Evaluation of an automated cell processing device to reduce the dimethyl sulfoxide from hematopoietic grafts after thawing

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BACKGROUND: The direct transfusion of thawed hematopoietic progenitor cells (HPCs) is associated to transfusion-related side effects that are thought to be dose-dependent on the infused dimethyl sulfoxide (DMSO). Both the effectiveness of a fully automated cell processing device to washing out DMSO and the effects of DMSO elimination over the recovered cells were evaluated.

STUDY DESIGN AND METHODS: Twenty cryopreserved peripheral blood HPC bags (HPC apheresis [HPC-A]) were thawed and processed for washing with an automated cell-processing device. Viability, colony-forming units (CFUs), and absolute count of recovered cells were evaluated by flow cytometry immediately after washing as well as at different times after washing and compared with a sample taken just after thawing (control) but maintained at 4°C. DMSO content was measured by high-performance liquid chromatography and the osmolarity with an osmometer.

RESULTS: The median recovery of viable total nucleated cells, viable CD34+ cells, and CFU colonies was 89 (range, 74-115), 103 (range, 62-126), and 91 percent (range, 46%-196%), respectively, in the washing group. Recovery of viable CD3+ cells was 97 percent (range, 42%-131%) and CD14+ cells was 82 percent (54%-119%). The percentages of DMSO elimination and osmolarity reduction were 98 (range, 96-99) and 90 percent (range 86%-95%), respectively. Moreover, elimination of the cryoprotectant improved CFU count, viability, and cell recoveries along the time when compared with the control group.

CONCLUSION: Washing out DMSO in thawed HPC-A by use of this approach is safe and efficient in terms of recovery and viability of nucleated and progenitor cells. Additionally, the removal degree of DMSO is very high and therefore might ameliorate the transfusion-related side effects.

Transplantation of hematopoietic progenitor cells (HPCs) from peripheral blood (HPC apheresis; HPC-A) is a commonly used treatment for hemopoiesis reconstitution following high-dose chemotherapy. Autologous HPC transplantation involves transfusion of postthawed cells, whereas allogeneic transplantation is performed with fresh or, less frequently, cryopreserved HPCs.

The freezing process requires the addition of cryoprotectants to prevent cell injury from low-temperature effects.[1] 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[2] during freezing. At time of transfusion, HPCs are thawed and usually transfused without manipulation. Consequently, cells, DMSO, and lysis products enter into the patient's blood.[1,3] 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[4-6]) to severe (renal failure and cardiovascular[7,8] as well as neurologic complications[9]). These transfusion-related complications are mainly attributed to DMSO content but also to the transfused cell debris and released intracellular products.

ABBREVIATIONS: 7-AAD = 7-aminoactinomycin D; HPC-A = hematopoietic progenitor cell apheresis; NC(s) = nucleated cell(s); TNC(s) = total nucleated cell(s).

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To minimize these complications, cryopreservation of grafts highly concentrated with nucleated cells (NCs),[10] the use of less DMSO in the preservation solution,[11,12] mixture of cryoprotectants,[13] fractionated transfusion,[14] and CD34+ cell selection techniques[15] have been proposed. All these techniques, however, may only reduce to a low extent the final content of cryoprotectant and lysis products.

HPC washing after thawing has been also proposed to remove DMSO and debris before transfusion[16,17] by applying centrifugation techniques, either manual or semiautomatic.[16,18] Again, these techniques result in variable and some times poor elimination of DMSO and released lysis products.[18-21] Indeed, suboptimal HPC-A washing might result in loss of progenitor cells, compromising the engraftment potential.

Recently a new benchtop, fully automated cell processing device (Cytomate, Baxter Oncology, Chicago, IL) consisting of a user-programmable, closed system with a spinning membrane designed for concentration and washing of blood samples has been made available.

This study was designed to evaluate the feasibility and efficacy of this device on the washing of HPC-A products regarding its potential on DMSO reduction and recovery of viable nucleated and progenitor cells. Additionally, the effects of this procedure on cell viability and recovery along the time were investigated.

