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Research article

Integrated Low-DMSO Cryopreservation and Expansion Strategies Enhance Cord Blood CD34+ Stem Cell Recovery and Function

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ABSTRACT

Umbilical cord blood (UCB) is a source of CD34+ hematopoietic stem cells (HSCs). The main challenges in handling them are the cytotoxicity of 10% dimethyl sulfoxide (DMSO) cryoprotection and insufficient cell number from a single cord blood unit. To address them, UCB-derived mononuclear cells (MNCs) were isolated by Ficoll density gradient centrifugation and cryopreserved in different formulations: 2.5% or 5% DMSO, each alone or supplemented with trehalose (25 µg/mL) or ascorbic acid (80 µg/mL). Post-thaw, viable MNC counts were assessed using trypan blue viability, and CD34+ immunocytochemistry was performed. To overcome the second challenge, MNCs were cultured for 7 days with 5%, 10%, or 25% placental extract, or for 4 days with 4 plant hormones, including kinetin, indole-3-acetic acid, naphthaleneacetic acid, and gibberellic acid at 2.5 and 5 mg/L. The MTT assay was used to evaluate the cytotoxicity. The results showed that 5% DMSO with trehalose yielded the highest post-thaw MNC count (4.70 ± 0.10 × 106 cells/mL) and viability of 54.50 ± 0.28%, whereas 5% DMSO with ascorbic acid produced the highest CD34+ retention (81.16 ± 0.60%), both significantly higher than in 2.5% DMSO formulations ($P < 0.05$). 5% placental extract increased cloning efficiency 5.7-fold compared with the control ($P < 0.05$). The hormones used depleted differentiated MNCs while preserving CD34+ content at 89-96%. These findings establish 5% DMSO with trehalose or ascorbic acid as an alternative to conventional 10% DMSO protocols, and 5% placental extract as a cost-effective HSC expansion supplement with direct translational importance for cord blood banking and transplantation medicine.

Keywords: Cord blood bank; CD34+, cryopreservation, hematopoietic stem cells, plant hormones.

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1. INTRODUCTION

Umbilical cord blood (UCB) is one of the most important sources of hematopoietic stem cells (HSCs) for allogeneic transplantation. It is collected non-invasively at birth; it differs from bone marrow or peripheral blood because it is widely banked in public registries, tolerates a greater degree of human leukocyte antigen (HLA) mismatch, and carries a substantially lower risk of graft-versus-host disease (GvHD) [1, 2]. It is co-enriched in CD34+ HECs and mesenchymal stem cells (MSCs), making it a powerful therapeutic resource for more than eighty malignant and non-malignant hematological illnesses [3].

There are two major technical defects that limit the broad clinical use of UCB transplantation. First, insufficient cells are collected,

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as a single UCB unit contains fewer total nucleated cells than an adult bone marrow harvest, often failing to meet the threshold for adult-sized recipients. The second is cryopreservation toxicity. Standard cord blood banking relies on dimethylsulfoxide (DMSO) at 10% as the cryoprotectant of choice. DMSO is associated with a wide spectrum of clinical toxicities following infusion, including nausea, bradycardia, and cardiac arrhythmias that threaten the life of the host. Fatal outcomes have been reported in previous literature [4,5]. DMSO also damages the HSCs, reducing pos-thaw clonogenic potential and engraftment efficiency [6].

The reduction of DMSO in cryopreservation protocols has become an important issue in stem cell banking. There are two biologically complementary types of adjuvants that have generated a great deal of interest recently: trehalose and ascorbic acid (vitamin C). Trehalose is a type of glucose disaccharide that does not reduce and is essentially found in nature in many organisms that go through extreme desiccation (drying out completely). Trehalose has three converging routes by which it protects cells during desiccation. First, it creates a highly viscous amorphous (glassy) substance at low temperatures, thus restricting molecular mobility, so recrystallization does not occur. Second, trehalose participates in direct hydrogen bonding with the polar head group of phospholipids found in cell membrane bilayers, thus preventing the lipid phase transition from occurring (which would rupture the cell membrane) during the freeze/thaw cycle. Third, trehalose decreases the intracellular nucleation of ice crystals [7, 8]. A previous study by Rodrigues et al. reported that trehalose combined with 2.5% DMSO yielded post-thaw HSC viability equal to that of the 10% DMSO protocol, representing a foundational proof-of-concept for trehalose to help reduce the DMSO percentage in cryopreservation protocols [9]. This result was approved by applying on different HSC sources and a cryopreservation platform [10].

