Effects of Cryopreservation Duration on the Outcome of Single-Unit Cord Blood Transplantation

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Abstract
Cryopreservation is widely used in umbilical cord blood (UCB) banking, yet its impact on progenitor cell function remains largely unaddressed. It is unknown whether long-term cryopreservation affects UCB transplantation outcomes. Herein, we evaluated the impact of UCB age on clinical outcomes and investigated the effect of cryopreservation duration of UCB on hematopoietic potency in 91 patients receiving single cord blood transplantations. UCB cryopreservation duration was 0.7 to 13.4 y. The most common indication of transplant was thalassemia (48%). There was no significant association between cryopreservation duration and neutrophil engraftment probability (P = 0.475). Cryopreservation duration did not affect the post-thaw viability and subsequent neutrophil engraftment rate. Therefore, UCB units can undergo cryopreservation for at least 8 y with no impact on clinical outcomes.

Keywords
umbilical cord blood, cryopreservation, transplantation

Introduction
Umbilical cord blood (UCB) is a viable source of stem cells, particularly for patients belonging to racial and ethnic minority whose genetic variations are often not included in unrelated volunteer donor registries¹,². With improving expertise and supportive care, UCB transplant outcomes are now comparable to those from unrelated or sibling donors³. The use of UCB as a donor source has increased over the past decade. Although cryopreservation is now universally practiced in cord blood banking, its impact on progenitor cell function has been only partially addressed⁴. It is essential to determine the reasons for the quantitative association and improve selection criteria for UCB grafts using indices that are strongly predictive of engraftment. Cord blood acquisition and expansion are precious, and maintaining a cord blood bank is expensive⁵. Volume reduction procedures concentrate progenitor cells to optimize the use of storage space. If long-term cryopreservation is detrimental to UCB transplantation outcomes, the current model of cord blood banking must be revisited. In this present study, we sought to determine whether cryopreservation duration influenced single UCB transplantation outcomes if the quality parameters and surrogate markers were not significantly different between groups.

Materials and Methods
All patients who underwent single UCB transplantation at Chang Gung Children’s Hospital between October 2003 and October 2015 were included in this single-center analysis. Informed consent was waived because of the retrospective nature of the study.

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Overall Study Design

We reviewed electronic medical records and selected patients according to our inclusion and exclusion criteria for unrelated donor cord blood transplantation (CBT) in children. Cord blood units for the present study were selected from two National Marrow Donor Program–affiliated UCB banks to ensure clarity of measurement. Initially, units were considered if their human leukocyte antigen (HLA)-A, -B, and -DRB1 phenotypes matched at least 4 of the patient’s submitted 6 antigen-HLA phenotypes at a serologic or intermediate DNA level of resolution. HLA-A,-B, and -DRB1 compatibility was further confirmed using polymerase chain reaction sequence-based typing to provide absolute HLA resolution to 2 digits and a high probability resolution to 4 digits. A single allele or antigen-level mismatch at HLA-DRB1 was considered acceptable.

Cord blood banks use two methods to store frozen UCB: plasma depletion (PD) or red cell reduction (RCR). The PD method removes plasma, saves all the cells, and freezes them in 10% dimethyl sulfoxide (DMSO). The RCR method centrifuges cord blood in hetastarch or albumin to isolate 21 mL of cord blood containing mostly white blood cells, adds 4 mL of 50% DMSO, and then freezes the resulting 25 mL of cell suspension. Data analysis using the Pediatric Hematopoietic Stem Cell Program was approved by the institutional review board of Chang Gung Memorial Hospital, and the requirement for informed consent was waived (Approval No.: 104-9676B).

Measures

UCB units delivered to Chang Gung Children’s Hospital were inspected, transferred, and maintained in vapor-phase liquid nitrogen storage until the day of infusion. ABO/Rh typing of the unit was performed before the wash. Data on total nucleated cell (TNC) count, CD34+ cell counts, and cell viability before freezing were retrieved from the bank’s database. TNC counts were repeated locally after thawing and dilution. CD34+ cell counts were measured at the time of unit collection, and CD34+ viability was determined using flow cytometry with 7-aminoactinomycin D (7-AAD). Flow cytometry was performed according to International Society of Hematotherapy and Graft Engineering specifica-

Biological Controls

TNC and CD34+ cell counts and CD34+ cell viability were determined for samples obtained from UCB before and after volume reduction. The counts were not corrected for nucleated red blood cells (RBCs). Flow cytometry was used to quantify the CD34+ cell content. TNC viability was measured using 0.4% trypan blue staining and 7-AAD by excluding the nonviable cells during CD34+ cell enumeration; 7-AAD is a membrane-impermeable dye that binds to double-stranded DNA by intercalating between base pairs in G-C-rich regions and is generally excluded from viable cells. The viability of CD34+ cells was measured using 7-AAD, which was excited at 488 nm with an argon laser.

