

Cryopreservation of cord blood after liquid storage

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Background

The processing of cord blood may result in delays prior to RBC depletion and cryopreservation. The overall objective of this investigation is to determine the influence of liquid storage prior to cryopreservation on the post-thaw viability.

Methods

UC blood supplemented with CPD anticoagulant (CB) was obtained from normal donors with informed consent. CB was stored undiluted, or diluted with 1:1 ratio of storage solution STM-sav for up to 72 h. The undiluted control samples were stored at room temperature. CB samples supplemented with STM-sav were stored at 4°C. After completion of the storage protocol, the sample was RBC depleted, frozen, stored, thawed, and assayed for viability. Nucleated cell counts, percentage of CD34⁺ cells, and frequency of colony formation were determined during liquid storage and after cryopreservation.

Introduction

Clinical banking of cord blood has increased considerably in recent years, with current estimates of the number of units stored being in excess of 70 000 world-wide [1–4]. Regional processing centers have been established for the processing of cord blood (RBC depletion, shipping, and freezing). It is not uncommon for collection site to be distant from the regional processing center. The distance between collection and processing, as well as timing of collections, necessitates the development of procedures for the short-term storage of cord blood, to provide optimum preservation of the samples after collection for transportation to the site of processing.

Previous studies have examined factors that influence the viability and recovery of cells obtained from UC blood (CB) after storage. Shlebak and colleagues observed that liquid storage of CB for over 9 h resulted in a decline in

Results

The post-thaw mononuclear cell recovery and viability of cord blood stored for 72 h was significantly lower than that of cord blood stored for 24 h prior to cryopreservation. This difference was true for cord bloods stored in STM-sav and controls. Dilution of the cord blood with STM-sav improved the frequency of CFU-GM observed.

Discussion

Liquid storage of cord blood for 24 h prior to cryopreservation does not adversely influence the post-thaw cell recovery. The use of a storage solution (STM-Sav) enhances the retention of colony-forming capabilities post-thaw. These and other studies provide an important foundation for the development of integrated protocols for cord blood banking.

Keywords

cord blood, cryopreservation, liquid storage.

post-thaw recovery of CFU-GM [5]. When comparing cryopreservation methods, they showed that both controlled rate and passive cooling of samples resulted in similar post-thaw viability. The samples that were frozen were not stored prior to cryopreservation.

Rogers and colleagues observed that the post-thaw viability of cord blood decreased when it was stored for 24 h of storage prior to cryopreservation [6]. In contrast, Beaujean and colleagues observed that PBPC could be effectively stored for 24 h prior to cryopreservation without significant loss of post-thaw viability [7]. Similarly, several studies of the short-term liquid storage of hematopoietic stem cells obtained from marrow [8–13] and peripheral blood [14–18] have established that tissue culture solutions with anticoagulants or anticoagulated plasma can satisfactorily preserve stem cells at 4°C or room temperature for ≥ 24 h or more. These studies do

not, however, report the effects of short-term storage on post-thaw recovery of progenitors.

In a recent series of studies, we have examined optimal liquid storage conditions and duration for PBSC [19], BM [20], and liquid storage of CB [21]. In general, these studies showed that dilution of the product with a storage solution (specifically, STM-sav) improved recovery of mononuclear cells, CD34⁺ cells, and colony-forming capability. Furthermore, favorable recovery was observed using storage solutions appropriate for human use. Significant losses in viability were observed for all products after 24 h of liquid storage. Storage at 4°C in general resulted in an improvement in the recovery of cells.

For most applications, CB will undergo additional processing after liquid storage (RBC and cryopreservation). Our previous studies did not examine the role of the liquid storage phase on the response of the cells to subsequent processing. Specifically, we are interested in determining the influence of liquid storage conditions on the response of CB cells to the stresses of freezing and thawing.

Thus, the objectives of the present study were to determine the influence of short-term liquid storage prior to cryopreservation on the post-thaw viability of CB. This information will be essential in establishing processing guidelines for the processing of CB that ensures optimum recovery and reduces costs associated with the operation of CB processing centers.

