

Cell loss and recovery in umbilical cord blood processing: a comparison of postthaw and postwash samples

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BACKGROUND: Engraftment after umbilical cord blood (UCB) transplantation is highly dependent on nucleated cell (NC) and CD34+ cell content. Current standard postthaw (PT) processing includes a wash step to remove dimethyl sulfoxide (DMSO), lysed red cells, and stroma. The contribution of the wash step to cell loss and ultimately the dose of cells available for transplant have yet to be systematically reported. This study examines the effect of the wash step as well as that of PT storage on various quality control variables of UCB units.

STUDY DESIGN AND METHODS: Ten units were thawed and washed based on the New York Blood Center method. Samples were removed from each unit at six time points: prefreeze (PF), immediately PT, immediately postwash (PW), and 1, 2, and 5 hours PW. On each sample, total nucleated cell (TNC) count, CD34+ cell enumeration, colony-forming unit (CFU)-granulocyte-macrophage, and viability assays (fluorescence microscopy [acridine orange/propidium iodide, or AO/PI] and flow cytometry [7-aminoactinomycin]) were obtained.

RESULTS: TNC counts decreased PT and at subsequent time points; the PT TNC recovery was 89 percent compared to 82 percent PW ($p < 0.01$). TNC recovery decreased to 90 percent of PW (82% of PT) values ($p < 0.01$) and 83 percent of PW (76% of PT) values ($p < 0.001$), at 2 and 5 hours PW, respectively. CD34+ cell loss PT was not significant. Viability by AO/PI decreased PT and plateaued over time. In contrast, viability by flow cytometry remained higher and increased slightly over time. CFUs were significantly lower PT, recovering PW.

CONCLUSIONS: Our data indicate that the thawing and washing results in a substantial loss of cells, with TNC loss approaching 20 percent when compared with PF counts; the wash step was responsible for nearly half of the cell loss. The reduced PT viability was expected. Elapse of time PW resulted in further loss of NCs but no detectable significant changes in CD34+ cell content and viability and/or CFU.

At present, umbilical cord blood (UCB) is being used as a source of hematopoietic progenitor cells (HPCs) mainly for pediatric patients and low-weight adult patients because of concerns about reaching a sufficient cell dose for engraftment in adult patients. Different approaches to increase cell dose are being considered, such as expansion of UCB-derived HPCs and by use of multiple units of UCB.¹ Despite these concerns, there is no official recommendation regarding the minimal nucleated cell (NC) and CD34+ cell dose for successful transplantation. Results from published studies are difficult to interpret, because some studies have examined the cell content determined before cryopreservation,²⁻⁴ whereas others have focused on infused (postthaw [PT]/postwash [PW]) cell counts.⁵⁻⁷ There is an emerging consensus, however, that a rate of neutrophil engraftment comparable to unrelated marrow transplantation can be achieved with an infused NC dose of 1.5×10^7 to 2.0×10^7 NCs per kg.^{1,8} Furthermore, one study⁵ has shown a correlation between the probability of neutrophil and platelet (PLT) engraftment and infused

ABBREVIATIONS: 7-AAD = 7-aminoactinomycin; AO/PI = acridine orange/propidium iodide; NC(s) = nucleated cell(s); NRBC(s) = nucleated red blood cell(s); PF = prefreeze; PT = postthaw; PW = postwash; TNC count = total nucleated cell count; UCB = umbilical cord blood.

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CD34+ cell dose and suggests a minimum infused dose of 1.2×10^5 to 1.7×10^5 per kg.

