

## Short-term liquid storage of umbilical cord blood

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**BACKGROUND:** Processing and banking of umbilical cord blood requires the development of methods for short-term liquid storage. This study examines the conditions (temperature, time, and storage solution) for optimal storage of cord blood.

**STUDY DESIGN AND METHODS:** Cord blood obtained from normal donors was collected and divided into aliquots. Some of the aliquots were supplemented with a storage solution and undiluted cord blood was used as a control. MNC counts, percentage of cells that are CD34+45+, frequency of CFU-GM, and solution pH were monitored for up to 72 hours in storage at 4°C and room temperature.

**RESULTS:** MNC counts, CD34+45+ cell recovery, and frequency of CFU-GM were all improved in samples diluted with a storage medium when compared to undiluted controls. MNC counts were higher when cells were stored at 4°C. MNC counts and the frequency of CFU-GM were reduced at 72 hours when compared with 24 hours.

**CONCLUSIONS:** These results indicate that the recovery of cells from cord blood can be improved if samples are stored using a storage solution for 24 hours without significant cell losses. Some of the solutions determined to be effective in maintaining viability are approved for human applications, although not specifically cord blood preservation.

Umbilical cord blood has continued to grow in importance as a source of HPCs. Clinical banking of cord blood has grown considerably in recent years, with current estimates for the number of units stored in excess of 70,000 worldwide.<sup>1-3</sup> Much of the research addressing the processing of cord blood has involved maximizing the progenitor cell content of stored units by maximizing the volume of cord blood collected and minimizing losses due to RBC depletion of the unit.<sup>4-9</sup> Similarly, studies have been performed to validate cryopreservation protocols for umbilical cord blood banking.<sup>10,11</sup>

Cord blood for transplantation may be collected at any time of the day or night. In addition, processing, freezing, and storing cord blood has become more centralized with regional centers being developed. To obtain the largest number of cord blood units cost effectively while also gaining the desired ethnic mix of donors, collection sites may be distant from the laboratory processing site. This 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.

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**ABBREVIATIONS:** PBPC = peripheral blood progenitor cell; RT = room temperature.

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Several studies of the short-term liquid storage of HPCs obtained from marrow<sup>12-17</sup> and peripheral blood<sup>18-22</sup> have established that tissue culture solutions with anticoagulants or anticoagulated plasma can satisfactorily preserve HPCs at 4°C or room temperature (RT) for 24 hours or more. The successful transplantation of marrow stored in the liquid state over several days demonstrates that such storage can be successful.<sup>18,20,21,23</sup> Despite this experience, there are no generally accepted guidelines or criteria for short-term liquid storage of HPCs, nor are there any preservative solutions that are FDA licensed for this purpose. Even fewer data are available for cord blood.

Few studies have been performed on short-term liquid storage of umbilical cord blood. Shlebak et al.<sup>24</sup> found that a significant reduction in frequency of CFU-GM for undiluted cord blood stored for 9 hours or more at either 4 or 25°C. Campos et al.<sup>25</sup> observed a greater than 95-percent recovery of total nucleated cells (TNC) and CFU-GM for cells preserved at RT for 24 hours and then cryopreserved. Storage at 4°C resulted in more rapid degradation. These contradictory results and the limited amount of data indicate that further study of the optimum short-term storage conditions of cord blood is warranted.

The objective of the present study was to establish optimal conditions (solution, time, temperature) for short-term liquid storage of umbilical cord blood before RBC depletion and cryopreservation. Establishing these conditions would make it possible to locate collection and processing sites that are ideal for each function without the need for proximity, insure optimum cell recovery, and reduce costs associated with the development and operation of cord blood banks.

**MATERIALS AND METHODS**

**Umbilical cord blood collection**

Human umbilical cord blood was aspirated with a 17-ga needle from volunteer donor cord veins using standard procedures.<sup>7,9</sup> All samples were collected with informed consent and with the approval of the local Institutional Review Board. Blood was collected into a 450-mL blood bag (Baxter-Fenwal, Deerfield, NJ) containing 20 mL of CPD (0.52 g sodium citrate, 0.51 g dextrose, 60 mg citric acid, and 44 mg monobasic sodium phosphate) while gently mixing.

