration of 2.5%.

Thawing and washing

Grafts were thawed in a water bath at 37° C, an aliquot immediately removed for pre-wash analysis and the bag immediately submitted to the washing protocol. An automated washing methodology was performed with the SEPAX 2™ system (Biosafe) using a specific kit for washing (CS 600.1 kit Biosafe) and following the *SmartWash* v.314 program protocol, as established by the manufacturer. For each bag, a dilution ratio of 1.0 and input and output volume of 100 mL were set, with a total procedure time of around 25 min per sample. For each patient, the thawing of two equal bags and each step of the washing procedure were performed in parallel, using each of the two solutions in two different SEPAX 2™ devices.

Product analysis

As criteria for protocol evaluation and validation, after thawing and washing, the recovery of TNC, number of $CD34^+/CD45^+$ viable cells, number of colonies forming units and cell clumping were accessed. TNC count was performed using an automated hematological counter (ABX Micros 60).

Quantification of HPC $CD34^+/CD45^+$ viable was carried out by flow cytometry, according to the ISHAGE protocol[20] modified to include the viability dye 7-aminoactinomicina D (7-AAD, BD Pharmingen™). *In vitro* diagnostics approved anti-CD45 FITC (2D1 clone, BD Biosciences) and anti-CD34 PE (8G12 clone, BD Biosciences) were used, and samples were evaluated in an Accuri™ C6 flow cytometer (BD Biosciences).

For evaluation of the number of granulocyte-monocyte colony forming units (CFU-GM), 10^4 cells were plated per well in the semi-solid methylcellulose-based Methocult™ H4034 media (Stem Cell Technologies). CFU-GM colonies were identified by their characteristic morphology.

The presence of macroscopic clumps after washing was assessed by visual inspection. As a further quality control of the washing procedure, products were tested for sterility, before the cryopreservation and at the end of the process.

Clinical follow-up

After validation of the washing protocol for DMSO removal, products from 3 patients were submitted to washing. In our center, DMSO washing is not a frequent procedure, however, the establishment of an effective protocol is necessary. Indication criteria are chosen based on experiences with adverse events associated with DMSO during infusion, for example, whether five or more cryobags per patient or patient clinical problems that may increase the risk of adverse events, for example, disease progression, chronic ischemia, heart disease and others. For the clinical follow-up of these patients, number of frozen bags and number of washed bags, source of CPH and number of CD34/Kg cells infused were independently analyzed. In the post-transplant period, it was mainly verified whether there was any adverse event related to the infusion of the washed bags or any change in the graft time. Adverse events evaluated include headache, nausea, vomiting, change in blood pressure, tachycardia, fever, mucositis or irritation of the throat and others. For graft evaluation, the first day was determined by blood counts showing more than 500/mm^3 of neutrophils and 20,000/mm^3 of platelets for 3 consecutive days after 7 days without transfusion.

Statistical analysis

TNC and CD34$^+$/CD45$^+$ cells recovery was calculated using the following fórmula: % Recovery = (Pre-cryo or Post-wash ÷ Post-thaw) x 100

Data was plotted and analyzed using Graph Pad Prism 8.0.1 (GraphPad Software, San Diego, CA, USA, www.graphpad.com) and EXCEL (Microsoft Inc). Descriptive analysis, including calculation of median or mean ± standard deviation (SD) was performed. For each solution, data is expressed as a percentage of the post-thaw results, and comparison between the two solutions was performed by Wilcoxon test's. Differences were considered statistically significant when *p* value was less than 0.05.

RESULTS

TNC, viable CD34$^+$/CD45$^+$ cells and CFU-GM of products washed with both solutions were evaluated pre-cryo or post-thaw and post-wash, Table 1. During the washing of two paired bags, a mechanical problem occurred in one of the Sepax™ device, which performed the washing of the product using solution 1, this data being excluded from the analyses. The parameters were compared in grafts washed with saline-based solution 1 and colloid-based solution 2. Recovery of TNC after thawing and before washing was 35.8 ± 12.1%. After washing, the recovery of CNT was 28.0 ± 11.0% for solution 1 (p = 0.0625) and 39.0 ± 15.1% for solution 2 (p = 0.0313) if compared to pre-cryopreservation, and 82.9 ± 14.9% for solution 1 (p = 0.0625) and 110 ± 30.7% for solution 2 (p = 0.0313) if compared to post-thaw, Table 2.

