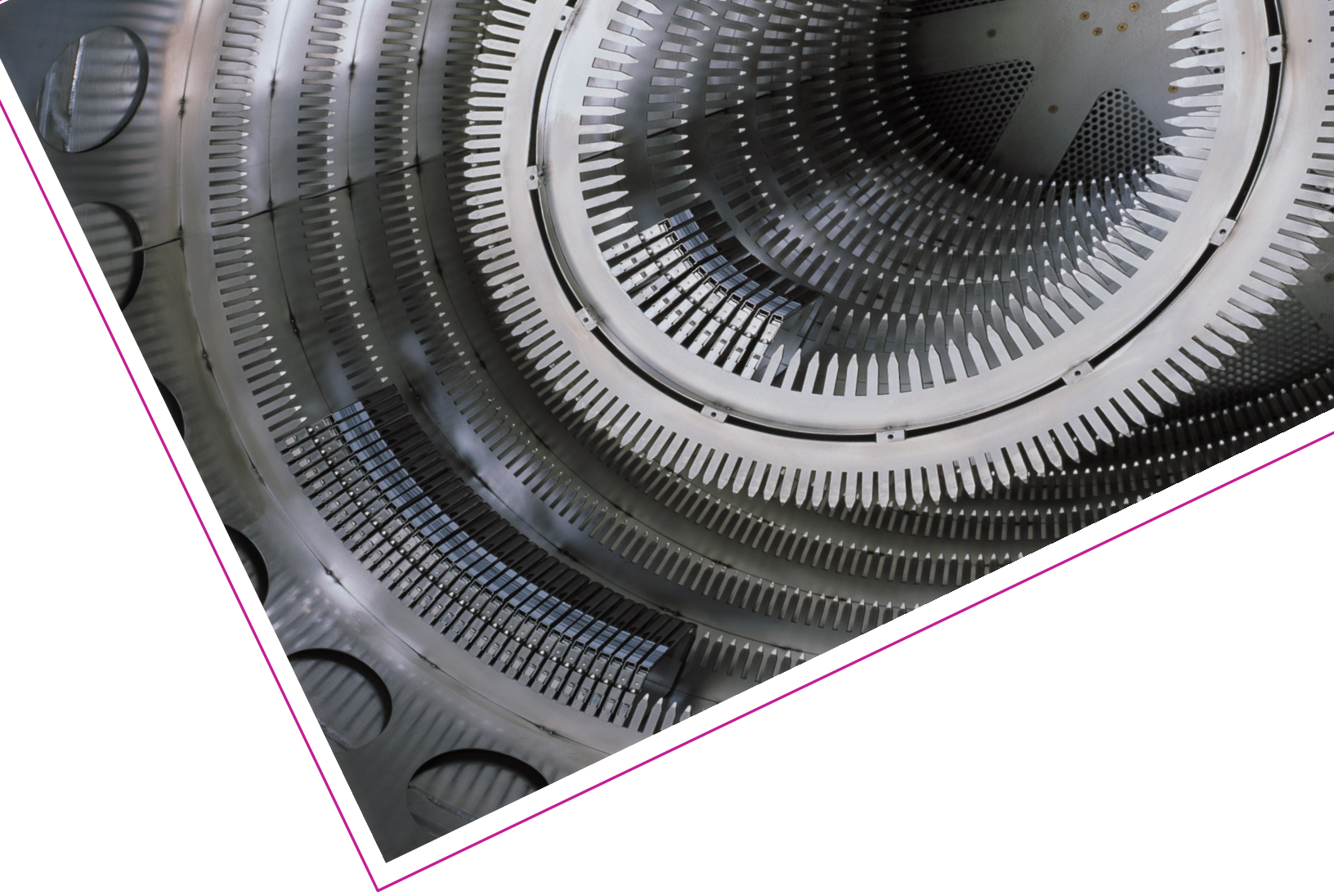

# EFFECT OF TRANSIENT WARMING EVENTS ON HEMATOPOIETIC PROGENITOR CELL VIABILITY IN CRYOPRESERVED UNITS OF UMBILICAL CORD BLOODS

