Factors influencing cord blood viability assessment before cryopreservation

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BACKGROUND: Cord blood (CB) viability determines product quality and varies with time and temperature of exposure before cryopreservation. Global viability assessment may not reflect viability of white blood cell (WBC) subsets, CD34+ cell viability, or hematopoietic stem/progenitor cells function.

STUDY DESIGN AND METHODS: We compared trypan blue (TB) and acridine orange/propidium iodide (AO/PI) staining with flow-cytometric (7-aminoactinomycin D [7-AAD]) viability in total WBCs (Tot-AAD), granulocytes, monocytes, lymphocytes, and CD34+ cells and total nucleated cell, CD34+, and colony-forming cell (CFC) recovery as a function of time and temperature (4, 24, and 37°C) before cryopreservation.

RESULTS: TB, AO/PI, and Tot-AAD viability was concordant up to 72 hours (4°C) and 48 hours (24°C) postcollection; however, CD34+ viability was significantly higher due to loss of viable granulocytes. In contrast, at "physiologic" temperature (37°C), the decline in TB, AO/PI, and Tot-AAD viability was significantly lower than the rate of viable CD34+ and CFC loss. At all times and temperatures, CFC recovery correlated best with CD34+ viability and recovery.

CONCLUSIONS: CB cell populations exhibit differential time- and temperature-dependent susceptibility to in vitro cell death; consequently, global viability measurements using TB, AO/PI, or 7-AAD (Tot-AAD) significantly underestimate (4-24°C) or overestimate (24-37°C) CD34+ viability and CFC recovery. Our results demonstrate the limitations of global viability assessment with TB, AO/PI, and total AAD; endorse the routine use of CD34+ cell viability measurements; emphasize the importance of temperature control during shipment; and have implications with regard to establishing acceptable "cutoff" values for viability measurements and CB collection through processing time.

The viability of white blood cells (WBCs) in cord blood (CB) products is an important indicator of product quality and suitability for use in transplantation. Conventional approaches to measure cell viability are generally based on subjective assessment of trypan blue (TB) or acridine orange/propidium iodide (AO/PI) dye uptake by individual cells using transmission or fluorescence light microscopy, respectively. Cellular uptake of large ionic dye molecules such as TB or PI reflects loss of surface membrane integrity, a characteristic feature of necrotic cell death and also seen as a late event in programmed cell death (apoptosis).[1,2] Because nuclear morphology is not clearly delineated and generally fewer than 10 to 20 nonviable cells are counted, TB and AO/PI methods do not allow reliable, precise assessment of dye uptake among different WBC populations. Accordingly, these methods provide a "global" measure of total WBC viability. 7-Aminoactinomycin D (7-AAD) is similar to TB and PI and is largely excluded from cells with an intact surface membrane; however, in contrast to TB and PI, 7-AAD will stain cells at an earlier stage in the programmed cell death

ABBREVIATIONS: 7-AAD = 7-aminoactinomycin D; AO/PI = acridine orange/propidium iodide; CB = cord blood; CD34-AAD = CD34 cell viability using 7-AAD; CFC(s) = colony-forming cell(s); Gr-AAD = granulocyte cell viability using 7-AAD; Ly-AAD = lymphocyte cell viability using 7-AAD; Mo-AAD = monocyte cell viability using 7-AAD; NRBC(s) = nucleated red blood cell(s); SSC = side scatter; TB = trypan blue; Tot-AAD = total white blood cell viability using 7-AAD.

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pathway in addition to necrotic cells.[3-5] Thus, when measuring total WBC viability with 7-AAD by flow cytometry, we would expect the results to be lower than total WBC viability determined with TB or AO/PI.

The WBC population in CB is primarily composed of mature granulocytes that generally account for approximately 55% to 65% of the total nucleated cell (TNC) content.[6,7]

The remaining mononuclear cell (MNC) population in CB (35%-45% of TNC) includes monocytes, lymphocytes, and CD34+ and CD34– hematopoietic stem/progenitor cells (HPC).[8] In contrast to mature granulocytes, MNCs are capable of engrafting in CB transplant recipients and thus make a significant contribution to transplant outcome.[8,9] Since granulocytes are the most abundant WBC subpopulation in CB, global assessment of cell viability using TB, AO/PI, or 7-AAD primarily reflects granulocyte viability, which may not be representative of engrafting MNC viability. Accordingly, in this study, we utilized multicolor flow cytometry combining 7-AAD staining with signals arising from forward and side scatter (SSC) and CD45 and CD34 expression to measure not only total (i.e., "global") cell viability but also the viability of granulocyte, monocyte, lymphocyte, and CD34+ cell populations separately.

In peripheral blood or CB in vivo, granulocytes are "programmed" to die by apoptosis and generally do so within 12 to 24 hours after collection in vitro.[10-12] In contrast to mature granulocytes, engrafting MNCs are not programmed for "self-destruction" by apoptosis in vivo. Thus, it seems reasonable to expect that MNCs will exhibit longer survival in vitro compared to granulocytes. Consequently, a major focus of this study is to compare TB and AO/PI viability with the viability of individual WBC populations determined by flow cytometry with 7-AAD and to correlate these changes in cell viability with cell recovery and HPC function as assessed by colony-forming unit (CFU) assay to enumerate colony-forming cell (CFC) content. These studies were performed as a function of time and temperature during storage (prefreeze) in the anticoagulant solution used for CB product collection to allow comparison of the differential rates of cell death in WBC populations in CB under conditions commonly encountered in CB banking (room temperature, 22-24°C), at a lower temperature with potential for better preservation of cell viability and function (4°C) and after exposure to "physiologic" temperature (37°C) where in vitro progression of programmed cell death would likely proceed at a rate similar to that seen in vivo.

