

Noncryopreserved Bone Marrow Storage in STM-Sav, an Infusible-Grade Cell Storage Solution

J.L. SCHMID,¹ J. McCULLOUGH,² S.R. BURGER,³ and A. HUBEL²

ABSTRACT

Liquid storage of hematopoietic stem and progenitor cells often is necessary in clinical cell engineering. The interval between cell harvest and transplantation or processing may be as long as 1 or 2 days. We previously developed an infusible-grade solution, STM-Sav, for nonfrozen storage of peripheral blood stem cells. This study addresses the validation of the storage conditions using STM-Sav for the short-term storage of bone marrow. Bone marrow from normal donors was stored in STM-Sav in gas-permeable Cryocyte and plasma transfer bags at 4°C and room temperature. Cell viability, number, and morphology, percentage of CD34⁺ cells, frequency of granulocyte/macrophage colony-forming units (CFU-GM), and solution pH were measured every 24 h for up to 72 h. More than 88% of viable nucleated cell recovery was observed at 72 h of liquid storage. CD34⁺ cell recoveries were unchanged over the 72-h storage period as well, and the frequency of CFU-GM per 10⁵ cells was also constant. The regulation of solution pH was improved with storage of the cells at 4°C in a plasma transfer bag. These results indicate that STM-Sav can be effective in preserving bone marrow viability over a 72-h period during liquid storage.

INTRODUCTION

L IQUID STORAGE OF hematopoietic stem and progenitor cells often is necessary in clinical cell engineering. The interval between cell harvest and transplantation or processing may be as long as 1 or 2 days, particularly if products are transported from collection centers to distant transplantation and processing centers. Delays also may occur between receipt of a specimen and availability of staff or equipment for processing. Thus, there is a need for the short-term liquid storage of hematopoietic cells.

Short-term liquid storage of hematopoietic cells from bone marrow has, in fact, been practiced for decades.¹⁻⁷ In addition to applications in graft transport and laboratory logistics, transplants have been successfully performed using noncryopreserved autologous bone mar-

row⁸⁻¹³ stored over several days. The favorable clinical outcome of these studies demonstrates the practical value of short-term liquid storage.

A broad variety of storage conditions have been used to maintain hematopoietic cells. Anticoagulated plasma has been used for short-term storage of peripheral blood progenitor cells (PBPCs) and bone marrow. Donor or autologous plasma is not always available and is inherently variable and undefined, and therefore in conflict with the increasing emphasis on control and consistency in clinical cell engineering. Tissue culture media with anticoagulant commonly are also used to maintain hematopoietic cell grafts, usually at either 4°C or ambient temperature for 24–72 h.^{2-5,14} Nearly all the tissue culture media in use are prepared for *in vitro* use only, however, and contain non-USP reagents. Because these he-

¹Department of Laboratory Medicine and Pathology, Tulane University School of Medicine, New Orleans, Louisiana.

²Department of Laboratory Medicine and Pathology, University of Minnesota Medical School, Minneapolis, Minnesota.

³Merix Biosciences, Durham, North Carolina.

matopoietic cell products are intended for infusion into patients, infusible-grade solutions, formulated from USP components, are more appropriate and consistent with regulatory requirements.

In addition, tissue culture media typically require a 5% CO₂ atmosphere to maintain appropriate buffering. Under normal atmospheric conditions, the buffering systems become ineffective, leaving cells without environmental pH control to compensate for metabolic effects. In a previous study,¹ we showed that PBPCs stored in several types of tissue culture media approached pH 8.0 after 48 h at ambient temperature. Shifts in pH have been shown to affect cloning efficiency and differentiation potential for hematopoietic progenitor cells.^{15,16}

For these reasons, infusible-grade solutions such as balanced electrolyte solutions such as PlasmaLyte A, Normosol, and STM-Sav are used increasingly for clinical cell storage and transport. As a harvest solution for bone marrow, PlasmaLyte A appeared equivalent to RPMI 1640,^{17,18} although this application did not involve a significant period of liquid storage. In contrast, we have shown previously that PlasmaLyte A is inferior to a variety of other solutions for the storage of PBPCs.¹ Furthermore, we showed that approximately 90% of the mononucleated cells in a PBPC product could be preserved when stored for 24 h at 4°C in STM-Sav. The present study evaluates the ability of STM-Sav to preserve function and viability of hematopoietic cells obtained from bone marrow as a function of time, temperature, and bag. Characterizing these parameters is essential to determining an appropriate preservation protocol for short-term liquid storage of bone marrow for different therapeutic purposes.

