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Analysis of the Factors Affecting the Stem Cell Yield and Post Thaw Cellular Viability in Cord Blood Units

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Abstract

Background: The role of cord blood for procuring stem cells for transplantation in various disorders became prominent in last few decades. However the processing methods like cryoprotectant concentration, volume reduction and viability assessment techniques remained a debate and it affected the yield of final cellular product for transplantation and clinical outcome.

Aims and Objective: To standardize and determine the factors affecting the cryopreservation procedure of cord blood units and post-thaw viability assessment methods to procure optimum yield of stem cells from the thawed cord blood units.

Materials and Method: In this prospective study, 120 cord blood units were collected by in-utero and ex-utero methods abiding by defined inclusion-exclusion criteria and were processed in two groups based on volume reduction done by HETA starch or not. Each group product were cryopreserved in – 80 o C mechanical deep freezer using Human albumin and varying concentrations of DMSO (2.5%, 5% and 10%). Final thawed products were assessed into two groups – with and without post thaw washing for cellular yields. Cellular profiles were asessed at three steps-post collection, pre cryopreservation (after volume reduction) and post thaw washing, using TNC, MNC and CD 34+ counts (flow cytometry). Cell viability was assessed using Trypan blue method. Maternal and fetal factors like maternal age, gestational age, placental weight, birth weight, sex of baby were assessed for their role in cellular yields. Effect of technical factors like hold over time, cord clamp interval, volume reduction, DMSO concentration and washing were studied by intergroup comparison.

Results and Conclusion: A maternal age over 25 years, fetal birth weight > 3 kg, in-utero collection method, cord clamp- collection interval < 1 minute showed better initial cellular yields. DMSO concentration of 5 % was found optimum for cryopreservation for best TNC or MNC recovery and 10 % for best recovery of CD34+ cells. Post thaw no wash technique appeared to be better method as cell loss due to washing was significant.

Keywords: Cryopreservation; Trypan blue method

Abbreviations: AABB: American Association of Blood Banks; ALL: Acute Lymphoblastic Leukemia; AML: Acute Myelogenous Leukemia; ASHRAE: American Society for Heating, Refrigeration, Air conditioning Engineers; BMT: Bone Marrow Transplant; CBT: Cord blood transplant; CD: Cluster differentiating; DMSO: Dimethylsulfoxide; FACT: Foundation for the Accreditation of Cellular Therapy; GVHD: Graft Versus Host Disease; GCSF: Granulocyte colony stimulating factor; HSCT: Hematopoietic stem cell transplant; HLA: Human Leucocytes Antigen; HETA Starch: Hydro ethyl starch; ISHAGE: International Society for Hemotherapy and Graft Engineering; LNG: Liquefied natural gas; MNC: Mononuclear cell; TNC: Total nucleated cell; TTI: Transfusion Transmitted Infection; UCB: Umbilical cord blood; FSC: Forward scatter; SSC: Side scatter

Introduction

For the last two and a half decade, cord blood has been looked as an alternative source to bone marrow for stem cell transplantation in various aggressive hematopoietic malignancies and immunodeficiency disorders. The results of the clinical trials comparing bone marrow versus cord blood as source of stem cells is encouraging and this has increased the interest of transplant scientists to look into various aspects of cord blood research[1,2]. Various maternal and neonatal factors like gravida status, gestational age, placental weight, birth weight and sex of baby, technical factors like collection method, hold over time, DMSO concentration [7], post thaw washing have been found to affect the final cellular yield derived from one cord blood unit (CBU) for transplantation[4,5,6]. Evident from various works, the TNC, MNC and CD 34+ cell counts of the product are found to correlate with the final cellular dosage and transplant outcomes.

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In view of the easy availability, low risk to donor, less constrained HLA matching, the need to standardize the collection, processing, storage and post thaw assessment has emerged [3].

Methods

The purpose of our study was aimed to analyse the various factors that affect the stem cell yield from cord blood units and assess and compare the post thaw viability among various methods of cryopreservation. Present study was done in the Department of Transfusion Medicine in collaboration with the department of Obstetrics and Gynaecology, PGIMER Chandigarh. The study included randomly selected 120 cord blood units harvested from the placenta immediately after live birth delivery in the clean labour room (CLR) of the Department of Obstetrics and Gynaecology, PGIMER from the mothers fulfilling the inclusion criteria of the study. Prior informed consent was taken from all the donors. Inclusion criteria included all live birth delivery (> 24 weeks of gestation), any gravida status, vaginal delivery as well as delivery by cesarean section, grossly normal placenta of all live births on physical (visual) examination. Donor exclusion criteria included chorio-amnionitis, placental tumour, antepartum haemorrhage, foetal death, donor on antibiotics, growth hormone, unlicensed vaccine, bovine Insulin, history of tissue transplantation, high risk behaviour. The cord blood units fulfilling the inclusion-exclusion criteria were analyzed for fetal, maternal and technical factors that attributed to cellular yield [35] (using TNC, MNC, CD 34+ cellular profiles and trypan blue assay for viability testing) and cryopreservation was compared using different concentrations of cryoprotectant.

