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Recovery and Post-Thaw Assessment of Human Umbilical Cord Blood Cryopreserved as Quality Control Segments and Bulk Samples

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A B S T R A C T

Quality control (QC) segments conjoined to a bulk sample container are used to evaluate the viability and quality of cryopreserved umbilical cord blood (UCB). Such QC segments are typically attached lengths of sealed tubing that are cooled concurrently with the bulk sample, both containing material from the same donor. QC segments are thawed independently of the bulk sample to assess the quality of the cryopreserved product. In current practice, there is typically post-thaw variation between the QC segment and the bulk sample which if suggestive of inadequate performance, could lead to material being needlessly discarded. In this study, these performance differences were quantified. Two cooling protocols in common use, 1 with and 1 without a "plunge" step to induce ice nucleation, gave equivalent results that maintained the QC segment versus bulk sample differences. Ice nucleated at significantly lower temperatures in the QC segments compared with the bulk samples, a consequence of their lower volume, thereby enhancing damaging osmotic stress. A reduction in total viable cells of approximately 10% was recorded in the QC segments compared with comparable bulk samples. It has been shown that $CD45^+$ cells are more adversely impacted by this lower ice nucleation temperature than $CD34^+$ cells, which can result in altered composition of the post-thaw cell population.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HCT) is a potential treatment for a wide range of conditions, including cancer immunodeficiency and autoimmune disease [1–3]. Historically, the primary sources for these cells have been bone marrow and peripheral blood, and in the last 10 years umbilical cord blood (UCB) has become recognized as an important additional source. UCB can be cryopreserved immediately postpartum and stored at ultra-low temperatures until required, and currently more than 700,000 UCB units are stored this way worldwide [1,4].

A key enabling step for successful cord blood transplantation is effective and reproducible cryopreservation of the UCB [1], yet the cryopreservation procedure is not standardized between centers, with each using their own, albeit similar, methods [1,5,6]. Typically, this will involve the addition of cryoprotectants, most commonly dimethyl sulphoxide (DMSO), dextran, and hydroxyethyl starch, to protect cells during the cryopreservation process [7–10]. Samples are cooled in a controlled-rate freezer (CRF) following a specific cooling profile, because the importance of a precisely controlled cooling rate in mammalian cell cryopreservation has long been recognized [11,12].

The volume of UCB available in the umbilical tissue obtained after birth is limited and can vary substantially between donations. Thus, it is pragmatic to cryopreserve a small QC segment together with the bulk UCB sample that can be thawed separately for recovery estimation or matching purposes. The bulk sample is typically approximately 20 mL, with QC samples ranging from 1 to 200 μL [5,9,10,13,14] (Fig. 1). It is imperative that the QC samples reflect, as accurately and consistently as possible, the properties of the bulk sample. Differences seen as indicating a significant reduction in post-thaw recovery between the QC segments and the bulk sample may lead to unnecessary discarding of valuable material. Few studies examining such differences are to be found in the literature, and yet, as cord blood transplantation becomes more common, the pressure to provide reliable methods giving accurate QC information increases [5,6,9,10,13,14].