Methods

CB collection

Human CB was aspirated with a 17 ga needle from volunteer donor cord veins using standard procedures (range of volumes obtained: 41–172 mL). A total of 21 CB units were studied, with 11 units stored for 24 h and 10 units stored for 72 h prior to cryopreservation. All samples were collected with informed consent and with the approval of the local Institutional Review Board. Blood was collected into a blood bag (Baxter-Fenwal; Deerfield, NJ, USA) containing approximately 20 mL of CPD (citrate–phosphate–dextrose: 0.52 g sodium citrate, 0.51 g dextrose, 60 mg citric acid and 44 mg monobasic sodium phosphate) while gently mixing.

Liquid storage protocol

Within 4 h of the blood collection time, the experiment was initiated. A blood sample of each CB unit was analyzed

for WBCC, MNC (mononuclear cell) counts, percentage and viability of CD45⁺/CD34⁺, total nucleated cell viability and progenitor [colony-forming units granulocyte–macrophage (CFU-GM)] assay at 0 and 24 or 72 h. These time-points were chosen based on a previous study [21], in which CB was stored for up to 72 h without a significant loss in cell number.

In order to have a realistic volume of blood and sufficient numbers of cells for the assays required, the CB unit was halved, with one portion stored at room temperature as a control arm of the study. Our previous study showed that the cell recovery for undiluted CB was roughly the same for samples stored at room temperature and at 4°C [21]. Thus, the control studies were performed at room temperature. The remaining half of the unit was diluted with an equal volume of STM-Sav [STM-Sav is composed of 1000 mL lactated Ringers (Baxter; Deerfield, IL, USA) solution with 50 Mm histidine and 1% HSA] [19,20] which was compounded at the Fairview-University Hospital Pharmacy (Fairview University Medical Center, Minneapolis, MN, USA). These units were stored at 4°C in a monitored blood bank refrigerator. Units held at room temperature were stored in a small cooler at room temperature, to minimize temperature fluctuations. As with the controls, these samples were stored for 24 or 72 h.

The samples were analyzed for WBCC, MNC counts, percentage and viability of CD45⁺/CD34⁺, total nucleated cell viability and progenitor (CFU-GM) assay after the liquid storage period. A schematic representation of the experimental protocol is given in Figure 1.

Cryopreservation

After the liquid storage period (24 or 72 h), the CB samples were processed for cryopreservation. Each unit was transferred to a separate 150 mL transfer bag (Terumo, Tokyo/Japan). A volume of 6% hetastarch (HES, Baxter) solution equal to 20% of the CB volume was added to each bag. The STM-Sav units had been diluted with the STM-Sav solution, the volume of HES added to these units was therefore approximately twice that added to the controls. After mixing, the resulting solutions were centrifuged at 90 *g* for 6 min (Sorvall, RC-3 series, Kendro Laboratory Products; Newtown, CT, USA). Units were centrifuged upright, and the supernatant was transferred to a separate bag using a plasma expressor. The final volume of the unit was reduced to 15 mL. A sample of the RBC-depleted product was assayed for WBCC, MNC counts, percentage

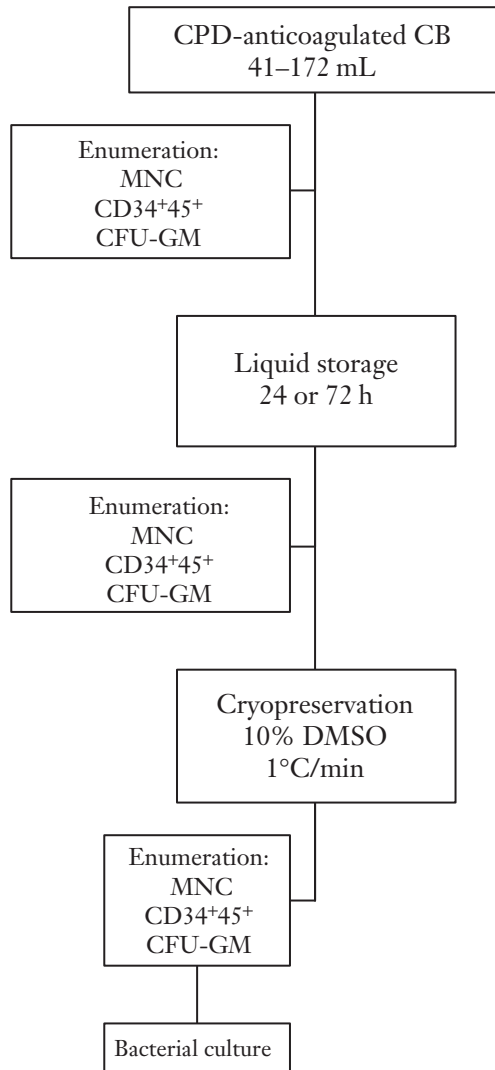


Figure 1. Schematic representation of the CB processing protocol.