MATERIALS AND METHODS

CB was collected via cannulation of the umbilical vein into a sterile anticoagulated (CPD) blood bag with the placenta in situ under institutional review board approval and donor informed consent, which allows use of CB products for research if the TNC content of the product is less than 1×10^9 total cells. Collected CB units were shipped at room temperature (22-24°C, with temperature monitoring) to a central processing laboratory where all processing, cryopreservation, and product characterization was performed as previously described.[13,14]

For our "prefreeze" studies, 10 CB products were analyzed without further processing. Processing was performed within 12 to 16 hours after collection. Upon receipt, aliquots were removed for the following "time zero" (T0) measurements: TB, AO/PI, and 7-AAD viability; TNC count; "viable" CD34+ cell count; and CFC content by CFU assay. Each CB product was then divided into three equal-volume aliquots. One aliquot was placed in a temperature-controlled refrigerator at 4°C, the second aliquot was placed at room temperature (24°C), and the third aliquot was placed in an incubator at 37°C. Additional measurements were taken at 6 hours (T0 + 6), 24 hours (T0 + 24), 48 hours (T0 + 48), and 72 hours (T0 + 72). TB and AO/PI percent viability at each time point was determined by scoring 100 cells (%viability = 100 – number of non-viable cells). 7-AAD viability was determined by flow cytometry as described below. TNC, viable CD34+ cell, and CFC percent recovery was calculated as

$$\%\text{recovery} = 100 \times [\text{variable value}/\text{variable value at T0}].$$

Details of specific methods are as follows:

TNC count

TNC measurements were obtained using an automated hematology analyzer (XE-2100, Sysmex, Mundelein, IL), which provides automated counting of total WBCs, automated five-part differential, and nucleated red blood cell (NRBC) count. TNC count was expressed as WBCs + NRBCs. All products used in this study contained less than 5% NRBC.

TB

Seven drops of 4% Tris-NH_4Cl lysing solution were added to one drop of blood. After 20 minutes, two drops of TB stain (0.4%, Gibco, Grand Island, NY) were added, and 100 cells were scored using a Neubauer hemocytometer at 400× magnification. Cells staining blue were scored as nonviable.

AO/PI

AO/PI stock solution was prepared using 1.0 mL of 1 mmol/L acridine orange stock solution, 2.0 mL of 0.5 mg/mL PI stock solution, and 47 mL Dulbecco's

phosphate-buffered saline (Sigma Chemical Co., St Louis, MO). Fifteen drops of AO/PI stock solution were added to one drop of blood, and 100 cells were examined on a Neubauer hemocytometer using a fluorescent microscope with a 100-W Hg bulb at 200× magnification. Cells fluorescing orange or red were scored as nonviable.

CFU assay

Diluted CB containing 1×10^5 TNCs was added to 3 mL of methylcellulose medium with erythropoietin (Methocult GF H4434, StemCell Technologies, Vancouver, BC, Canada) and 1.5 mL plated in two separate wells of a six-well plate. The plates were incubated for 14 days at 37°C in 5% CO_2/95% air, and CFC (BFU-E, CFU-GM, CFU-GEMM, and CFU-E) counts were made and expressed as CFCs/10^5 TNCs by adding the total CFC count in the two separated wells. In our experience, reproducibility between CFC counts in the two separate wells is within ±10%.

Flow cytometric determination of total, granulocyte, lymphocyte, monocyte, and CD34+ cell viability using 7-AAD and viable CD34+ cell content

A 100-µL sample of CB was added to 300 µL of reagent (Isoflow, Coulter, Hialeah) to create a 1:4 dilution. To 100 µL of the diluted cell suspension, 20 µL of CD45-fluorescein isothiocyanate (FITC)/CD34-phycoerythrin (PE) and 20 µL of 7-AAD dye reagents (Coulter) were added. After a 20-minute incubation at room temperature, 2 mL of lysing solution (NH_4Cl) was added. After 10 minutes' lysing time, 100 µL of fluorospheres was added, and after thorough mixing, the tube was placed in the flow cytometer sampling rack. Flow cytometric analysis was performed using a flow cytometer (EPICS XL-MCL, Coulter), according to the manufacturer's recommendations[15] to obtain total 7-AAD and CD34+ cell viability (see Fig. 1). To determine absolute viable CD34+ cell counts, a "background" total CD34+ cell count is obtained from measurements in a separate "isoclonic control" tube prepared by substituting a reagent containing a large excess of unlabeled anti-CD34 in addition to anti–CD34-PE reagent to control for nonspecific binding of anti–CD34-PE as described by the reagent manufacturer.[15] Absolute CD34+ cell concentration is obtained by subtracting the control total CD34+ cell count from the sample total stem cell count and using the ratio of total CD34+ cell count events and total fluorosphere events as described.[15] This approach conforms to the recommendations commonly referred to as the ISHAGE protocol.[16,17]

To obtain separate lymphocyte, monocyte, and granulocyte viabilities, further analysis of list mode data was performed using software for a flow cytometer (StemCXP 2.0 and FC500, respectively, Beckman Coulter; see Fig. 2). A two-dimensional scattergram was displayed based on SSC and CD45-FITC fluorescent signals to identify the total WBC population (low-high SSC, CD45 low-high) with the threshold for SSC and CD45 signals set to exclude platelets, "nonlysed" NRBCs, and cell debris. In the CD45-FITC versus SSC scattergram, the SSC axis allows discrimination of separate lymphocyte (low SSC, high CD45), monocyte (intermediate SSC, intermediate CD45), and granulocyte (high SSC, CD45 low) regions within the total CD45+ (total WBC) cell population. Total WBC and individual WBC cell populations are then separated along the 7-AAD axis into 7-AAD low (viable) and 7-AAD high (nonviable) uptake regions on a separate two-dimensional scattergram of SSC versus 7-AAD fluorescence. For each of the appropriately gated regions, total WBC, granulocyte, lymphocyte, and monocyte percent viability is defined as

$$100 \times \text{7-AAD low events}/(\text{7-AAD low} + \text{7-AAD high})\ \text{events}.$$

For analysis, 75,000 total CD45+ events are analyzed. In a typical CB sample, 75,000 total WBC events corresponds to 150 to 600 total stem cell events (i.e., approx. 0.2%-0.8% CD34+ cells).