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## ABSTRACT

**Background:** Transient Warming Events ("TWEs") are brief exposures of a frozen blood component to ambient air during post-freeze processing, storage, shipping, and transfer between cryogenic containers. The purpose of this study was to test the hypothesis that the viability of cryopreserved hematopoietic progenitor cells from cord blood units may be reduced as a result of non-thawing TWEs.

**Study Design and Methods:** We noted differences in cell recovery and viability data reported by the transplant centers on cord blood units that had incurred multiple TWEs and others that had not. As a result of these differences, we initiated three experiments. We measured cell viability after multiple exposure of frozen (-196°C) to different temperatures that could realistically occur in a cord blood bank setting. Cord blood cells were controlled-rate-frozen in 10% DMSO to –196°C in 1 ml aliquots. Vials were transferred to alcohol baths maintaining -25°C, 40°C or -80°C, for 4 minutes and then returned to liquid nitrogen. After 5 minutes in liquid, the exposed aliquots were returned to the same alcohol baths. Each aliquot underwent a total of five identical cycles from liquid nitrogen to either -25°C, -40°C or -80°C and back into liquid nitrogen. Control aliquots were maintained at –196°C. All exposed and control aliquots were thawed and DMSO removed. Subsequent experiments were performed on 25 ml volume reduced cord blood samples with transient exposure to room air (2-15 minutes). Recoveries of total and viable leukocytes were assessed by acridine-orange and ethidium bromide staining. Recoveries of viable progenitor cells were ascertained by double staining of $CD34^+$ cells and formation of hematopoietic cell colonies, (CFU-C).

**Results:** The TWEs were observed to cause and increase in loss of cell viability of progenitor cells as measured by CFC and $CD34^+$ cells as well as leukocytes. The loss of cell viability increases with multiple warming events. For example, repeating the TWE cycle from -196°C to -40°C five times instead of one time increased the CFU cell loss from 28% to 52% using 1 ml aliquots of cryopreserved peripheral cord blood cells. In 25 ml volume reduced cord blood, significant loss of CFU were variable. In one experiment, a significant increase in CFU loss was reproducibly observed after ambient air exposure of 6 minutes. In a second study, comparable treatment did not effect CFU treatment but $CD34^+$ cell viability was markedly effected. This data suggests that there may be significant variation from unit to unit of cryopreserved volume reduced cord blood units in their susceptibility to TWE.

**Conclusions:** Cell viability loss was demonstrated in several of the TWE modes evaluated in this study. The effect of the TWEs were more pronounced with increased duration and frequency. These losses occurred under conditions that could readily occur in a cord blood bank setting. The results suggest that units that may be used for transplantation should be monitored for TWE with all incidents recorded as to their time and temperature change.

## INTRODUCTION

The first public cord blood bank, now known as the NYBC National Cord Blood Program (NCBP) collected its first donated cord blood unit in February 1993 and provided the first unrelated cord blood graft in August 1993. To date, the NCBP has collected more than 30,000 units, all stored in liquid nitrogen, and provided more than 1,900 units for transplant for the treatment of malignant and genetic hematological diseases.

Initially, the entire volume of collected units, cryoprotected at 10% Dimethylsulfoxide (DMSO), were placed in standard freezing bags (Baxter) and aluminum canisters, frozen in -80ºC freezers and stored in racks of 5 units under liquid nitrogen in conventional Dewars. Manual transfer of units from the -80ºC freezer to the conventional Dewar and, subsequently, for retrieval prior to shipment to transplant centers requires removal of the entire racks. Thus, these units are exposed to ambient air and then returned to the liquid nitrogen, resulting in multiple transient warming events (TWEs).