and viability of CD45⁺/CD34⁺, total nucleated cell viability and progenitor (CFU-GM). The remaining processed blood was transferred to a 50 mL Cryocyte bag (Baxter)

The cryopreservation medium containing 55% DMSO w/v and 5% dextran 40 w/v (Protide; St Paul, MN, USA) was added to the RBC-depleted blood to a final concentration of approximately 11% DMSO. The sample was transferred to a controlled rate freezer (Planer, Kryo 10, series III; Middlesex, UK) that was pre-cooled to 0°C. The sample was then cooled at 1°C/min to -12°C, cooled at 20°C/min to -60°C, followed by warming of the sample at 15°C/min to -18°C, cooled at 1°C/min to -60°C and finally, 3°C/min to -100°C. After completion of the freezing protocol, the units were removed from the

controlled rate freezer and stored in the vapor phase of nitrogen.

Thawing protocol and post-thaw viability assessment

After a minimum of 14 days of storage in liquid nitrogen, units were thawed in a 37°C water bath (National; Portland, OR, USA) for analysis. Prior to further processing, samples were drawn from every unit for viability assessment. A minimum of 15 mL each of dextran 40 (Abbott Laboratories; North Chicago, IL, USA) and 5% human albumin (Baxter Healthcare Corporation; Glendale, CA, USA) were slowly added to each unit. The resulting solutions containing the thawed cells were centrifuged in a pre-cooled centrifuge (Sorval, RC series, Kendro Laboratory Products). After removal of the supernatant, cells were re-diluted for analysis.

As with previous studies, WBCC, MNC counts, percentage and viability of CD45⁺/CD34⁺, total nucleated cell viability and progenitor (CFU-GM) assay were performed. In addition, samples were also taken for bacterial culture to determine if the sample had been contaminated.

Assays

MNC counts were performed manually using a Zeiss Axioskop (Zeiss; Jena/Germany) or Leitz Laborlux S microscope (Leica; Wetzlar/Germany) at 100× magnification with a fluorescent light source. All samples were diluted with AO/PI (acridine orange-propidium iodide) [22]. A Hausser Scientific (Hausser Scientific; Horsham, PA, USA) brightline hemacytometer was used to make all counts. All cells fluorescing green were scored as viable. MNC count was determined based on the staining pattern of the cell and the morphology of the cell using brightfield microscopy. Manual MNC counts were confirmed using a Coulter ACT-T diff counter (Coulter, Hialeah). MNC recovery was calculated by dividing the viable MNC counts at a given time point (24, 72 h or post-thaw) by the initial viable MNC count.

CD45⁺/CD34⁺ percentage, viability and total nucleated cell viability were analyzed by flow cytometry.

RBC were lysed and samples were stained with anti-CD45-FITC Hle-1 and anti-CD34-PE HPCA-2 (Becton-Dickinson Immunocytometry Systems; San Jose, CA, USA). The cells were stained with Via-Probe (7-AAD, BD Pharmingen; San Jose, CA, USA) to assay viability.

Utilizing a Becton-Dickinson FACSCalibur flow cytometer, specimens were then evaluated using ISHAGE gating [23]. Briefly, this included primary gating region based on CD45 and 90° light scatter (SSC), and secondary gating based on CD34 expression and SSC. True CD34⁺ cells were defined as cells that express strongly CD34 Ag, are dim CD45⁺ and show low complexity (low SSC value). The number of CD34⁺/45⁺ cells was calculated by multiplying the total number of nucleated cells times the percent of CD34⁺/45⁺ cells. The fraction of dead cells was determined based on the fraction of cells that incorporated the viability dye. The recovery of CD34⁺45⁺ cells was calculated by dividing the number of viable CD34⁺45⁺ cells at a given time-point by the number of viable CD34⁺45⁺ cells at Time 0 h.