In the analysis of viable CD34$^+$/CD45$^+$ cells, the mean recovery after thawing was 84.2 ± 58.4%. After washing, the recovery was 43.4 ± 38.3% for solution 1 (p = 0.0625) and 68.7 ± 54.9% for solution 2 (p = 0.0313) if compared to pre-cryopreservation, and 64.6 ± 26.1% for solution 1 (p = 0.0625) and 81.9 ± 18.9% for solution 2 (p = 0.0313) if compared to post-thaw.

The results show recovery of CNT and viable CD34$^+$/CD45$^+$ cells generally higher in products washed with solution 2, being statistically significant.

When comparing the number of CFU-GM colonies after washing with the number obtained after thawing, we observed a greater recovery of the number of CFU-GM in products washed with solution 2, 114.6 ± 21.4% (p = 0.0625), versus solution 1, 73.3 ± 31.0% (p = 0.1250), although there is no statistical significance.

Considering that the storage period in the freezer (-80 ºC) of the analyzed samples was between 1 and 13 years, the viability of CD34$^+$/CD45$^+$ cells was on mean of 67.2% ± 19.2% after thawing. In correlation analysis between CD34$^+$/CD45$^+$ cell viability after thawing and storage period, the results showed a moderate correlation, $R^2 = 0.66$.

Analysis of clumps formation during the wash protocol was performed by visual inspection. Significant cell clumping, that could not be dissolved by manual homogenization, was recurrently observed post-wash in all products washed with solution 1, although these were not a significant problem in products washed with solution 2 (Figure 1). An aliquot of 10 mL of each product was transferred to a conical tube after homogenization, in order to better evaluate the amount of clumps, confirming that they were present in great quantity in products washed with solution 1 (Figure 2A), although nearly absent in those washed with solution 2 (Figure 2B).

All samples selected for this study had negative pre-freeze blood cultures for aerobic and anaerobic bacteria and for fungi. No contamination was observed after washing.

Based on the results obtained, solution 2 was established in the laboratory washing routine to remove the DMSO, in an automated way, using the SEPAX 2 ™ system. In our institution, washing for DMSO removal is not routine, but it is required in situations where there is a need to reduce toxicity related to DMSO due to the patient's clinical condition at the time of transplantation or the excessive number of bags to be infused. Thus, after validation, 3 patients needed to have their product washed before infusion, and the results for each procedure are detailed in table 3. The products cryopreserved for an average of 107 ± 63 days at -80 ºC. After thawing, in a 37 °C water bath, each bag was subjected to the washing process following the established protocol described above.

After washing, TNC recovery and viability were evaluated. For these cases, the analysis of cell recovery after washing was calculated in relation to the number of cells before cryopreservation. Therefore, considering the total number of bags washed for each patient, the mean recovery of TNC, comparing before cryopreservation, was 100 ± 0% for patient 1, 92.9 ± 5.8% for patient 2 and 82.7 ± 15.4% for patient 3. For total cells viability after washing, the means were: 91.5 ± 6.9% for patient 1, 95.7 ± 0.8% for patient 2 and 97.6 ± 0.1% for patient 3.

To guarantee the efficacy and safety of the washing procedure, adjusting it to the number of bags to be thawed and infused and ensuring the quality of the product intended for the patient, this process was performed by 3 professionals. While one operator performed the washing protocol, the second performed the necessary quality control assays, such as evaluation of TNC recovery and viability analysis. A third member of staff was available at the patient's bedside to monitor adverse events during infusion and manage and coordinate the beginning of the washing procedure for the posterior bags, so that all products were infused within 1 hour of thawing.

In clinical follow-up, regarding the proportion of bags submitted to the washing protocol in relation to the total of infused bags, patient 1 only 5% were washed, while patient 2, 60% and patient 3 had all bags submitted to the washing protocol. Due to the small number of patients followed, variability of infusion conditions of washed bags and clinical characteristics of each patient, the impact of the washing procedure on transplantation was evaluated through the graft recovery time for each type of HPC source, occurrence of problems during thawing or washing and adverse events during the infusion. Our team followed each patient during infusion and post-transplantation to assess graft time. There were no problems thawing or washing the bags. For all patients followed, no adverse events were observed during the infusion of the washed bags and, in the post-transplant follow-up, the neutrophil and platelet engraftment times occurred within the expected time for each type of CPH source, 11 days to neutrophils in autologous MPB transplants and up to 32 days for cryopreserved BM, considering a minimum dose of 2-3 x10^6 $CD34^+$ cells/kg[21].