In 1994, NCBP developed a method to reduce the frozen volume of each unit while retaining nearly all the mononuclear cells (MNC), adding Hespan (hetastarch) followed by centrifugation to remove excess red blood cells and plasma and achieve a uniform final volume of 20 mL.[1] Dimethylsulfoxide (DMSO) (5 mL of 50% solution) was added as a cryoprotectant. Units collected from October 3, 1994 to May 3, 1999 were volume reduced (VR) in this manner and stored in racks of 10 units under liquid nitrogen. The larger number of units in each rack increases the probable number of TWE that each volume reduced unit will incur over time.

Since May 4, 1999, all units at the NCBP were volume reduced and all were placed in specially designed freezing bags (Pall Corp., East Hills, NY), frozen and stored in canisters at individual "addresses" in a BioArchive® System (ThermoGenesis Corp., Rancho Cordova). The BioArchive System performs the initial controlled rate freezing in the gas phase above liquid nitrogen, records the freezing curves and robotically transfers the unit to its assigned storage address under liquid nitrogen within the same Dewar. When units are retrieved for shipment, the BioArchive removes the individual unit robotically, transfers the unit from liquid nitrogen into insulated sleeves in the gas phase. The insulated unit is then transferred to a liquid nitrogen cooled dry shipper for shipment to the transplant center. Thus, once frozen, BioArchive units are not exposed to ambient temperature and, thus, are not exposed to TWEs.

One of the challenges in the clinical use of cord blood is the limited number of stem cells present in each cord blood collection as reviewed by Lane.[2] The pace of engraftment is strongly influenced by the total nucleated cell dose and $CD34^+$ cell content.[3] It is essential to minimize the losses in viability of the stem and progenitor cells, especially those that occur after the prefreeze cell count is recorded, as these losses are invisible to the transplant physicians, as they are typically discovered only after the patient is ablated and the unit is thawed.

Most, if not all, of the nucleated cells that survive the freezing are reported to remain viable over a decade, or more, of time.[4,5,6] The critical question addressed here is whether moderate TWE of units of cryopreserved cord blood that could occur in routine practice of cord blood banking could have an adverse effect on recovered cell viability. In order to remove from consideration any differences in losses of cell viability due to the freezing of whole volume units and the volume reduced units, we report on the cell viabilities reported by participating transplant centers following the thawing of our two types of volume reduced cord blood units and additional studies using both cryovials and full scale 25 mL cryostorage bags containing volume reduced cord blood cells prepared according to routine clinical practice.[1]

## MATERIALS AND METHODS

### Placental Cord Blood (PCB) Collection from the Delivered Placenta

PCB Blood from delivered placenta and umbilical cord was retrieved using protocols approved by the Institutional Review Boards of the New York Blood Center and Mount Sinai School of Medicine. The procedure, described previously (Ref 10), consists of inserting the 16-gauge needle of a standard 450-ml blood donor set containing CPD A anticoagulant (citrate/phosphate/dextrose/adenine) (Baxter Health Care, Deerfield, IL) into the umbilical vein of the delivered placenta and letting PCB drain by gravity into the blood bag. The 63 ml of CPD A used in the standard blood transfusion bag, calculated for 450 ml of blood, is reduced to 23 ml by draining 40 ml into a graduated cylinder just prior to collection. This volume of anticoagulant matches better the PCB volumes usually retrieved (<170 ml). To facilitate collection and reduce the likelihood of contamination with maternal blood and secretions, the placenta is placed in a plastic-lined absorbent cotton pad suspended from a specially constructed support frame.