The activity of ascorbic acid (vitamin C) complements that of trehalose through a separate protective mechanism. Reactive oxygen species (ROS) stability increases dramatically under low-moisture, subfreezing conditions, leading to oxidative damage that destroys lipid bilayers, membrane proteins, and nucleic acids. Ascorbic acid can neutralize superoxide radicals ($O2^-$) and hydroxyl radicals, thereby protecting the integrity of macromolecules during the freeze–thaw process [11]. Limaye and Kale provided initial clinical data on using ascorbic acid as an adjunct to HSCs that had been frozen/thawed in an effort to regenerate increased specific colonies, and subsequent evaluations provided clinical evidence supporting the use of antioxidants to preserve primitive CD34+ HSCs, which have been shown to be more susceptible to oxidative stress than later progenitors [12, 13]. The complementary mechanisms of action of trehalose (protects structurally) and ascorbic acid (protects against oxidative stress) indicate that the combination of these two agents leads to a synergistic effect and warrant testing through an experimental assessment of each agent compared to a control.

Previous research has been conducted to find ways to expand HSCs outside of the body because of insufficient cell doses. Using recombinant cytokines and combinations of recombinant cytokines, such as Stem Cell Factor (SCF), Thrombopoietin (TPO) Fms-like tyrosine kinase 3 (Flt3) ligand, interleukin (IL-) 3, and Granulocyte Colony-Stimulating Factor (G-CSF), may be able to significantly expand the number of HSCs through short-term culture, but due to cost, the shelf life of reagents, and the fact that these will push HSCs toward becoming committed rather than remaining self-renewing, researchers are interested in exploring biological alternatives that are more complex and less expensive [14]. Placental extracts provide the majority, or all, of the HSC support in the embryo from conception through birth. The placental extracts are known to sustain the quiescence and self-renewal of HSCs through a rich mixture of SCF, CXCL12, angiopoietin-1, and Wnt ligands, and extracellular matrix (ECM) forms that support HSCs in the placenta [15]. Following up on a study of Alkhaldi et al. [16], who reported that the use of placenta-derived exosomes increased total nucleated UCB cells and CD34+ cells after 10 days of culture while also maintaining primitive colony-forming ability, demonstrating that placental paracrine factors in exosomes can facilitate the generation of significant numbers of HSCs.

There are another way uses plant hormones. Different phytohormones, including ascorbic acid (ABA), cytokinins such as kinetin, the auxins indole-3-acetic acid (IAA) and naphthaleneacetic acid (NAA), and gibberellic acid (GA_3), was reported to impact mammalian cell proliferation, differentiation, and apoptosis by mechanisms which overlap partially with conserved eukaryotic signaling pathways [17]. A previous study investigated that ABA is endogenously synthesized by human CD34+ HSCs, and exogenous ABA enhances CD34+ colony establishment at physiological concentrations. That demonstrates the plant hormone signaling machinery is functionally present in human hematopoietic progenitor populations, suggesting a wider role for phytohormones in HSC biology than earlier recognized.

The present study was designed to address both barriers, DMSO toxicity and cell dose insufficiency, in a single integrated experimental framework. Six cryopreservation formulations were systematically compared for their ability to maintain UCB-derived MNC count, viability, and CD34+ phenotype over 1 month of liquid-nitrogen storage. Placental extract and plant hormones were evaluated as ex vivo HSC expansion stimuli, with $CD34^+$ phenotype maintenance assessed throughout. The results are interpreted in the context of the mechanistic framework summarized in Figure 1 and provide a data-driven basis for the design of next-generation UCB banking and cell-therapy manufacturing protocols.

2. MATERIALS AND METHODS

2.1. UCB Collection and Ethical Considerations

UCB specimens were obtained following written informed consent from donors at Fattema Al-Zahrraa Hospital, Baghdad, Iraq. All samples were collected from full-term normal vaginal deliveries. A closed in utero (*in situ*) collection system was employed immediately following cord clamping, wherein 100–120 mL of blood was aspirated from the umbilical vein into sterile bags containing citrate-phosphate-dextrose-adenine-1 (CPDA-1) anticoagulant. Samples were transported at 4°C and processed within 24 hours. The in utero closed-system approach was selected based on its documented superiority over open ex utero collection in terms of total nucleated cell yield, hematopoietic progenitor concentration, and microbiological safety [19, 20].