To consistently achieve an adequate cell dose, UCB processing methods must minimize cell losses. By use of colony-forming unit (CFU) assays, Broxmeyer and coworkers⁹ initially reported poor recovery of UCB progenitor cells separated into a low-density fraction by Ficoll-Hypaque before cryopreservation compared to unseparated cells. Cell loss ranged from 30 to 40 percent before cryopreservation and removal of red blood cells (RBCs) before density gradient separation increased cell loss to 50 to 90 percent. After cryopreservation, the immediate PT recoveries were similar between unseparated and separated cells at 80 to 100 percent. Washing after thaw, however, increased cell losses (not quantified). A few years later, Rubinstein and associates¹⁰ published a processing method that became widely applied. Their method allowed RBC depletion before cryopreservation and washing of the thawed UCB units before infusion while getting good recoveries. The rationale behind RBC depletion was to reduce the total volume of the product being frozen to maximize storing capacity. 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. With this method, they reported a cell loss of 9 percent in mean white blood cell (WBC) count per unit and a 2 percent loss of progenitor cells (CFU assay) after RBC depletion. After cryopreservation, and after thawing and washing, they reported an additional loss of 8 percent of mean WBC count per unit, 39 percent of viable WBCs, and no loss of progenitor cells. Subsequent publications presenting data on post-RBC depletion processing have shown comparable results, with total cell losses falling in the range of 20 to 30 percent.¹¹⁻¹⁴

Each additional manipulation of a cellular product potentially leads to further loss of cells. With UCB being used increasingly as an alternative source of HPCs in older children and adults, cell losses that were not significant for smaller children may become critical in reaching a suitable dose for transplantation. To our knowledge, there is no recent systematic review of the cell content and function of UCB units throughout the different steps of processing in the published literature. With the goal of optimizing UCB processing, it is therefore difficult to know the extent to which the wash step contributes to total cell loss and whether it is necessary in all cases. Hence, the goal of this study was to document the effect of thawing and washing on the cell content and function of UCB products. In addition, because the thawed and washed UCB units need to be delivered to the patient care unit and delays may occur, we examined the effect of elapsing time after wash on cell counts, viability, and clonogenicity.

MATERIALS AND METHODS

This study was performed at the Clinical Cell Therapy Laboratory of Fairview-University Medical Center in October 2003. Ten UCB units collected for the American Red Cross Cord Blood Bank in St Paul, Minnesota, were used.

UCB collection, processing, cryopreservation, and storage¹⁵

After obtaining consent, the potential donors are screened with a medical questionnaire and infectious disease testing. UCB units were collected from eligible mothers with an *ex utero* procedure. Briefly, after natural delivery of the placenta, the cord and placenta are sent to the collection room where a needle is inserted into a placental vein and the cord blood is allowed to flow by gravity. The cord blood is collected in a bag containing citrate phosphate dextrose anticoagulant. After collection, the bag is sent to the processing facility. The UCB product is then mixed with hydroxyethylstarch in a 5:1 ratio and centrifuged at $90 \times g$ for 6 minutes. The WBC-rich plasma is expressed in a separate bag and centrifuged at $450 \times g$ for 10 minutes. The WBC-poor plasma is expressed and discarded. DMSO and dextran 40 are added to the remaining product at a final concentration of 10 and 1 percent, respectively. The product is then cryopreserved with a controlled-rate freezer. After freezing, the bags are transferred to a liquid nitrogen storage container.

UCB thawing and washing

UCB processing methods are based upon those originally described by Rubinstein and colleagues¹⁰ (New York Blood Center). For this study, we selected 10 units that could not be used for transplantation because of disqualifying medical history. These units were thawed and washed. Briefly, UCB units were taken out of the liquid nitrogen storage container, put in a plastic bag, and immersed in a 37°C water bath. After inspection, 10 percent dextran 40 was slowly added followed by 5 percent human albumin and the bag was left to equilibrate for 5 minutes. The product was then transferred to a transfer bag and centrifuged at $400 \times g$ for 15 minutes at 4°C. Approximately three-fourths of the wash supernatant was expressed and transferred in a second transfer bag. The wash supernatant was then centrifuged at $800 \times g$ for 15 minutes at 4°C. Again, three-fourths of the wash supernatant was expressed, and the cell pellets obtained from the two centrifugation steps were combined in one bag. The combined cell pellets were resuspended in 10 percent dextran and 5 percent human albumin. Finally, the product was passed through a standard blood filter (170-260 μm).¹⁶

Sampling

Samples were taken from each UCB unit before cryopreservation (after RBC depletion) and at five different time points during the thaw-wash procedure: immediately PT, immediately PW, and 1, 2, and 5 hours PW.