MATERIALS AND METHODS

Study design

A total of 15 experiments were performed including 10 procedures processing one and 5 processing two HPC-A bags, which are the most common format employed in cell-processing laboratories. The inclusion criteria for this study were products excluded for transplantation (patient's relapse or death) and fully characterized, including total NCs, CD34 cell number, and clonogenic assays data before cryopreservation. Two sets of experiments were carried out: first, one HPC-A bag (n = 10) was washed after thawing, and the evolution of the cell populations was monitored after thawing, after washing, and 1, 2, 4, and 24 hours after washing. Second, two HPC-A bags (n = 5) were sequentially washed after thawing, and the same testing strategy was applied. In every case, an aliquot (30 mL) of the thawed apheresis was maintained in a bag (Lifecell, Baxter) at 4°C without washing and served as a control. At every point of the study, viability, total NC count, clonogenic assay, absolute cell count by flow cytometric analysis, osmolarity, and DMSO quantification were assessed in the washed and the unwashed control products. Sterility tests were also performed just before and after wash.

Processing and cryopreservation

HPC-A from patients with various hematologic malignancies mobilized with chemotherapy and/or recombinant human (rHu) granulocyte–colony-stimulating factor were used. HPC-A were processed according to standard validated methods.[10] Briefly, HPC-A was centrifuged at $400 \times g$ for 15 minutes to remove plasma and adjust volume, chilled to 4°C, and then mixed with 4°C chilled cryopreservation solution consisting in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), human serum albumin (HSA), and DMSO to obtain a final concentration of 10 percent DMSO (vol/vol) and 2 percent HSA (vol/vol). Cells were then cryopreserved in a controlled-cooling-rate freezer up to –120°C (rate of 1°C/min up to –40°C, 2°C/min up to –60°C, and 10°C/min up to –120°C) and stored in liquid nitrogen.

Thawing and washing procedures

HPC-A bags were thawed by immersion in a 37°C water bath. Immediately after thawing, an aliquot was obtained, as a control of the experiments, and kept at 4°C. The remaining product was connected to the Cytomate device for processing. This machine is a user-definable, computer-controlled system that ensures cell washing and concentration. The sterile and single-use disposable set consists of a spinning membrane connected to a filtered wash bag, a waste bag, and several spikes to connect buffers and product bags. Washing program settings were an initial 1:1 dilution of the HPC-A at 10 mL per minute with a cold PBS buffer containing 5 percent dextran 40, 5 percent HSA, and 5 percent sodium-citrate followed by 5 minutes at 4°C in continuous agitation. After this period, the washing procedure started with a cooled PBS buffer containing 2.5 percent HSA and 5 percent sodium citrate. Washing proceeded in a continuous cycling of cell concentration-wash steps through the spinning membrane. The "residual fold reduction" setting, which defines the efficiency of the procedure, was set at 50.

Automatic cell count and viability

NC count was assessed for each sample by use of the automatic cell counter (ACT-diff, Coulter, Miami. FL). Each sample was tested for viability by use of the trypan blue dye-exclusion method (0.4% solution; Sigma Chemical Co., St Louis, MO) at every time point of study.

Flow cytometric assay

Samples (1 mL) drawn just before and after the washing process were incubated for cytometric absolute count with anti-human fluorescein isothiocyanate–conjugated CD45 monoclonal antibody (MoAb; BD Pharmingen, San Diego, CA). Double staining was performed by use of one anti-human phycoerythrin-conjugated CD34, CD3, CD14, or CD15 on each tube (Pharmingen, BD Biosciences) in PBS containing 1 percent albumin and 0.1 percent

sodium azide for 30 minutes at 4°C in the dark. After this, samples were lysed in ammonium chloride for 10 minutes, and 20 µL of commercially available fluorescent microbeads[22] was added and mixed with the sample just before acquisition for single-platform determination according to manufacturer's instructions (Perfect-Count, Cytognos, Salamanca, Spain). Absolute cell counts were measured and analyzed in a flow cytometer (FACSCalibur, BD Biosciences) with software (Cellquest, BD Biosciences). Cell viability was measured staining the samples with the vital dye 7-aminoactinomycin D (7-AAD) as proposed by the ISHAGE guidelines.[23]

Median (range) recoveries were calculated for all the analyzed cell subsets by use of the formula

$$\text{Percent recovery} = ((\text{absolute cell count after wash})/\text{absolute cell count after thaw})) \times 100.$$

Clonogenic assay

Progenitor cell assays were performed at every time point of study with the commercially available methylcellulose-based H4431 medium supplemented with rHu stem cell factor, interleukin-3, granulocyte-monocyte–colony-stimulating factor, and rHu erythropoietin (Stem Cell Technologies, Vancouver, British Columbia, Canada). Briefly, 10^5 cells were seeded directly from the samples on the methylcellulose-based medium, gently mixed, and then plated on petri dishes. Cultures were incubated at 37°C and in 5 percent CO_2. Assays were performed in triplicate. Colony-forming units–granulocyte-macrophage (CFU-GM), burst-forming units–erythroid, and CFU–Mixed colonies were identified and counted according to standard criteria[24] after 14 days of culture.