2.2. Mononuclear Cell Isolation

MNCs were isolated by Ficoll-Paque (density 1.077 g/mL) density gradient centrifugation at 400 × g for 30 minutes at room temperature. The buffy coat interface was carefully aspirated, washed twice in phosphate-buffered saline (PBS), and resuspended in Minimum Essential Medium (MEM)

supplemented with 10% fetal calf serum (FCS). Cell viability and count were assessed by trypan blue dye exclusion (Viable % = [viable cells / total cells] × 100). Only preparations exhibiting ≥90% viability were used. Baseline $CD34^+$ content was established by immunocytochemistry (Section 2.3) prior to any cryopreservation or culture manipulation.

2.3. $CD34^+$ Immunocytochemistry

The collected mononuclear cells were centrifuged, smeared onto positively charged microscope slides (10 µL per slide), air-dried, and fixed with DAKO fixation solution for 10 min at 21 °C. Slides were stored at −20°C until staining. Subsequently, immunocytochemical staining was carried out using a (monoclonal) anti-human CD34 primary antibody and d-diaminobenzidine (DAB) as the chromogen as per the DAKO manufacturer protocols. The positive cells had received a deep brown cytoplasmic color, plus the presence of the nucleus with the counter-staining of the negative cells using hematoxylin blue for visualization. The percentage of CD34+ cells present was recorded for 200 minimum cells per slide at 40× magnification. The expression of CD34+ is a definitive marker for identifying human HSC/progenitor cells [21].

2.4. Cryopreservation Protocol

Six experimental cryopreservation formulations were evaluated: (1) 2.5% DMSO alone; (2) 2.5% DMSO + 25 µg/mL trehalose; (3) 2.5% DMSO + 80 µg/mL ascorbic acid; (4) 5% DMSO alone; (5) 5% DMSO + 25 µg/mL trehalose; (6) 5% DMSO + 80 µg/mL ascorbic acid. All formulations were prepared in MEM supplemented with 20% FCS. MNCs were resuspended at 6 × 10^6 cells/mL. Cryotubes (1 mL) were pre-chilled, layered with an equal volume of cryoprotective medium, gently mixed, and equilibrated on ice for 1 minute. Cryotubes were then held at 4°C for 10 minutes, transferred to −80°C for 24 hours (uncontrolled-rate freezing), and subsequently plunged into liquid nitrogen (−196°C) for a storage period of one month.

Thawing was performed by rapid immersion in a 37°C water bath for 5 minutes. Post-thaw MNC count and viability were recorded directly after thawing (pre-wash). Cells were then diluted 1:4 in MEM/10% FCS, rested for 1 hour, centrifuged at 1000 rpm for 10 minutes, and resuspended in fresh medium. Post-wash count, viability, and $CD34^+$ percentage were reassessed.

2.5. In Vitro Expansion, Placental Extract

Freshly isolated MNCs (1.62 × 10^6 cells/mL) were seeded in 25 cm^2 tissue culture flasks in MEM/10% FCS supplemented with 5%, 10%, or 25% (v/v) placental extract, or without extract (control). Cultures were incubated for 7 days at 37°C in a humidified 5% CO_2 atmosphere and inspected daily by inverted microscopy. Cloning efficiency (CE) was calculated from the mean number of colonies observed in 10 randomly selected 40× fields per flask. Colonies were classified as small (20–40 cells), medium (41–100 cells), or large (>100 cells). $CD34^+$ percentage was assessed before and after culture.

2.6. *In Vitro* Expansion Plant Hormones

Four plant hormones, kinetin (K), indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA), and gibberellic acid (GA_3) were tested at concentrations of 2.5 mg/L and 5 mg/L. MNCs (1.1 × 10^5 cells/well) were seeded in 96-well plates in MEM/10% FCS with each hormone and incubated for 4 days at 37°C / 5% CO_2. Cell metabolic activity was quantified using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay: MTT solution (5 mg/mL, 10 µL/well) was added, incubated for 4 hours at 37°C, and the resultant formazan crystals were solubilized in DMSO and measured at 620 nm. MNC counts were interpolated from a standard curve. $CD34^+$ percentage was assessed at day 0 and day 4.