Testing

Samples from each time point were analyzed for total nucleated cell (TNC) count, CD34+ cell count, CFU-granulocyte-macrophage (CFU-GM) assay, and viability.

TNC count. TNC count was obtained with a hematology analyzer (Coulter MDII, Beckman Coulter, Fullerton, CA). To avoid wrongly attributing cell loss due to sampling to the washing step, the TNC count of the product was adjusted by subtracting the volume of sampling from the presampling volume (initially used to calculate the product's TNC). For the second part of the experiment, we adjusted the 1-, 2-, and 5-hours-PW TNC count to compensate for the loss of volume due to sampling, again to avoid attributing the loss to time rather than sampling.

CD34 analysis. CD34+ cell enumeration was determined with a dual platform protocol and the gating strategy was based on ISHAGE guidelines. Samples were incubated with phycoerythrin-conjugated anti-CD34 (8G12), fluorescein isothiocyanate-conjugated anti-CD45 (2D1) monoclonal antibodies (BD Biosciences, San Jose, CA), and 7-aminoactinomycin D (7-AAD, Sigma Chemical Co., St Louis, MO). Flow cytometry analysis was performed with a flow cytometer (FACSCalibur, BD Biosciences). The percentage of CD34+ cells was determined in the 7-AAD-negative population by side scatter and CD34 positivity with initial gating on CD45+ cells. The percentage of CD34+ cells was then multiplied by TNC count to obtain the absolute number of CD34+ cells. Adjusted TNC count was used to avoid wrongly attributing cell loss due to sampling to the washing step.

Viability assays. Viability assays for TNCs were performed by acridine orange/propidium iodide (AO/PI) dual staining and by flow cytometry. Briefly, AO/PI solution was prepared as 1 mmol per L AO and 1 mmol per L PI in phosphate-buffered saline (Sigma Chemical Co.). A quantity of 190 μ L of AO/PI solution was added to 10 μ L of the sampled UCB unit. Two-hundred cells were counted in a Neubauer hemocytometer with a Zeiss compound fluorescence microscope at 250 \times . Cells expressing green fluorescence (AO) were counted as viable, whereas cells expressing orange fluorescence (PI) were considered non-viable. 7-AAD was used for viability testing of TNCs and CD34+ by flow cytometry. CD34+ cell-specific viability was also obtained by looking at the proportion of 7-AAD-negative cells among the CD34-positive cell population.

CFU-GM assay. The CFU-GM assay was performed as is standard in the Clinical Cell Therapy Laboratory. Meth-

ocult GF H4534 "complete" methylcellulose medium containing recombinant cytokines without erythropoietin (StemCell Technologies, Vancouver, British Columbia, Canada) and an in-house progenitor assay medium with 2 percent fetal calf serum were the reagents used. Samples were diluted with 2 percent fetal calf serum to achieve a concentration of between 0.2×10^6 to 0.4×10^6 per mL. The final cell count of the sample was verified with the Coulter MDII. An aliquot of Methocult was added to the sample. After 5 minutes, a sample of the Methocult mixture (20-40,000 cells) was plated in two wells of the culture plate and incubated for 14 to 16 days at 37°C in 5 percent CO₂. Plates were examined under the dissecting microscope at 2 \times to 2.3 \times . All CFU-GMs colonies with approximately more than 40 cells were counted. The mean number of colonies in the two wells was used to calculate the number of colonies per 10⁶ cells plated.