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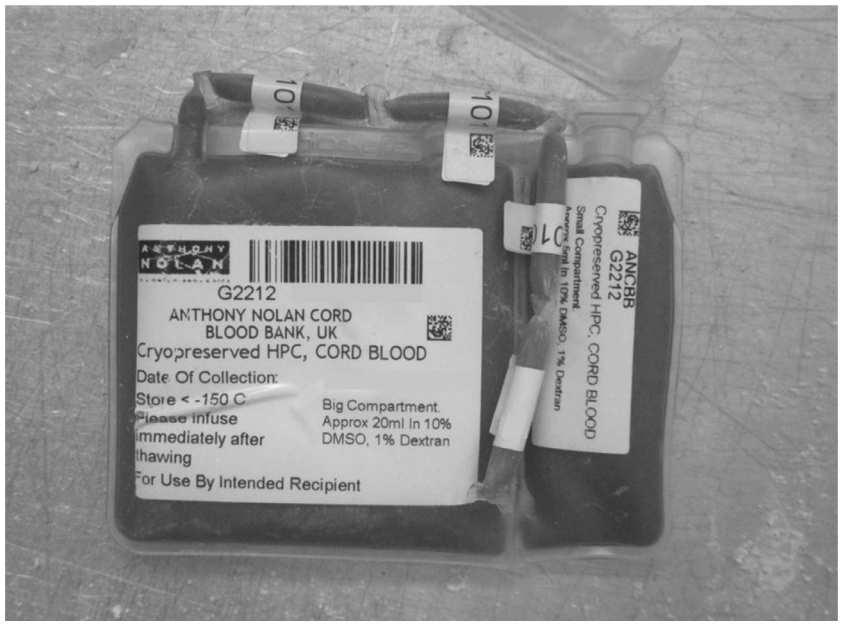

Figure 1. An example of a cryobag (CryoSC-Db, Biosafe) with a bulk sample compartment and 4 small segments used for quality control.

A significant complicating factor in attempts to align QC and bulk samples of cryopreserved UCB is that ice nucleation temperature is volume-dependent, with smaller volumes (as found in QC samples) experiencing greater supercooling before ice nucleation compared with bulk samples [15,16]. Excessive supercooling is known to be harmful to many cell types [16] and can give results for a QC sample that do not reflect the likely performance of the comparable bulk sample after thawing [9,10,13–16]. Cord blood samples contain a heterogeneous population of cells, and because different cell types can respond differently to ice nucleation, a difference in post-thaw cell populations between recovered QC and bulk samples may also occur [9,11,16,17]. Although such QC segments are commonly used in cord blood cryopreservation, their use in other procedures must be considered as the use of cryopreserved products in cell therapies increases [15,18,19].

When cryopreserving UCB, some centers will use a cooling protocol, in which initially the sample is rapidly cooled to a limited extent to induce ice nucleation at a specific time (sometimes referred to as a "plunge" or "shock cooling" step, the former term used hereinafter). Originally developed for bone marrow cryopreservation, these programmed protocols [20–22] can define the time window, but not the precise temperature, when ice first nucleates. Alternatively, current protocols may use a "linear" rate of cooling (eg, 1°C min^{-1}), which allows nucleation to occur spontaneously during cooling.

This study is seen as a step toward optimizing USB cryopreservation and assessment by considering differences in post-thaw performance between QC segments and their bulk samples. Both plunge and linear cooling protocols were selected to be typical of those currently used in UCB cryopreservation centers in the absence of a unified cryopreservation protocol for UCB [1,2,8,10].

To evaluate the difference in post-thaw performance between QC segments and the bulk sample in both overall nucleated cell yield and outgrowth, $CD34^+$ and 7AAD/annexin-V fractions were examined to provided a rounded assessment of cell health and apoptosis, as is typically done with UCB and wider blood-based cell therapies [8,17,23–25].

METHODS

UCB Collection and Processing

Informed donor consent was obtained, and cord blood was collected ex utero in a dedicated bag (MSC 1200PU; Macopharma, Mouvaux, France), with 33 mL of anticoagulant citrate-phosphate-dextrose solution added. The collection volume varied from patient to patient. Up to 3 UCB samples were pooled and processed to achieve a total nucleated cell (TNC) content of 1.4×10^9 nucleated cells (the minimum for processing at an Anthony Nolan cell therapy center). The sample was then mixed and split equally into 2 collection bags. Both bags were processed on a closed-cell separation and centrifugation system (Sepax; Biosafe SA, GE Health Care, Eysins, Switzerland) to reduce the sample volume to approximately 20.5 mL and concentrate the nucleated cell fraction using the CS-540.4b accessory (10036, Biosafe SA). A total of 15 pairs of pooled samples were used in this study.