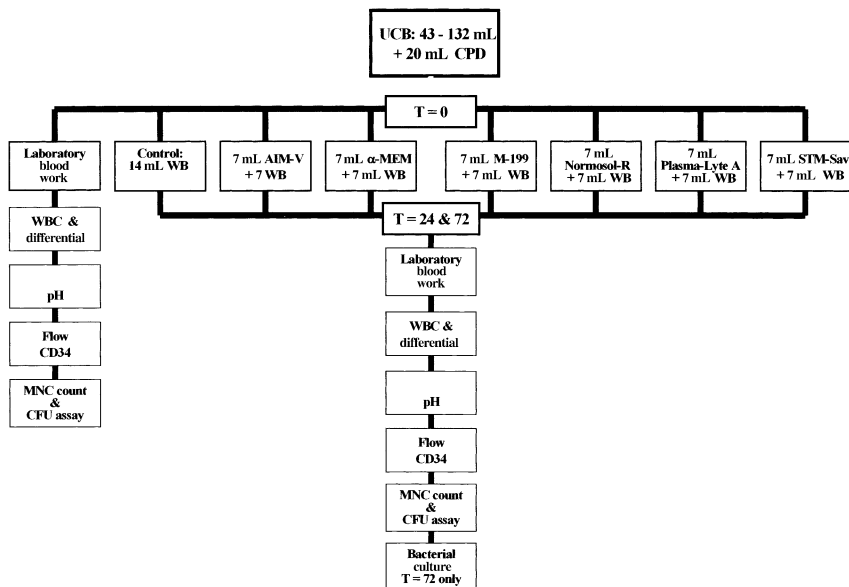
**Storage protocol**

Within 4 hours of the blood collection time, the experiment was initiated. Whole blood was analyzed for WBC counts, WBC differential, pH, MNC counts, percentage and viability of CD34+45+, total nucleated cell viability, and progenitor (CFU-GM) assay. Blood samples were also taken at 24 and 72 hours from both the control and solutions for testing as described below. At 72 hours, a blood or a blood-solution sample from all bags tested were submitted for bacterial culture.

The 150 mL transfer bags (Baxter-Fenwal) were adapted (heat sealed) to simulate the size of a typical clinical cord blood unit. This involved heat-sealing the bag at about its middle. Then, 14 mL of CPD anticoagulated cord blood (control) or 7 mL of CPD anticoagulated cord blood and 7 mL of preservation solution were injected into each of seven adapted bags. Solutions used were AIM-V, (GibcoBRL, Grand Island, NY) α-MEM (GibcoBRL), MEM (GibcoBRL), Normosol (Abbott Laboratories, North Chicago, IL), Plasmalyte-A (Baxter-Fenwal), and STM-Sav (Baxter-Fenwal; STM-Sav is composed of 1000 mL Lactated Ringers solution with 50 mm histidine and 1% HSA<sup>26,27</sup> and was compounded at the Fairview-University Hospital Pharmacy). A schematic of the experimental protocol is given in Fig. 1.

Each cord blood unit was randomly assigned to be stored at a temperature of 4°C (3-5°C) or RT (22-26°C). Units stored at 4°C were in a monitored blood bank re-

**Cord blood flow chart**



**Fig. 1. Schematic representation of experimental protocol. Cord blood unit is divided into aliquots. One aliquot is removed for testing of the initial unit. Additional aliquots are supplemented with the storage solution (or undiluted for the control arm). Samples of the solutions are removed at 24 and 72 hours for testing. WB = whole blood.**

frigerator, and units stored at RT were stored in a small cooler at RT to minimize temperature fluctuations. At appropriate times, each unit was mixed thoroughly and a sample was removed from an injection port by syringe.

### Assays

MNC counts were performed utilizing a microscope (Zeiss Axioskop, Jena, Germany) at 100 $\times$  magnification with a fluorescent light source. All samples were diluted with acridine orange (AO) PI.<sup>28</sup> A brightline hemacytometer (Hausser Scientific, Horsham, PA) was used to make all counts. All cells fluorescing green were scored as viable. The MNC recovery was calculated by dividing the MNC counts at a given time point (24, 72 hr) by the MNC count at time 0 hours.