Statistical analysis

Computer software (GraphPad InStat version 3.01 for Windows 95/NT, GraphPad Software, San Diego, CA; <http://www.graphpad.com/>) was used for statistical analysis. Means were calculated with 95 percent confidence intervals (CIs) for each quality control (QC) variable at each time point. Ordinary one-way analysis of variance with Tukey's posttest was performed to compare results from different time points. For analysis of the effect of time, the 1- and 2-hour-PW results were compared to each other and to the PW results. In a separate analysis, the 5-hour-PW results were compared with the PW results only. The reason for this is the difference in time interval between each time point. Because we use time as the variable against which our endpoints (TNCs, CD34+, CFUs) are compared, the 5-hour-PW results cannot be considered equivalent to the 1- and 2-hour-PW results that are all 1 hour apart.

RESULTS

For the 10 units used in this study, the mean volume of UCB collected was 107 mL (range, 90-125 mL). The mean TNC content before RBC depletion was 1.71×10^9 NC (1.55-1.87) and the mean recovery post-RBC depletion was 85 percent (81-89). The mean volume of the product going into cryopreservation was 28 mL, including a mean of 5.6 mL of cryoprotectant. Mean values for all QC variables (TNC, CD34, viability, and CFU) at each of the testing points are summarized in Table 1.

Effects of processing

The mean TNC count decreased significantly at each step of processing. The largest drop occurred after thaw, with loss of 0.15×10^9 NCs ($p < 0.001$). The PT and PW TNC

TABLE 1. Pre- and postcryopreservation QC variable data

Variable	PF	PT	PW	Time (hr) PW		
				1	2	5
TNC ($\times 10^9$)*	1.32 (1.18-1.46)	1.17 (1.04-1.31)	1.08 (0.97-1.19)	1.03 (0.92-1.14)	0.98 (0.87-1.09)	0.91 (0.77-1.04)
CD34+ cell ($\times 10^6$)*	6.80 (3.72-9.87)	5.36 (2.31-8.41)	9.29 (5.04-13.54)	8.49 (5.54-11.44)	9.46 (4.96-13.97)	10.93 (5.63-16.24)
TNC AO/PI viability†	97 (95-99)	62 (54-69)	53 (45-62)	51 (45-58)	54 (48-60)	52 (44-59)
TNC flow viability†	NA‡	68.6 (62.4-74.8)	87.4 (84.3-90.5)	90.4 (87.2-93.6)	92.5 (90.3-94.7)	94.3 (92.4-96.2)
CD34+ cell flow viability†	NA	83.3 (72.5-94.1)	95.1 (93.5-96.7)	96.1 (94.0-98.2)	94.4 (91.1-97.7)	94.9 (91.0-98.8)
CFU-GM (per 10^6 cells plated)*	904 (598-1210)	152 (94-211)	577 (339-814)	577 (423-730)	566 (311-821)	504 (225-784)

* Data are reported as number (95% CI).
† Data are reported as percent (95% CI).
‡ NA = not available.

recoveries (relative to prefreeze content) were 89 percent (95% CI, 84%-94%) and 82 percent (95% CI, 79%-84%) ($p < 0.01$), respectively; the PW recovery relative to PT TNC content was 92 percent (95% CI, 86%-98%). Of the 18 percent total loss of TNCs associated with standard postcryopreservation processing, the washing step accounted for 7 percent. CD34+ cell counts decreased after thawing and increased after washing, although the differences we observed were not significant. The recovery of CD34+ cells was 97 percent (95% CI, 50.1%-143.1%) PT and 148.9 percent (95% CI, 112.8%-185%) PW (p value NS). The majority of the increase in CD34+ cells occurred after washing, although this difference is not significant. Analysis of individual UCB results did not reveal any significant trend, because some units had a lower CD34+ cell content PW than PT whereas others increased or remained stable. CFUs decreased from 904.1 per 10^6 cells plated prefreeze (PF; 95% CI, 598-1210) to 152.5 per 10^6 cells plated PT (95% CI, 94.4-210.6) but increased PW with 576.6 per 10^6 cells plated (95% CI, 339.6-813.7; $p < 0.001$). PW CFUs, however, remained significantly lower than their PF value ($p < 0.01$).