Statistical analysis

Mean, standard deviation, and standard errors of the mean (SEM) were computed for each group and data in the figures presented as mean ± SEM values. One-way analysis of variance (ANOVA) techniques with post hoc analysis by the Neuman-Keuls method were used to establish statistical significance between groups and p values less than 0.05 were considered significant. All statistical analyses were performed using software from the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL).

RESULTS

Time- and temperature-dependent alterations in global measures of cell viability before cryopreservation (pre-freeze) using TB, AO/PI, and Tot-AAD are shown in Fig. 3. For each temperature examined, data are presented by grouping results according to the time elapsed since collection. Group O represents the viability results obtained upon receipt (i.e., Time 0 values). These products were previously exposed to room temperature conditions (22-24°C in an insulated container) for 12 to 16 hours after collection before processing in the laboratory. Data shown for Groups A through D are the results of additional viability measurements on the same products in Group O after additional exposure for 6, 24, 48, or 72 hours at 4, 24, or 37°C as shown in Fig. 3. For products incubated at 4 and 24°C, there was general agreement between all three

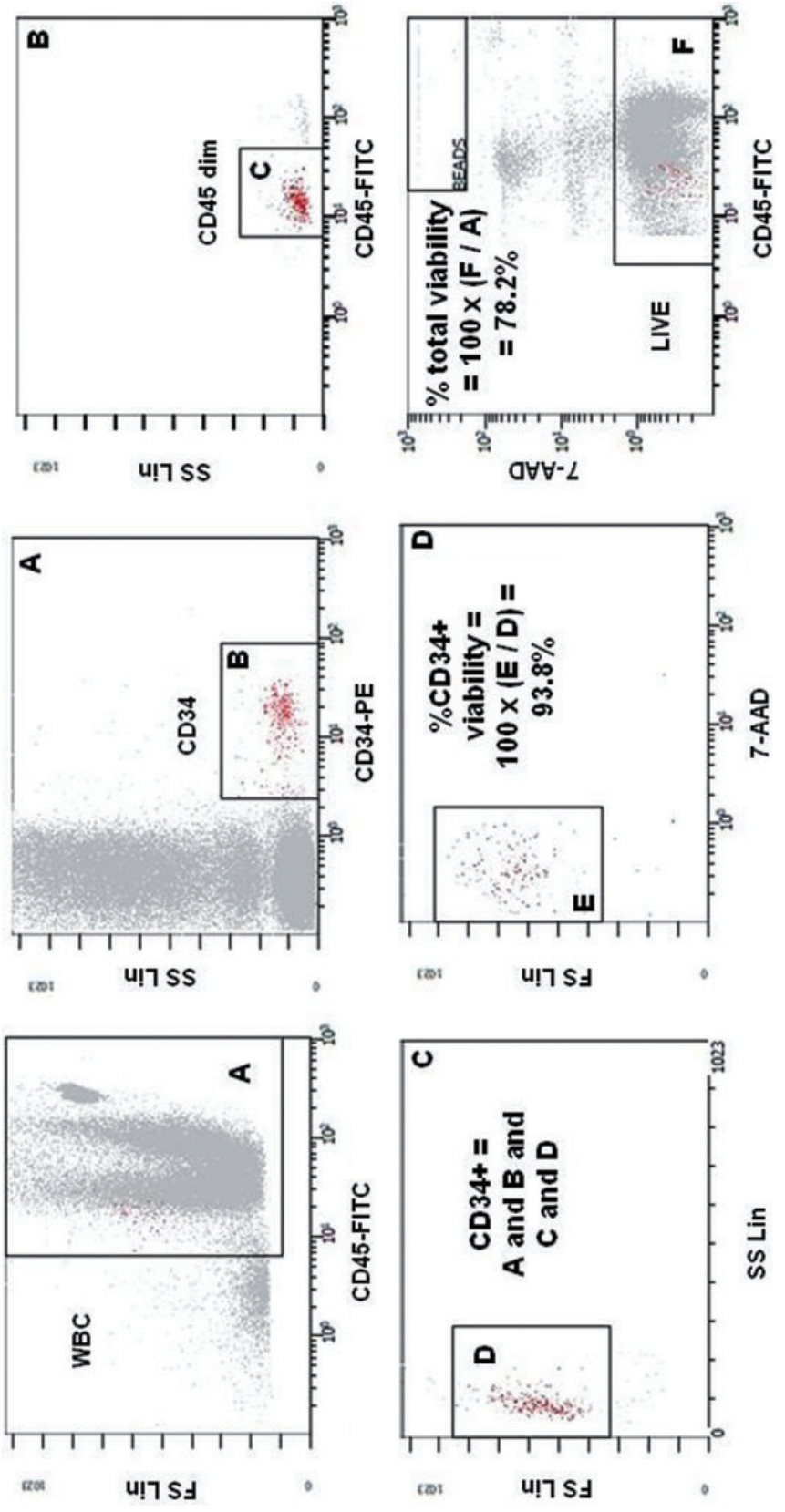


Fig. 1. Strategy for flow cytometric analysis to determine Tot-AAD and CD34+ cell viability and absolute viable CD34+ cell counts. Details of the viable CD34+ cell gating strategy and calculation of viable CD34+ cell counts are described under Materials and Methods.