MATERIALS AND METHODS

STM-Sav solution

STM-Sav consisting of lactated Ringer's solution (Baxter-Fenwal, Deerfield, IL), buffered with 50 mM histidine (Sigma Chemical Co., St. Louis, MO) and supplemented with human serum albumin (Baxter-Hyland, Glendale, CA) for a final concentration of 1% (wt/vol)¹ was

prepared by the Fairview-University Medical Center pharmacy.

Cells and processing

Liquid bone marrow was aspirated from the posterior iliac crest of a normal donor with informed consent and with Institutional Review Board approval. A total of approximately 80 mL of bone marrow was collected from five different normal donors using 35-mL syringes, each containing sodium heparin (Elkins-Sinn, Cherry Hill, NJ) and anticoagulant-citrate-dextrose (ACD; Baxter-Fenwal) anticoagulants. Anticoagulated bone marrow contained approximately 1,000 U heparin/mL and ACD at a 1:6 volume ratio of ACD:bone marrow. Anticoagulated bone marrow from each syringe was transferred to a 300-mL plasma transfer bag (Baxter-Fenwal) and gently mixed. STM-Sav solution was used to dilute the anticoagulated bone marrow to a final concentration of 5.0×10^6 nucleated cells/mL. A 7-mL sample of the diluted cell suspension, representing the initial or $t = 0$ time point, was withdrawn from the bag for assays. The bone marrow cell suspension was divided into four equal aliquots of 30–40 mL, stored in two plasma transfer bags and two 50-mL Cryocyte bags (Baxter-Fenwal). Cells in one plasma transfer bag and one Cryocyte bag were maintained in a blood bank refrigerator at approximately 4°C (range 2–6°C), while cells in the other plasma transfer and Cryocyte bags were stored in a biological safety cabinet at ambient temperature (22–24°C).

Samples were taken at 0, 24, 48, and 72 h, and total nucleated cell count, percent viable cells, percent CD34⁺/45⁺ cells, and suspension pH were measured at these time points. Myeloid progenitor [granulocyte/macrophage colony-forming units (CFU-GM)] frequency was measured at 0-, 24-, and 48-h time points. Wright-stained smears of each sample also were examined.

Assays

Total nucleated cell counts were performed on bone marrow cell suspensions using a Coulter STKS automated cell counter (Coulter Electronics, Hialeah, FL).

Viability was determined using fluorescent dyes. All samples were diluted with acridine

orange-propidium iodide. The suspension was loaded into a Neubauer hemacytometer and read at 400 \times using a Leitz Laborlux fluorescence microscope (Leitz, Wetzlar, Germany). Cells fluorescing green were scored as viable. Cells demonstrating orange or red nuclear fluorescence were scored as nonviable. The mean percentage of viable cells was determined based on scoring at least 300 cells.

CD45⁺/CD34⁺ percentage, viability, and total nucleated cell viability were analyzed by flow cytometry. Red blood cells 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). Utilizing a Becton-Dickinson FACSCalibur flow cytometer, specimens were then evaluated using International Society of Hemotherapy and Graft Engineering gating.¹⁹

Progenitor assays were performed using methylcellulose (Methocult GF H4434; Stemcell Technologies, Vancouver, BC, Canada) as per the manufacturer's instructions. Red blood cells were lysed with ammonium chloride. Following red blood cell lysis, cells were suspended in MethoCult medium and plated in duplicate 35-mm-diameter culture dishes at 1.0×10^5 nucleated cells per plate. Plates were incubated at 37°C in a humidified 5% CO₂ incubator for 15 days and then read using an Olympus SZ60 inverted microscope at 15–25 \times power. Mean frequency of CFU-GM per 10⁵ nucleated cells was calculated based on the mean number of CFU-GM for two duplicate plates and the number of nucleated cells plated. CFU-GM results are the mean average number of colonies per 10⁵ nucleated cells represented by the following for-

mula: (number of CFU-GM colonies counted/number of cells plated) \times 100,000.

Solution pH was measured using a Corning Model 125 pH meter (Corning Glassworks, Medfield, MA).

Statistical methods

The percentage of viable cells, percentage of viable CD34⁺ cells, CFU-GM per 10⁵ cells, and solution pH were monitored as a function of experimental conditions (time, temperature, bag). Differences in the parameters with time were determined by determining the statistical significance of the slope. The influence of bag and/or temperature was determined by analysis with repeated-measures ANOVA. All analysis was performed using SAS version 8 software (SAS Institute, Cary, NC).