Collection Procedure

Cord blood units were collected in the side room of Clean labour room (Ex utero = 23) or on the delivery table (In utero = 97). Umbilical cord blood was collected using single bags (make Terumo Penpol Pvt Ltd) of 100 ml capacity using aseptic precautions by cleaning the cannulation site with povidone iodine and spirit [10,26]. Each bag contained 14 ml Citrate Phosphate Dextrose Adenine (CPDA) as the anticoagulant with the ratio of CPDA to cord blood being around 1:7. Blood was collected by inserting needle proximal to the clamp applied to the cord and keeping the cannulation site below the placenta level – allowing free flow of blood. Units were labelled and carried to Transfusion Medicine department using thermocol box at room temperature and stored at 40C till processing. Needle and placenta were discarded following biomedical waste disposal rules. Units were labelled mentioning unit number, date/time of collection, collector's name, donor's name, volume, blood group, TTI screening status. Volume of collected cord blood (ml) was calculated as = [Weight of collected bag (gm) - Weight of empty bag (gm)] / Specific gravity of cord blood (≈1.053).

Volume Reduction

Units were transferred to bio safety cabinet class 2 A (BIOAIR Safe flow 1.2, Euro clone, Siziano, Italy) and volume reduction was done in 50 ml Falcon tubes after adding 6% Hydroxyethyl starch weight/volume (commercial) to each tube in a ratio of HES : Blood = 1 : 5 by volume and mixed well [8,11]. In comparison to conventional techniques we followed a no centrifugation technique, as the samples were kept erect vertically undisturbed for a period of 2 hours after addition and mixing of HES. After a holdover time of 2 hours most samples showed appreciable separation of plasma column under gravity depending mostly on the hematocrit of the sample. The cap of the vials was gently opened without disturbing the interface and supernatant was taken out using syringes (without needle) from the undisturbed tubes. The Buffy coat layer was aspirated from the interface. About 10-15 ml of the final PBS suspended Buffy coat was cryopreserved and 0.5 ml sample taken for pre cryopreservation quality control assessment i.e. TNC, MNC, viability and Flow cytometry and suspended in PBS in another test tube.

Cryopreservation Protocol

The cryoprotectant mixtures were prepared at 3 different strengths of DMSO [7] concentration – 2.5%, 5% and 10% depending on the volume of Plasmalyte A added [14,17]. While preparing the mixture at first cold sterile pyrogen free 99.99% DMSO (CRYO-SURE, WAK-Chemie, Medical GmbH, Steinbach, Germany) was added to cold Plasmalyte A (Baxter International Inc Pvt Ltd Gurgaon, Haryana, INDIA) in falcon tube to counter effect the exergonic reaction and heat generation mediated by DMSO. Then cold 20% Human albumin (ALBUREL 20gm RELIANCE Life Sciences, Ravale, Navi Mumbai, India) stored at 40C was added to the mixture slowly. After volume reduction the final product was transferred to a Fresh tube and product volume noted. To it the cold cryoprotectant mixture was added in equal volume slowly by directly trickling from the side wall of the Falcon tube and the final pre cryopreservation product mixture was quickly transferred to mechanical deep freezer -80 0 C and stored for 3-6 months.[9,14] Temperature of the deep freezer was continuously monitored using a data logger (Log Tag).

Thawing and Washing

Thawing was done in 370C water bath. A mixture of 10% Dextran and 20% Human albumin in a ratio of 5:1 by volume pre cooled to 40C in Falcon tubes was used for final washing of thawed products [15, 18, 19]. Three washings were given using hard spin at 2000 rpm for 10 minutes. After each wash, supernatant was removed carefully using syringe leaving behind the white cell button. Final cell button was dissolved in 5 ml of Dextran – Albumin wash solution and used for post thaw parameter assessment like TNC, MNC count, viability assessment with Trypan blue dye[12], Flow cytometry[13].

Quality Control

Quality control [20] of the units included ABO and RhD typing, TTI screening and viability testing[23], TNC [16] and MNC count, Flow cytometry for CD 34+ cell estimation-done at three stages: Post collection, post processing/pre cryopreservation, and post thawing (washing done/not done).

CD34+ Cell Estimation

Dual platform modified ISHAGE protocol [13, 21] was used

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to enumerate CD34+ cells by flow cytometry along with the help of TNC [22] and MNC (Haematology Analyser – ORION Ocean Medical Technologies, Delhi, India) counts at the three stages and percentage recovery after each step was calculated to identify amount of cell loss. Ethical clearance was taken from Institute Ethics Committee for Stem Cell Research.

Results

TNC [20], MNC, CD34+ cellular profiles and their percentage recovery was used to assess the role of factors affecting the cryopreservation procedure and post thaw product yield. Factors were classified as source related (maternal and foetal) and process related (collection process and cryopreservation related) variables.