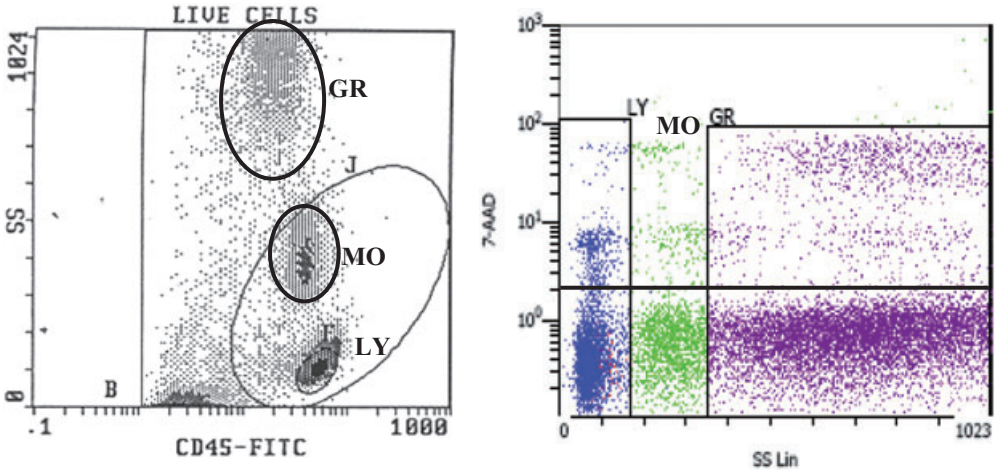


Fig. 2. Strategy for flow cytometric analysis to determine Gr-AAD, Mo-AAD, and Ly-AAD. After identifying separate granulocyte (GR), monocyte (MO), and lymphocyte (LY) cell clusters based on CD45 expression and SSC signals (left scattergram), another scattergram was constructed displaying SS and 7-AAD signals to identify nonviable (7-AAD high) and viable (7-AAD low) cells (right scattergram). Percent viability was then calculated based on the ratio of 7-AAD low events to total events in the corresponding region of the scattergram.

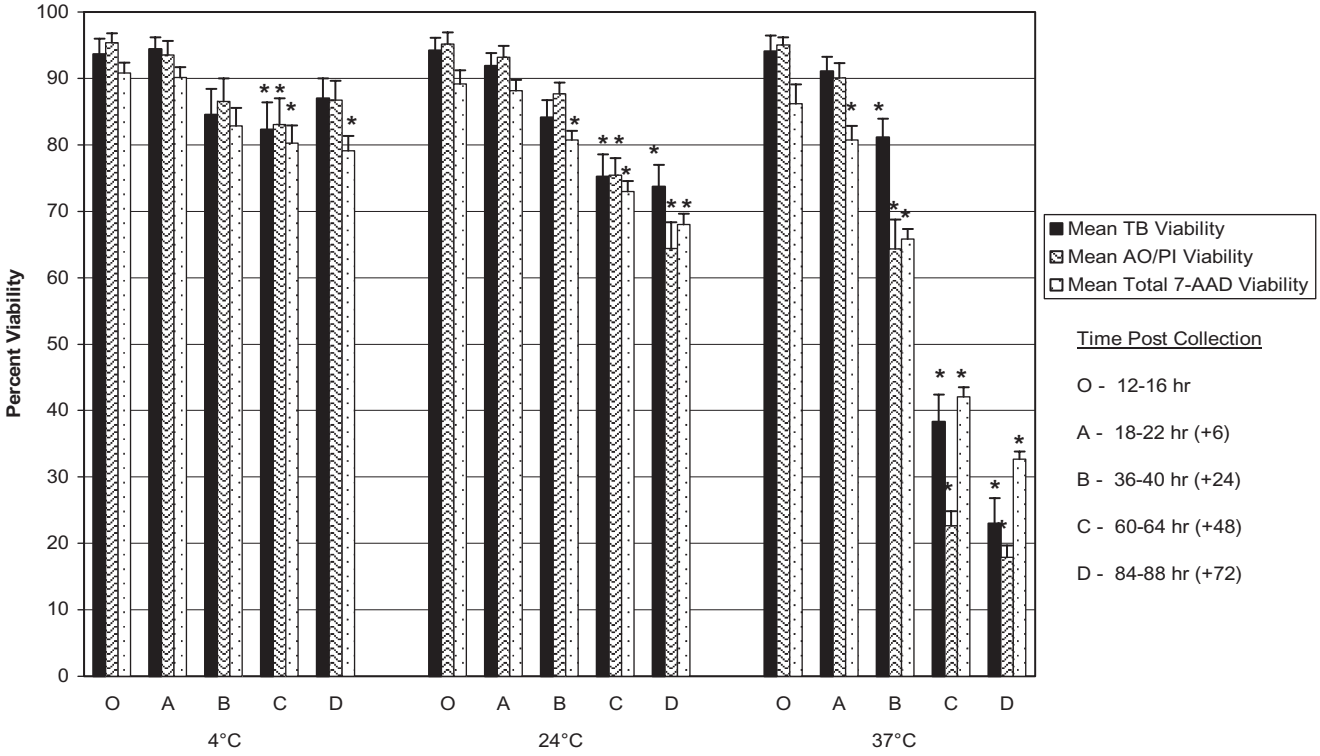


Fig. 3. Time- and temperature-dependent alterations in global measures of CB WBC viability using TB, AO/PI, and 7-AAD (Tot-AAD). Upon receipt in the laboratory, products were separated into three different aliquots; incubated at 4, 24, or 37°C; and analyzed in groups (O, A, B, C, and D) based on time after collection as shown in the figure (e.g., Group O represents data obtained immediately after receipt in the laboratory, which was 12 to 16 hours after collection and transport at 22°-24°C). The numbers in parentheses preceded by the "+" sign indicate the additional time of incubation (hr) at the indicated temperature after receipt in the laboratory. Cell viability measurements were made as described under Materials and Methods. Values shown represent the mean ± SEM of 10 different products in each group. Statistical comparisons were made by ANOVA techniques. *$p < 0.05$ for comparison with the corresponding Group O values.