RESULTS

Nucleated cell recovery

The average nucleated cell concentration for fresh bone marrow obtained from the donors was 18.66×10^6 cells/mL. Following dilution with STM-Sav, the mean cell concentration averaged $4.9 \pm 0.4 \times 10^6$ cells/mL. Over the period of storage tested, both the concentration of cells and the viability of those cells exhibited a downward trend, but the differences were not statistically significant (Fig. 1). Further analysis showed that there was no difference in the nucleated cell recovery for either of the temperatures tested. There was a slight downward trend in the nucleated cell recovery for cells

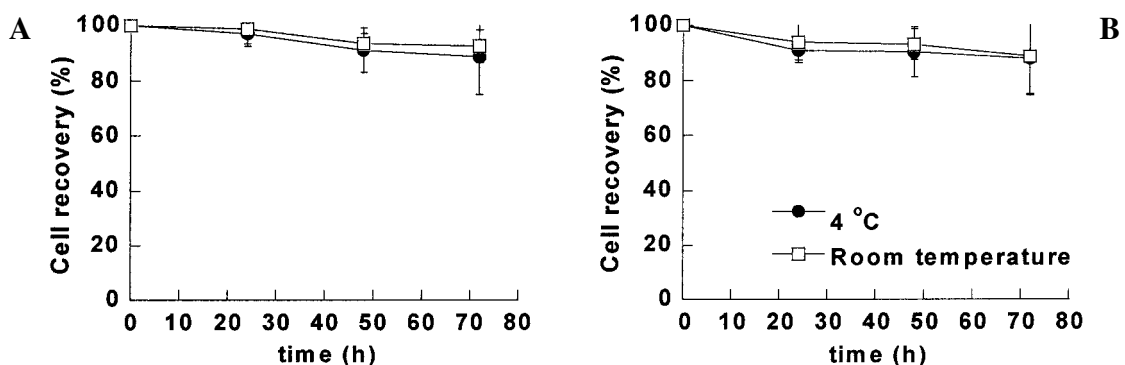


FIG. 1. Percentage of nucleated cell recovery as a function of time for bone marrow supplemented with STM-Sav stored at 4°C and room temperature in (A) plasma transfer bags and (B) Cryocyte bags.

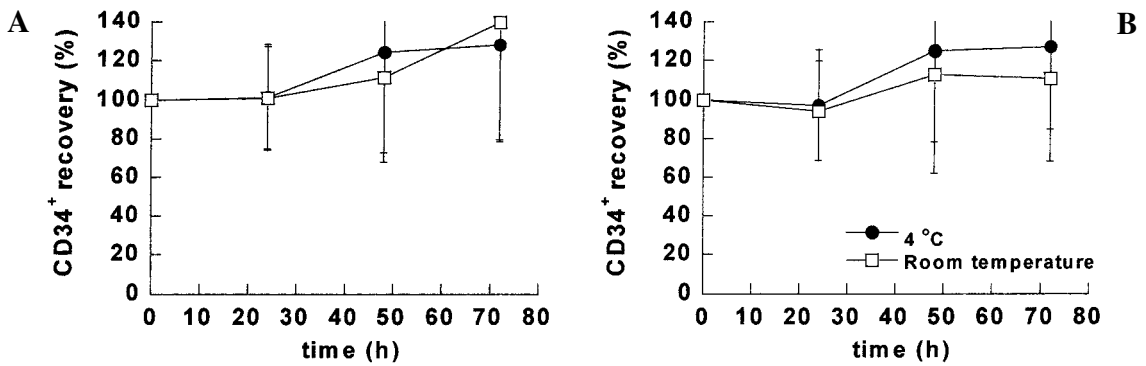


FIG. 2. Percentage of CD34⁺ cell recovery as a function of time for bone marrow supplemented with STM-Sav stored at 4°C and room temperature in (A) plasma transfer bags and (B) Cryocyte bags.

stored in Cryocyte bags, but the trend was not statistically significant. In addition, there was a general downward trend in nucleated cell recovery for cells stored at 4°C, but the differences were not statistically significant.

Viable CD34⁺/45⁺ cell recovery

There was no statistically significant decrement in viable CD34⁺ recovery with time (Fig. 2). There appears to be a generally upward trend in the data, but these differences are not statistically significant. In general, there was no statistically significant difference in CD34⁺ recovery between either bag tested for either temperature. CD34⁺ cells stored in Cryocyte bags at room temperature had a downward trend in recovery, but it was not statistically different.

