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COMPARISON OF PRE-CRYOPRESERVED AND POST THAW AND WASH NUCLEATED CELL COUNT ON MAJOR OUTCOMES FOLLOWING UNRELATED CORD BLOOD TRANSPLANT IN CHILDREN

Meghann Pine McManus, DO, MPH¹, Li Wang, MS³, Cassie Calder², Becky Manes², Misty Evans², Kathryn Bruce, PharmD², Richard H. Ho, MD², Jennifer Domm, MD², and Haydar Frangoul, MD²

¹Pediatric Stem Cell Transplant Program, Primary Children's Medical Center, Salt Lake City, UT, United States

²Pediatric Stem Cell Transplant Program, Monroe Carell Jr. Children's Hospital at Vanderbilt, Nashville, TN, United States

³Department of Biostatistics, Vanderbilt University, Nashville, TN, United States

Abstract

Engraftment and overall survival after umbilical cord blood transplant is highly dependent on the total nucleated cell count (TNC). The contribution of the wash step to cell loss and ultimately the dose of cells available for transplant is not well described. To investigate the amount of cell loss after washing and its impact on major outcomes compared to pre-cryopreserved TNC, we analyzed data from patients prospectively enrolled on a National Heart, Lung and Blood Institute sponsored cord blood transplant study between 1999 and 2003. There were 310 patients 18 years of age with malignant (N=218) or non-malignant (N=92) disease enrolled on this trial. Only single cord blood units were used. All cord blood units were thawed and washed using an identical process. The median TNC after thawing and washing (PTW) was $5.43 \times 10^7/\text{kg}$ (79% recovery of cells). The cumulative incidence of neutrophil engraftment was significantly higher in patients receiving a PTW TNC $< 2.5 \times 10^7/\text{kg}$ ($p=0.01$). The cumulative incidence of transplant related mortality was higher among patients receiving post thaw and wash TNC $< 2.5 \times 10^7/\text{kg}$ ($p=0.039$). In conclusion, receiving a PTW TNC of $< 2.5 \times 10^7/\text{kg}$ resulted in worse neutrophil engraftment and increased transplant related mortality compared to a PTW TNC of $> 2.5 \times 10^7/\text{kg}$.

Keywords

Cord blood transplant; Pediatrics; Cell dose; Washing and Thawing

Introduction

Umbilical cord blood is being used more frequently as a source of hematopoietic stem cells for transplantation, especially in children. Engraftment and overall survival after umbilical

Correspondence to: Haydar Frangoul, MD, Division of Pediatric Hematology Oncology, Vanderbilt University, 2200 Pierce Ave, 397 PRB, Nashville, TN 37232-2573, United States, Tel: 615-936-1762, Fax: 615-936-1767, Haydar.Frangoul@vanderbilt.edu.

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cord blood transplant is highly dependent on the total nucleated cell count (TNC) and human leukocyte antigen (HLA) matching (1, 2). These are the two main factors used to select the appropriate cord donor unit. The TNC for each cord blood unit is measured before the cells are processed and preserved. Therefore, it is a reasonable concern to worry about the effects that freezing, thawing and washing have on the TNC. It is widely known that choosing a TNC 2.5×10^7 per kilogram is associated with improved overall survival (OS), decreased time to engraftment and decreased risk for graft failure (1). There are multiple reports comparing cell dose data before and after cryopreservation. (3–5). These studies have shown that there is a significant loss of cells, with an overall TNC loss approaching 35% (4). The main limitation of prior studies include using various methods of washing and thawing cord blood units and using different preparative regimens, graft versus host disease (GVHD) prophylaxis and supportive care protocols. The impact of the post thaw and wash (PTW) cell count on major outcomes following cord blood transplant is not known. We sought to determine if the PTW cell count had a more significant impact on major outcomes following cord blood transplantation in children compared to pre-cryopreserved cell counts.

Material and Methods

The cord blood transplant (COBLT) study is a multi institutional trial of cord blood transplants sponsored by the National Heart, Lung and Blood Institute (NHLBI) branch of the National Institutes of Health. A total of 364 adults and children were prospectively enrolled on the study between 1999 and 2003. The protocol was approved by the institutional review board (IRB) of all participating centers. Data from the study were made available by the NHLBI for analysis via a limited data agreement with Vanderbilt University. The analysis was approved by the Vanderbilt University Medical Center IRB. Three hundred and ten patients 18 years of age who lacked HLA matched related donors with malignant and non-malignant diseases were enrolled. Only subjects receiving one cord blood unit (CBU) were eligible. Searches for CBU were conducted by using low/intermediate resolution molecular typing for HLA class I (A and B) and high resolution molecular typing for HLA DRB1. Selection of the unit was based on providing the highest number of TNC per kilogram of body weight and matching at a minimum of 3/6 HLA loci. All cord blood units were thawed and washed using an identical process developed by Rubinstein et al. (6).