Cryopreservation Procedure

The processed UCB cryobags were placed in an automated mixing and cooling device (Coolmix; Biosafe SA), set at 4°C, for 10 minutes for cryoprotectant addition. Then 5 mL of the cryoprotectant consisting of 55% DMSO (v/v) and 5% (w/w) dextran 40 (CryoPur-D; Origen Biomedical, Austin, TX) were added over 10 minutes through a syringe pump attached to the cryobag, to reach a final concentration of 10% (v/v) DMSO. The cryobag tubing was filled with sample and segmented into 4 parts, each approximately 1.25 cm long, using a tube heat sealer (2380; Sebra, Tucson, AZ), creating QC segments of ~120 μL (Figure 1). The cryobags were accurately weighed to complete the cell number determination.

The prepared cryobags were inserted into metal cassettes (Aviamax, Robertsbridge, UK), transferred to a metal rack, and then placed into the appropriate CRF. The protocol was initiated immediately, to prevent any further warming of the sample during transfer.

Two controlled cooling profiles were tested, one including a limited rapid cooling/rewarming phase ("plunge cooling") to induce ice nucleation at a specific time and a linear profile. The 2 profiles are described in Table 1. The plunge profile was run on an LN_2 CRF (Kryo 560; Planer, Sunbury-On-Thames, UK), and the linear profile was run on an LN_2-free, Stirling engine-based CRF (VIA Freeze, Asymptote, GE Health Care, Cambridge, UK).

After completion, the frozen UCB units were transferred to a portable cryogenic device (MVE CryoCart; Chart Industries, Canton, GA) and then stored in the vapor phase of liquid nitrogen in a cryotank (MVE Series 1500; Chart Industries) until thawing.

Thawing Procedure

After a minimum of 7 days of storage, the UCB units were removed from cryogenic storage. QC segments were separated from the bulk sample, and both were thawed by immersion in a 37°C water bath until no visible ice was present.

Table 1
Details of the "Plunge" and "Linear" Cooling Profile Used for the Cryopreservation of Processed Cord Blood

Step	Plunge Protocol			Linear Protocol		
	Rate, °C min^{-1}	End temperature, °C	Hold, min	Rate, °C min^{-1}	End temperature, °C	Hold, min
1		4	5		2	10
2	-1	-10		-2	-35	
3	-20	-50			-35	15
4	15	-18		-2	-70	
5	-1	-40				
6	-2	-60				
7	-3	-80				
8	-10	-120				

Ice Nucleation Measurements

To determine the temperature at which samples nucleated, cryobags were prepared with a bulk sample and 3 QC segments containing cryoprotectant medium but without cells. A T-type thermocouple (RS Components, Corby, UK) was placed into each QC segment and the bulk sample, and the cryobag was cooled in each CRF using the protocols described in Table 1. Measurements were recorded using a TC-08 thermocouple data logger (Pico Technology, St Neots, UK), with nucleation observed as a sharp, rising temperature discontinuity. This was repeated 4 times for each protocol.

Hematologic Cell Count

A volume of 0.2 mL was removed from the cryobags prefreeze and from both bulk samples and QC segments post-thaw. These samples were transferred to a automated hematology analyzer (XE-2100; Sysmex, Kobe, Japan) for counting of TNCs, nucleated red blood cells (NRBCs), and white blood cells (WBCs) and determination of WBC differential counts.