The CD34+45+ percentage, viability, and total nucleated cell viability were analyzed by flow cytometry. RBCs 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). The cells were stained (Via-Probe, BD Pharmingen, San Jose, CA) to assay viability. Utilizing a flow cytometer (Becton-Dickinson FACSCalibur), specimens were then evaluated using International Society of Hematology and Graft Engineering gating.<sup>29</sup> 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 hours.

Progenitor assays were performed using methylcellulose (Methocult GF H4434, Stemcell Technologies, Vancouver, British Columbia, Canada).<sup>30</sup> RBCs were lysed with ammonium chloride. Cells were then washed, diluted, and then added to methylcellulose to result in final cell concentrations of 30,000 and 50,000 nucleated cells per mL. One milliliter of cells per methylcellulose were plated in duplicate into 22-mm diameter nontissue culture-treated, hydrated 12-well plates. Plates were incubated in a 37°C temperature 99-percent humidity, 5-percent CO<sub>2</sub> incubator for 14 days. An inverted variable magnification microscope (Olympus SZ60, Melville, NY) 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 number of colonies per 10<sup>5</sup> nucleated cells represented by the formula: CFU-GM colonies counted/cells plated  $\times$  100,000.

A blood gas machine (Radiometer Copenhagen 725, Radiometer Medical, Denmark) was used to measure pH.

### Statistical methods

The percentage of viable cells, percentage of viable CD34+ cells, CFU-GM per 10<sup>5</sup> cells, and solution pH were monitored as a function of experimental conditions. The correlation of within-unit measurements as well as the correlation of repeated measures required the doubly repeated measures regression analysis. The measures at 24 and 72 hours were assumed to have an unstructured correlation, and the correlation within experimental units assumed unstructured correlation. The unit measurement was the primary statistical endpoint. The type of solution, temperature of the solution, baseline unit measurement (measurement at  $t = 0$ ), and time of measurement were evaluated in the model. The Bonferroni adjustment was used to make multiple comparisons between solutions.<sup>31</sup> All analysis was performed using software (SAS V8, SAS Institute, Cary, NC).

## RESULTS

The results described in this section will compare the behavior of cord blood stored in the various storage solutions versus the control. For the purpose of this study, we defined "control" as undiluted cord blood containing 20 mL of CPD anticoagulant.

### MNC recovery

The MNC recovery for undiluted cord blood containing CPD stored at RT was 88  $\pm$  12 percent at 24 hours and 56  $\pm$  12 percent at 72 hours. The nucleated cell recovery for cord blood in STM-Sav is 101  $\pm$  16 percent at 24 hours and 72  $\pm$  10 percent at 72 hours. The nucleated cell recovery was greater for all of the solutions tested at 24 and 72 hours when compared with control ( $p < 0.01$ ), but none of the solutions showed higher MNC recovery than the others (Fig. 2). The MNC recovery was greater at 24 than 72 hours for all the solutions tested ( $p < 0.01$ ). At 4°C,

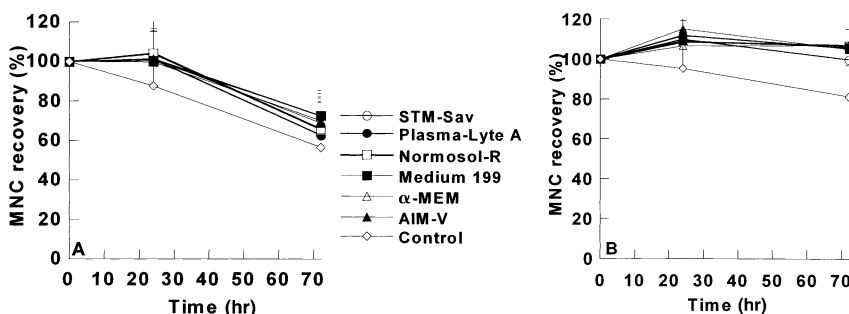


Fig. 2. Percentage of MNC recovery as a function of time and solution composition for umbilical cord blood stored at (A) RT and (B) 4°C. Positive error bars indicate SD for a minimum of 14 measurements.

the MNC recovery was greater than that observed for cells stored at RT ( $p < 0.01$ )