### Preparation of volume reduced cord blood units:

PCB Blood from delivered placenta and umbilical cord was retrieved using protocols approved by the Institutional Review Boards of the New York Blood Center and Mount Sinai School of Medicine. The procedure, described previously (Ref 10), consists of inserting the 16-gauge needle of a standard 450-ml blood donor set containing CPD A anticoagulant (citrate/phosphate/dextrose/adenine) (Baxter Health Care, Deerfield, IL) into the umbilical vein of the delivered placenta and letting PCB drain by gravity into the blood bag. The 63 ml of CPD A used in the standard blood transfusion bag, calculated for 450 ml of blood, is reduced to 23 ml by draining 40 ml into a graduated cylinder just prior to collection. This volume of anticoagulant matches better the PCB volumes usually retrieved (<170 ml). To facilitate collection and reduce the likelihood of contamination with maternal blood and secretions, the placenta is placed in a plastic-lined absorbent cotton pad suspended from a specially constructed support frame.

### Cell Viability:

Viability is measured with a computer-driven Patimed microfluorimeter (Leica) by using DNA enhancement of ethidium bromide fluorescence as the index of cell death. One microliter of cell suspension, containing 5000-10,000 nucleated cells, is exposed to ethidium bromide at a final concentration of 1μg/ml for 30 min prior to reading fluorescence emission in the 550- to 580-nm range. Viability is determined by least-squares linear regression: the coefficient of correlation (r2) with known mixtures of heat-killed and live cells is ≥0.991. With appropriate magnification, the morphology of the fluorescent nucleus of dead cells permits easy visual differentiation of mononuclear cells from segmented granulocytes.

### CFC Assay:

The Colony forming unit (CFU) assays of the PCB was performed by the previously described method (Migliaccio). Erythroid bursts and granulocyte/macrophage and mixed-cell colonies are identified by microscopic examination and counted separately in the same dish, but data are presented as the total number of colonies in the PBC unit.

## Flow Cytometric Analysis:

Immunofluorescence staining and FACS analysis to detect residual lineage marker- positive cells in the $CD34^+$ population was performed on a FACScan (Becton Dickinson, San Jose, CA). Staining was performed at $4^oC$ using 20 ul of antibody in 50 ul of cell suspension in flow cytometry buffer (PBS/4% fetal calf serum/ 0.1% azide). Cell staining included: anti-CD45 FITC/CD34 PE monocloncal antibodies. Forward and $90^o$ light scatter gates and background fluorescence were determined using unstained $CD34^+$ cells. Vital staining of $CD34^+$ cells was accomplished using 7 amino-actinomycin D (7AAD).

## Cryopreservation:

DMSO (Cryoserv, Research Industries, Salt Lake City) is used at a final concentration of 10% (vol/vol). The required volume of sterile, chilled DMSO solution is added to the blood bag over the course of 15 min by using a syringe pump and an orbital mixer to assure smooth but vigorous mixing. The final volume of each red cell and plasma reduced cord blood unit with DMSO was a uniform 25 ml. Cryoprotectant and cord blood are kept cold with wet ice throughout the addition. When the concentration of DMSO reaches 10%, cell suspensions are transferred to freezing containers, overwrapped in Teflon bags, placed in stainless steel canisters, and then deposited in the BioArchive System.

## Thawing Units after Storage in liquid nitrogen:

To thaw the unit, the bag is held in nitrogen gas phase for 15 min and then exposed to ambient air for 5 min to allow the plastic to regain some elasticity. The bag is then placed in a Ziploc bag and immersed in 37°C water for thawing as rapidly as possible, usually <2 min.

## Removal of Hypertonic Cryoprotectant:

Immediately after being thawed, each PCB unit is diluted with an equal volume of a solution containing 2.5% (wt/vol) human albumin (Cutter) and 5% (wt/vol) Dextran 40 (Baxter Health Care) in isotonic salt solution, with continuous mixing, and then centrifuged at 400 x g for 10 min. The supernatant is removed, and the sedimented cells are re-suspended slowly and viable leukocytes, CFU-C (2) and $CD34^+$ cells counted.