Assessment of Total, Viable, Apoptotic, and Dead CD34$^+$ and CD45$^+$ Cells

A volume of 14 to 33 μL was removed from the cryobags prefreeze, as well as from the bulk samples and QC segments post-thaw, to perform stem cell analysis. The extracted volume was varied to ensure that 0.6×10^6 nucleated cells were tested. The antibody cocktail used for CD34$^+$ enumeration comprised 10 μL of CD34-PE (555822; BD Biosciences, San Jose, CA), 3.3 μL of CD45-FITC (555482; BD Biosciences), and 37 μL of FACS buffer per test. For annexin V, the cocktail comprised 10 μL of CD34-PE, 5 μL of CD45-APC (555485; BD Biosciences), and 35 μL of FACS buffer. FACS buffer comprised 1% AB serum (v/v) and 0.01% sodium azide (w/v) in PBS. BD Trucount tubes (BD Biosciences) were used for CD34 enumeration, and standard 3.5-mL tubes were used for the annexin V assay.

The volume removed was made up to 50 μL with FACS buffer, and then 50 μL of the test cocktail wasadded. The tubes were incubated out of direct light for 15 minutes. Thereafter 1:10 lysis buffer was added (Pharm Lyse; BD Biosciences) and incubation was continued for another 10 minutes. For CD34$^+$ enumeration, 5 μL of 7-AAD (559925; BD Biosciences) was added to the tube immediately before analysis.

For the annexin V assay, the tubes were then centrifuged at $800 \times g$ for 4 minutes (Rotina 420R; Hettich, Tuttlingen, Germany). After centrifugation the supernatant was removed and the pellet resuspended in 1:10 diluted Annexin V buffer (Ref: 556454, BD Biosciences), with 3.1 μL of Annexin V (Ref: 556419, BD Biosciences) and 5 μL of 7-AAD added separately. The tubes were then incubated for 15 minutes prior to analysis.

The stained samples were analysed using a flow cytometry system (BD FACSCanto II, BD Biosciences). 7-AAD-negative cells and Annexin V-negative cells were separately used to calculate CD34+ cell viability, while 7-AAD-negative, Annexin V-positive cells were considered as apoptotic, and 7-AAD-positive, Annexin V-positive cells were considered as dead.

Cloning Efficiency Assay

The colony-forming ability of stem cells was determined by adding 150 stem cells (based on pre-freeze assessments) to 1.1 mL per well of a semisolid methylcellulose medium (Methocult, STEMCELL Technologies, Vancouver, BC, Canada). Duplicate 6-well plates were established for each sample. The plates were incubated at 37°C in a humidified environment with 5% CO_2 and were counted under a microscope after 14 days of incubation. Clonogenic efficiency was calculated as the scored colony-forming units divided by the number of viable CD34$^+$ cells seeded. Viability was calculated as the total number of living cells divided by the total number of cells (alive and dead). Recovery was calculated as total number of living cells compared with the number of cells pre-freeze, to take into account cells destroyed by the freezing process and no longer identifiable.

Statistical Analysis

Statistical analyses were performed in R version 3.4.2 [26] using the R Commander 2.4-1 package [27], and P values $<.01$ were considered significant. The Shapiro-Wilk test was used to test for the normality of distributions. Data were compared using the paired t test if the data followed a normal distribution and the Wilcoxon signed-rank test otherwise.

RESULTS

Ice nucleation in the bulk sample was recorded at -10.5 ± 3.8°C in the linear profile and at -13.6 ± 5.9°C in the plunge profile (n = 4). Comparable temperatures in the QC segments were significantly lower ($P < .01$), at -23.3 ± 2.7°C and -19.6 ± 3.9°C, respectively (n = 12). There was no significant difference in nucleation temperature ($P > .01$) between the plunge and linear cooling profiles.

The results of cell recovery and potency for thawed UCB are presented as Figure 2. The linear and plunge cooling profiles are presented for bulk samples in Figure 2A and for QC segments in Figure 2B. No significance differences ($P > .01$) were seen between any of the parameters examined; however, when the data from both cooling profiles was combined, TNC recovery, viable MNC counts, and viable CD45$^+$ counts were significantly higher in the bulk sample compared with the QC sample ($P < .01$) (Figure 3A).