Progenitor assays were performed using methylcellulose (Methocult GF H4434, Stemcell Technologies; Vancouver, BC, Canada) [24]. RBCs were lysed with ammonium chloride for samples taken at $t = 0$ and after liquid storage. Post-thaw samples were not lysed because the cryopreservation process resulted in RBC lysis, so no further depletion was necessary. All cell samples were diluted and then added to methylcellulose to result in final cell concentrations of 30 000 nucleated cells/mL. One mL of cells/methylcellulose was plated in duplicate into 22 mm diameter non-tissue culture treated, hydrated 12-well plates. Plates were incubated in a 37°C temperature, 99% humidity, 5% CO₂, incubator for 14 days. An Olympus SZ60 (Olympus; Tokyo, Japan) inverted variable magnification microscope was used to identify and count progenitor assay colonies. Each well was scored for total colony and CFU-GM counts. CFU-GM results are the mean average number of colonies per 10⁵ nucleated cells represented by the formula:

$$\text{CFU-GM colonies counted/No. cells plated} \times 100\,000$$

Statistical analysis

The percentage of viable cells, percentage of viable CD34⁺CD45⁺ cells, and CFU-GM per 10⁵ cells were determined as a function of the experimental conditions (time period for liquid storage and storage in STM-Sav compared to control). The frequency of CFU-GM, CD34⁺ recovery, and MNC recovery were analyzed using the Student's *t*-test. Due to non-normal distribution of CD34⁺ counts, a log transformation of the data was performed prior to analysis using the *t*-test.

Results

Mononucleated cell recovery

Twenty-one units of CB were studied. The volume of CB averaged 67 ± 29 mL (range 41–172 mL) and the average initial MNC count for CB prior to storage was $3.4 \pm 0.9 \times 10^6$ cell/mL. The processing of the cells for the protocol results in significant changes in volume and cell concentration. As such, we calculated the recovery of MNCs at different time-points in the protocol. The recovery is defined as the total number of MNCs at a given time-point divided by the total initial number of MNCs.

For the liquid storage phase of the study, the MNC recovery for samples stored in STM-sav was $150 \pm 37\%$ and $140 \pm 23\%$ at 24 and 72 h, respectively. For control samples, the MNC recovery was 140 ± 36 and $95 \pm 23\%$ at 24 and 72 h, respectively (Figure 2). There was no difference in MNC recovery after 24 h of liquid storage prior to cryopreservation (control and CB stored in STM-sav $p = 0.76$). After 72 h of liquid storage prior to cryopreservation, the MNC recovery for control samples was significantly less than that determined for samples stored in STM-sav ($p < 0.01$).

The addition of a freeze–thaw step in the processing of the CB resulted in further losses. For control samples held for 24 or 72 h prior to cryopreservation, the post-thaw MNC recovery is 61 ± 13 and $51 \pm 21\%$, respectively. For samples stored in STM-SAV for 24 or 72 h prior to cryopreservation, the post-thaw MNC recovery is 64 ± 10 and $51 \pm 21\%$, respectively. For samples stored either 24 or

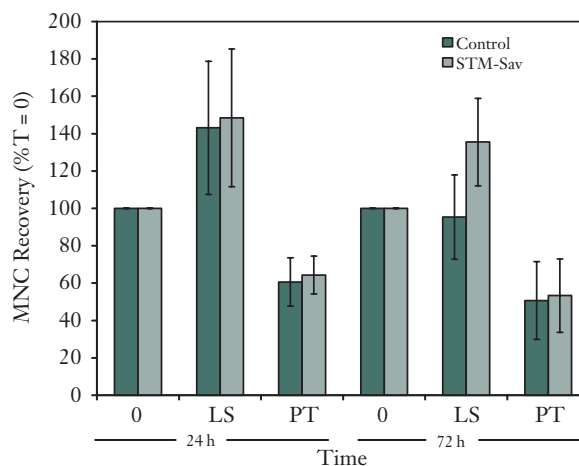


Figure 2. MNC recovery percentage during liquid storage (LS) and post-thaw (PT) for CB stored in STM-sav, and undiluted control CB stored for either 24 and 72 h prior to cryopreservation. Error bars correspond to the SD of the mean.