2.7. Statistical Analysis

All experiments were performed in triplicate (n = 3). Data are expressed as mean ± standard error (SE). Statistical comparisons used one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) post-hoc test, implemented in SAS statistical software version 7.0 (SAS Institute Inc., Cary, NC). A threshold of $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. UCB Collection & Baseline Characteristics

The closed in utero system yielded 100–120 mL UCB per collection without observable contamination. Following Ficoll density gradient centrifugation, all MNC preparations met the viability threshold (≥95.1% by trypan blue). Baseline $CD34^+$ content was 97.5% in all preparations, confirmed by immunocytochemistry. Positive cells were identified by deep brown DAB staining; hematoxylin-counterstained negative cells appeared blue. These baseline characteristics confirmed the suitability and consistency of the isolation protocol for downstream comparative experiments [21].

3.2. Post-Thaw MNC Count

Post-thaw MNC recovery differed markedly across the six cryopreservation formulations (Table 1). All groups showed a significant ($P < 0.05$) reduction from the pre-freeze baseline of 6 × 10^6 cells/mL. The 5% DMSO + trehalose formulation achieved the highest pre-wash MNC count (4.70 ± 0.10 × 10^6 cells/mL) and remained the best performer after washing (4.06 ± 0.06 × 10^6 cells/mL). By contrast, 2.5% DMSO alone yielded only 0.63 × 10^6 cells/mL post-wash, a recovery of barely 10.5% of the original input. DMSO washing caused additional significant MNC depletion in all groups, with the magnitude of loss inversely correlated with initial cryoprotective efficacy.

Table 1. Post-thaw MNC count (×10^6 cells/mL) across six cryopreservation formulations.

Group	Pre-freeze	Post-thaw pre-wash	Post-thaw post-wash
2.5% DMSO only	6.00	2.26±0.14*	0.63±0.06*
2.5% DMSO + Trehalose	6.00	2.20±0.05*	0.76±0.08*
2.5% DMSO + Ascorbic acid	6.00	2.46±0.03*	0.67±0.03*
5% DMSO only	6.00	4.23±0.12*	2.83±0.16*
5% DMSO + Trehalose ★	6.00	4.70±0.10*	4.06±0.06*
5% DMSO + Ascorbic acid	6.00	4.13±0.08*	3.50±0.11*
LSD	–	0.302	0.305

**P<0.05 vs. pre-freeze baseline. ★Best performer. Mean ± SE, n=3.*

3.3. Post-Thaw MNC Viability

Viability fell significantly from the 95.1% baseline in all groups, but the magnitude of decline was strongly dependent on the cryoprotective formulation (Table 2). The 5% DMSO + trehalose group maintained the highest pre-wash viability (54.50 ± 0.28%) and post-wash viability (48.50 ± 0.28%). All 5% DMSO formulations significantly outperformed their 2.5% DMSO counterparts ($P < 0.05$). The 2.5% DMSO-alone group showed the lowest viability (21.06 ± 0.06% pre-wash; 13.16 ± 0.08% post-wash), consistent with insufficient colligative cryoprotection at this DMSO concentration. Post-wash viability decrements were significant across all groups, confirming osmotic stress-related cell loss during DMSO removal.

Table 2. MNC viability (%) by cryopreservation formulation.

Group	Pre-freeze	Post-thaw Pre-wash (%)	Post-thaw Post-wash (%)
2.5% DMSO only	95	21.06±0.06*	13.16±0.08*
2.5% DMSO + Trehalose	95	26.06±0.23*	18.73±0.14*
2.5% DMSO + Ascorbic acid	95	26.10±0.10*	14.56±0.23*
5% DMSO only	95	50.50±0.28*	42.16±0.16*
5% DMSO + Trehalose ★	95	54.50±0.28*	48.50±0.28*
5% DMSO + Ascorbic acid	95	44.50±0.28*	41.96±0.24*
LSD	—	0.710	0.631

*P<0.05 vs. pre-freeze baseline. ★Best overall viability. Mean±SE, n=3.