Using the plunge cooling protocol (Figure 3B) there was a limited level of improved post-thaw outcome in the bulk sample over the QC segments in each assay, although statistical significance was not reached. The data from the linear cooling protocol (Figure 3C) indicated that the bulk sample was significantly better than the QC segments for TNC recovery and viable MNCs and CD45$^+$ cells. No significant differences were recorded elsewhere.

DISCUSSION

This study has shown significant differences in ice nucleation temperature and post-thaw recovery between the QC segment and bulk sample of cryopreserved UCB material, using cryobags and 2 widely adopted cryopreservation protocols, No significant differences in post-thaw performance were gained by using the protocol that included a plunge step to initiate ice nucleation.

No significant difference in ice nucleation temperatures between the linear and plunge cooling protocols was recorded. Clearly, the plunge influences the timing of nucleation yet provides no advantage over linear cooling as the nucleation temperature (the critical parameter) is unchanged. This is of importance, because typically UCB banks may cryopreserve their samples using a profile including [9] or excluding [7,8,23] plunge cooling.

The TNC recovery (total viable cells) was consistently lower in the QC segments than in the bulk sample (~10% TNCs; Figure 3A). Within the TNC population, the mean recovery of viable MNCs and CD45$^+$ cells was also significantly reduced in

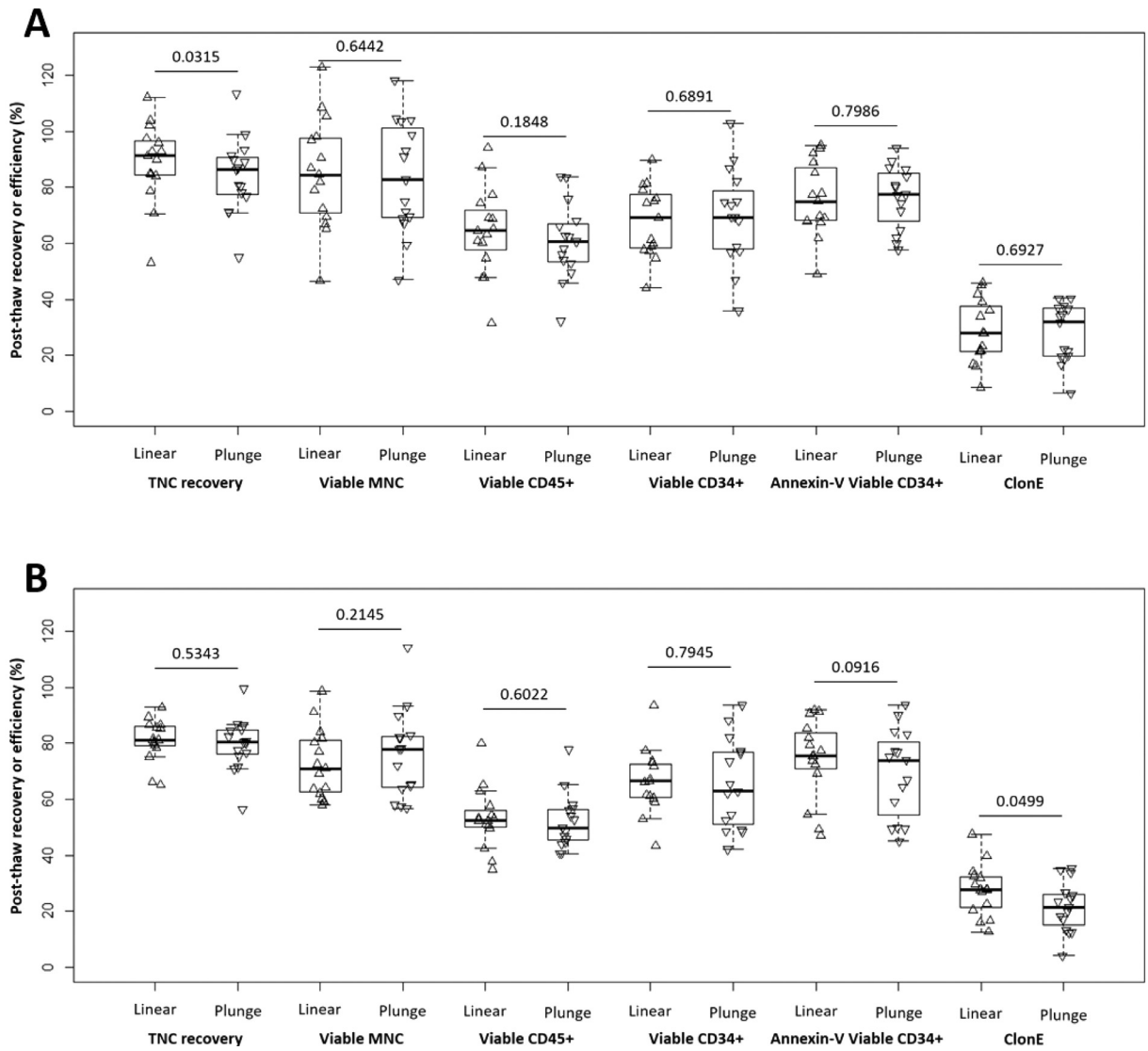

Figure 2. Cell recovery and potency assays for post-thaw UCB bulk samples (A) and QC segments (B), with data normalized to prefreeze values (n = 15). Upward-pointing triangles represent the "linear" protocol; downward-pointing triangles, the "plunge" protocol. Indicated are recovery of total nucleated cells (TNC recovery), recovery of viable mononuclear cells (viable MNCs), recovery of viable $CD45^+$ cells (viable $CD45^+$), recovery of viable $CD34^+$ cells (viable $CD34^+$), percentage of viable $CD34^+$ cells by annexin-V assay (annexin-V viable $CD34^+$), and clonogenic efficiency (ClonE). The *P* value for each parameter is indicated above the horizontal lines.