Transplant procedure

The conditioning regimen for patients with non-malignant diseases consisted of busulfan, cyclophosphamide and equine antithymocyte globulin (ATG). For patients with malignant diseases the conditioning regimen consisted of total body irradiation (TBI), cyclophosphamide and ATG. One stratum evaluated the safety of busulfan, melphalan and ATG in young patients and those who were not able to tolerate TBI. The details of these preparative regimens have been previously reported (7–9). GVHD prophylaxis consisted of methylprednisolone 0.5 mg/kg twice daily on days +1 through +4 and then 1 mg/kg twice daily from days +5 to day +19 or until the first day the absolute neutrophil count (ANC) reached 500/ μ L, at which time the dose was tapered at the rate of 0.2 mg/kg/wk. Cyclosporine was started on day -3 and continued until at least day 180. The dose was then tapered at the rate of 5% of the initial dose per week, if the recipient had no evidence of GVHD.

Definitions

Neutrophil engraftment was defined as achieving an ANC of at least 500/ μ L for three consecutive measurements on different days and demonstrating donor chimerism of > 90%.

Platelet engraftment was defined as achieving a platelet count above 50,000/mm³ for three consecutive measurements on different days and not requiring platelet transfusions for a minimum of seven days. The grading of acute GVHD followed the GVHD consensus grading scheme (10). An algorithm calculated the maximum GVHD clinical grade based on the weekly organ staging in skin, upper and lower gastrointestinal tract, and liver. This calculated organ stage was decreased by one stage if a listed specific differential diagnosis was reported for either gastrointestinal tract or liver. An independent panel reviewed all weekly records and assigned each patient a final maximum grade, similar to the methods described by Weisdorf et al (10).

Statistical analyses

To assess the association between TNC and the OS, we used Cox proportional hazards model while controlling for multiple risk factors that have been shown to influence the outcome. Separate multivariate Cox models were generated for pre-wash cell TNC and PTW TNC. We used regression modeling of subdistribution functions in competing risks to assess the association between TNC and other outcome variables including transplant related mortality, neutrophil engraftment, platelet recovery, acute GVHD, and chronic GVHD, while controlling for other risk factors and considering death as the competing event. All models included age, gender, disease (malignant versus nonmalignant), performance status (< 90 versus ≥ 90), HLA Match (3–4/6 versus 5–6/6 match) and CMV status. The nonlinear effects of age and the total cell count were accounted for by including a quadratic term. To test for a difference in the predictive value of the models containing pre-wash cell TNC and PTW TNC, we used the likelihood ratio (LR) χ^2 test for nested models to assess whether pre-wash cell TNC adds predictive value to a model that includes PTW TNC and whether PTW TNC adds predictive value to a model that includes pre-wash cell TNC. The adequacy index was also calculated, which is the fraction of the total LR χ^2 explained by a set of variables that could be explained by omitting the competing variables. Adequacy (A) index can be used to quantify the predictive information contained in a subset of predictors compared to the whole set of predictors, which takes value between 0 and 1. When A=1, the subset of the predictors contains all the predictive information as in the whole set of predictors; which means, the subset is adequate by itself and the additional predictors contain no independent information. On the other hand, when A=0, the subset of predictors contains no predictive information by itself. Therefore, A index can be used to select from competing predictors. All statistical analyses were performed using R software (version 2.10.0, Vienna, Austria).

Results

There were 310 patients enrolled with a median age of 4.6 years (range 0.04 – 17.9) who were included in this analysis. Sixty three patients (20%) had acute myelogenous leukemia (AML), 121 patients (39%) had acute lymphoblastic leukemia (ALL), 71 patients (23%) had a storage disease, 34 patients (11%) had other malignant diseases and 21 patients (7%) had other non-malignant diseases. One hundred and forty-four patients (46%) received a HLA matched or single antigen mismatch cord blood. The median follow up of surviving patients is 21 months (range 3–64). The patients were comparable with respect to gender, race, performance status, TNC per kilogram infused, degree of graft mismatch and CMV status. (Table I).