Osmolarity quantification

Samples osmolarity (mOsm/L) was measured by depression of freezing point in a automatic osmometer (Roebling 1313, Hermann Roebling Messtechnick, Berlin, Germany). Before osmometric determination samples were centrifuged for 5 minutes at 11,750 × g to obtain cell-free solutions and were diluted 1 in 10 in distilled water; after that, the samples were analyzed.

DMSO quantification

DMSO content of the samples was determined by high-performance liquid chromatography (HPLC) following a reported method.[25] Previous experiments were performed to confirm that calibration plot of DMSO peak area versus DMSO concentration was linear and samples had only one peak of DMSO. Samples were centrifuged for 5 minutes at 11,750 × g to obtain cell-free solutions and diluted as needed to obtain DMSO contents falling within the linear range of the calibration plot. Samples taken just after thawing were diluted at 1 in 1000 in 10 percent (vol/vol) methanol whereas samples taken just after washing were diluted 1 in 10. The HPLC device employed was a Beckman System Gold HPLC (Beckman, San Ramon, CA) with multiple solvent delivery system coupled to a variable-wavelength detector (Beckman-168, Beckman). The column used was an Ultrasphere ODS2 C_{18} (5 µm) measuring 4.6 × 25 cm. The mobile phase contained 10 percent (vol/vol) methanol in high-purity deionized water, 20 µl of each sample was injected, the flow rate was set to 1.0 mL per min, and the detector was set at 214 nm. With these conditions, DMSO peak eluted after 3 minutes.

Statistical analysis

Data are shown as medians and ranges unless otherwise indicated. Statistical analysis was performed with the Wilcoxon's rank test and comparing continuous variables. p values of less than 0.05 were considered as significant. Statistical analyses were performed with computer software (SPSS 9.0, SPSS, Chicago, IL).

RESULTS

Cell recoveries after washing

Data from 15 washing procedures performed with a total of 20 HPC-A bags are shown in Table 1. The median total NC (TNC) count loaded into the Cytomate device for washing was 8.7×10^9 (range, 0.8×10^9-21.6×10^9). The median volume was 82 (range, 72-123) and 245 mL (range, 184-257 mL) in the one-bag and two-bag experiments, respectively. Viability of NCs measured by 7-AAD staining was 75 percent (range, 73%-89%) after thawing. After washing, viability was 73 percent (range, 60%-91%) in the control group and 87 percent (range, 71%-93%; p = 0.04) for the washed cells. Cell viability calculated from trypan blue staining showed similar results for both groups of study (data not shown).

Immediately after washing there were no differences in the recovery of viable NCs, CD3+ cells, CD14+ cells, and CD15+ cells between the washing and the control group. CD34+ cells loss was negligible in both groups. The percentage of viable CD34+ cells (CD34+/7-AAD–) was 90 percent (range, 25%-99%) after thawing and 95 (75-99) and 92 percent (61%-99%) in the washing and control groups, respectively. Loss of CFU-GM or total CFU was approximately 10 percent in both groups. Final volumes obtained were 109 mL (range, 91-134 mL) in the one-bag experiments and 206 mL (range, 183-230 mL) in the two-bag experiments. The washing method described here takes approximately 30 minutes per bag.

TABLE 1. Results of the washing procedure*

	Content after thawing	Control		After washing		
		Content	Recovery (%)	Content	Recovery (%)	p value†
TNCs ($\times 10^9$)	8.7 (0.8-21.6)	8.6 (0.7-19.4)	95 (61-127)	8.4 (0.7-21.2)	89 (74-115)	0.9
CD34 ($\times 10^6$)	17.3 (10.7-64)	17.5 (7.5-65.7)	94 (75-110)	18.2 (7-66.5)	103 (62-126)	0.04
CD3 ($\times 10^8$)	42 (0.2-590)	44 (0.2-530)	97 (61-112)	36 (0.1-620)	97 (42-131)	0.09
CD14 ($\times 10^8$)	24 (0.02-41)	19.1 (0.02-40)	85 (61-104)	17 (0.02-42)	82 (54-119)	0.7
CD15 ($\times 10^8$)	1.7 (0.03-12.4)	1.4 (0.02-13)	70 (9-124)	0.11 (0.02-15)	63 (24-121)	0.17
CFU-GM ($\times 10^6$)	3.3 (0.8-23.1)	2.8 (0.8-18.2)	88 (21-142)	3.5 (0.95-25)	91 (46-196)	0.2
Osmolarity (mOsm/L)	3000 (1545-3000)	3000 (1545-3000)		302 (227-402)	10 (5-14)	0.001
DMSO (g/L)	130 (97-171)	130 (91-171)		2 (0.5-4)	2 (1-4)	0.002

* Data are reported as median (range).
† p value from Wilcoxon rank test statistic comparing recovery values from control and washing groups.