## Experimental Design:

**Transplant Center Cell Recovery and Viability Reports**

As of December 2002, Transplant centers had reported on post-thaw TNC recovery and cell viability on 651 units of cord blood that was volume reduced and stored in conventional Dewars and 139 cord blood units volume reduced and stored in BioArchive freezers.

As a result of the differences in recovery and viability reported for the two types of volume reduced cord blood units, the following 3 experiments were conducted at the New York Blood Center's National Cord Blood Program laboratories.

### *Experiment 1*

PCB leukocytes were controlled-rate-frozen in 10% DMSO to –196°C in 1 ml aliquots. Vials were transferred to alcohol baths maintaining 40°C or -80°C, for 4 minutes and then returned to liquid nitrogen. After 5 minutes in liquid nitrogen, the exposed aliquots were returned to the same alcohol baths. Each aliquot underwent a total of five identical cycles from liquid nitrogen to either -25°C, -40°C or -80°C and back into liquid nitrogen. Control aliquots were maintained at –196°C. All exposed and control aliquots were thawed and DMSO removed as described previously. Recoveries of total and viable leukocytes were assessed by acridine-orange and ethidium bromide staining. Recoveries of viable progenitor cells were ascertained by double staining of $CD34^+$ cells and formation of hematopoietic cell colonies, (CFU-C).

### *Experiment 2*

Placental blood collections, matched for ABO group, were combined into two separate pools (Pool 1 and Pool 2), each containing $>10^9$ total viable nucleated cells. Hydroxyethyl starch was added to 1% w/v (standard technique, ref 1). After 6 minutes centrifugation at 50 x g, the leukocyte-rich plasma from each was collected. The supernatant from each pool was aliquoted into five processing bags and concentrated to a volume of 20 ml per bag by centrifugation (400 x g, 13 minutes). 5 ml of 50% DMSO/5% Dextran 40 was added and each mixture transferred to a freezing bag (Pall Model 791-05, Figure 1) and overwrapped. The cryoprotected units were controlled-rate-frozen and stored (immersed in liquid nitrogen) in a BioArchive® System (THERMOGENESIS CORP.) for at least 48 hours. Units were retrieved from liquid nitrogen and kept in ambient air for either 2, 4, 6 or 8 minutes (21°C, 60% humidity) and then put back into liquid nitrogen. Warming was monitored by thermocouples from a validated temperature logger. Exposed and control bags, were thawed, DMSO washed off and the number and viability of leukocytes and hematopoietic progenitor cells determined.

### *Experiment 3: Cumulative effect of multiple timed exposures to ambient air*

Units from two additional pools (Pool 3 and Pool 4) were retrieved from liquid nitrogen and kept for either 1, 2 or 3 minutes, at room temperature (21°C) and then returned to liquid nitrogen for 2 minutes. The same units were again brought to air for the same time period and back to liquid nitrogen for 2 minutes for a total of five identical cycles. All exposed units and unexposed controls were kept in liquid nitrogen until tested. Temperature was monitored for each unit. All units were then thawed, DMSO washed off and viable leukocytes, CFU-C measured and $CD34^+$ cells counted. Cell recoveries were then calculated.

## RESULTS

### Transplant Center Reports

Post-thaw TNC recovery was significantly higher for BioArchive units than for volume reduced units that were stored in conventional Dewars (mean proportion of cells recovered $\pm$ standard error of the mean = 0.82 $\pm$ 0.03 and 0.69 $\pm$ 0.01, respectively. $P < 0.001$). Similarly, the viability of cells recovered post-thaw was higher in BioArchive units than in volume reduced unit that were stored in conventional Dewars (mean

percent of viable cells ± standard error of the mean = 86.9% ± 1.4 and 81.7% ± 0.8, respectively. P = 0.003).