the QC sample compared with the bulk sample. These data reflect earlier reports of lower results in QC segments compared with bulk samples [9,10,13,14].

The mean reduction in viability of 10% reported here can only be seen as indicative; the random nature of ice formation will produce variation between QC segments. However, a value of this size becomes significant when considering the use of QC segments in UCB cryopreservation or other blood-based therapies [18,25]. Material may be rejected due to apparent low cell recovery indicated by the QC segment or dosage, as cells/mL, being calculated incorrectly due to variation between the QC segment and bulk sample.

The reduced post-thaw outcome in the smaller QC segments (for both cooling protocols) results from the significantly lower nucleation temperatures observed compared with the bulk samples. This occurs because ice nucleation temperature decreases as solution volume is reduced, due to a concomitant reduction in ice nucleating sites [16]. When a cryopreservation medium cools substantially below its equilibrium melting point before ice nucleation (supercooling), significant cellular injury can result, reducing post-thaw recovery [16,28–30]. Consequently, because QC segments are commonly approximately 1% of the volume of the bulk sample, an increase in cellular damage to their contents can be expected compared wih their associated bulk sample.

A major cause of damage is the increasing hypertonicity of the cryopreservation medium as ice quickly forms throughout the sample after nucleation. Ice crystals exclude solutes from their structure, so the residual solution containing the cells becomes increasingly concentrated as ice increases [31–33]. A rapid increase in potentially lethal osmotic stress results. Postnucleation cooling can also trigger injury and different cell types each have optimal cooling rates [12,34]. Following nucleation, temperature rapidly rises as the latent heat of fusion of ice is released. Once ice has formed throughout the sample, the temperature will drop rapidly to equilibrate with its surroundings and these temperature excursions can have lethal consequences [16,31,33–35]. Such effects are compounded in smaller sample volumes, in which there is less thermal mass to dampen the cooling rate