The median pre-cryopreserved TNC per kilogram infused was $6.93 \times 10^7/\text{kg}$ (range $1.5\text{--}80.9 \times 10^7/\text{kg}$). The median PTW TNC per kilogram infused was $5.43 \times 10^7/\text{kg}$ (range $1\text{--}31.6 \times 10^7/\text{kg}$). The average cell recovery was 79% after thawing and washing. There were 15 patients (5%) who received a CBU which had a pre-wash TNC of $<2.5 \times 10^7/\text{kg}$. Of the

295 patients who received a CBU with a pre-wash TNC $2.5 \times 10^7/\text{kg}$, there were 24 patients (8%) who subsequently had a measured PTW TNC of $<2.5 \times 10^7/\text{kg}$. The cumulative incidence of neutrophil engraftment was significantly higher among patients receiving a PTW cell dose of $2.5 \times 10^7/\text{kg}$ compared to those receiving a PTW dose of $<2.5 \times 10^7/\text{kg}$ ($p=0.01$). The cumulative incidence of neutrophil engraftment at day 42 for those receiving a PTW TNC $<2.5 \times 10^7/\text{kg}$ is 56% (95% CI: 41–72) compared to 75% (95% CI: 69–80) for those receiving a PTW dose of $2.5 \times 10^7/\text{kg}$. Interestingly the cumulative incidence of neutrophil engraftment was similar in those receiving a pre-wash TNC of $<2.5 \times 10^7/\text{kg}$ compared to those receiving a PTW TNC of $<2.5 \times 10^7/\text{kg}$, 53% (95% CI: 27–80) and 56% (95% CI: 41–72) respectively. The results were similar for cumulative incidence of neutrophil engraftment at day 100 (data not shown). The cumulative incidence of TRM was significantly higher ($p=0.039$) in patients who received a PTW TNC $<2.5 \times 10^7/\text{kg}$ compared to those who received a dose $2.5 \times 10^7/\text{kg}$. The cumulative incidence at 1 year of TRM for those receiving PTW TNC of $2.5 \times 10^7/\text{kg}$ was 17% (95% CI: 13–22) compared to 31% (95% CI: 16–45). The TRM at 1 year for patients receiving a pre-wash TNC of $<2.5 \times 10^7/\text{kg}$ was 33% (95% CI: 08–58).

Using multivariable analysis using pre and post wash TNC as a continuous variable, neutrophil engraftment was significantly associated with PTW TNC (HR 1.55, 95% CI: 1.16–2.08) but not pre-cryopreserved TNC infused (HR 1.17, 95% CI: 0.96–1.44). Both pre-cryopreserved and PTW TNC infused were not predictive for platelet engraftment. The risk of grade II-IV, III-IV acute GVHD was significantly higher among patients receiving higher pre or post wash TNC. There was a trend towards worse TRM in patients receiving lower PTW TNC ($p=0.049$) but not a lower pre-cryopreserved TNC ($p=0.56$). The predictive values measured by the LR χ^2 of PTW TNC are higher for both transplant related mortality and neutrophil engraftment. For transplant related mortality, using the LR χ^2 test for nested models, pre wash TNC did not add predictive value to a model that includes PTW TNC (LR $\chi^2 = 0.63$, $df = 2$, $p = 0.731$), whereas PTW TNC added more predictive value to a model that includes pre wash TNC (LR $\chi^2 = 5.5$, $df = 2$, $p = 0.064$). The adequacy index for pre wash TNC and PTW TNC were 0.88 and 0.99, respectively. For neutrophil engraftment, both pre wash TNC and PTW TNC added more predictive values (LR $\chi^2 = 9.1$, $df = 2$, $p = 0.010$ and LR $\chi^2 = 19.9$, $df = 2$, $p < 0.001$, respectively). The adequacy index for pre wash TNC and PTW TNC were 0.59 and 0.81, respectively indicating that PTW TNC has more predictive value to neutrophil engraftment. For the other outcome variables, we did not observe a differences between pre wash TNC and PTW TNC.