### *Experiment 1. Effect of Single and Multiple TWEs in Culture Vials at Subfreezing Room Temperature*

Significant losses in cell viability and functionality were observed for cells stored in tissue culture vials when exposed to transient warming events as shown in Table 1. The loss in cell viability was comparable between leukocytes and $CD34^+$ cells with no evidence of a greater sensitivity of one cell type to TWEs. The temperature gradient of the TWE was found to be a significant factor in determining the amount of cell loss that was observed. For example, the degree of cell viability loss was consistently greater with vials transiently exposed to -40°C as compared to - 80°C. In addition, there was an increase in the loss of CFU cells when the aliquots were challenged with repetitive TWE events as compared to a single TWE. For example, repeating the TWE cycle from -196°C to -40°C five times instead of one time increased the CFU cell loss from 28% to 52%.

Table 1: Effect of Single and Repetitive Transient Warming Events (TWE) on Cord Blood Cell Viability and Functionality at Subfreezing Temperature Gradients

| Temperature Used for TWE* | | -80 °C | -80 °C | - 40 °C | - 40 °C |
|---|---|---|---|---|---|
| Number of TWE Cycles | | x 1 | x 5 | x 1 | x 5 |
| Leukocyte Viability (% Loss) | Median | 0** | 6 | 10 | 13.5 |
| | Range | 0 – 4 | 3 - 10 | 9 – 11 | 13 - 14 |
| | | (n=3) | (n=3) | (n=2) | (n=2) |
| $CD34^+$ Cell Viability (% Loss) | Median | 2 | 6.5 | 10 | 21 |
| | Range | 0 – 4 | 2 - 11 | 9 – 11 | 18 - 24 |
| | | (n=2) | (n=2) | (n=2) | (n=2) |
| CFU-C (% Loss) | Median | 9 | 22 | 28 | 52 |
| | Range | 3 - 14 | 18 - 25 | 18 – 38 | 48 - 55 |
| | | (n=2) | (n=2) | (n=2) | (n=2) |

* A Transient Warming Event (TWE) was generated in this study by transferring 1 ml aliquot of cells in a vial from a temperature of -196°C to either -80 °C or -40 °C for 4 minutes before returning the vial back to -196 °C.
**Data are presented as the median value and range of increased % of non-viable cells (cell loss) as compared to control (liquid nitrogen storage without TWE).

### *Experiment 2. Effect of Single TWE on 25 ml volume reduced Cord Blood Units Exposed to Room Temperature*

The duration of the TWE event was found to be a significant variable affecting cell viability and CFU cell number remaining in cord blood units stored in freezing containers under intended clinical use conditions. The data in Table 2 demonstrates that the placement of the freezing bags containing 20 ml of cord blood at ambient room temperature from their storage at -196°C for 2 to 8 minutes resulted in progressive nucleated cell viability and CFU cell loss. Figure 1 is a drawing of the cryostorage bag employed in this study and

Figure 2 shows an example of the temperature change it underwent following an 8 minute exposure to ambient air after its storage at -196°C.

Table 2. Effect of Single TWE on 25 ml volume reduced Cord Blood Units exposed to Room Temperature*

| | | CFU Control Post-Thaw | Control % | Exposed Time 2 min | 4 min | 6 min | 8 min |
|---|---|---|---|---|---|---|---|
| % Total nucleated cells Viability (% Loss) | Pool 1 | | 76 | 75 (1) | 75 (1) | 69 (7) | 63 (13) |
| | Pool 2 | | 87 | 81 (6) | 79 (8) | 71 (16) | 67 (20) |
| % CFU Recovery | Pool 1 | 450,000 | 100 | 100 | 94 | 78 | 67 |
| | Pool 2 | 650,000 | 100 | 72 | 69 | 65 | 46 |

* A Transient Warming Event (TWE) was generated by transferring a 25 ml cord blood unit from a temperature of -196°C to ambient room temperature for the indicated minutes before returning the storage bag back to -196 °C.

**Data are presented as the observed value of cell viability for total nucleated cells and % CFU recovery as compared to the control not subjected to a TWE.