measures of total WBC viability (TB, AO/PI, and Tot-AAD) throughout the entire 72-hour incubation period. Compared to the Group O value, there was no significant, persistent trend in viability changes in the other groups. Accordingly, we conclude that at 4°C there is no significant reduction in TB or AO/PI viability throughout the entire 72-hour incubation period. In contrast, Tot-AAD viability demonstrated a declining trend after 48 and 72 hours' incubation at 4°C. The "increased sensitivity" of 7-AAD staining to identify nonviable cells may be explained by the fact that 7-AAD staining occurs at an earlier stage in apoptosis than for TB and AO/PI dye uptake. At 37°C, significant reduction in Tot-AAD was seen by 6 and 24 hours' incubation for TB and AO/PI viability. Viability continued to decline rapidly throughout the remainder of the 72-hour incubation period at 37°C.

Figure 4 (top) summarizes the results of flow cytometric characterization of Tot-AAD viability and of separate granulocyte (Gr-AAD), monocyte (Mo-AAD), lymphocyte (Ly-AAD), and CD34+ (CD34-AAD) cell populations in products incubated at 4°C. As seen in Fig. 4, the time-dependent decline in Tot-AAD viability shown in Fig. 3 predominantly reflects the decline in Gr-AAD where granulocyte viability decreases from 85% at Time 0 to 60% after 72 hours' incubation. At Time 0, we see that CD34-AAD > Ly-AAD > Tot-AAD ~ Mo-AAD > Gr-AAD viability. The rate of decline in viability over time follows the order granulocytes > monocytes ~ lymphocytes, which reflects the differential rate of cell death in these WBC subpopulations during 4°C storage. In sharp contrast, the viability of CD34+ cells showed no significant change over the entire 72-hour incubation period at 4°C (84-88 hr postcollection) in all products evaluated.

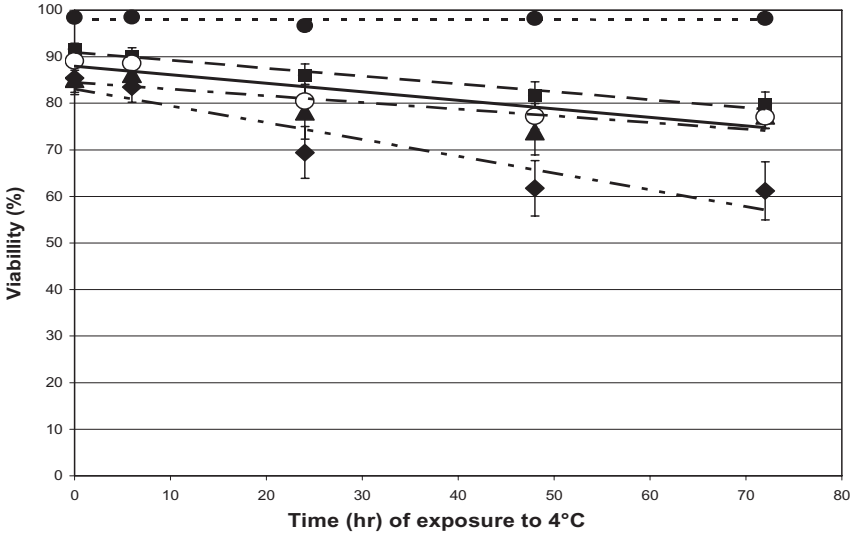
Examination of the data at 24°C (Fig. 4, middle) shows the same qualitative pattern as observed at 4°C; however, the rate of decline in Ly-AAD and Mo-AAD viability is greater at 24°C than at 4°C and comparable to the decline in Gr-AAD viability. Interestingly, Time 0 CD34-AAD viability was 97% with minimal decrease to 93% during the entire 72-hour incubation at 24°C (84-88 hr postcollection). At 37°C, there was a rapid decline in Tot-AAD, Gr-AAD, and Mo-AAD (Fig. 4, bottom). CD34-AAD viability was greater than 90% after 6 hours but rapidly decreased to an estimated value of significantly less than 20% after 72 hours' incubation. It was necessary to estimate CD34-AAD after 48 hours at 37°C since fewer than 20 to 40 total events (i.e., $CD34^{high}$ $7\text{-}AAD^{low}$ events corresponding to viable CD34+ cells) were identified in some products. The decrease in viable CD34+ cells was related to two factors: 1) apoptotic/necrotic cell death causing high 7-AAD signals and 2) increase in "nonspecific" binding of anti–CD34-PE by necrotic cells (i.e., increased "background" CD34+ cell count in the isoclonic control tube). At 37°C, Ly-AAD cell viability was better preserved than all other WBC populations including CD34+ cells.