Cell recoveries at several time points after washing

In a set of 10 experiments, the state of cells at different times after washing was assessed and compared to the control, unmanipulated sample (see Fig. 1).

One hour after washing, recovery of viable CD34+ cells ($p = 0.04$) and CFU-GM ($p = 0.02$) significantly decreased in the control group when compared to the washing group. This difference was observed in both CD34+ cells and CFU-GM at 2 hours ($p = 0.04$), 4 hours ($p = 0.02$ and $p = 0.04$), and 24 hours ($p = 0.02$) after washing. A similar figure was observed for total CFU along the time between the washing and control group. In addition, viability of CD34+ cells measured by 7-AAD was higher in the washing than in the control group at 1 hour (96% vs. 87%; $p = 0.03$), although there was no difference at 2 hours (94% vs. 87%; $p = 0.06$), 4 hours (97% vs. 95%; $p = 0.2$), and 24 hours (96% vs. 95%; $p = 0.06$) after washing. Viability of TNC (CD45+/7-AAD– events) was higher in the washing than in the control group at 1 hour (79% vs. 74%; $p = 0.04$), 2 hours (82% vs. 68%; $p = 0.04$), 4 hours (82% vs. 78%; $p = 0.04$), and 24 hours (87% vs. 65%; $p = 0.008$).

DMSO reduction after washing

Median quantity of DMSO after thawing was 130 g per L (range, 97-171 g/L), equivalent to 12 percent (vol/vol; range, 9%-14%). After washing, DMSO content dropped to 2 g per L (range, 0.5-4 g/L) or 0.2 percent (vol/vol; range, 0.05%-0.21%) as measured by HPLC, which represents a median DMSO reduction of 98 percent (96%-99.7%). Consistently, osmolarity of the same cell suspensions had a median value of 3000 mOsm per L (range, 1545-3000 mOsm/L) after thawing and 302 mOsm per L (range, 227-402 mOsm/L) after washing, resulting in a median osmolarity reduction of 90 percent (range, 84%-91%).

DISCUSSION

Direct transfusion of thawed cells is commonly used in the setting of HPC therapy for hematopoietic reconstitution. Such a method, however, implies transfusion of DMSO and cell lysis products into the patient's blood and might be in part responsible for the transfusion-related complications observed in many patients.[4-8] Some of these complications are thought to be derived from DMSO-induced histamine release, whereas others could be directly related to the amount of DMSO transfused.[6] To improve patient comfort and safety at time of transfusion, we evaluated the Cytomate system (Baxter Oncology) to obtain grafts with minimized DMSO and cell lysis products content.

In this study, we demonstrate that this approach is feasible and allows the HPC-A washing without major problems during processing as well as a highly effective DMSO reduction. A reproducible 98 percent elimination of DMSO was determined by HPLC. This chromatographic technique was previously used in tissue samples[25] and has been adapted in our laboratory for DMSO quantification in thawed HPC-A. Accordingly, final osmolarity of the washed cells suspension was reduced to 300 mOsm per L, close to that of plasma. Moreover, removal of cryoprotectant by this technique was associated to an irrelevant loss of HPCs.

Other investigators have used different strategies to wash DMSO from HPC-A. Del Mastro and coworkers[17] reported a direct manual centrifugation method keeping the cells in saline solution containing deoxyribonuclease. They did not observe any side effects related to transfusion of thawed and washed HPCs. Unfortunately, they did not give data about HPC recovery nor DMSO reduction. Syme and associates[26] have recently reported results from a series of HPC-A washed by use of a manual centrifugation method. They reported a median NC loss of 15 percent but with a CD34+ cells loss of 29 percent, which is slightly higher when compared to our results. These differences might be due to the different processing protocol used but also to the analysis methods because they use a double platform cytometry instead of the single platform used in this work.