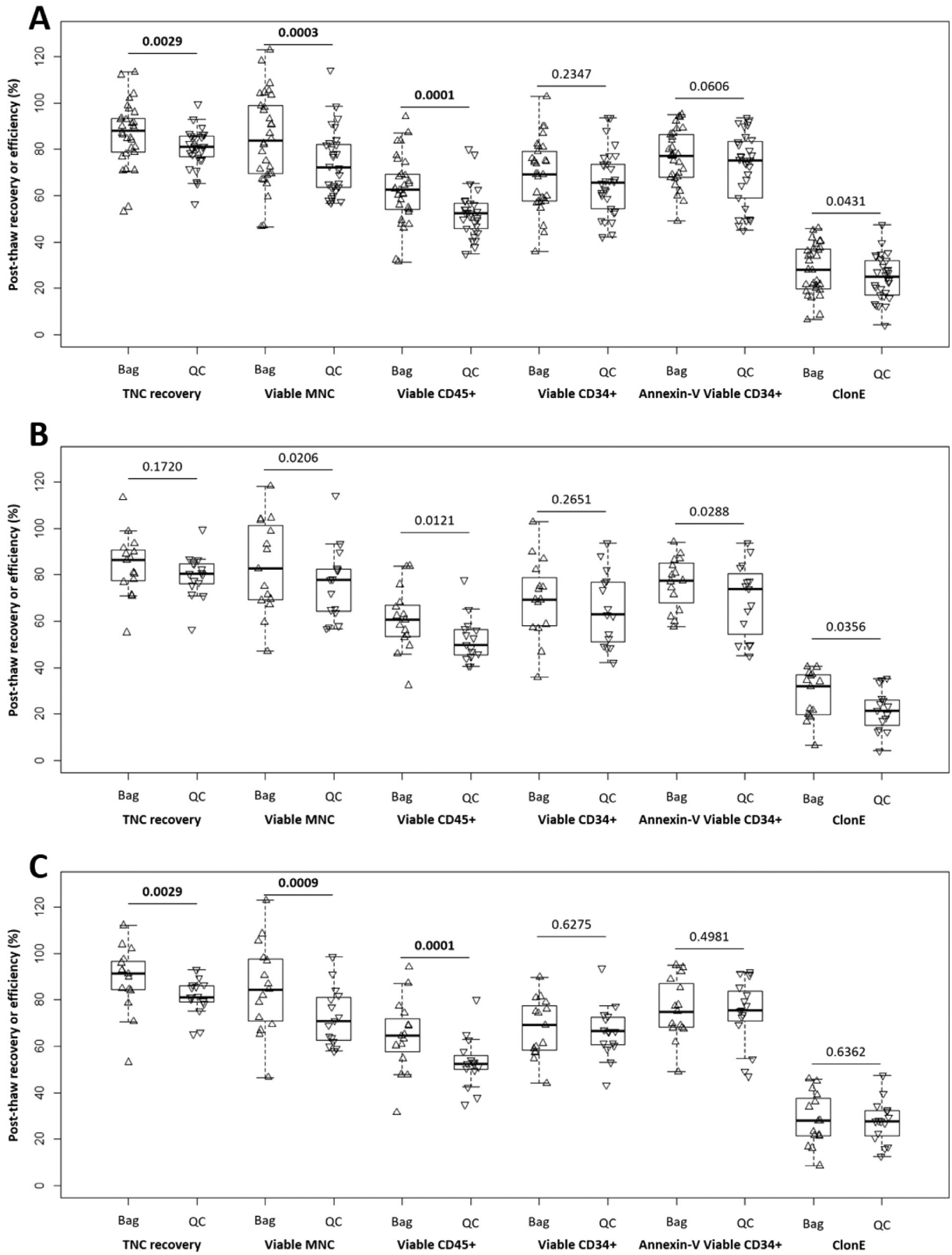

Figure 3. Boxplots of cell recovery and potency assays for post-thaw UCB units comparing bulk sample (upward-pointing triangles) to QC segments (downward-pointing triangles). Data are normalized to prefreeze values. Shown are TNC recovery, viable MNCs, viable CD45$^+$, viable CD34$^+$, annexin-V viable CD34$^+$, and ClonE. Boxplots in (A) present the data from all 30 samples; those in (B) show those from the "plunge" protocol only, and those in (C) show those from the "linear" protocol only. The *P* value for each parameter is indicated above the horizontal lines.