### Viable CD34+45+ cell recovery

The recovery of CD34+45+ cells was greater for all of the solutions tested when compared with the control cord bloods ( $p < 0.01$ ) (Fig. 3). For undiluted cord blood stored at RT, the recovery of CD34+45+ cells was  $96 \pm 15$  percent at 24 hours and  $84 \pm 15$  percent at 72 hours at RT and  $109 \pm 25$  percent and  $101 \pm 30$  percent after 24 and 72 hours storage at  $4^{\circ}\text{C}$ . The recovery of CD34+45+ cells stored in STM-Sav at RT was  $108 \pm 19$  percent at 24 hours and  $110 \pm 16$  percent at 72 hours. CD34+45+ cell recovery for samples stored in Normosol-R was significantly greater ( $124 \pm 27$  percent) than the recovery observed for cells stored in  $\alpha$ -MEM ( $111 \pm 29\%$ ;  $p < 0.01$ ). No other differences were observed between CD34+45+ cell recovery between the other solutions tested. No difference was observed between the recovery of CD34+45+ cells at 24 and 72 hours ( $p = 0.29$ ) at  $4^{\circ}\text{C}$  or RT ( $p = 0.09$ ).

### Frequency of CFU-GM

The number of CFU-GM was  $124 \pm 64$  per  $10^5$  cells at time 0 hours,  $127 \pm 47$  per  $10^5$  cells at 24 hours, and  $95 \pm 35$  per  $10^5$  cells at 72 hours for controls stored at RT (Fig. 4). At  $4^{\circ}\text{C}$  storage, the number of CFU-GM was  $112 \pm 41$  per  $10^5$  cells initially,  $111 \pm 51$  per  $10^5$  cells after 24 hours, and  $93 \pm 46$  per  $10^5$  cells after 72 hours of storage. No difference was observed between the number CFU-GM at  $4^{\circ}\text{C}$  and RT ( $p = 0.13$ ) for the control. The number of CFU-GM was greater for all of the solutions tested when compared with controls ( $p < 0.01$ ) at both 24 and 72 hours. The number of CFU-GM for cord blood stored in STM-Sav was  $118 \pm 48$  per  $10^5$  cells at 24 hours and  $108 \pm 47$  per  $10^5$  cells at 72 hours. The frequency of CFU-GM for cells stored in Normosol-R was greater than all solutions except Plasmalyte-A ( $p < 0.01$ ). The number of CFU-GM was greater at 24 than 72 hours for all the solutions tested ( $p < 0.01$ ).

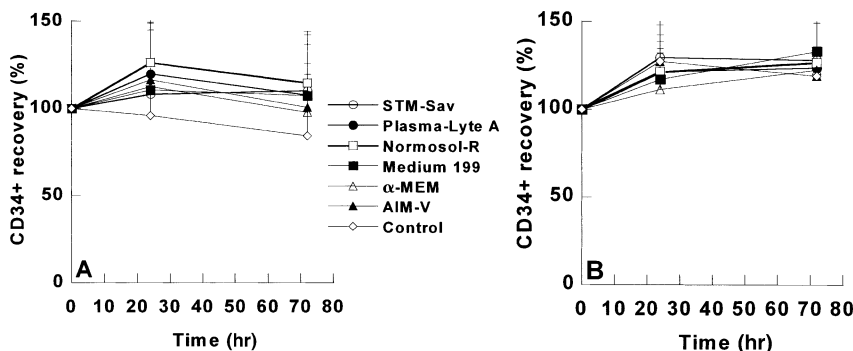


Fig. 3. Percentage of CD34+ cell recovery as a function of time and solution composition for umbilical cord blood stored at (A) RT and (B)  $4^{\circ}\text{C}$ . Positive error bars indicate SD for a minimum of 14 measurements.

### Suspension pH

The initial pH for the control cord blood was acidic ( $6.39 \pm 0.14$  pH). The addition of a storage solution resulted in an increase in the solution pH when compared with controls ( $p < 0.01$ ). The pH increased slightly from 0 to 24 hours, but that increase is not significant (Fig. 5). The pH decreased from 24 to 72 hours ( $p < 0.01$ ). The pH for cells stored in M 199 was greater than all other solutions tested ( $p < 0.01$ ). The pH for STM-Sav was higher than Normosol-R, control and Plasmalyte A ( $p < 0.01$ ). The pH was higher for cells stored at  $4^{\circ}\text{C}$  compared with RT ( $p < 0.01$ ).