DISCUSSION

To preserve the potential of CPH during cryopreservation, it is necessary to use a cryoprotective agent such as DMSO, which is the most used[22]. However, adverse events may occur during infusion of thawed products and most of them are with DMSO toxicity[5-7]. Nevertheless, there are studies showing that the DMSO removal in MPB samples after thawing, by washing, reduces adverse events without adversely affecting the grafting[10, 15, 16].

To avoid HPC losses as well as to reduce the risk of contamination, several techniques and methodologies have been developed for DMSO removal, replacing the conventional method which is based on manual removal after centrifugation [23]. An automated washing system, such as demonstrated by the present study, provides time sensitive alternative, optimizes the washing process and reduces the risk of contamination due to the closed fluid path[7, 16, 17].

In 2011, Scerpa et al, showed that the automated washing procedure for DMSO removal, using the Sepax™ S-100 system, guarantees a better result in terms of recovering TNC, $CD34^+$/$CD45^+$ cells and total CFU without affecting cell functionality, when compared to the manual centrifugation procedure[24]. In this study, the automated Sepax2™ system has proven an effective method for routine removal of DMSO from MPB cryopreserved grafts after thawing, with the mean recovery of TNC, viable $CD34^+$/$CD45^+$ cells, CFU-GM count as well as maintaining test sterility of cell products. These results are compatible with those obtained by Rodriguez et al, 2004 showing that the wash for DMSO removal from umbilical cord blood units using the Sepax™ system is a secure method for maintaining cell function and a viable option for clinical routine[15]. In addition, according to Huvarová et al 2021[25], washing of cryopreserved transplants using Sepax 2 showed high recovery of hematopoietic cells, did not influence time to engraftment, and resulted in a satisfactory reduction of adverse effects and improved tolerance to the procedure.

Considering the long-term storage time of the samples used in the present work, studies show that MPB subjected to long-term storage at -80 ºC with uncontrolled freezing rate and cryopreservation with 5% DMSO combined with HES, can support hematopoietic reconstitution when compared to that of controlled rate freezing and liquid or vapor nitrogen storage[26-28]. In our analyses, the lower rates of $CD34^+/CD45^+$ cell viability were not associated with longer storage period in freezer -80 °C, the sample with the longest storage period used in the study, 164 months, had 92% cell viability $CD34^+/CD45^+$ after thawing.

For a reliable evaluation of the results, the criterion established for sample selection was that two discarded bags from the same collection day should be available for each patient and that the proper authorization for their use was obtained. With this approach, it was possible to guarantee that the analysis of each evaluated solution was comparable. Therefore, the number of samples available, according to the established criteria, limited the number of samples evaluated in this study.

Our results showed that the washing solution containing HES showed a statistically significant increase in the recovery of CNT and CD34+/CD45+ cells, in addition to a greater number of CFU-GM colonies when compared to the solution of sodium chloride 0.9%. Furthermore, washing solution containing HES also prevented the significant clumping, unlike what was observed in the wash with the sodium chloride 0.9% solution.

Literature shows that cell clumping is a major problem when centrifugation of thawed HPC products is performed[13] and this is likely due to DNA release from the fragilized cells, since DNAse treatment was shown to reduce clumping of cells during the thawing procedure[29]. Since the presence of these cell clumps is associated with clinical toxicity of infused products[6], the use of HES-based washing solutions is clearly advantageous over those constituted of isotonic saline. Thus, Larrea et al 2021, concluded in their study that HES can be used by observing the recipient's renal function to assess the need to adjust the proportion of HES to be used in washing DMSO[30].

Despite the small number, the post-transplant clinical follow-up of patients who had products submitted to the washing protocol showed that solution 2 did not harm the patient. Within the established criteria for analysis, no adverse events were observed in any of the patients during the infusion of washed products or delays in the recovery hematopoiesis. Therefore, the HES-based lavage protocol established in the study can be considered safe for patients, with no impact on infusion or HPC transplant outcomes.

In summary, this study shows that HES-based washing solution is a good choice to remove DMSO from cryopreserved MPB grafts, because it is satisfactory in maintaining the viability and functionality of HPC after thawing and washing. Furthermore, the Sepax 2™ automated system is a good alternative for thawed HPC products wash, allowing a high rate of cell recovery after the procedure and ensuring sterility of the samples. Thus, the proposed washing protocol can be effectively used in clinical HPC transplantation routine and, considering the increasing advances in cell therapy, we can prospect the use of this washing protocol for any product intended for cell therapy. Nevertheless, this HES based solution can even be adapted and validate for manual processing protocols, not restricting the use to automated systems.