Two different groups[27,28] have recently reported their results of washing out DMSO from HPC-A and cord blood by use of the automated Cytomate device in a series of

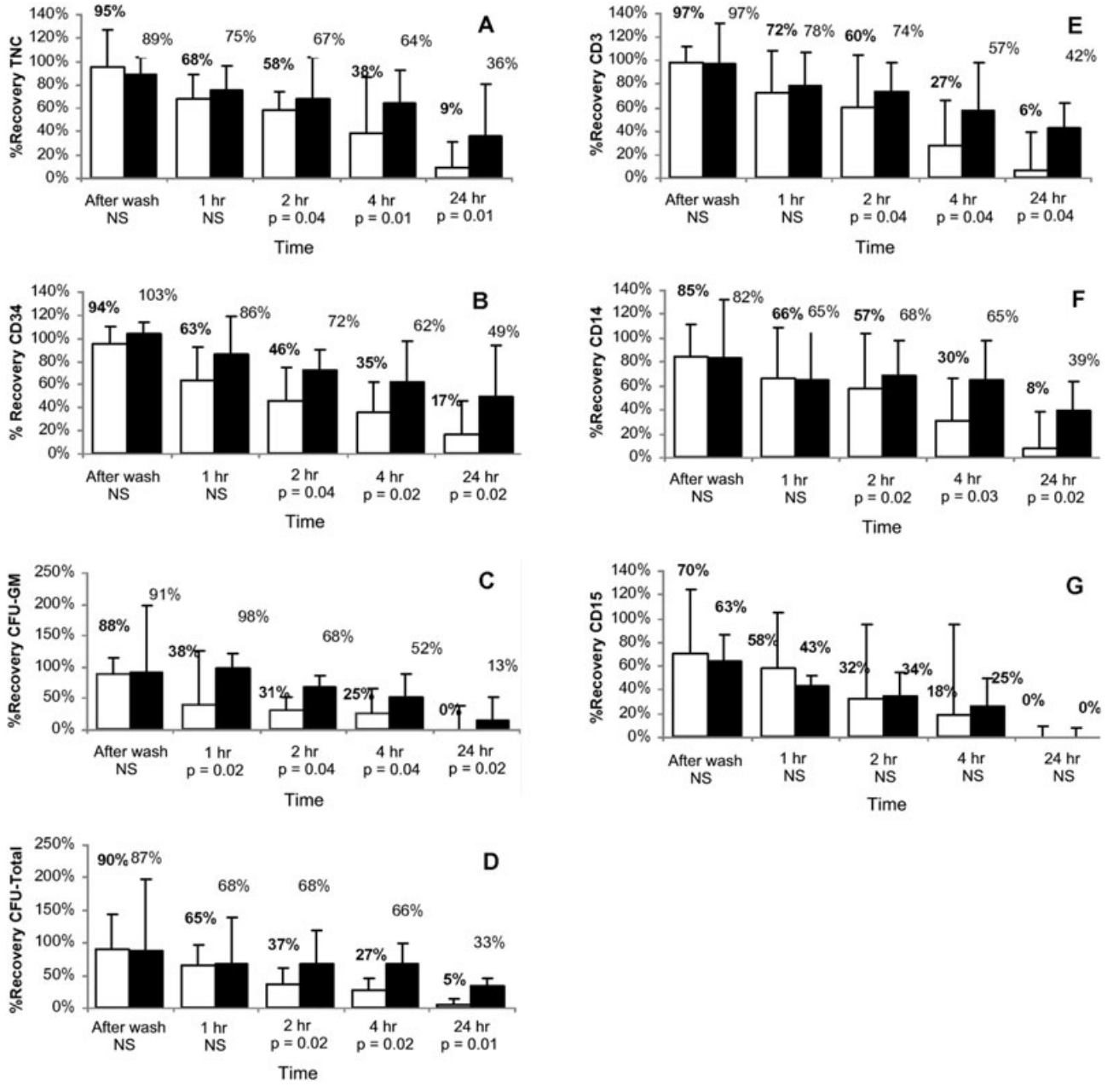

Fig. 1. Samples evolution after thawing with or without washing. Recoveries of viable NCs (A), CD34+ cells (B), CFU-GM (C), total colony-forming units (D), CD3+ cells (E), CD14+ cells (F), and CD15+ cells (G) are shown in washed (■) and unwashed (□) groups, immediately after washing, and at 1, 2, 4, and 24 hours after washing.

preclinical experiments. They also reported a very high elimination of DMSO, more than 98 percent. Moreover, Calmels and colleagues.[27] reported median viable CD34+ cell, CFU-GM, and CD3+ T-cell recoveries of 66, 63, and 77 percent in HPC-A products, similar to those results obtained by Perotti and associates[28] in cord blood but lower than those obtained in our study. These differences in cell recoveries among works could have been influenced by the quality of the grafts after thawing. In this regard, mean viability of CD34+ cells after thawing was 66 percent as reported by Calmels and coworkers and 86 percent in our work. Also, Perotti and colleagues[28] reported a NC viability after thawing below 70 percent.