Discussion

To our knowledge, this is the first study to compare the effect of pre-wash and PTW TNC on the major outcomes following unrelated CBT. It was recognized early on in the development of unrelated CBT, that the obstacle of cell processing and storage would have to be overcome to make this donor source practical (6). Rubinstein et al. described a processing and cryopreservation method which allowed for a good recovery of hematopoietic colony-forming cells and storage in smaller volumes, saving both in cost and space (6). Laroche et al. reported on the amount of cell recovery in post thaw and post wash cord blood samples, as well as various time points after the wash step. His group showed that although there was generally good recovery of cells with currently used methods, the TNC recovery was 89% post thaw, which decreased to 82% post wash and even further at two and five hours after the washing step (4). Wagner et al. compared measurements at their transplant center to those provided by the cord blood bank to determine if there were significant differences in the reported information. They found that the TNC correlated well between their transplant center and the cord blood banks, although the TNC was often

lower, with a median difference of 10.4% to 16.6%, upon thawing and infusion, respectively (5).

To further examine the effect of storage conditions on the cellular content of processed cord blood units, Kurtz et al. performed various studies. They observed that both the TNC and CD34+ cell levels were well retained after samples were stored in test tubes after processing at 1 to 6° Celsius for three days. The authors also found, in concordance with the results of Laroche et al. and Wagner et al. that there were reductions in TNC levels of cryopreserved thawed samples compared to prefreeze samples (3–5). In our current analysis the TNC recovered after the thawing and washing of cells was 79%, which is comparable to previously reported studies. There are several methods to thaw and washing cord blood units. Most of these methods have a recovery ranging from 77–86% (11). Rubinstein et al. evaluated outcomes among 562 recipients of unrelated cord blood transplant and reported that receiving a pre-washed cell dose of $<2.5 \times 10^7/\text{kg}$ resulted in an increased risk of complications and death (12). Similar results were reported in children with acute leukemia where recipients of a pre-wash TNC of $<3 \times 10^7/\text{kg}$ had worse outcome (13). The prior two studies report data from multiple centers that have different practices in thawing and washing the units, different preparative regimens, GVHD prophylaxis and supportive care. Advantages of our current study include the use of identical GVHD prophylaxis and supportive care as well as all centers were trained and used an identical thawing and washing procedure for the units (6).

In our analysis patients receiving a PTW TNC of $<2.5 \times 10^7/\text{kg}$ had similar risk of graft failure and TRM as those receiving a pre-wash cell dose of $<2.5 \times 10^7/\text{kg}$. Interestingly, we were able to identify 24 (8%) patients who had an adequate pre-wash cell dose ($2.5 \times 10^7/\text{kg}$) but after thawing and washing, the TNC was $<2.5 \times 10^7/\text{kg}$. Those patients had identical outcomes to patients receiving a pre-thaw and wash cell count of $<2.5 \times 10^7/\text{kg}$ with increased TRM. In our multivariable analysis, the higher PTW TNC was significantly associated with improved neutrophil engraftment. One limitation of our study is the lack of CD34 cell count that has been associated with engraftment (2). CD34 cell counts were not collected on the PTW samples, so we were unable to evaluate the recovery of these cells or determine how this variable influences major outcomes in our study. Since our analysis is based on transplants using a single cord blood unit, it is unclear what would the effect of thawing and washing is on the outcome following double cord blood transplant. For transplant centers the choice of cord blood units is based on the pre cryopreserved cell dose reported by the cord blood banks. To avoid using a cord blood unit with a PTW TNC of $<2.5 \times 10^7/\text{kg}$, it might be reasonable to choose a single unit with a TNC $3 \times 10^7/\text{kg}$ to account for the expected average cell loss of 20–25% during processing. We conclude that when using single cord blood unit, a PTW TNC of $2.5 \times 10^7/\text{kg}$ of recipient weight is associated with improved neutrophil engraftment and decreased TRM.

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Table 1

Patient Characteristics

Characteristics of Patients	Total, N (%)
Number of patients	310
Age, median (range), years	4.6 (0.04 – 17.9)
Gender	
Male	188 (61%)
Female	122 (39%)
Performance Status	
90	249 (80%)
80	61 (20%)
Median TNC ($\times 10^7/\text{kg}$)	
Pre-wash (range)	6.93 (1.5 – 80.9)
Post wash (range)	5.43 (1 – 31.6)
Diagnosis	
ALL	121 (39%)
AML	63 (20%)
Storage Disease	71 (23%)
Other Malignant Diseases	34 (11%)
Other Non-Malignant Diseases	21 (7%)
HLA match	
6/6 or 5/6	144 (46%)
4/6 or 3/6	166 (54%)
Recipient CMV	
Positive	130 (43%)
Negative	174 (57%)
Not tested	6

HLA = Human Leukocyte Antigen; TNC = Total Nucleated Cell Count; CMV = Cytomegalovirus