3.4. CD34⁺ HSC Retention After Cryopreservation

Post-thaw CD34⁺ percentage declined significantly from the 97.5% baseline in all groups ($P < 0.05$), reflecting freeze-thaw-mediated attrition of the HSC compartment (Table 3). However, 5% DMSO formulations preserved a substantially greater CD34⁺ fraction than 2.5% DMSO counterparts. The 5% DMSO + ascorbic acid group achieved the highest post-wash CD34⁺ retention (81.16 ± 0.60%), followed by 5% DMSO + trehalose (79.00 ± 0.57%). Among 2.5% DMSO groups, trehalose supplementation (59.67 ± 0.88%) outperformed ascorbic acid (49.16 ± 0.60%) and DMSO alone (49.66 ± 0.33%), highlighting that at sub-optimal DMSO concentrations, membrane-structural protection by trehalose is more critical than antioxidant defense for preserving HSC identity.

Table 3. CD34⁺ percentage before and after cryopreservation.

Group	Before Freezing (%)	After Freezing Post-wash (%)
2.5% DMSO only	97.5	49.66±0.33*
2.5% DMSO + Trehalose	97.5	59.67±0.88*
2.5% DMSO + Ascorbic acid	97.5	49.16±0.60*
5% DMSO only	97.5	69.16±0.44*
5% DMSO + Trehalose	97.5	79.00±0.57*
5% DMSO + Ascorbic acid ★	97.5	81.16±0.60*
LSD	—	1.839

*P<0.05 vs. pre-freeze baseline. ★Highest CD34⁺ fidelity. Mean±SE, n=3.

3.5. *In Vitro* Expansion with Placental Extract

Placental extract produced a dose-dependent and concentration-limited effect on HSC colony formation (Table 4). The 5% concentration yielded the highest cloning efficiency (CE = 0.00027 ± 0.00001), a 5.7-fold increase over the untreated control (CE = 0.000047; $P < 0.05$). The 10% concentration produced an intermediate effect (CE = 0.00015 ± 0.00002). The 25% concentration was overtly cytotoxic: no colonies formed and the culture medium showed no pH-related color change, indicating complete metabolic quiescence and cell death. CD34⁺ percentage declined modestly in all viable groups post-culture (from 92% to 85–89%), with the 5% extract group showing the largest decline, consistent with growth-factor-driven differentiation alongside self-renewal expansion. Morphologically, expanded cultures contained bipolar spindle-shaped cells (MSC-like) and multipolar star-shaped cells with dendritic projections, consistent with monocyte/macrophage lineage commitment.

Table 4. Effect of placental extract on cloning efficiency (CE) and CD34⁺% after 7 days.

PE (%)	CE (Mean±SE)	CD34⁺ Before (%)	CD34⁺ After (%)
5 ★	0.00027±0.00001*	92	85.00±0.57*
10	0.00015±0.00002*	92	87.00±0.57
25	0.00 (cytotoxic)	92	—
0 (Control)	0.000047±0.0000004	92	89.00±2.08
LSD	0.000000043*	—	3.646*

*P<0.05. ★Best cloning efficiency. PE = placental extract. Mean ± SE, n=3.

3.6. Effects of Plant Hormones on MNC Viability and CD34⁺ Phenotype

All four plant hormones significantly reduced total MNC count relative to the untreated control (1.289 ± 0.018 × 10^5 cells/well; $P < 0.05$; Table 5). The auxin IAA at 5 mg/L produced the least depletion (0.668 ± 0.034 × 10^5 cells/well), while GA_3 and NAA caused greater reductions. Critically, CD34⁺ percentage was dramatically better preserved in all hormone-treated cultures (89–96%) compared to the untreated control, which collapsed from 98% to 27.33 ± 3.71% by day 4 due to spontaneous differentiation without HSC-supportive factors. This selective pattern, mature MNC depletion alongside CD34⁺ preservation was consistent across all four hormones and both concentrations tested.

Table 5. Plant hormone effects on MNC count (×10^5/well) and CD34⁺% after 4-day culture.

Hormone	MNC 2.5 mg/L	MNC 5 mg/L	CD34⁺ 2.5 mg/L (%)	CD34⁺ 5 mg/L (%)
NAA	0.50±0.02*	0.43±0.01*	89.83±0.16	92.33±0.33
GA_3	0.35±0.01*	0.35±0.00*	94.83±0.16	96.66±0.33
Kinetin	0.51±0.00*	0.36±0.01*	91.60±0.20	89.83±0.28
IAA	0.66±0.01*	0.67±0.03*	94.16±0.60	93.83±0.16
Control	1.29±0.02	1.29±0.02	27.33±3.71	27.33±3.71

*P<0.05 vs. control; green cells = CD34⁺ preservation. Mean±SE, n=3.