Additionally, Calmels and coworkers[27] performed a simultaneous washing of bags instead of the sequential strategy followed in this work. In fact, we tested in previous experiments the washing of cells pooled from two thawed HPC-A bags, but cell aggregates and clumps occurred

during manipulation, resulting in a progenitor cells loss up to 50 percent including CFUs (data not shown). Moreover, we introduced, immediately after thawing and dilution, an equilibrium step of 5 minutes with a hyperosmotic buffer as described by Rubinstein and colleagues[29] for cord blood. The rationale of such a step is the control of the osmotic gradient suffered by cells by adding a buffer with high content in macromolecules and protein, which associate free water. In this regard, osmotic shock might also be affecting thawed cells when directly enter the blood causing changes in their osmotic environment. This situation could be prevented by applying the automatic washing described here as shown by osmolarity values obtained after washing. In fact, additional experiments introducing washed and unwashed cells into an isotonic environment ($n = 10$, data not shown) showed that thawed samples directly diluted in PBS suffered a loss of 25 ± 21 percent of total CD34+ cells as well as a reduction of 15 percent in viability as measured by 7-AAD staining. In contrast, washed cells from the same samples showed a 99 ± 21 percent recovery without significant changes in viability after the same manipulation.

Whether washing out DMSO after thawing affects the speed of engraftment is not clear. Recently, Syme and coworkers[26] compared the time to hemopoietic engraftment in a series of 35 patients with breast cancer autotransplanted with HPC-A DMSO-washed compared with a control group of 21 patients autotransplanted with HPC-A containing DMSO. They did not find differences in hemopoietic recovery between groups but at time of transfusion, the washed group had a higher number of CD34+ cells and CFU-GM than the group containing DMSO. Nevertheless, the authors noted a significant lower incidence of transfusion related side effects in the DMSO-washed group.

Although automatic and easy handling, the washing protocol described here was time-consuming compared to direct transfusion after thawing. If washing out of DMSO and cell debris is translated in lesser transfusion-related reactions and reduced workload over the nurse staff, however, this technique might be considered for routine thawed cell processing.

Another interesting question is whether DMSO is toxic for the cells at different times after thawing. Based on cell viability studies with marrow, Branch and coworkers[30] found no toxicity of DMSO over CFU-GM up to 2 hours at 4°C. In contrast, Douay and coworkers[31] reported a loss of CFU-GM of 85 percent when marrow HPCs were kept in DMSO at 4°C for a period of 120 minutes. Additionally, Humpe and associates[32] have reported a significant reduction of the clonogenic potential of HPC-A in the presence of DMSO with loss of CFU-GM up to 40 percent only 30 minutes after thawing. Our results also suggest a real negative effect of DMSO translated into a loss of HPCs of approximately 50 percent within 90 minutes after thawing. In this context, we observed a rapid reduction of viable CD34+ cells and CFU-GM, with less than 50 percent recovery in the control group compared to 70 percent in the washed arm 2 hours after washing. The same behavior was observed at every time of study after washing affecting counts of CD3 and CD14+ cells, which were lower in the group with DMSO. Such differences in cell counts increased proportionally with time and favored in each case the group where DMSO was washed out.

Taken together, our results suggest that unmanipulated grafts should be transfused as soon as possible after thawing and that it should be recommended that the deadline of 1 hour not be exceeded. Additionally, if a time delay from thawing to transfusion is expected, it should be recommended that DMSO be washed out to obtain better cell recoveries and viability. Moreover, washing HPC-A after thawing could be performed under laboratory-controlled conditions rather than at the bedside, improving synchronization among cell-processing laboratories and the clinical staff. Finally, HPC-A wash with this automated device allows the user to define the final volume of the product and, thus, a large volume of cryopreserved HPC-A could be reduced to a suitable volume for transfusion.

In conclusion, thawed HPC-A washing by use of this fully automated cell-processing device and the described protocol is highly effective and reproducible in reducing the DMSO content with a negligible loss of viable HPCs. Moreover, the washed HPC-A products remain more stable along time than those containing DMSO. A further step will be a pilot clinical study to assess the clinical safety and efficacy of this approach.

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