UCB units were analyzed by comparing their cryopreservation durations. We grouped UCB units in tertiles based on cryopreservation durations (<4 y, 4.1 to 8 y, and >8 y) with an arbitrary lower limit cutoff of 4 y. TNC recovery was defined as the total TNC counts recovered at thaw, expressed as a percentage of the total TNC count reported before freezing. Neutrophil and platelet engraftments were defined as the first of 3 consecutive days with an absolute neutrophil count of >0.5 × 10^9/L and a platelet count of >20 × 10^9/L unsupported by transfusion. Variables assessed for their association with neutrophil engraftment included cryopreservation duration, pre-freeze TNC/kg, pre-freeze CD34+/kg, post-thaw viability, UCB unit–recipient HLA match, recipient sex, recipient age, and primary diagnosis of the recipients.

Statistical Analysis

All patients were followed longitudinally until death or until the final follow-up. Statistical analysis was performed using SPSS Statistics version 18 (SPSS Inc., Chicago, IL, USA). UCB durational tertile data were tested using Kruskal–Wallis nonparametric analyses. Cox regression analysis was used to perform univariate and multivariate analyses of patient and UCB unit factors and their influence on outcomes. Mann–Whitney U test was used for statistical comparisons of continuous data. A P value of <0.05 was considered statistically significant.

Results

UCB units were classified based on cryopreservation duration: <4 y, 20 units; 4.1 to 8 y, 39 units; and >8 y, 32 units. UCB units were analyzed in tertiles based on cryopreservation duration. Of the 91 enrolled patients, 22 who received UCB were aged >10 y (UCB recipients aged >12 y = 6, 11 to 12 y = 8, and 10 to 11 y = 8). The cryopreservation duration of UCB units ranged from 0.7 to 13.4 y (Fig. 1). UCB units were obtained from StemCyte Cord Blood Registry (n = 79) and the Buddhist Tzu Chi Cord Blood Bank (n = 12), both of
which are accredited by the National Marrow Donor Program. The former one stores cord blood units (CBUs) frozen with the PD method and the latter with RCR method. The median TNC dose for the 91 patients was \(7.5 \times 10^7\) NC/kg (range = 2.5 to 19.3 \(\times 10^7\) NC/kg), and the median CD34\(^+\) cell dose was \(4.3 \times 10^5\) kg (range = 0.6 to 23.5 \(\times 10^5\) kg). Thalassemia was the most common indication for transplant (48%). Of the 91 transplants, 79 (86%) achieved neutrophil engraftment at a median of 17 d (range = 12 to 36 d). Median time to RBC transfusion independence was 35 d (range = 10 to 63 d), and median time to platelet engraftment was 48 d (range = 24 to 117 d).

No association was found between cryopreservation duration and neutrophil engraftment probability. Cryopreservation duration did not significantly impact recovery rates of TNCs and CD34\(^+\) cells (Table 1). None of these attribute variables were statistically significant by univariate analysis (Table 2).

**Discussion**

Treatments that use patient-derived cells or patient-generated induced pluripotent stem cells have dramatically increased the need for cord blood banking. Previous reports suggested that short-term cryopreservation had no significant impact on clinical outcomes. A recent study evaluating clinical outcomes using cryopreserved units only documented 15 UCB units that were older than 5 y, and no differences were observed for duration of hospitalization and storage duration or storage year.

Minimum TNC doses may vary with the degree of HLA disparity. We hypothesized that superior unit quality, as measured by a higher percentage of viable cells post-thaw, would determine the outcome of CBT as evaluated by engraftment. The CBU quality may also be due to the confounding effect of age on transplant outcome. However, these procedures are based on plasma and RBC depletion, and cell recovery is highly variable. Washing techniques can also result in unnecessary cell loss of 20% to 25% during processing, further reducing the infused cell dose. The present study mainly focuses on cellular recovery.