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TABLE 1. Descriptive statistical analysis

	Pre-cryo (n=6)		Post-thaw (n=6) Solution 1 (n=5)			Post-wash Solution 2 (n=6)					
	Mean ± SD	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range
TNC (x10^9)	43.4 ± 13.1	30.8 - 67.9	14.7 ± 4.4	13.5	10.7 - 21.4	11.2 ± 3.9	12.1	6.8 - 16.8	15.9 ± 5.1	18.9	8.3 - 19.7
Total CD34$^+$/ CD45$^+$ viable (x10^6)	69.8 ± 40.1	8.1 - 128.8	55.3 ± 53.4	46.6	6.4 - 148.2	28.6 ± 29.9	14,0	3.3 – 65.6	48.3 ± 51.8	35.3	5.7 - 142.0
CFU-GM (x10^4)	a	a	34.0 ± 17.1	22.5	21.0 - 53.0	23.4 ± 19.5	16.8	8.5 - 51.5	40.7 ± 24.9	29.0	17.0 - 70.5

[a]Not shown because CFU-GM test was not performed on pre-cryopreservation product

Legend: This table shows descriptive statistics, including mean, standard deviation (SD), median and range of the number of TNC, the number of viable CD34+/CD45+ cells and the number of CFU-GM in the pre-cryo, post-thaw samples and after each washing procedure.

TABLE 2. Descriptive statistical analysis of recovery cells

Cell recovery pre-cryo vs Post-thaw (%)				Cell recovery pre-cryo vs Post-wash (%)						Cell recovery post-thaw vs Post-wash (%)					
			Solution 1 (n=5)			Solution 2 (n=6)				Solution 1 (n=5)			Solution 2 (n=6)		
	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range
TNC (x10^9)	35.8 ± 12.1	38.0	15.7 - 49.6	28.0 ± 11	30.0	10.1 - 38.2	39.0 ± 15.1	43.5	15.5 - 57.3	82.9 ± 14.9	79.3	63.9 - 102.5	110.0 ± 30.7	100.9	77.6 - 161.9
Total CD34$^+$/ CD45$^+$ viable (x10^6)	84.2 ± 58.4	81.6	11.6 - 163.8	43.4 ± 38.3	40.6	7.5 - 106.4	68.7 ± 54.9	54.4	11.3 - 157.0	64.6 ± 26.1	64.7	31.4 - 100.6	81.9 ± 18.9	86.1	54.6 - 100.6
CFU-GM (x10^4)	a	a	a	a	a	a	a	a	a	73.3 ± 31.0	75.3	40.5 - 102.4	114.6 ± 21.4	121.7	81.0 - 134.3

[a]Not shown because CFU-GM test was not performed on pre-cryopreservation product

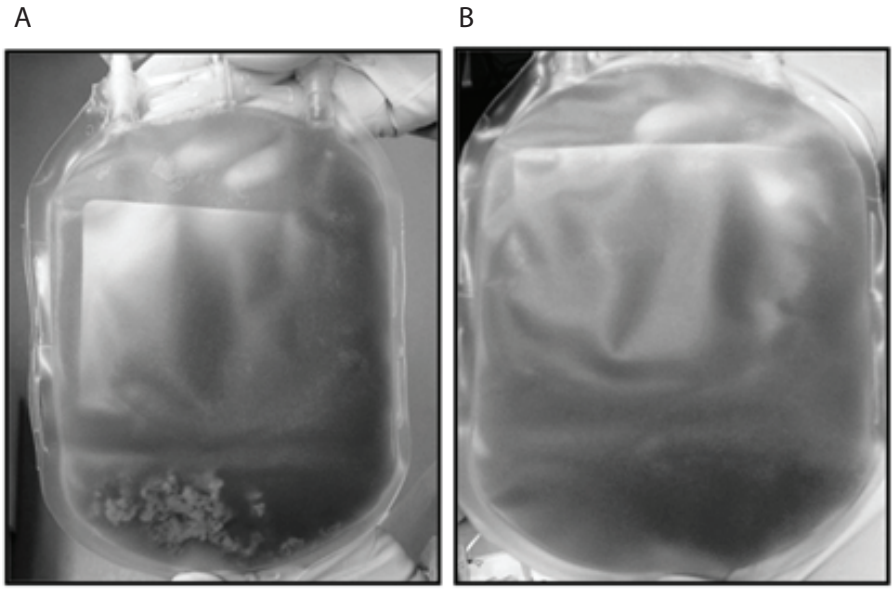
Legend: This table shows descriptive statistics including mean, standard deviation (SD), median and recovery rate range of TNC, CD34+/CD45+ and CFU-GM cells comparing pre-cryo, post-thaw and after each wash procedure.