Viability, as assessed by AO/PI staining, decreased after thawing, from 97 percent (95% CI, 95%-99%) PF to 62 percent (95% CI, 54%-69%) PT ($p < 0.001$). There was no significant impact of washing on viability. With flow cytometry, we observed an increase in TNC viability from 68.6 percent (95% CI, 62.4%-74.8%) PT to 87.4 percent (95% CI, 84.3%-90.5%) PW ($p < 0.001$). The effect of thawing and washing on viable TNCs and viable CD34+ cells was also examined by multiplying viability results by the absolute number of TNCs and CD34+ cells at each time point. Viable TNC counts with AO/PI decreased from 1281.7×10^6 (95% CI, 1133.9×10^6 - 1429.5×10^6) PF to 724.5×10^6 (95% CI, 603.1×10^6 - 845.9×10^6) PT ($p < 0.001$) and 579.9×10^6 (95% CI, 443.6×10^6 - 716.1×10^6) PW ($p < 0.001$ from PF and $p < 0.05$ from PT). Viable TNC

counts with flow cytometry, however, increased from 804.9×10^6 (95% CI, 697×10^6 - 912.8×10^6) PT to 935.8×10^6 (95% CI, 849.3×10^6 - 1022.2×10^6) PW ($p < 0.01$). CD34+ cell-specific viability by flow cytometry and 7-AAD staining increased from 4.40×10^6 (95% CI, 2.11×10^6 - 6.68×10^6) PT to 8.89×10^6 (95% CI, 4.77×10^6 - 13.02×10^6) PW (p value NS).

Effects of time

TNC counts continued to decrease over time, becoming significantly lower at 2 hours ($p < 0.05$) and 5 hours ($p < 0.001$) PW. Two- and 5-hour-PW TNC recovery decreased to 90 percent (95% CI, 86%-94%; $p < 0.01$) and 83 percent (95% CI, 77%-90%) of immediate PW values ($p < 0.001$), respectively. One-hour-PW TNC recovery was 94.7 percent (95% CI, 91.3%-98.1%) of PW values (p value NS). When compared to PT results, TNC recovery was 86 percent (95% CI, 78.9%-93.3%) at 1 hour, 82 percent (95% CI, 75%-89%) at 2 hours, and 76 percent (95% CI, 67%-84%) at 5 hours. This decrease became significant at 2 hours ($p < 0.01$) and 5 hours ($p < 0.001$). There was no significant difference in CD34 counts and CD34 recovery over time after wash. The significant increase in CFU values PW compared to those of PT was maintained through 5 hours PW. No further significant increase in CFUs occurred, however, with elapse of time when compared to immediate PW results.

AO/PI viability remained unchanged over time after wash with values between 45 and 60 percent (p value NS). Flow viability, however, continued to increase, reaching statistical significance at 5 hours ($p < 0.01$). The observed decrease of TNCs over time after wash was no longer apparent when examining viable TNCs by both methods (AO/PI and flow cytometry). Analysis of CD34+ cell-specific viability by flow cytometry did not reveal any significant changes at any point after wash.

DISCUSSION

This study was designed to examine two specific questions: 1) what is the influence of the washing step on cell counts and function and 2) what is the influence of time after wash on cell counts and function? We found an 18 percent loss of TNCs and a 55 percent loss of AO/PI-viable TNCs after thawing and washing. The washing step accounted for nearly half of the loss of TNCs (7%) as well as that of AO/PI-viable TNCs (21%). When compared to PT results, the loss of both TNCs and viable TNCs induced by washing is a significant loss. Conversely, we were unable to find a significant effect on total and viable CD34+ cells. In the 5 hours that followed washing, TNCs continued to decrease, reaching significance after 2 hours. We found no difference, however, in viable TNCs as well as CD34+ cells and CFUs.