4. DISCUSSION

This study presents a comprehensive, mechanistically grounded evaluation of strategies to simultaneously improve the safety of UCB cryopreservation and the ex vivo expandability of UCB-derived $CD34^+$ HSCs. The dual experimental framework, schematically summarized in Figure 1 below, reveals that both challenges are tractable with accessible, low-cost biological agents and provides a clear hierarchy of cryoprotective formulations based on three independent functional readouts.

The consistently superior performance of 5% versus 2.5% DMSO across all three endpoints reflects the threshold-dependent nature of colligative cryoprotection. At 2.5%, DMSO osmolality is insufficient to adequately offset the transmembrane water activity gradient during freezing, resulting in inadequate suppression of both the solution effect (hyperosmotic salt concentration in remaining unfrozen water) and intracellular ice nucleation [6]. At 5%, sufficient cryoprotective potency is achieved while the DMSO dose remains well below the 10% level associated with clinical cardiotoxicity and neurotoxicity [4, 5]. This distinction is clinically significant for cord blood banking: stored units may be infused years after collection in emergency settings, where pre-infusion DMSO washing may not be feasible, particularly in paediatric recipients where the permissible DMSO dose per kilogram body weight is more restrictive [4].

The superiority of 5% DMSO + trehalose for total MNC recovery (67.7% of pre-freeze input retained post-wash, compared to 10.5% for 2.5% DMSO alone) is explained by trehalose's ability to protect bulk membrane integrity during both the freeze and the wash. By forming an amorphous vitreous matrix that immobilizes phospholipid headgroups in their native lamellar configuration, trehalose prevents the lipid phase-transition, from liquid-crystalline to gel phase, that occurs as cells cool through the phase-transition temperature and is the primary driver of freeze-thaw membrane lysis [7, 8]. Crucially, trehalose provides this protection extracellularly; full cytoprotective efficacy requires intracellular trehalose as well, which may explain why recovery, while markedly improved over controls, did not achieve complete preservation [9, 30]. Future protocols employing liposomal or electroporation-based intracellular trehalose loading merit evaluation in this system [22].

The result of the current study confirms 5% DMSO + ascorbic acid resulted in the highest CD34+ retention (81.16 ± 0.60%) compared to trehalose (79.00%), with lower total cell recovery after washing, indicating a mechanistically significant difference in these cryoprotectants. While ascorbic acid did not preserve overall membrane structure during storage at low moisture and subfreezing temperatures, it specifically neutralized reactive oxygen species (ROS) generated under these conditions and preferentially affected the oxidatively vulnerable primitive hematopoietic stem cell (HSC) population [11]. Primitive HSCs have an increased production of mitochondrially derived ROS than committed progenitors and lower levels of constitutive antioxidant enzymes [12], making primitive HSCs disproportionately susceptible to oxidative damage that is preventable by ascorbic acid. Therefore, as compared to trehalose per unit of total cell yield, ascorbic acid maintains the CD34+ fraction of primitive HSCs more effectively. The original study by Limaye and Kale demonstrated this effect using 10% DMSO; however, the current study confirms this finding with 5% DMSO and indicates that it generalizes to clinically acceptable DMSO doses. Thus, for cord blood banks, if maximum absolute yield of HSC is of primary concern (e.g., adult transplant recipients needing large numbers of cells), then 5% DMSO + trehalose should be used; conversely, if maximum preservation of CD34+ phenotype is the most critical factor (e.g., research studies, gene therapy applications or quality control evaluation), then 5% DMSO + ascorbic acid should be used.

I noticed that after thawing, when DMSO was washed out, there was further loss of MNC's (leukocytes) from each of the six groups, with the greatest loss seen with 2.5% DMSO formulations. This indicates that DMSO removal by centrifuge after thawing (to reduce the infusion toxicity in the clinical transplantation) exposes already compromised cryopreserved cells to a second round of osmotic and mechanical stress. This observation was first made by Broxmeyer et al., where they documented nucleated cell loss due to washing of UCB preparations and this continues to be a significant practical problem [1]. The 5% DMSO + trehalose formulation uniquely retained the highest percentage (86.4%) of nucleated cells from the post-thaw/pre-wash group, indicating that trehalose helps to stabilize the integrity of the cellular membranes against osmotic perturbations during washing. Because of this benefit, there are implications for the use of the 5% DMSO + trehalose formulation in clinical transplantation. Specifically, even when DMSO washing is done for recipient safety, the use of the 5% trehalose formulation allows for a much larger number of transplantable HSC's to be available than with other formulations.