72 h prior to cryopreservation, there was no difference in post-thaw MNC recovery between control and cord blood stored in STM-sav ($p > 0.48$).

Viable CD34⁺/45⁺ cell recovery

The average initial concentration of CD34⁺CD45⁺ cells was $11 \pm 6 \times 10^3$ cells/mL. There was no statistically significant decline in CD34⁺ recovery over the time-period of liquid storage (up to 72 h, $p > 0.21$) (Figure 3). For control samples held for 24 or 72 h prior to cryopreservation, the post-thaw CD34⁺ recovery was 150 ± 52 and $120 \pm 51\%$, respectively. For samples stored in STM-SAV for 24 or 72 h prior to cryopreservation, the post-thaw CD34⁺ recovery was 140 ± 39 and $130 \pm 56\%$, respectively. The differences in post-thaw recovery for CB stored in STM-sav and control samples were not statistically different, and did not vary with time in liquid storage prior to cryopreservation ($p > 0.22$).

Progenitor assays

The frequency of CFU-GM per 10^5 cells was 93 ± 20 for fresh CB samples. During the liquid storage phase of the protocol, the frequency of CFU-GM per 10^5 cells remained constant over the 72 h time period for both control and CB stored in STM-sav (Figure 4). There was no difference in the post-thaw frequency of CFU-GM for either time-point (24 or 72 h) or control versus STM-sav ($p > 0.34$).

For control samples held for 24 or 72 h of liquid storage prior to cryopreservation, the post-thaw frequency of CFU-GM per 10^5 cells decreased to 86 ± 40 and 73 ± 28 , respectively. For samples stored in STM-SAV for 24 or 72 h prior to cryopreservation, the post-thaw frequency of CFU-GM per 10^5 cells was 100 ± 28 and 110 ± 36 , respectively. The post-thaw frequency of CFU-GM per 10^5 cells was higher for cells diluted in STM-Sav prior to cryopreservation ($p = 0.002$). There was not, however, a significant difference in the frequency of CFU-GM per 10^5 cells for samples stored in STM-SAV for 24 or 72 h ($p = 0.76$).

The microbial testing of all the samples for the study was negative, indicating that no samples had become contaminated during processing.

Discussion

Optimizing liquid storage conditions and then determining the influence of those conditions on post-thaw viability is

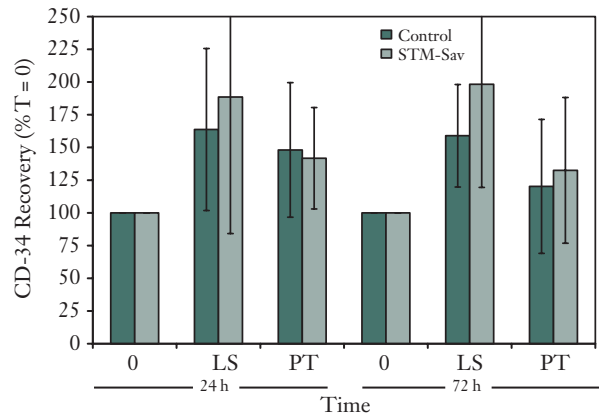


Figure 3. CD34⁺ CD45⁺ recovery percentage during LS and PT for CB stored in STM-sav and undiluted CB stored for either 24 and 72 h prior to cryopreservation. Error bars correspond to the SD of the mean.

essential in the development of integrated protocols for the processing of CB. This study describes the use of an infusible-grade cryopreservation solution for the short-term storage of CB prior to cryopreservation — a solution more appropriate for use than culture media. We have shown previously that hematopoietic cells can be stored for at least 24, and possibly up to 72 h [19,20], and could be effectively preserved in liquid storage. In this study, we determined the influence of liquid storage on the post-thaw viability of CB.