### *Experiment 3: Effect of Multiple TWEs on 25 ml volume reduced Cord Blood Units exposed to Room Temperature*

The effect of repeated TWE events to the same volume reduced cord blood unit (20 mL) was demonstrated to be a significant variable affecting cell viability and functionality of cord blood units prepared in freezing containers under intended clinical use conditions. The data in Table 3 demonstrates that the repetitive placement of cord blood in the freezing bags at -196°C and ambient room temperature for five cycles of TWE resulted in progressive nucleated cell viability and $CD34^+$ cell loss. The magnitude of loss under this treatment regimen was less dependent upon the duration of the ambient air exposure with the 1 minute excursion regimen causing similar losses as observed in 3 minute excursion regimen. In Experiment 3, the effect of the treatment regimens were minor on CFU cell recovery but $CD34^+$ cell viability was significantly reduced. This result suggests that variability can exist in the sensitivity of different preparations of cord blood to TWEs.

Table 3. Effect of Multiple TWEs on 25 ml volume reduced Cord Blood Units exposed to Room Temperature

| | | CFU Control Post-Thaw | Control % | Exposed Time | | |
|---|---|---|---|---|---|---|
| | | | | 1 min. x 5 | 2 min. x 5 | 3 min. x 5 |
| % TNC Viability (% Loss) | Pool 1 | | 72 | 63 (9) | 62 (10) | 58 (14) |
| | Pool 2 | | 74 | 72 (2) | 70 (4) | 65 (9) |
| CFU Recovery (%) | Pool 1 | 533,333 | 100 | 100 | 94 | 87 |
| | Pool 2 | 300,000 | 100 | 100 | 100 | 89 |
| % $CD34^+$ Viability (% Loss) | Pool 1 | | 87 | 82 (5) | 81 (6) | 82 (5) |
| | Pool 2 | | 65 | 64 (1) | 76 (0) | 61 (4) |

* Repetitive Transient Warming Events (TWEs) were generated by transferring a 25 ml cord blood unit from a temperature of -196°C to ambient room temperature for the indicated minutes before returning the storage bag back to -196 °C. This process was repeated five times for each container.

**Data are presented as the observed value of cell viability for total nucleated cells, % CFU recovery and % $CD34^+$ cell viability as compared to the control not subjected to a TWE.

## DISCUSSION

Quality control issues in the processing of cord blood has become a subject of recent interest as cord blood banking is transitioning from research-based laboratory to the clinical cellular therapy laboratory[7,8,9,10] The long-term effects of cryopreservation on hematopoietic progenitor cell viability in human bone marrow has been studied, but there are no studies to our knowledge on the effect of transient warming events on cord blood cell viability. The major findings of our experiments are: 1) TWE well below the freezing point of blood and within the range of routine cord blood bank practices can cause an inadvertent loss of cell viability; 2) the loss of cell viability increases with duration of the warming event; and 3) the loss of cell viability increases with multiple warming events as measured by $CD34^+$ cell viability. In 25 ml volume reduced cord blood, the loss of CFU were observed to be variable. In Experiment 2, a significant increase in CFU loss was reproducibly observed after ambient air exposure of 6 minutes (approximately half of control). In Experiment 3, comparable treatment did not affect CFU treatment. This indicates that there may be significant variation in cryopreserved volume reduced cord blood units in their susceptibility to TWE. The data indicate that TWE should be recognized as a potential risk for the integrity of the stored blood sample. Because viable cell dose is critical variable for transplant success, all measures taken to maintain viable cell

recovery are likely to have clinical relevance and should be controlled and documented as part of current Good Tissue Practices.

