Cryoprotective Effect of Disaccharides on Cord Blood Stem Cells with Minimal Use of DMSO

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Abstract Umbilical cord blood (UCB) is an extremely attractive source of stem cells for the treatment of various benign and malignant hematological and non-hematological disorders. To facilitate the preservation of these stem cells, 10 % dimethylsulfoxide (DMSO) is widely used as cryoprotectant in cord blood banks. But it is found to be toxic at this concentration with the result of serious side effects in recipients after infusion of DMSO-cryopreserved cells. Evaluation of viability and functionality of cryopreserved hematopoietic stem cells is needed with either inclusion of nontoxic additives alone or with reduced DMSO concentration. We assessed the post thawing viability of UCB stem cells in the freezing medium containing disaccharides (sucrose or trehalose) alone and in combination with reduced amount i.e. 2 % DMSO by trypan blue staining. The functionally active progenitor cells content of the optimized media was then evaluated and compared with 5% DMSO by a colony forming unit assay using methylcellulose based media. The freezing solution containing 0.2 M trehalose with 2 % DMSO came out to be superior in the evaluation of viability and generation of hematopoietic colonies of erythroid and myeloid lineage than 5 % DMSO alone. While the percentage of viability was lower than 2 % DMSO, as observed in the medium containing 0.2 M trehalose or sucrose alone, with poor outcome of generation of myeloid lineage based colonies. Our study results suggest that trehalose (0.2M) with the

inclusion of reduced concentration of DMSO(2%) can better replace 5%DMSO rather than complete removal of DMSO from the freezing medium.

Keywords Sucrose · Trehalose · Cryoprotective medium · Viability · Hematopoietic colonies

Introduction

Clinically relevant cryopreservation and banking of adult stem cells are advantageous to preserve their functionality and effectuation of these cells in safe stem cell transplantation. Umbilical cord blood (UCB) is usually stored by either public or private cord blood banks. For all biological systems, the freezing rate is a significant factor in the determination of viability following cryopreserved storage. Slow freezing protocols seem to be the consensus among most of the laboratories for cryopreservation of adult stem cells [1–4].

Significant draw back with vitrification or faster freezing or cooling rate, is the chemical toxicity and extreme osmotic stresses caused due to the high concentrations of permeable and/or nonpermeable cryoprotective agents (CPAs) loading before plunging into liquid nitrogen affecting cell viability significantly [5, 6]. Donaldson et al. [7] even reported a statistically significant difference in viability between cooling at 1 and 5 °C/min. Vitrification is also not a suitable method of cryopreservation for large sample volumes. The toxicity of CPAs has been a limiting step for the use of high CPA concentrations, and thus for betterment of cryopreservation protocols. One strategy to deal with this issue is to utilize less toxic but reasonably efficient CPAs. As DMSO, most commonly used CPA in cord blood banking, is associated with adverse effects upon infusion [8–11]. Many cosolutes which act as efficient

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cryoprotectants fail to protect the protein from drying/desiccation-induced stress. The only exceptions are disaccharides [12–14]. Disaccharides such as sucrose and trehalose have been widely used as natural cryoprotectants [15]. The inclusion of sucrose as an additive to media for cryopreservation and hypothermic storage of rat hepatocytes allows high viability rate to be achieved [16–18].

Trehalose, offers both cryo- as well as lyoprotection during freeze-drying. It stabilizes the partially unfolded state of the protein (rather than stabilizing the native structure) [19]. It is also thought to increase the cytoplasmic viscosity, thus decreasing the possibility of formation of intracellular ice crystals [20]. Increased colony formation of cryopreserved human HSC derived from cord blood and fetal liver was observed after supplementation of trehalose to DMSO-based cryopreservation media [21]. Similarly, the combination of DMSO and trehalose for cryoprotection of hematopoietic cells derived from cord blood resulted in increased numbers of LTCIC (long-term culture-initiating cells). These cells also retained the potential to multilineage engraftment in nonobese diabetic-SCID mice [22]. Extracellular and intracellular loaded trehalose also proved to be an effective CPA for HSCs as suggested by one of the lab report [23]. Trehalose, and to some extent sucrose can stabilize proteins as well as lipid bilayer. Trehalose has also been found to act as a stabilizer to improve the shelf-life of therapeutic proteins [24] along with sucrose [25]. With the utilization of reduced concentration of DMSO (i.e. $\frac{1}{4}$ parts of 10 % DMSO) some investigators assessed the ability of disaccharides (trehalose and sucrose) as CPA for the improvement of the viability & Functionality of post thawed UCB HSCs [26]. So far in Indian Scenario, Limaye et al. [21] demonstrated that the inclusion of both bio-antioxidant Catalase and trehalose in the conventional freezing medium provided better cryoprotection of HSCs derived from cord blood and fetal liver. The above supplementation is also more effective than conventional freezing medium in rendering better preservation of growth factor receptors, adhesion molecules and functionality of HSCs [27]. Further investigation by the same lab reported that the above aforementioned combination improved cryo-protection of human HSCs with reference to in vitro migration and adhesion properties [28]. Fuller and Paynter [29] suggested that the area of CPA toxicity remains one of considerable uncertainty and is ripe for re-evaluation.