Our results compare with previously published reports that included some pieces of processing data. Rubinstein and coworkers¹⁰ reported an 8 percent loss of WBCs and 39 percent loss of viable WBCs (ethidium bromide); they did not report data on CD34+ cells. Their hematopoietic progenitor assay, equivalent to our CFU assay, showed similar results with a significant decrease of counts in the unwashed PT sample and a PW recovery above 100 percent (but CI overlaps with those of PF). The better recovery we report here may be explained by differences in our wash procedure. For example, we use a slightly different washing reagent concentration and different centrifugation variables. We also add a second centrifugation step to maximize cell recovery. Additionally, we use a different viability assay. Alonso and coworkers¹³ published a study on UCB processing before cryopreservation. This group analyzed QC variables from 4055 UCB units and showed a median yield of 87 percent for TNCs, 97 percent for CD34+ cells, and 84 percent for CFUs. Of those 4055 UCB units, PT and PW data were available from 25 units that were used for transplantation. Recoveries for the 25 units were 81 percent for TNCs, 71 percent for viable TNCs, and 76 percent for CFUs. Recovery data for CD34+ cells were not reported. There was also no mention of the method used for thawing and washing, because this was not the aim of the study. Finally, another study evaluated the impact of the wash step by washing a sample taken from a thawed unit and comparing QC variables between the washed sample and the unwashed counterpart.¹¹ This group reported a TNC loss of 29.6 percent PW compared with 14.8 percent PT.

In part, the loss of TNCs could be explained by loss of nucleated RBCs (NRBCs). We did not document the proportion of NRBCs in the TNC content of our studied UCB units, contrary to what we do with our units used for transplantation. This would be particularly relevant to the question of whether the TNC loss we observed has any clinical significance because NRBCs do not have hemato-

poietic regeneration potential. Therefore, if loss of TNCs happened preferentially in the NRBCs population, it may not have affected the engraftment potential of the UCB units. Although we have no data to refute or support this hypothesis, we believe that it is unlikely that loss of NRBCs explains most or all of the loss of TNCs based on findings of the St Louis and Ohio Cord Blood Bank where NRBC recovery after thawing and washing reached 67 percent.¹³ Nevertheless, engraftment data would likely provide a more definitive answer to the clinical significance of our results. Unfortunately, the UCB units processed in our study were not used for transplantation. One recent study compared outcome of UCB transplantation with washed versus unwashed UCB units.¹⁷ They did not find any difference in days to neutrophil and PLT engraftment between the two arms. There was no difference, however, in the baseline TNC and CD34+ cell content of the washed and nonwashed UCB products before infusion.

Because of its impact on engraftment, concern for loss of cells due to the wash step is warranted, because cell (TNC and CD34+) counts will be of primary importance in graft selection. By use of a PF NC dose of 1.5×10^7 NC per kg as the minimum recommended cell dose for transplantation, we evaluated the inventory of the American Red Cross Cord Blood Program to see what impact cell loss related to processing might have on the availability of UCB. Our analysis suggests that cell loss associated with processing significantly impacts the number of UCB units suitable for transplantation in patients weighing 40 kg or more (Table 2). Patients weighing between 40 and 60 kg would seem to gain more if the washing step was removed. Further studies are needed to accurately establish whether UCB processing has a significant impact on the number of suitable units available in cord blood banks.