## DISCUSSION

Coordinating collection, processing, and transportation to central banking facilities necessitates the development of methods for short-term liquid storage of umbilical cord blood. The storage protocol should specify temperature, solution, and maximum duration of storage. Many laboratories use culture media that is not suitable for human infusion, and there is no consensus on the optimum temperature or maximum acceptable duration of storage. It is not clear whether the composition of the storage container, the surface-to-volume ratio, and the method of storage (static vs. mixed) are important. We have previously shown that peripheral blood progenitor cells (PBPCs) can be stored for at least 24 and possibly up to 72 hours, and the recovery of MNCs and CFU-GM is slightly better maintained at  $4^{\circ}\text{C}$  rather than RT.<sup>26</sup> We have also shown that marrow HPCs can be preserved successfully for up to 72 hours in a solution specifically designed for PBPC storage.<sup>27</sup>

The present study evaluated the recovery and viability of cord blood cells in six different solutions, three of which are intended for tissue culture and three for human infusion. There was good recovery of MNCs at 24 hours, with the control averaging 88 percent at RT and 95 percent at  $4^{\circ}\text{C}$  and with all test solutions showing higher recoveries than the control. The recovery of viable control

MNCs decreased with time in storage over the 72 hours but still was 81 percent when stored at  $4^{\circ}\text{C}$ , but had fallen to 56 percent when stored at RT. Thus MNC recovery was significantly better with storage at  $4^{\circ}\text{C}$  and resulted in a satisfactory recovery for at least 24 and possibly up to 72 hours. The MNC recovery observed in this study was considerably higher than that observed in a study by Shlebak et al.,<sup>24</sup> who reported that the number of MNCs in cord blood stored at RT decreased by 51 percent within 24 hours of storage. The differences in recovery between this study and the study by Shlebak et al.<sup>24</sup> may

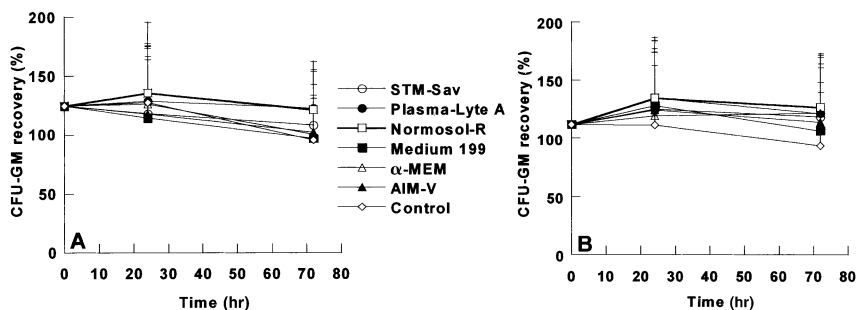


Fig. 4. CFU-GM per  $10^5$  cells as function of time and solution composition for umbilical cord blood stored at (A) RT and (B)  $4^{\circ}\text{C}$ . Positive error bars indicate SD for a minimum of 14 measurements.

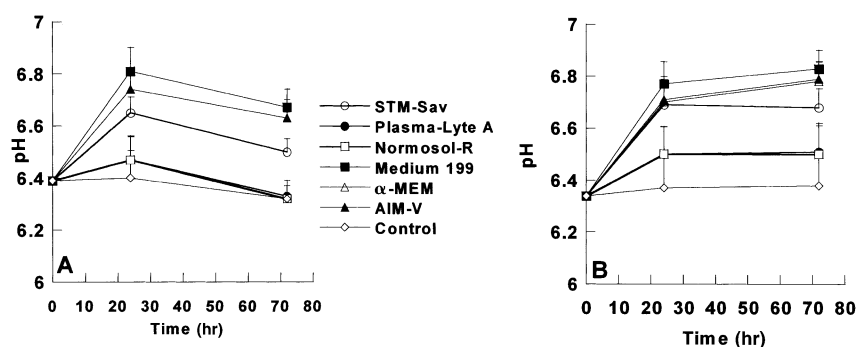


Fig. 5. Solution pH as a function of time and solution composition for umbilical cord blood stored at (A) RT and (B)  $4^{\circ}\text{C}$ . Positive error bars indicate SD for a minimum of 14 measurements.