10 % DMSO demonstrated highest viability of 93.0 ± 3.02 % in comparison to 5 % DMSO in our previous study [30]. Therefore we are analyzing the efficacy of two disaccharides, trehalose and sucrose in cryopreservation medium to maintain the quality of HSCs from UCB with the aim of eliminating or reducing the amount of DMSO to 2 % DMSO in comparison to 5 % DMSO in this present study.

Methodology

The investigational study was carried out in the Department of Biochemistry in collaboration with Department of Obstetrics and Gynecology, S.C.B. Medical College, Cuttack, where the Cord blood collection was accomplished.

Collection, Processing of the Human UCB Sample

With the informed consent from Mothers, approved by Institutional Ethical Committee, 65–70 mL of UCB was collected in a sterile sample container having anticoagulant citrate–phosphate–dextrose (CPD), from the cords of the full term deliveries (gestation weeks: 37–40). The eligible participants were above 20 years of age with no indication of septicemia, any other Hematological infections or complications and negative for VDRL, human immunodeficiency, hepatitis B, hepatitis C viruses. The processing time was within 7 h of collection without any sign of hemolysis or coagulation. The whole sample blood was diluted with phosphate buffered saline (PBS) in 1:1 ratio. UCB mononuclear cells (MNCs) were recovered from the interface obtained by density gradient centrifugation (1.077 g/mL Histopaque, Sigma). Then the collected MNCs were washed twice with PBS and the number of MNCs was measured by cell counting through Haemocytometer.

Experimental Design

Our experimental study involved the cryopreservation medium containing two types of disaccharides i.e. sucrose and trehalose separately as additives in different concentrations. 0.2 M concentration and its 1/10th part i.e. 0.02 M amounts of disaccharides were taken as high and low concentration of both sugars to evaluate their cryo-protective effect. While 2 % DMSO inclusion to the above disaccharide concentrated solutions was carried out separately to compare its effect on cryopreserved cells than solution containing disaccharide alone.

Our study was accomplished with two phases which were sequentially carried out. Optimization of the variable disaccharide based cryoprotective media with or without DMSO was performed by analyzing the viability of post thawed cells in the first phase of cryopreservation, while the effect of the selected optimal concentrations of disaccharides with and without 2 % DMSO (from the first phase of study) on the hematopoietic functionality of the post thawed cells of the above optimized media was subsequently studied in the second phase by colony forming unit (CFU) assay.

Table 1 Preparation of two different groups of disaccharide based freezing media for cryopreservation

Group-I freezing media Disaccharides with reduced concentration of DMSO (2 %)	Group-II freezing media Disaccharide based media without DMSO
0.02 M sucrose with 2 % DMSO	0.02 M sucrose
0.02 M trehalose with 2 % DMSO	0.02 M trehalose
0.2 M sucrose with 2 % DMSO	0.2 M sucrose
0.2 M trehalose with 2 % DMSO	0.2 M trehalose

Preparation of Freezing Media

The basic composition of the freezing or cryoprotective media was 10 % Bovine Serum Albumin (BSA, PAN-BIOTECH, Germany) in Dulbecco's Modified Eagle's Medium (DMEM, PAN-BIOTECH, Germany) after the addition of sucrose or trehalose alone or with 2 % DMSO in its final concentration. Here we took two different concentrations of disaccharides i.e. lowest 0.02 M & its 10 times concentrated 0.2 M into consideration for our experimental study to evaluate their cryoprotective effect on HSC as presented in Table 1. All of these freezing media were freshly prepared and chilled at 4 °C.