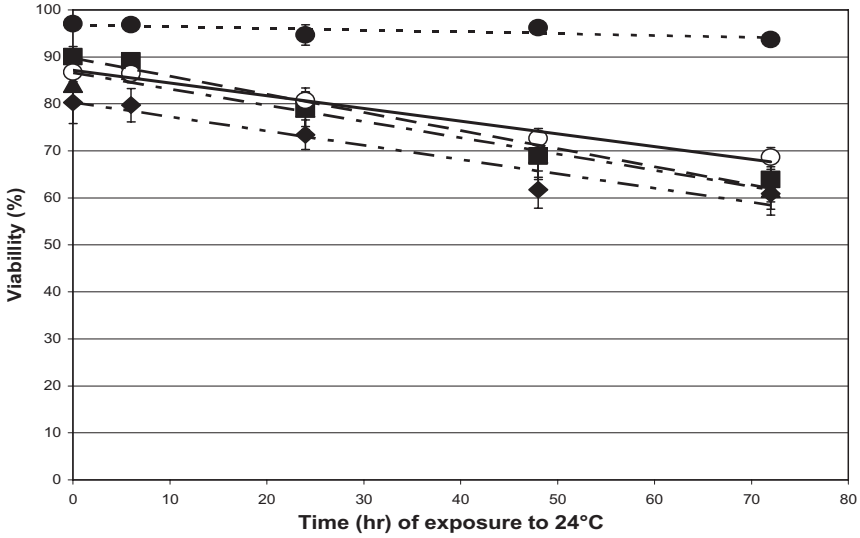
The results of our cell recovery experiments are shown in Fig. 5 where the designation of Groups A through D corresponds to the same group designations shown in Fig. 3. For cell recovery measurements, we expressed results as a percentage of the Time 0 values obtained upon receipt of the products in the lab (Group O in Fig. 3). Comparing cell recovery (Fig. 5) with viability (Fig. 3) shows that global measures of cell viability using TB and AO/PI are strongly correlated with TNC recovery and stable for at least 72 hours' incubation at 4°C. The modest decline in Tot-AAD viability at 4°C (Fig. 3), which results from loss of granulocyte viability (Fig. 4, top) is not accompanied by a reduction in TNC recovery (Fig. 5). This is explained by the fact that automated hematology analyzers count both viable and nonviable cells.[18] Comparison of CD34+ cell viability (Fig. 4, top) with viable CD34+ cell recovery and CFC recovery (Fig. 5) demonstrates that all three of these measurements provide comparable results for assessment of HPC content and function when cells are maintained at 4°C.

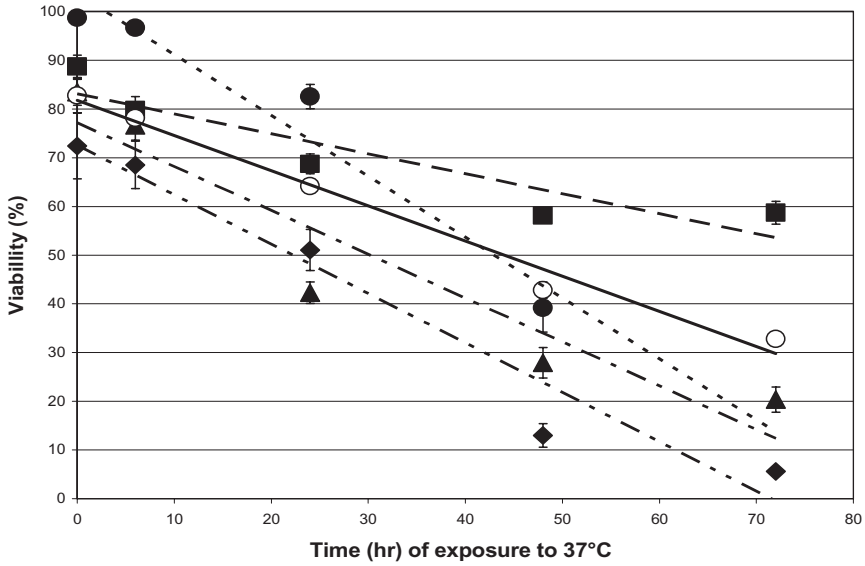
At 24°C, we observed a significant decline in viable CD34+ cell recovery (to 85%) by 24 hours' incubation (36-40 hr postcollection), a decline in CFC recovery (to 81%) by 60 to 64 hours postcollection, and a decline in TNC recovery (to 94%) by 84 to 88 hours postcollection (Fig. 5). When these results are compared to those presented in Fig. 3, it is apparent that global measures of cell viability (TB, AO/PI, and Tot-AAD) decline more rapidly than the observed decline in TNC, viable CD34+ cell, and CFC recovery at 24°C.

At 37°C, based on flow analysis (Fig. 4, bottom), loss of viable granulocytes and monocytes accounts for the decrease in TB, AO/PI, and Tot-AAD viability (Fig. 3) and fragmentation of these apoptotic/necrotic cells likely accounts for the decline in TNC recovery over time (Fig. 5). At 37°C, after only 6 hours' incubation, CFC recovery had declined to 84% and by 72 hours' incubation, CFU activity was completely abolished. This was accompanied by a parallel, rapid decrease in viable CD34+ cell recovery

Fig. 4. Time- and temperature-dependent alterations of viability of granulocytes (◆), monocytes (▲), lymphocytes (■), and CD34+ cells (●) as determined by flow cytometry using 7-AAD and the gating and data analysis strategy described under Materials and Methods. Data shown in this figure were obtained by flow cytometric analysis of CB incubated at 4°C (top), 24°C (middle), or 37°C (bottom) for the time indicated in the figure. Time shown on the *x*-axis represents time after receipt in the laboratory (i.e., Time 0 on the *x*-axis corresponds to 12 to 16 hours after collection and transport at 22-24°C). Data were analyzed at 6, 24, 48, and 72 hours to correspond to the time at which global measures of cell viability were performed and the values shown represent the mean ± SEM of the same 10 products described in the legend to Fig. 3. (○) Tot-AAD.