The 5.7x increase in cloning efficiency due to 5% placental extract demonstrates proof-of-concept for the usefulness of biological factors derived from a placenta as accessible and cost-effective supplements for HSC expansion. The placenta is the major HSC niche during embryogenesis, supplying SCF, CXCL12, angiopoietin-1, Wnt ligands and many others; all of these factors, together with the large variety of extracellular matrix proteins, provide the necessary conditions for HSC self-renewal [15]. The work done by Alkhaldi et al., 2023 [16], further supported this concept through findings that were obtained through studying the effects of exosomes derived from MSCs isolated from the placenta. The exosomes contained paracrine factors secreted into the tissue culture medium and were able to significantly expand UCB-HSC numbers in culture while allowing primitive colony-forming capacity to be preserved [23]. Our results using whole extract support the independent findings of Alkhaldi et al. and establish that 5% is the optimal concentration of extract; using an extract above 10% produces inhibitory or cytotoxic signals at a greater rate than growth-promoting signals and 25% is lethal. The decrease of CD34+ cells (from 92% to 85%) following culturing in 5% extract exemplifies the anticipated compromise between self-renewal and early lineage commitment, a principle that is true for all successful protocols for HSC expansion, but in no way diminishes the net increase in absolute CD34+ cell number [24]. The novel finding of the study is the selectivity of plant hormones' effects: The four hormones significantly depleted differentiated, mature MNCs while maintaining CD34+ percentages of 89-96% in contrast to the untreated control, in which CD34+ collapsed to 27.33% through self-differentiation by day 4. It was reported that kinetin and cytokinin ribosides stimulate differentiation and mitochondrial-pathway apoptosis preferentially in committed myeloid cells, through mechanisms, including ROS accumulation and ATP depletion [17,18]. Primitive $CD34^+$ HSCs are protected from these mechanisms by their high BCL-2/BAX ratio, elevated drug efflux transporter (ABCG2/MDR1) expression, and intrinsically low mitochondrial activity in the quiescent state [25]. In a 2024 study conducted by Meaker and Wilkinson [25], the researchers found that FOXO3a