Cryopreservation of UCB MNCs

After the preparation of the above freezing media, 2×10^6 MNCs were suspended with 1.8 mL of the freezing media of each group separately in a 2 mL of cryovial tube and immediately transferred to the isopropanol container (Tarson) for freezing at -80 °C overnight with the cooling rate of 1 °C/min. Finally it was transferred to the canister and stored in vapour phase of liquid nitrogen (at -156 °C) for 1 month until further analysis.

Thawing

For thawing of the cryopreserved samples, DMEM media with heat inactivated 10 % fetal bovine serum (FBS) was used. The cryovials, removed from liquid nitrogen tank, were immediately placed in a water bath maintained at 37 °C until ice crystals were just disappearing and transferred to a 50 ml centrifuge tube & the thawing media was added slowly in the ratio 2:1 to the cell suspension. The MNCs were recovered by centrifugation at 1,500 rpm for 6 min at room temperature. Excess supernatant was decanted and cell pellet was suspended in the above pre-warmed (warmed for 5–6 min at temperature of 37 °C) thawing media.

Further washing of recovered MNCs twice with thawing media by centrifugation was followed by viability assay of the above samples.

Viability Assay

The evaluation of cell viability was conducted using 0.4 % Trypan blue (Hi-media, India) by Dye exclusion method. Afterwards, the cells were enumerated using an inverted microscope. The non-viable cells stained blue as they took up the dye while the viable cells remained colorless excluding the dye. The cell viability was calculated by the following formula:

% Viability = number of viable MNCs after thawing $\times$ 100/total number of UCB MNCs before freezing.

Colony Forming Unit (CFU) Assay

Hematopoietic progenitor cell proliferation of post thawed cells was quantified by an in vitro method using MethoCult Classic (H4434), a semisolid methylcellulose system containing stem cell factor (SCF), GM-CSF, IL-3 and Erythropoietin (Stem Cell Technologies, Vancouver, BC, Canada) and measuring the clonogenic activity via CFU. The cell samples with the concentration of 1×10^5 were suspended in 4 ml of the methylcellulose medium for triplet cultures with sterile water containing in the culture plate and cultures were incubated at 37 °C, in 5 % CO₂ with ≥ 95 % humidity for 2 weeks (14 days). Colonies were scored as erythroid lineage based burst forming unit—erythroid/colony forming unit—erythroid (BFU-E/CFU-E), myeloid lineage based colony forming unit—granulocyte, macrophage (CFU-GM) and the mixed type colony forming unit—granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) under the inverted microscope and evaluated on the basis of their relative distribution in the culture plate. The culture plates were then discarded. Colony forming unit-erythroid (CFU-E) are clonogenic progenitors that generate only one or two red colored clusters with each cluster containing from 8 to approximately 200 erythroblasts. Burst forming unit-erythroid (BFU-E) colonies containing erythroblasts can be described as small (3–8 clusters), intermediate (9–16 clusters), or large (more than 16 clusters) according to the number of clusters present. Colony forming unit-granulocyte, macrophage (CFU-GM) are progenitors that give rise to colonies containing a heterogeneous population (>40 numbers) of macrophages and granulocytes including eosinophils, basophils, or neutrophils. Colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM) are multi-lineage progenitors that give rise to a colonies containing erythroid, granulocyte, macrophage and megakaryocyte cells.

Fig. 1 Viability percentage as shown by different media containing disaccharides alone and with 2 % DMSO

For this experimental analysis, we took sample size three ($n = 3$) for each of optimized freezing media as determined in the first phase of our study.

Statistical Analysis

Statistical analysis was performed using the GRAPHPAD PRISM 5 software. Data were presented as mean $\pm$ standard deviation and analyzed by ANOVA and Student's t test with significant $*p$ value <0.05 .

Results

We selected ten cord units for the whole experimental study after fulfilling our exclusion and inclusion criteria. The total number of MNCs derived from single cord unit was 10.6×10^8 after processing of blood samples. For each of the single experiment of Viability Assay, MNCs derived from same cord unit were aliquot for 10 times in order to compare their viability in ten different freezing media (0.02 M sucrose, 0.02 M sucrose + 2 % DMSO, 0.02 M trehalose, 0.02 M trehalose + 2 % DMSO, 0.2 M trehalose, 0.2 M trehalose + 2 % DMSO, 0.2 M sucrose, 0.2 M sucrose + 2 % DMSO, 5 % DMSO and 2 % DMSO). This above single experiment was repeated 10 times with 10 different cord units.