This is the first report to our knowledge of an investigation into the significance of TWEs on frozen cord blood cell viability. Future research will be required to determine whether stem cells exposed to transient warming events and then stored for prolonged periods of time have sustained accelerated rates of cell viability loss. The practicality of assessing the significance of TWE on clinical outcomes is problematic due to the large number of variables involved in cord blood transplantation as reviewed by Lang. Even in the absence of clinical data, it is evident that every reasonable effort to maximize the viability of the limited number of cord blood progenitor cells in each unit should be taken. This study suggests that avoidance of TWE should be part of the cell processing practices of cord blood to the extent possible and when they occur they should be documented.

Figure 1: CryoBag 25 ml (Model 791-05) employed in Experiments 2 and 3 denoting positions of temperature probes for monitoring temperature fluctuations

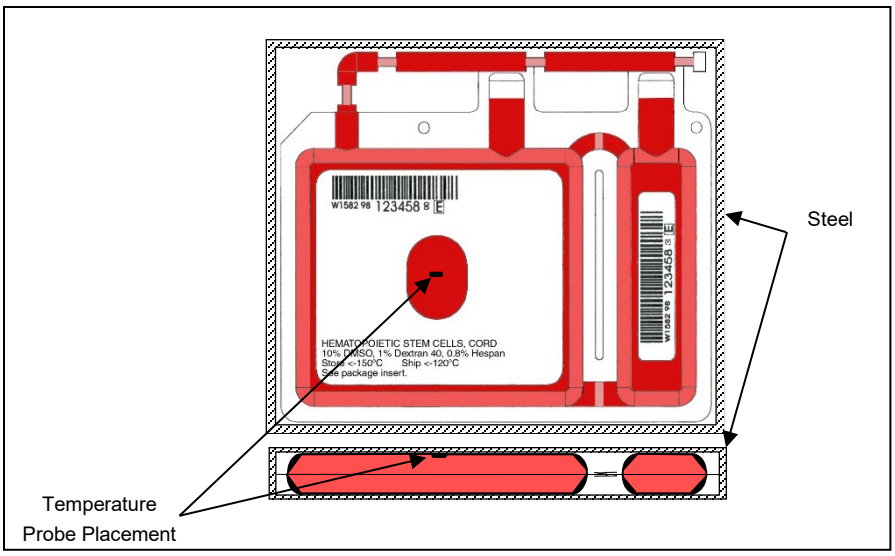
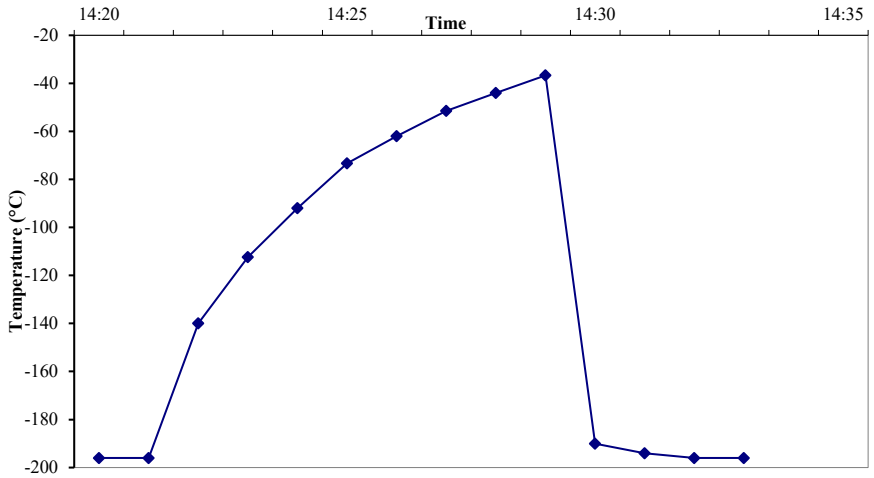



Figure 2: Temperature kinetics of a one TWE, 8-Minute Exposure to Ambient Air of a Pall Medical (Model 791-05) CryoBag containing 25 ml of volume reduced cord blood in an overwrap bag enclosed in a stainless steel canister



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