Viabillity (%)
Time (hr) of exposure to 4°C


Viabillity (%)
Time (hr) of exposure to 24°C


Viabillity (%)
Time (hr) of exposure to 37°C

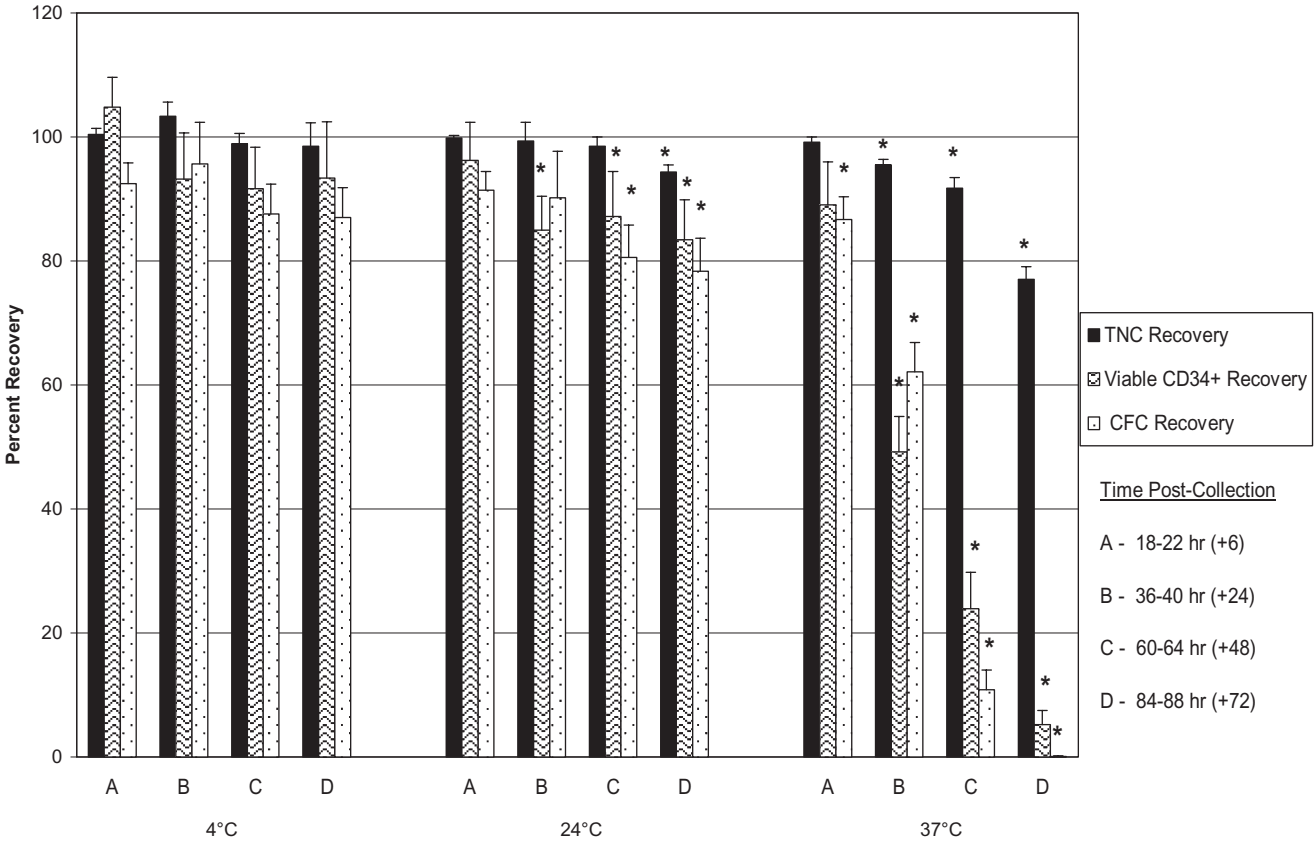


Fig. 5. Recovery of TNCs, viable CD34+ cells, and CFCs before freezing. Percent recovery for each variable was calculated relative to the corresponding value obtained immediately after receipt of the product in the laboratory (baseline value) as described under Materials and Methods. Data were analyzed in groups (A, B, C, and D) corresponding to the group designations described in the legend to Fig. 3. Values shown represent the mean ± SEM of the same 10 products described in the legends to Figs. 3 and 4. Statistical comparisons were made by ANOVA techniques. *p < 0.05 for comparison with the corresponding baseline (Time 0) values.

(Fig. 5). Comparison of these results with those of Figs. 3 and 4 (bottom) demonstrates that loss of viable CD34+ cells and CFC occurs at a much faster rate than loss of TB, AO/PI, or Tot-AAD viability at 37°C in distinct contrast to what was observed at 4 and 24°C.

DISCUSSION

Measurement of cell viability is an essential component of CB product quality assessment. Conventional approaches using TB or AO/PI to measure total WBC viability have significant limitations including 1) interpretation is subjective; 2) the endpoint is not stable, causing variation of results over time (particularly for TB);[19] 3) TB and AO/PI stains necrotic cells or cells in the late stages of apoptosis (nonviable cells in the early stages of apoptosis are not distinguished from viable cells);[1,4] 4) TB and AO/PI staining reflects the viability of mature cells, which may be significantly different from the viability of CD34+ cells or functional HPC; and 5) TB and AO/PI staining cannot be used to reliably distinguish live and dead cells in different WBC populations in CB. This is a particularly important limitation of TB and AO/PI viability assessment since our data show that mature WBC populations in CB exhibit differential susceptibility to apoptotic/necrotic cell death in vitro. This study is the first to compare TB and AO/PI staining with total 7-AAD staining as global measures of cell viability by separately evaluating the viability of the granulocyte, monocyte, lymphocyte, and CD34+ cell populations using flow cytometry with 7-AAD in CB. By making these comparisons as a function of time and temperature before freezing we are able to examine how the differential susceptibility of WBC populations to necrotic/apoptotic cell injury affects global measures of cell viability. We also reasoned that since engraftment and overall survival are related to TNC dose,[8,9] viable CD34+ cell dose,[20] and CFC content,[21,22] by comparing cell viability with recovery of TNCs, viable CD34+ cells, and CFCs we could determine which global viability measurements might be predictive of engraftment and overall survival following CB transplantation.