Viability of the cryopreserved cells

The thawed samples preserved in different cryoprotective media were analysed for viability and compared with 5 % DMSO (control) as graphically presented in Fig. 1.

None of the above freezing media containing disaccharides alone reached up to the viability percentage as exhibited by the Control media (5 % DMSO), while disaccharides only with 0.2 M concentration along with DMSO showed significant improvement in the viability of stem cells.

The highest mean percentage of viability in our study was 74.3 ± 1.64 %, observed more significantly ($p < 0.0001$) in

0.2 M trehalose protected cells in combination with 2 % DMSO, than cells protected by 0.2 M trehalose alone. 2 % DMSO also enhanced the viability of UCB derived mononuclear cells observed in case of trehalose at its lower concentration (0.02 M) with p value <0.0001 .

In case of sucrose (0.2 M) with DMSO provided mean 65 ± 1.4 % viability, more than that of 0.02 M sucrose based media with 2 % DMSO. Significant (p value = 0.0009) improvement in viability was also observed in 0.2 M sucrose protected cells in combination with 2 % DMSO than 5 % DMSO.

The presence of high or low concentrations of sucrose and trehalose alone in the cryoprotective medium did not substantially increase stem cell viability. But the inclusion of 2 % DMSO to the disaccharides based cryoprotective medium enhanced the viability of cells significantly ($p < 0.0001$) as shown in Fig. 1.

The above analysis of viability study suggests that out of the two different groups of freezing media as mentioned above, medium containing 0.2 M disaccharides can be used as optimal cryo-protective media than that of 0.02 M concentrated media, which could effectively reduce the DMSO concentration from 5 to 2 %. Thus 0.2 M disaccharides alone and with 2 % DMSO were used for the next phase of our analytical study of stem cells considering 5 % DMSO as control.

CFU-assay

The selected optimized freezing media along with the control (0.2 M trehalose, 0.2 M trehalose + 2 % DMSO, 0.2 M sucrose, 0.2 M sucrose + 2 % DMSO, 5 % DMSO and 2 % DMSO), from the viability assay (first phase of the experiment), were chosen for the highly cost-effective CFU Assay. This assay was performed only with the post thawed MNCs (cryopreserved during first phase) from three different cord blood samples of optimized media. Hence, CFU assay for each optimized media was performed in triplicates, indicating total 18 CFU assays that were carried out in our study.

Table 2 Total number of hematopoietic colonies as generated during CFU assay

Type of cryoprotective media	Total no. of colonies in mean $\pm$ SD	Range (min–max)
5 % DMSO	138 $\pm$ 3.21	6 (136–142)
2 % DMSO	107 $\pm$ 6.11	12 (100–112)
0.2 M sucrose	62 $\pm$ 3.0	6 (59–65)
0.2 M sucrose + 2 % DMSO	93 $\pm$ 5.0	10 (88–98)
0.2 M trehalose	138 $\pm$ 2.52	5 (136–141)
0.2 M trehalose + 2 % DMSO	183 $\pm$ 3.61	7 (179–186)

Three types of colonies were identified under inverted microscope as BFU-E (including CFU-E), CFU-GM and CFU-GEMM, after 14 days culture with methylcellulose according to the standard procedures [31, 32]. The incubated plates of each type of optimized cryoprotective media were scored for colonies separately and then discarded.

The total number of colonies were expressed in mean $\pm$ SD and range and presented in Table 2.

There was significant decrease in the total number of hematopoietic colonies in 2 % DMSO based media than 5 % DMSO one ($p = 0.0015$). Trehalose 0.2 M with 2 % DMSO media provided highest mean 183 ± 3.61 number of colonies than all other media (Table 2).

Trehalose 0.2 M in combination with DMSO exhibited better number of erythroid colonies (99.7 ± 2.52), than that of 5 % DMSO as presented in Fig. 2. But 0.2 M trehalose protected cells did not demonstrate significant number of BFU-E colonies even after inclusion of 2 % DMSO ($p = 0.1013$), the similar result was observed between 0.2 M sucrose alone and with DMSO ($p = 0.1215$).

While there was a great decline in the mean number of CFU-GM colonies as observed in all types of

cryoprotective solution except the trehalose solution having DMSO. 0.2 M trehalose protected cells showed significant mean number of 82.7 ± 4.73 of CFU-GM colonies ($p = 0.0001$) after the inclusion of DMSO. But there was no significant difference observed between two solutions containing 0.2 M sucrose and 2 % DMSO alone ($p = 0.6890$). The result of 0.2 M sucrose based solution after the inclusion of DMSO also was similar from that obtained with 5 % DMSO alone ($p = 0.8280$).