This investigation describes the recovery of cells post-thaw as a function of the liquid storage conditions prior to cryopreservation. It is noteworthy that the MNC and

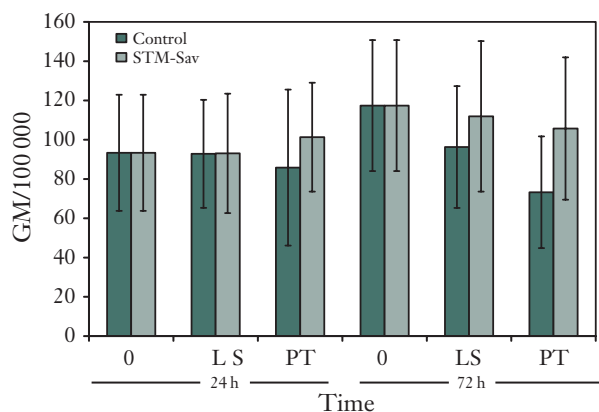


Figure 4. Frequency of CFU-GM per 10^5 cells during LS and PT for CB stored in STM-sav and undiluted control CB stored for either 24 or 72 h prior to cryopreservation. Error bars correspond to the SD of the mean.

CD34⁺45⁺ cell recovery determined in this investigation are defined as the number of viable cells post-thaw divided by the number of viable cells initially. This method avoids the common bias of determining viability. Specifically, it is common to assay fraction of cells that are viable post-thaw without accounting for cells that may have lysed during the freezing process. As such, post-thaw viability may appear to be higher than it actually is.

The present study has shown that post-thaw MNC recovery decreased significantly by 72 h for the control compared to cells stored in STM-sav (95% recovery for control versus 136% recovery for STM-sav). The MNC recovery that we observed during the liquid storage of CB was comparable to that observed in a previous study [21]. MNC recovery decreased after cryopreservation, with MNC recoveries post-thaw comparable to those observed in other studies where CB was stored for 24 h prior to cryopreservation [5,7].

The recovery of CD34⁺ cells remained unchanged over the duration of the protocol (liquid storage and cryopreservation), and did not vary with experimental conditions (duration of liquid storage and control versus STM-sav diluted samples). This result is consistent with our previous studies with liquid storage of CB [21] and PBSC [19]. Rogers and colleagues observed a similar result when analyzing recovery for CB with variable times of liquid storage prior to cryopreservation [6]. The statistical analysis of the data showed:

- The variance of the measurements increased with time
- The data was not normally distributed and a log transformation was required to perform the statistical analysis.

Another parameter of interest is the frequency of CFU-GM. During the liquid storage phase of storage, little difference in the frequency of colony formation was observed with time in storage (188 versus 198 per 10⁵ cells at 24 and 72 h, respectively) for cells stored in STM-sav. Similar results were observed with control samples. For cells cryopreserved after 24 h in storage, there was no difference in frequency of CFU-GM post-thaw between CB stored in STM-sav and controls ($p = 0.33$). This result is consistent with that observed by Beaujean and colleagues [7]. For cells cryopreserved after 72 h prior to cryopreservation, there was a distinct difference in the post-thaw frequency of CFU-GM for samples stored in STM-sav versus control ($p = 0.04$). Shlebak and collea-

gues observed a decline in post-thaw recovery of CFU-GM with time in liquid storage for a 24 h period [5]. The results of our study suggest that there is a threshold time of storage beyond which post-thaw recovery of CFU-GM will be diminished.

These studies were designed to address liquid storage of CB under realistic conditions. The CB units used in this study were typically too small to be accepted as clinical CB units. The units were then divided in half, further reducing the total volume of solution. Full, clinical size units may be considerably larger than those used in this study. The volume of solution will influence the transport of oxygen, and the diffusion of nutrients and products of metabolism. Additional studies to examine the influence of sample volume may be needed to clarify what effect, if any, differences in storage volume have on cell recovery.

The measures of viability described in this investigation have been limited to short-term *in vitro* studies. Clearly, additional studies should be performed to assay the frequency of LTCIC or engraftment of stored CB cells into animal models. Short term *ex vivo* culture of hematopoietic cells has been shown to influence the ability of those cells to engraft [25]. Additional studies should be performed with cells that have been stored to determine if a similar effect is observed.

In conclusion, liquid storage prior to cryopreservation can influence the post-thaw recovery of cells. Specifically, liquid storage for > 24 h prior to cryopreservation can have an adverse effect on post-thaw recovery. However, the use of a short-term storage solution for the liquid storage of CB prior to cryopreservation can improve the recovery of colony-forming cells and viable cell recovery over controls for cells stored beyond 24 h.

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