The importance of CD34+ cell content is also apparent, because recent studies have shown a correlation between the number of CD34+ cells in the product and either time to complete engraftment^{5,17} or time to only PLT engraftment.⁶ We were unable to show any significant effect of UCB processing on CD34+ cell content. Although

TABLE 2. Impact of cell loss by processing on number of suitable UCB units for transplantation by recipient's weight*

Variable	Recipient's weight (kg)						
	10	20	30	40	50	60	70
Cryopreserved units (%)	100	100	99	94	83	69	43
Thawed-washed units (%)	100	100	94	68	42	42	10
Thawed-only units (%)	100	100	99	83	54	42	10

* Shown in this table are the percentages of units banked at the American Red Cross Cord Blood Bank (St. Paul, MN) meeting the minimum NC dose of 1.5×10^7 NCs per kg, with the cryopreserved cell dose. The results are presented by hypothetical recipient's weight category (courtesy of American Red Cross Cord Blood Bank.)

an obvious explanation for these results would be that UCB processing has little impact on CD34+ cell content, we think that one should be cautious before drawing such a conclusion for the following reasons. First, the standard deviation (SD) in our sampling is elevated. Rogers and associates¹² have shown in a study looking at 4000 banked UCB samples that there is a large interdonor variation in CD34+ cell counts measured before cryopreservation. They have also observed a significant correlation among CD34+ counts, WBC counts, and UCB volume collected. In our study, the collected volumes and pre-RBC depletion WBC counts showed little variability. Therefore, if we assume their analysis to be true, then the higher variability we observe exclusively in CD34+ cell counts cannot be explained by variability in volumes collected or TNCs. Our elevated SD, however, can be explained by the low number of UCB units included in this study coupled with a large variability in CD34+ cells collected. Second, for each UCB unit, there was no trend in PF CD34+ content when compared with PW. Some individual UCB units see their CD34+ content apparently increase while others decrease or remain unchanged. We did observe an apparent increase in CD34+ content from the PT to the PW time point in 8 of 10 units, which we cannot explain. Up regulation of CD34 surface expression related to cryopreservation and thawing of HPCs has not been well documented in the literature. Activation of the protein kinase C pathway, however, has been shown to increase CD34 surface expression *in vitro* in marrow CD34+ cells.¹⁸ To our knowledge, whether the thaw and wash cycle could cause up or down regulation of CD34 surface expression through the protein kinase C pathway or another pathway has not been studied. It remains an interesting theoretical possibility that could explain this apparent yet not significant increase in CD34+ cell content from PT to PW. For these reasons, we cannot rule out that UCB processing has no impact on UCB CD34+ content based on our results. In fact, the significant loss of TNCs and viable TNCs that we observed raises the possibility that UCB processing could influence CD34+ cell recovery but this would need to be studied with a higher number of UCB units. Other explanations for our results could be the use of a dual-platform for CD34+ cell enumeration, which likely increases the variability in measurements.

CFU assay or related assays have also been shown to correlate with time to engraftment.² In our study, CFU results showed a pattern similar to CD34+ cells: they decreased PT and increased PW. CFU results are significant probably because the relative importance of the changes observed are greater at each step than they were for CD34+ cells and also because of less unit-to-unit variability. We suspect that an inhibitory effect of DMSO on cell culture¹⁹ is responsible because the samples taken PT were not washed or significantly diluted. When DMSO was removed, as it was in the PW samples, the CFUs increased.

An additional factor that could have had a negative impact on culture was the presence of dead cells and cell debris in PT samples. These were removed along with DMSO during the washing step. Therefore, the CFU results probably do not allow us to make a valuable comparison between PT and PW samples.

For viability assessment, we used two acceptable but different methods. AO/PI staining, initially described for pancreatic islet cell viability,²⁰ has been adapted to HPC viability assessment²¹ and shown to correlate with CFU-GM frequency and is an easy and reliable method. Dual staining allows a comprehensive assessment of both dead and viable cells. The large difference in percentages between AO/PI and 7-AAD (flow cytometry) viability reported in this study is difficult to explain, but we see it consistently even outside of this study and it probably reflects the difference in technique. Whereas the AO/PI viability involves a global assessment of the whole sample, including cellular debris and apoptotic cells, flow cytometry viability involves exclusion of cellular debris at the data acquisition step, before gating on the 7-AAD-negative population. Debris is not usually counted in the AO/PI viability assessment. Furthermore, with flow cytometry, the degree to which dead cells are excluded along with debris is thought to be marginal. We cannot exclude the possibility that exclusion of dead cells is responsible for the higher viability observed by flow cytometry, however.