Progenitor assays

Myeloid progenitor cells (CFU-GM) did not decrease in frequency over time (Fig. 3). There

was a trend of increased CFU-GM frequency, but it was not statistically significant. Similarly, the storage of bone marrow at either temperature retained CFU-GM equally effectively.

Suspension pH

The solution pH remained slightly acidic throughout the time period studied. The solution pH for bone marrow stored at room temperature in either bag decreased in a statistically significant manner ($p < 0.04$). With storage at 4°C, the pH remained constant for both bags studied (Tables 1 and 2).

DISCUSSION

Delays between bone marrow collection and processing and the occasional needs for diluents or plasma replacement in processing necessitate the use of a short-term liquid storage solution that will preserve the number and

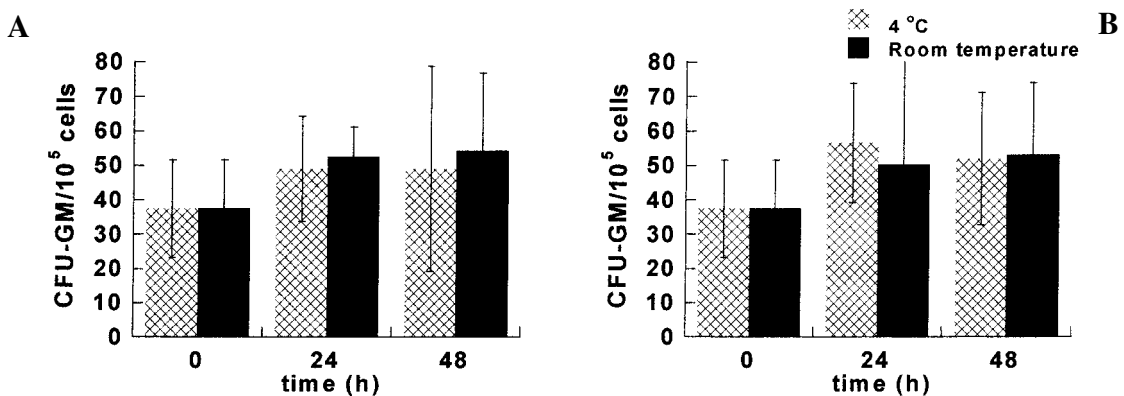


FIG. 3. CFU-GM per 10⁵ cells as a function of time for bone marrow supplemented with STM-Sav stored at 4°C and room temperature in (A) plasma transfer bags and (B) Cryocyte bags.

TABLE 1. pH OF BONE MARROW STORED IN STM-SAV IN A PLASMA TRANSFER BAG AS A FUNCTION OF TIME AND TEMPERATURE

	t (h)			
	0	24	48	72
4°C	6.87 ± 0.12	6.88 ± 0.11	6.89 ± 0.10	6.87 ± 0.10
Room temperature	6.87 ± 0.12	6.85 ± 0.10	6.79 ± 0.09	6.72 ± 0.09

function of hematopoietic progenitor cells. STM-Sav has been shown previously to preserve progenitor cells derived from granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood for up to 48 h in liquid suspension.¹ STM-Sav is composed entirely of FDA-approved infusible-grade reagents and utilizes a novel buffer system highly effective in atmospheric conditions. This study evaluated the ability of STM-Sav to preserve viable, functional progenitor cells derived from bone marrow and stored for up to 72 h under routine conditions of storage.

Nucleated cell recovery

The recovery of nucleated cells for all conditions tested was at minimum 88% of the original nucleated cell density for all bags and temperatures tested. This nucleated cell recovery is considerably higher than those observed for G-CSF-mobilized PBPCs stored in STM-Sav over the same period of time.¹ As with our previous study using bone marrow,¹ there appears to be a slight advantage to storage of the cells at 4°C rather than room temperature, although it is not statistically significant.

CD34⁺ cell recovery

As with nucleated cell counts, CD34⁺ recovery did not change with time in storage over the 72-h time period tested. In a recent study, de Kreuk et al.¹⁴ observed a steady decline in the recovery of CD34⁺ cells during liquid storage of

G-CSF-mobilized blood. In contrast, Kraai et al.²⁰ observed enrichment in primitive hematopoietic cells with time in storage for umbilical cord blood. Our previous studies with G-CSF-mobilized PBPC products did not exhibit a statistically significant decrease in the percentage of CD34⁺ cells with time in storage for up to 72 h.¹ A consistent observation that we have made in our studies with both liquid storage of PBPCs and bone marrow is that the variance in the measurement of CD34⁺ number increases with time in culture. Changes in morphology and cell surface antigen expression with time in storage may contribute to the increase in variance observed. A study by Bhatia et al.²¹ showed that after brief periods of *ex vivo* culture (72 h), the percentage of CD34⁺ cells had not changed, but the cells were unable to engraft in an immunodeficient mouse model. These studies as a whole suggest that the use of CD34⁺ enumeration using flow cytometry to evaluate preservation of hematopoietic potential of cells should be interpreted cautiously.