reflect differences in the processing of the cord blood. The samples in the study by Shlebak et al.<sup>24</sup> calculated the recovery after Ficoll separation of the MNCs. This study measured the cell recovery in unseparated cord blood. The MNC recovery for cord blood stored in STM-SAV is comparable to the MNC recovery for marrow stored in STM-SAV (101% for cord blood and 91% for marrow stored in STM-SAV for 24 hr). MNC recoveries for marrow or cord blood stored in STM-SAV were also similar after 72 hours in storage (72% for cord blood and 88% for marrow).<sup>26</sup> For HPCs stored in STM-SAV, the MNC recovery was comparable to that observed in this study after 24 hours in storage (101% for cord blood and 93% for PBPCs). By 72 hours in storage, the MNC recovery for PBPCs was 39 percent and cord blood was 72 percent. The differences in MNC recovery after 72 hours in storage between cord blood and PBPCs may reflect the influence of cytokine stimulation on the cells.

**CD34+ cell recovery**

CD34+45+ cell recovery did not decrease significantly during 72 hours of storage at either RT or  $4^{\circ}\text{C}$ . This result is consistent with the results obtained by Rogers et al.<sup>32</sup> and Kraai et al.<sup>33</sup> who observed enrichment in primitive

hematopoietic cells with time in storage for umbilical cord blood. Our previous studies with G-CSF mobilized PBPCs and marrow did not exhibit a significant decrease in the percentage of CD34+ cells during storage for up to 72 hours.<sup>26,27</sup>

**Colony formation**

The frequency of CFU-GM decreased after 72 hours in storage and does not seem to be influenced by storage temperature. Similar results were observed by Broxmeyer et al.<sup>34</sup> who observed little difference in the colony formation from cord blood stored for up to 3 days at both  $4^{\circ}\text{C}$  and RT. This result differs from a study by Tron de Bouchony et al.<sup>35</sup> in which colony formation for cord blood was improved for cord blood stored at  $4^{\circ}\text{C}$  for up to 48 hours. As with nucleated cell counts and CD34+ recovery, there was little variation in CFU-GM per  $10^5$  cells measured. In general, there was an upward trend in the number of CFU-GM with time in culture, but this trend was not significant. Other studies have observed a general downward trend in frequency of CFUs with time in liquid storage<sup>13,15,26</sup> for both marrow and PBPCs. The retention of colony-forming capability observed in this study was greater than that observed by Machalinski et al.<sup>36</sup> for cord blood stored at both RT and  $4^{\circ}\text{C}$ .

**Solution pH**

In general, the values of the pH measured were slightly acidic. The initial pH values probably reflect the use of CPD as an anticoagulant. The shifts in pH reflect the influence of products and metabolism and the buffering system for the solution. Tissue culture medium (M-199,  $\alpha$ -MEM, and AIM-V) used phosphate buffering, which is only effective in 5-percent  $\text{CO}_2$  incubators. These solutions experience rapid shifts in pH (within 30 min) after removal from a 5-percent  $\text{CO}_2$  incubator. Plasmalyte A and Normosol-R are not buffered at all. STM-Sav is buffered and the buffering system is designed to operate under atmospheric conditions.

The decrease in pH between 24 and 72 hours is small but significant. The shift in pH with time in storage is consistent with a previous study performed in our laboratory with marrow stored in STM-Sav.<sup>27</sup> We also observed a downward trend in pH with time in liquid storage. In another study using G-CSF-mobilized PBPCs, we observed little variation in solution pH with time.<sup>26</sup> Pre-

vious studies have shown that deviation in pH can influence cloning efficiency and differentiation potential of progenitor cells.<sup>37,38</sup> These results would imply reduced colony formation at 72 hours, which was not observed in this investigation. None of the units in this study had a pH below 6.3, and this still provided satisfactory viability and colony formation. Thus, the minimum pH that must be maintained is not yet established.

In conclusion, the use of a short-term storage solution for the liquid storage of cord blood improves the retention of viable cells compared with storage in CPD anticoagulant. Cord blood can be stored for at least 24 hours before an important loss in the number of MNCs, CD34+ cells, or CFU-GM occurs. Storage at 4°C may be preferable to RT, but further studies are needed to establish this conclusively. All of the preservation solutions tested in this study gave satisfactory results but only three, Normosol, Plasmalyte A, and STM-Sav are suitable for human use. One or more of these solutions should be further developed suitable for licensure by the FDA for HPC preservation.

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