discontinuities. In addition, the high water content of cells can result in lethal intracellular ice formation if the rate of cooling is excessively rapid [36].

During thawing, an overly rapid reduction in hypertonicity can also cause potentially lethal damage to cells [31,33]. These osmotic changes can be lethally damaging.

Larger volumes (as in the bulk sample) will suffer less from these factors as a consequence of ice forming at a higher average temperature than in the QC segment and because a larger mass acts as a thermal buffer to limit more rapid changes in temperature.

Cell types within the UCB samples were affected differently by cryopreservation. $CD45^+$ cells, an important marker of leukocyte population and overall cord blood composition [36–38], were more adversely affected in QC segments than $CD34^+$ cells, in agreement with data published by Lee et al [9,11,17]. This difference will result in a deviation in composition of the cell population in the QC segment compared with the bulk sample; for example, the $CD34^+$:$CD45^+$ ratio reported here was increased due to an increase in damage to the $CD45^+$ cells in the QC segments. This deviation has been reported previously [9,17], along with other variations in post-thaw populations dependent on the cooling profile used [11]. Minimizing post-thaw population deviations is necessary to ensure that the thawed product for UCB transfusions is acceptable for use. Further work is needed to determine the extent of population deviation that is due to ice nucleation per se, and techniques to better control ice nucleation temperature need to be developed and refined [16]. In this study, a plunge step (used in the LN_2 CRF) did not decrease the supercooling in the QC segments compared with a protocol in which such a step was omitted. However, other techniques, such as manual nucleation or the addition of ice nucleating agents [16], may be more effective.

Despite the post-cryopreservation differences between them, QC segments are of obvious clinical value in the estimation of the expected performance of the bulk sample. This investigation has shown that, using currently adopted freezing protocols, the cell recovery of the bulk sample routinely exceeds estimates drawn from the QC segments. Consequently, the outcome for the bulk UCB sample after thawing will be underestimated. If the performance differences between the QC segment and bulk samples were to become predictable, using an improved protocol and/or modified sample containers, then critical decision making would be improved.

In practice, for UCB material, the choice between plunge and linear cooling profiles can be based on other resource issues, because their post-thaw outcomes are comparable. For example, a conventional liquid nitrogen system has the advantage of being able to cool rapidly to accommodate the plunge step. However, liquid nitrogen may present a risk of contamination of the cryopreserved product [22,39–41] and has recognized user risks. Stirling engine coolers, typically cannot produce a plunge step but do not have contamination or safety concerns [30]. The higher final temperature of the cryopreservation cycle in a Stirling engine-based protocol does not affect the eventual biological outcome [8,18,42].

CONCLUSIONS

Following cryopreservation, significantly worse post-thaw outcomes are associated with excessive supercooling of UCB in the QC segments compared with the bulk sample in the conjoined cryobag. Improving UCB cryopreservation procedures to ensure comparable nucleation temperatures for both QC and bulk elements of a frozen product will lead to better, more consistent results. In our study of the impact on post-thaw outcomes of cryopreserved UCB samples using 2 commonly used cryopreservation procedures, no difference between them was noted despite the variations in cooling rates, cooling time, and temperature of transfer to long-term storage inherent in the protocols. This indicates that, fortuitously, the acceptable range of cooling rates for UCB cryopreservation is relatively broad, and the 2 protocols are equally effective.

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