TABLE 3. Detailed product information of patient washed for DMSO removal

	Patient 1	Patient 2	Patient 3
PATIENT INFORMATION			
Age (years)	60	62	14
Sex	Female	Male	Male
Diagnosis	LNH	MM	LLA-T/B
Transplantation Type	Autologous	Autologous	Alo-NR
Pre-transplant			
Mobilization Regimes:			
1ª mobilization	G-CSF only	G-CSF only	G-CSF only
2ª mobilization	G-CSF only	G-CSF only	-
PRODUCT INFORMATION			
HPC source	MPB/BM	MPB	BM
Total CD34/Kg (106)*	1.56	2.56	4.62
Total dose DMSO(g)/kg	0,85	0,81	0,31
Total cryopreserved bags	10	5	2
THAWING/WHASING/INFUSION			
Problem during thawing	No	No	No
Problem during whashing	No	No	No
Number of washed bags	2	3	2
Adverse reaction during infusion	No	No	No
TNC recovery post-wash (% - per bag)	100 /100	95.7/95.7/87.4	93.6/71.7
TNC viability post-wash (% - per bag)	86.6/96.3	96.2/96.2/95.1	97.7/97.5
Storage time at -80 °C (days)	199	92	64
POST-TRANSPLANT			
Grafting time (days):	14	11	24
GRAFT DATA:			
leukocytes (cels/mm3)	6100	3530	3670
neutrophils (cels/mm3)	3520	2676	1993
monocytes (cels/mm3)	1891	286	1369
Platelets (k/mm3)	32	37	65
erythrocytes (106/mm3)	3.15	2.73	3.26
hemoglobin (g/dL)	8.8	8.24	9.8
hematocrit (%)	27.1	24.27	26.5

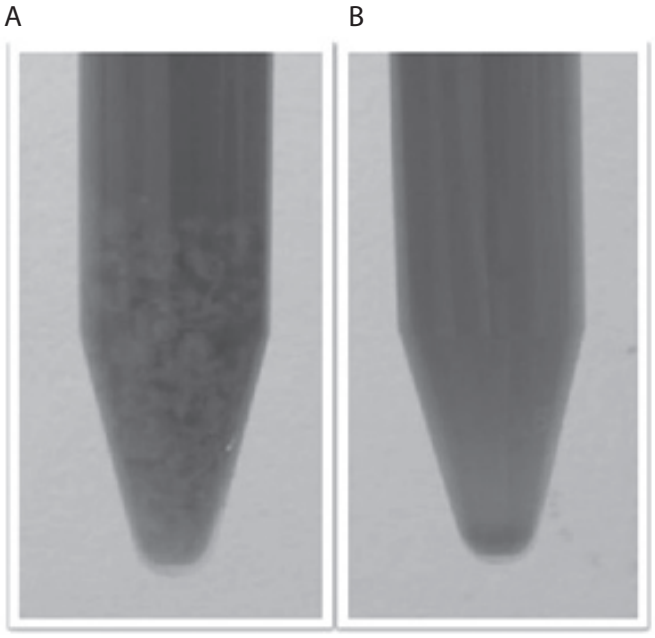
Abbreviations: MM, multiple myeloma; LLA-T/B, acute T and B cell lymphoblastic leukemia; BM, Bone marrow; G-CSF, Granulocyte colony-stimulating factor human; Alo-NR, Unrelated allogeneic.
*Pre-freezing enumeration
Legend: This table shows detailed information about the patients, infused product and post-transplant results.

FIGURE 1. Visual evaluation of macroscopic cell clumps.



Legend: *Figure A shows a large amount of cell clumps in the bottom of a bag of a representative sample subjected to washing with saline solution (solution 1). Figure B shows the absence of clumps in a bag of a representative sample subjected to washing with hydroxyethyl starch solution (solution 2). This result was consistently observed in all samples.*

FIGURE 2. Visual evaluation of macroscopic cell clumps.



Legend: *An aliquot of 10 mL of each sample was transferred to a 15 mL conical tube to better evaluate the amount of cell clumps in each washing protocol. Figure A shows nearly 2 mL of clumps in the bottom of a conical tube containing a representative sample subjected to washing with saline solution (solution 1). Figure B shows the absence of clumps in a conical tube containing a representative sample subjected to washing with hydroxyethyl starch solution (solution 2). This result was consistently observed in all samples.*

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