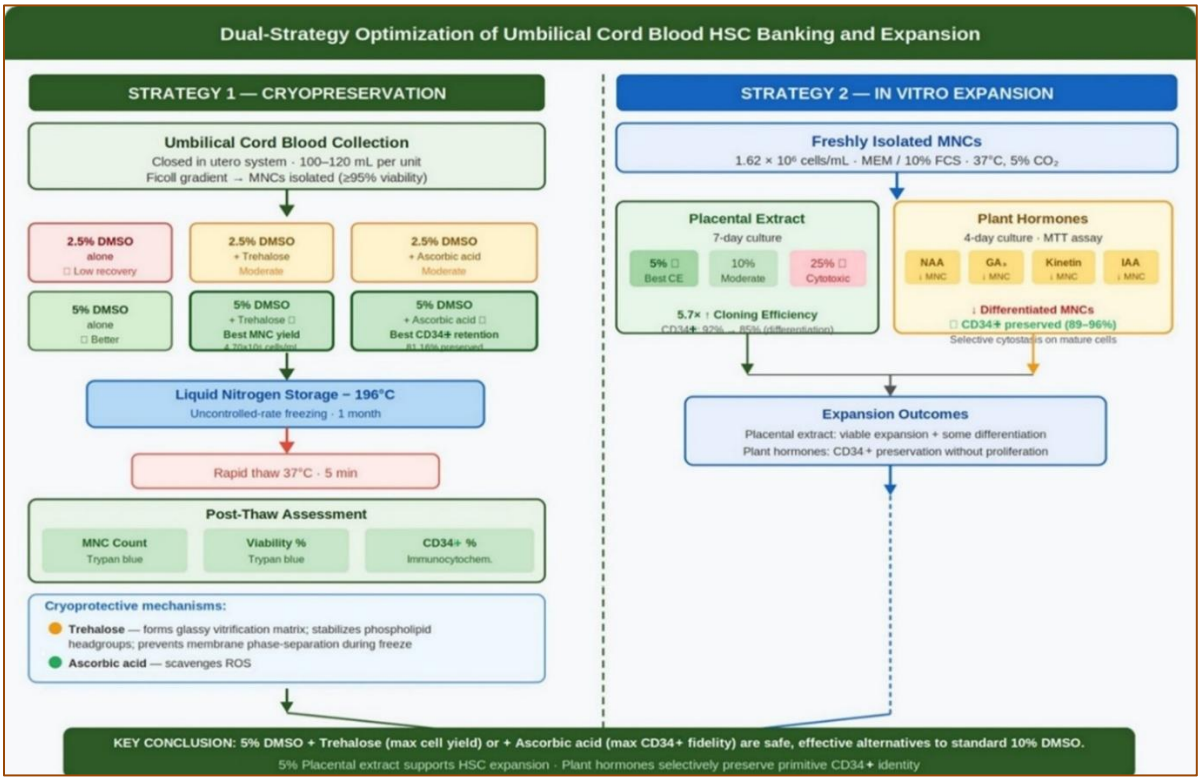


Fig. 1. The two experimental strategies are shown. Left panel: Six DMSO-based cryopreservation formulations at two concentrations (2.5% and 5%), in the presence and absence of trehalose (membrane stabilizer) or ascorbic acid (antioxidant), evaluated for post-thaw MNC count, viability, and $CD34^+$ retention. Right panel: Two in vitro expansion approaches, placental extract (dose-dependent HSC colony formation) and plant hormones (selective MNC depletion with $CD34^+$ phenotype preservation). Both strategies converge on the overarching goal of enhancing the clinical safety and therapeutic potency of UCB-derived HSC products.

and HIF-1α mediated the transcriptional regulation of both quiescence-related survival for HSCs (hematopoietic stem cells) and the mechanisms by which plant hormones preferentially affect committed vs. primitive MNCs (mononuclear cells) [26]. Thus, the data also explain why plant hormones can inhibit spontaneous differentiation of CD34+ MNCs in the absence of exogenous growth factors and provide an avenue for using these compounds to help maintain HSC quiescence prior to transplantation or manipulation, particularly when HSCs are stored temporarily before transplantation. Serum-free culture and RNA sequencing will be required for sorted CD34+ populations to fully validate this application [27-30].

5. CONCLUSION

The study approved 3 principal conclusions with direct translational relevance for UCB banking and medicine of transplantation. First, 5% DMSO supplemented with trehalose or ascorbic acid is a clinically safer alternative to the standard 10% DMSO cryopreservation protocol, producing meaningful preservation of MNC count, viability, and CD34+ phenotype over 30 days of liquid nitrogen storage. Trehalose is the adjuvant for elevating total cell production and post-wash recovery, while ascorbic acid most effectively preserves HSC phenotypic purity, a distinction that should inform formulation selection based on the clinical application. Second, low-concentration (5%) placental extract supports significant short-term expansion of CD34+ HSC colonies, establishing placenta-derived biological factors as a viable and cost-effective alternative to recombinant cytokine cocktails for ex vivo expansion. Third, plant hormones (kinetin, IAA, NAA, GA_3) selectively deplete differentiated MNCs while preserving the primitive $CD34^+$ compartment, a property that warrants further investigation as a potential HSC quiescence-maintenance strategy.

Together, these findings advance the rational, evidence-based design of next-generation UCB cryopreservation and expansion protocols that are simultaneously safer for recipients, more economical for cord blood banks, and more effective at delivering transplantable $CD34^+$ HSC doses for both pediatric and adult patients.

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Conflict of interest

The authors declare no conflicts of interest.

Ethical Approval

This review was approved by the Scientific Committee of the University of Baghdad, Baghdad, Iraq (104; 16-04-2009).

Author contributions

Shamkhi NF: Investigation; Methodology; Project administration; Resources; Supervision; Roles/Writing - original draft; and Writing - review & editing.
Sabbah MA: Conceptualization, Data curation, and Formal analysis, Roles/Writing - original draft; Visualization and Writing - review & editing.
Alwachi SN: Investigation; Project administration; Supervision; Validation.

Data availability

Data will be made available on request.

AI declaration

The authors used Claude AI to improve the manuscript's academic tone and readability. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

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