Colony formation

As with nucleated cell counts and CD34⁺ recovery, there was little variation in CFU-GM per 10⁵ cells measured. In general, there was an upward trend in the frequency with time in culture, but this trend was not statistically significant. Other studies have observed a general downward trend in frequency of colony-forming units with time in liquid storage^{1,4,14} for both bone marrow and PBPCs. In our previous

TABLE 2. pH OF BONE MARROW STORED IN STM-SAV IN A CRYOCYTE BAG AS A FUNCTION OF TIME AND TEMPERATURE

	t (h)			
	0	24	48	72
4°C	6.87 ± 0.12	6.90 ± 0.12	6.89 ± 0.09	6.91 ± 0.10
Room temperature	6.87 ± 0.12	6.86 ± 0.09	6.83 ± 0.08	6.80 ± 0.08

study with PBPCs, the decrease in frequency of CFU-GM was not statistically significant.¹

Solution pH

The solution pH remained constant for cells stored at 4°C and decreased for cell stored at room temperature. There was a slight downward trend in pH for cells stored in the Cryocyte bag over 72 h, but the difference was not statistically significant. In general, the values of the pH measured were slightly acidic (pH 6.72–6.90). The solution pH observed in this study is slightly lower than that measured in our previous study using PBPCs (pH 7).¹ The samples in this study contained both heparin and ACD as anticoagulants. The pH observed in this study may reflect the amount and composition of anticoagulant used. Previous studies have shown that deviation in pH can influence cloning efficiency and differentiation potential of progenitor cells.^{15,16} These results would imply reduced colony formation at 72 h, which was not observed in this investigation. De Kreuk et al.¹⁴ observed that the selection of anticoagulant played an important role in the preservation of G-CSF-mobilized whole blood stored for up to 7 days. Thus, selection of anticoagulant may also be a factor in the short-term storage of hematopoietic cells.

Cell concentration

In this study, bone marrow cells were diluted to a nucleated cell concentration of approximately 5×10^6 cells/mL. Previous work with G-CSF-mobilized PBPCs showed that cells could be effectively maintained in STM-Sav at cell concentrations as high as 2.5×10^8 cells/mL. It is reasonable to expect that bone marrow-derived nucleated cells can be concentrated higher than the 5×10^6 cells/mL used here. Indeed, Kohsaki et al.³ found comparable, acceptable survival with unstimulated bone marrow cells concentrated to 2×10^6 and 1.6×10^7 cells/mL. Unstimulated bone marrow was evaluated in the present study as well. G-CSF stimulation produces increased numbers of myeloid cells with predominance of primary granules. Whether and to what extent this change in cellular enzymatic composition may exert an effect on solution pH or preservation of stored stem and progenitor cells has not been

determined. Future study can address optimal concentration for successful storage of unstimulated as well as stimulated bone marrow-derived progenitor cells.

Recent studies have shown that cells isolated from the bone marrow (hematopoietic and non-hematopoietic) may differentiate into skeletal muscle myoblasts^{22,23} and endothelium^{24–26} and into cardiac myoblasts when transplanted in an infarcted heart^{24,25} and contribute to hepatic and biliary epithelial cells,^{27,28} liver,²⁹ lung, gut, and skin epithelia,³⁰ and neuroectoderm *in vitro* or *in vivo*.^{31–34} Clearly, short-term preservation of these cells is important for applications beyond bone marrow transplantation. Future studies should be performed to validate STM-Sav for use with nonhematopoietic cells present in the bone marrow.

In conclusion, this study validates STM-Sav for noncryopreserved short-term preservation of viable and functional progenitor cells from bone marrow. Bone marrow cells are effectively maintained in STM-Sav for at least 72 h without special processing, using routinely available storage conditions, in suspension with infusible-grade reagents.

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Address reprint requests to:

Allison Hubel, Ph.D.

Department of Laboratory Medicine and
Pathology

University of Minnesota

Mayo Mail Code 609

420 Delaware Street, SE

Minneapolis, MN 55455

E-mail: hubel001@umn.edu