It is appropriate to emphasize some of the analytical limitations of our study and other studies using CB with regard to viable CD34+ cell measurements by flow cytometry and CFC measurements by CFU assay. For flow cytometry, it is customary to analyze at least 50,000 to 75,000 CD45+ events. This is necessary to obtain reasonably precise measurements of viable CD34+ cell content. Since many CB products contain only 0.2% to 0.4% CD34+ cells,[23] this represents only 100 to 300 total CD34+ cell events. Based on Poisson statistics, for 100 and 300 total events, the coefficient of variation (CV) from sampling error is 10 and 5.8%, respectively.[24] For our CFU assay, optimal plating density on six-well plates and counting reproducibility is achieved when approximately 100 total colonies are counted (Dr Emer Clarke, Stem Cell Technologies, unpublished data). Thus, when counting 100 colonies, the "theoretical best" CV for CFC measurement is 10%. For our studies, the observed CVs of approximately 8% for viable CD34+ cell measurements by flow cytometry and approximately 15% for CFC measurements are certainly within acceptable limits particularly considering the additional contributions of biologic variability between samples and the analytical variability arising from pipetting and other manual steps. Thus with acceptable CVs and a sample size of n = 10, this study is sufficiently "powered" to detect significant differences and identify significant trends in the measured variables.

Our prefreeze studies at 4°C show that TB, AO/PI, and total 7-AAD staining as global measures of cell viability produce similar results; however, although TB and AO/PI viability was quite stable throughout the entire 72-hour 4°C incubation period, total 7-AAD viability showed a gradual downward trend during the same period, which was predominantly due to a decline in granulocyte viability. Thus, at 4°C, TB, AO/PI, and total 7-AAD (initially) as global measures of cell viability have similar values (approx. 86%-96%) that are comparable in magnitude to the observed recovery of TNC (97%-103%), viable CD34+ cells (91%-103%), and CFCs (86%-95%). CD34+ cell viability using 7-AAD (approx. 98%) was always greater than AO/PI, TB, and total 7-AAD viability and remained essentially unchanged throughout the incubation period at 4°C. At 24°C, a similar qualitative pattern relating global measures of cell viability and cell recovery was observed. During the first 24-hour incubation at 24°C (36-40 hr postcollection), TB, AO/PI, and total 7-AAD viability and TNCs, viable CD34+ cell and CFC cell recovery were all comparable in magnitude (86%-102% range) but declined over the next 48 hours. There was a more rapid decline in global measures of cell viability than the decline in viable CD34+ cell and CFC recovery that was due to the combined loss of viable granulocytes and MNCs. Again, CD34+ cell viability remained stable at 95% to 98%. Thus, after prolonged exposure at 24°C (>60 hours postcollection), global measures of cell viability "underestimate" product quality based on recovery of viable CD34+ cells and functional HPCs. At 37°C, there was a modest decline in TNC recovery due to loss of necrotic cells by fragmentation; however, there was a marked decline in global measures of cell viability and CD34+ cell viability using 7-AAD. In contrast to the results seen at 24°C, there was an even more rapid and parallel decline in recovery of viable CD34+ cells and CFCs. Thus, at 37°C, cell viability measurements "overestimate" product quality based on recovery of viable CD34+ cells and functional HPC.

The results of our study are in complete agreement with the findings of Humpe and coworkers.[25] in autologous and allogeneic growth factor–mobilized peripheral blood demonstrating consistently higher CD34+ cell viability compared to other WBC populations. Although we did not evaluate apoptotic cell injury directly, our prefreeze results can be explained by the differential susceptibility of mature WBCs, CD34+ cells, and functional HPCs to both apoptotic and necrotic cell death. CD34+ cells have been shown to express higher levels of the antiapoptotic genes Bcl-2 and Bcl-x and exhibit lower levels of caspase-3 activation (a marker of apoptosis) compared to other MNCs in "fresh" CB exposed to the same conditions as in our study (4-24°C).[5] Accordingly, the results of our prefreeze studies at 4 and 24°C can be explained by the greater susceptibility of mature granulocytes and MNCs to apoptotic cell death compared to CD34+ cells and functional HPCs. It is very interesting that the situation at 37°C is just the opposite. At this "physiologic" temperature, we find that CD34+ cell viability and recovery of viable CD34+ cells and CFCs declines faster than the global decrease in cell viability with TB, AO/PI, or 7-AAD. We propose that the increased susceptibility of CD34+ cells and HPCs to apoptotic/necrotic cell death at 37°C is the result of the fact that normal metabolic activity and function of these cells at physiologic temperature is critically dependent on the presence of growth factors and cytokines such as stem cell factor, GM-CSF, and IL-3 in CB plasma, which are essential for maintenance of CB progenitor cell function, in vitro.[26] Thus, dilution of CB with anticoagulant after collection dilutes these potentially labile compounds in CB plasma effectively resulting in growth factor/cytokine withdrawal and accelerated progression of apoptotic cell death at 37°C. At 4 to 24°C, CD34+ cell and progenitor cell function is better preserved for the reasons described above and because the cells are partially metabolically arrested and less susceptible to growth factor withdrawal. Of course, additional studies are needed to substantiate our hypotheses.

Our data showing optimal preservation of cell viability and cell recovery at 4°C also corroborate the results of other studies[27] and have important practical implications. For example, these results demonstrate the feasibility of transporting products at 4°C over long distances for an

extended period of time without compromising product integrity and suggest that if processing must be delayed, storage in a refrigerator at 4°C overnight will not have any adverse impact on product quality. Although current standards from FACT and AABB require processing within 48 hours of collection, storage at 4°C after receipt in the laboratory could extend acceptable processing time up to 80 hours or more postcollection. For products maintained at room temperature (22-24°C), we recommend that processing be completed within 48 hours and if products are exposed to temperatures between 24 and 37°C for more than 6 to 12 hours, significant compromise of product quality should be expected. Our studies also demonstrate that for products exposed to elevated temperatures (i.e., >24°C), rapid loss of viable CD34+ cells and CFCs that is out of proportion to the decline in TB or AO/PI viability (which are more easily measured) provides sufficient rationale for monitoring temperature during transport if the decision is made not to transport on ice or other cooling agent.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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