The passage of time after completion of thaw-wash brings further loss of TNCs. Even though this becomes significant after 2 hours, the CFU results do not change. This suggests that a few hours between the end of processing and infusion probably have little impact on the quality of the product infused and presumably on the risk of graft failure. Because all units were washed, we did not look at QC variables of unwashed cord blood products over time. We are not aware of published studies specifically examining DMSO toxicity on cord blood cells. DMSO toxicity on other sources of HPCs (marrow and peripheral blood), however, has been studied, and two reports suggest that CFU results are not significantly affected when HPCs are suspended in a solution containing 8 to 10 percent DMSO for up to 2 hours.^{22,23}

Although our study is limited in size, we believe that our results support the need for an assessment or reevaluation of the rationale for washing in cord blood processing. The potential benefits of removing debris and DMSO should be weighed against the impact of cell loss in the infused product, particularly if the product is to go to a heavier individual. From a biologic standpoint, cord blood CD34+ cells have been shown to be more sensitive to hypoosmotic conditions as opposed to hyperosmotic conditions with CFU assays.²⁴ This would indicate more sensitivity to removal of cryoprotectant. In autologous transplantation with cryopreserved marrow or peripheral blood progenitor cells (PBPCs), the products are not rou-

tinely washed. Noncardiovascular toxicities such as nausea, chills, and headache are reported in up to 70 percent of cases and cardiovascular toxicities, including transient hyper- or hypotension, are observed in 20 to 90 percent in various series.²⁵ Serious toxicities, however, are uncommon. Alessandrino and associates²⁶ showed that noncardiovascular toxicities occurred in 8 percent of PBPCs and 19 percent of marrow products and cardiovascular toxicities in 60 percent of PBPCs and 37 percent of marrow products.²⁶ In this study, the mean DMSO infused was 0.6 mg per kg and the mean volume infused was 400 mL. A retrospective analysis of infusional reactions due to UCB at our institution²⁷ (n = 82) showed that reactions with UCB infusions are less frequent and less severe than those associated with PBPCs^{28,29} and marrow.^{30,31} This could be explained in part by the fact that the UCB units were washed, whereas PBPCs and marrow in the cited studies were not washed.

With UCB transplantation, the actual amount of infused DMSO per kg is usually less than 0.6 mg per kg. Although the final DMSO concentration in both PBPCs and UCB products is 10 percent, the mean volume of thawed UCB in our study was only 28 mL, which included 5 to 6 mL of cryoprotectant solution. Syme and colleagues³² compared infusional reactions from autologous PBPCs and marrow in adult recipients between regular (unwashed) and washed products. There was significantly less gastrointestinal toxicity in patients receiving the washed products but there was no difference in cardiovascular or other serious toxicities. This was achieved at the expense of a 29 percent loss of CD34+ cells, 15 percent loss of TNCs, and a 3- to 4-hour procedure. With this in mind, even though there is evidence that DMSO can produce serious toxicity at higher doses, it remains to be proven that washing away the small quantity of DMSO in UCB products reduces the risk of serious infusional reactions, justifying significant losses of progenitor cells.

The overall loss of TNCs due to standard UCB processing (before cryopreservation and PT/PW) approaches 35 percent. More than half of this loss occurs when thawing and washing the products after cryopreservation; the washing step is responsible for close to half of the TNC loss. Although our results suggest that most of the TNC loss attributable to washing is nonviable cells, the loss of viable TNCs assessed by AO/PI remains significant. We were not able to show any significant effect of UCB processing on CD34+ cells recovery. Elapse of time after washing entails additional significant loss of TNCs. In conclusion, our results suggest that consideration should be given to removing the washing step from UCB processing altogether. At a minimum, the wash step should be omitted in certain categories of patients where the loss of cells may have a detrimental effect on the number of suitable units available for transplantation.

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