TNC dose, CD34\(^+\) cell counts, and colony-forming cell (CFC) assays are currently considered to be the most important parameters associated with CBT outcomes. CFC assays were not routinely performed before 2005. While
large interlaboratory variability exists in the latter 2 variables, TNC is a well-standardized surrogate marker of hematopoietic progenitor cell content\(^\text{18}\). Association of the speed of engraftment with the TNC dose is overshadowed by its relationship to the CFC dose, which constitutes a more predictive index\(^\text{19}\). The lack of a standardized method for CD34\(^+\) cell quantification makes it difficult to compare results among banks20. CD34\(^+\) enumeration may be unreliable unless performed using single-platform flow cytometry in accordance with International Society for Hematotherapy and Graft Engineering specifications21.

Clonogenic cultures produce approximate results because of the low number of cases.

The present study has several limitations. First, variation in the UCB collection technique between the 2 different cord blood banks made it difficult to assess the effectiveness of the overarching program delivery processes. Second, the use of 7-AAD is only limited to cell viability and does not provide qualitative information regarding apoptosis status or cell necrosis. Third, we could not analyze the impact of administering cord blood that shares a noninherited maternal HLA antigen (NIMA) with a mismatched HLA antigen in the recipient. These NIMAs may be “permissive” HLA mismatches and could be used to extend the genotypes of suitable matches for specific donors or CBUs22. The fact that there were no significant differences between TNC and CD34+ counts should be cautiously interpreted.

Our data suggested that, although the cryopreservation and thawing process may damage cord blood cells and lead to cell loss, it did not affect the neutrophil engraftment rate. Future studies on CBT may proceed on the assumption that cryopreservation duration does not affect hematopoietic potency; however, this is a small retrospective study in a heterogeneous cohort. Future, well-powered investigations should further validate the efficacy of cryopreserved UCB cells.

### Authors’ Note

T. H. Jaing contributed to study concept and design. Y. C. Wen, S. H. Chen, and T. Y. Chang contributed to data acquisition. P. K. Tsay contributed to data interpretation and analysis. T. H. Jaing, Y. C. Wen, and Y. C. Yang drafted the manuscript. T. H. Jaing critically revised the manuscript.

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### Ethical Approval

Data analysis using the Pediatric Hematopoietic Stem Cell Program was approved by the institutional review board of Chang Gung Memorial Hospital (Approval No.: 104-9676B).

### Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects, rather data analysis of human medical records.

### Statement of Informed Consent

Informed consent was waived because of the retrospective nature of the study.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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### Table 2. Univariate Analysis for Neutrophil Engraftment.*

| Parameter                  | Engraftment Rate (%) | 95% CI         | P Value |
|----------------------------|----------------------|----------------|---------|
| UCB unit cryopreservation, y |                      |                |         |
| ≤4                        | 85                   | 0.74 to 0.92   | 0.475   |
| 4.1 to 8                  | 90                   | 0.70 to 0.97   |         |
| 8.1 to 13.4               | 78                   | 0.45 to 0.97   |         |
| Pre-freeze TNC (\(\times 10^7/kg\)) |                |                |         |
| ≤5                        | 100                  | 0.61 to 1.00   | 0.716   |
| >5                        | 86                   | 0.77 to 0.92   |         |
| TNC viability             |                      |                |         |
| <75%                      | 85                   | 0.73 to 0.92   | 0.748   |
| ≥75%                      | 92                   | 0.79 to 0.97   |         |
| Pre-freeze CD34\(^+\) (\(\times 10^5/kg\)) |                |                |         |
| ≤3.5                      | 81                   | 0.66 to 0.91   | 0.307   |
| >3.5                      | 91                   | 0.80 to 0.96   |         |
| CD34\(^+\) viability      |                      |                | 0.999   |
| <75%                      | 86                   | 0.67 to 0.99   |         |
| ≥75%                      | 87                   | 0.77 to 0.93   |         |
| HLA matching              |                      |                | 0.992   |
| 6/6 or 5/6                | 88                   | 0.77 to 0.94   |         |
| 4/6 or less               | 85                   | 0.70 to 0.94   |         |
| Recipient gender          |                      |                | 0.233   |
| Male                      | 83                   | 0.71 to 0.90   |         |
| Female                    | 94                   | 0.80 to 0.98   |         |
| Recipient age, y          |                      |                | 0.999   |
| <3                        | 90                   | 0.60 to 0.98   |         |
| ≥3                        | 84                   | 0.80 to 0.98   |         |
| Primary diagnosis         |                      |                | 0.788   |
| Thalassemia               | 85                   | 0.72 to 0.92   |         |
| Others                    | 89                   | 0.77 to 0.95   |         |

Abbreviations: HLA, human leukocyte antigen; CI, confidence interval; TNC, total nucleated cell.

*P value of <0.05 was considered statistically significant.
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