Regarding the mixed type of hematopoietic colonies like CFU-GEMM generation, even inclusion of DMSO did not result in increased number of colonies in case of trehalose. Only sucrose (0.2 M) with DMSO though demonstrated an increase in the mean 22.3 ± 3.51 number of colonies, still the number was less than that obtained with the use of 5 % DMSO alone.

Discussion

The toxicity of CPAs has been considered as the single most limiting factor to the development of effective cryopreservation protocols for challenging cells and tissues [33]. In contrast trehalose is a disaccharide produced by several microorganisms; its cryoprotective potential has been evaluated in a variety of tissues and cells that do not produce trehalose by themselves [34–36].

In our previous study standard 10 % DMSO protected cells demonstrated more number of viable cells and myeloid lineage based colonies [30]. But toxic effect of this 10 % DMSO concentration is still a matter of debate in the transplantation field.

Scheinkö nig et al. evaluated the cryoprotective potential of trehalose at its different concentrations alone in comparison to 10 % DMSO. Trehalose at its 0.5 M concentration contributed similar results with that of 10 %

Fig. 2 Comparison of three types of hematopoietic colonies generation in response to optimized storage media with or without DMSO inclusion

DMSO and could possibly act as an valuable alternative or additive to DMSO for effective cryopreservation of HSCs [37]. But the results of our investigational study reflect the improvement in post thawing viability of cells by lowering the DMSO concentration to 2 % in the medium containing disaccharides than the one with disaccharides alone. When we assessed the influence of disaccharide based freezing media on the formation of three types of hematopoietic colonies, 0.2 M trehalose in combination with 2 % DMSO came out as the successful candidate for the generation of highest number of BFU-E/CFU-E colonies. The 0.2 M disaccharides protected cells still did not exhibit any better result in the number of BFU-E/CFU-E colonies significantly after the inclusion of DMSO. While larger number of myeloid based CFU-GM colonies was observed in case of trehalose in combination with DMSO than control. Highest numbers of CFU-GEMM colonies were generated with freezing solution containing 5 % DMSO alone. Sucrose (0.2 M) demonstrated some improvement with 2 % DMSO in mixed type colonies (CFU-GEMM) formation.

Rodrigues et al. in their investigational study, compared the cryo-protective effect of various combination of concentrations of disaccharides with 5 or 2.5 % DMSO to that of 10 % DMSO after the storage of cell samples under various freezing temperatures in a pre-established programme in a controlled rate freezing system for 2 weeks. They concluded that 30 mmol/L trehalose with 2.5 % DMSO or 60 mmol/L sucrose with 5 % DMSO produced results similar to those for 10 % DMSO in terms of the clonogenic potential of progenitor cells, cell viability, and numbers of CD45⁺/CD34⁺ cells in post-thawed cord blood cryopreserved for a minimum of 2 weeks [26]. In contrast, our study is purely carried out using conventional slow freezing rate for cryopreservation without the use of controlled freezer for which our cells frozen even with 0.2 M trehalose with 2 % DMSO did not demonstrate similar results with that of 10 % DMSO rather they showed increased viability and colony forming ability than that of 5 % DMSO.

Previously we observed that 10 % DMSO based freezing media exhibited mean number of 256 ± 7.55 total hematopoietic colonies [30], which was significantly higher to that of 5 or 2 % DMSO alone. Only trehalose at its 0.2 M concentration with 2 % DMSO produced better results than 5 or 2 % DMSO alone as evident from the CFU Assay of the present study. Though our study demonstrated the measurement of viable MNCs by Haemocytometer counting, CD34⁺ cell count by flowcytometry, both before freezing and after thawing would be rather useful in the evaluation of the functional viable cells for future effective engraftment of transplanted cells.

Conclusion

As the UCB transplantation is gaining momentum in stem cell therapy, optimization of cryopreservation medium with minimal use of DMSO or without it is now becoming a primary requisite for safe therapeutics. In this regard our study concludes with the improvement in functional activity of UCB stem cells after the inclusion of 2 % DMSO to cryoprotective medium containing the nontoxic disaccharides in order to minimize the concentration of DMSO. Further investigation on the long term storage of these cells using disaccharides with reduced amounts of DMSO might potentially ameliorate the engraftment outcomes of UCB transplants.

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