Source Related Variables

Among the maternal factors [5], gravida status and gestational age did not affect the initial total TNC and MNC yield per bag significantly, taking into consideration the variability in volume of cord blood collected. A maternal age of >25 years significantly added to better initial TNC and MNC yield (p values 0.014 and 0.021). A placental weight >500 grams although did not significantly affect the cellular yields, yet the MNC yield was better (close to significance, p value 0.087) in heavier placenta. A neonatal birth weight of >3 kg gave significantly better cellular yield. In male foetuses MNC counts were better than females and were close to significance (p value 0.083) [5, 6, 21, 24, 33, 34] (Table 1).

Table 1: enlists the source related factors in the study

Parameters		Initial Total Cell Count/Bag (MEAN)		P value
		TNC $\times 10^8$	MNC $\times 10^6$	
Gravida status	PGR (n=58)	7.193 ± 3.16	70.853 ± 42.39	0.13 (TNC)
	MGR (n=62)	8.466 ± 4.64	84.790 ± 64.43	0.314 (MNC)
Gestational Age	Preterm (n=30)	7.84 ± 5.59	71.97 ± 90.0	0.325 (TNC)
	Term (n=90)	7.86 ± 3.39	80.08 ± 55.24	0.299(MNC)
Mother's Age	>25 years (n=66)	8.73 ± 4.54	85.58 ± 52.99	0.014 (TNC)
	<25 years (n= 51)	6.83 ± 3.05	69.48 ± 58.11	0.021(MNC)
Placental Weight	>500 gms (n=87)	8.32 ± 4.03	80.17 ± 55.3	0.125 (TNC)
	<500 gms (n=33)	6.61 ± 3.80	72.48 ± 55.10	0.087(MNC)
Neonatal Sex	Male (n=62)	8.81 ± 4.49	85.99 ± 62.64	0.195 (TNC)
	Female (n=58)	6.83 ± 3.2	69.57 ± 44.74	0.083(MNC)
Birth weight	>3 kg (n=43)	9.06 ± 5.08	90.87 ± 55.29	0.040 (TNC)
	<3 kg (n=77)	7.18 ± 3.15	70.90 ± 54.06	0.019(MNC)

Cord Blood Collection Related Variables

In Utero method of collection and cord clamp- collection interval of < 1 minute both yielded significantly better initial TNC and MNC per bag. Hold over time [27] of >12 hours did not significantly reduce the TNC and MNC counts when kept at 40 C overnight after collection (Table 2).

Cryopreservation Related Variables

Volume reduction (manual method with HES) led to significant loss of TNC and MNCs in our study population. The percentage recovery of progenitor cells reduced to 30.71 ± 19.6 % (TNC) and 38.37 ± 26.5% (MNC) and the cell loss was appreciably high [31,32] (Table 3).

Table 2: Enlists the technical factors related to cord blood collection in the study

Parameters		Initial Total Cell Count/Bag		P value
		TNC $\times 10^8$	MNC $\times 10^6$	
Collection method	Ex Utero (n=23)	6.10 ± 2.4	58.41 ± 35.96	0.015 (TNC)
	In Utero (n=97)	8.29 ± 4.28	83.29±58.14	0.018(MNC)
Clamp -Collection Interval time	60 s< (n=79)	8.60 ± 4.42	87.51 ± 61.83	0.003(TNC)
	>60 s (n=41)	6.41 ± 2.65	59.84 ± 32.67	0.005(MNC)
Hold over Time	<12 hrs (n=86)	7.80 ± 3.96	77.70 ± 54.50	0.882 (TNC)
	>12 hrs (n=34)	7.97 ± 4.25	78.94 ± 57.47	0.729(MNC)

Table 3: Comparitive analysis of TNC and MNC % recovery after volume reduction in various studies

Study (Volume Reduction)	TNC % Recovery	MNC % Recovery
RUBINSTEIN P et al (Manual – HES, 1995)	90.7	98.1
SOLVES et al (Manual – HES, 2005)	74.7 ± 8.2	-
THEUNISSEN et al (SEPAX, 2004)	87.6 ± 10	87.7 ± 9.7
YASUTAKE et al (FILTER, 2001)	73.9 ± 13.9	80.8 ± 16.3
EICHLER et al (FILTER, 2003)	62.3 ± 11.6	80.7 ± 12.6
OUR STUDY (VOLUME REDUCTION ARM)	30.71 ± 19.6	38.37 ± 26.5
OUR STUDY (NO VOLUME REDUCTION ARM)	90.767 ± 6.9	90.767 ± 6.9

Role of Washing and DMSO Concentration on Cellular Profiles

The role of DMSO and post thaw washing on cellular profiles have been summarised in the (figure 3).

Role of DMSO Concentration and Washing on Post Thaw Mean Tnc % Recovery

Washing led to significant TNC loss at 5% and 10% DMSO concentration. Mean TNC % recovery was found best in No wash arm with 5% DMSO followed by 10% DMSO. Post thaw mean viable TNC count in 2.5% arm was much less compared to 5% and 10% DMSO arms. Results shown in (Table 4).

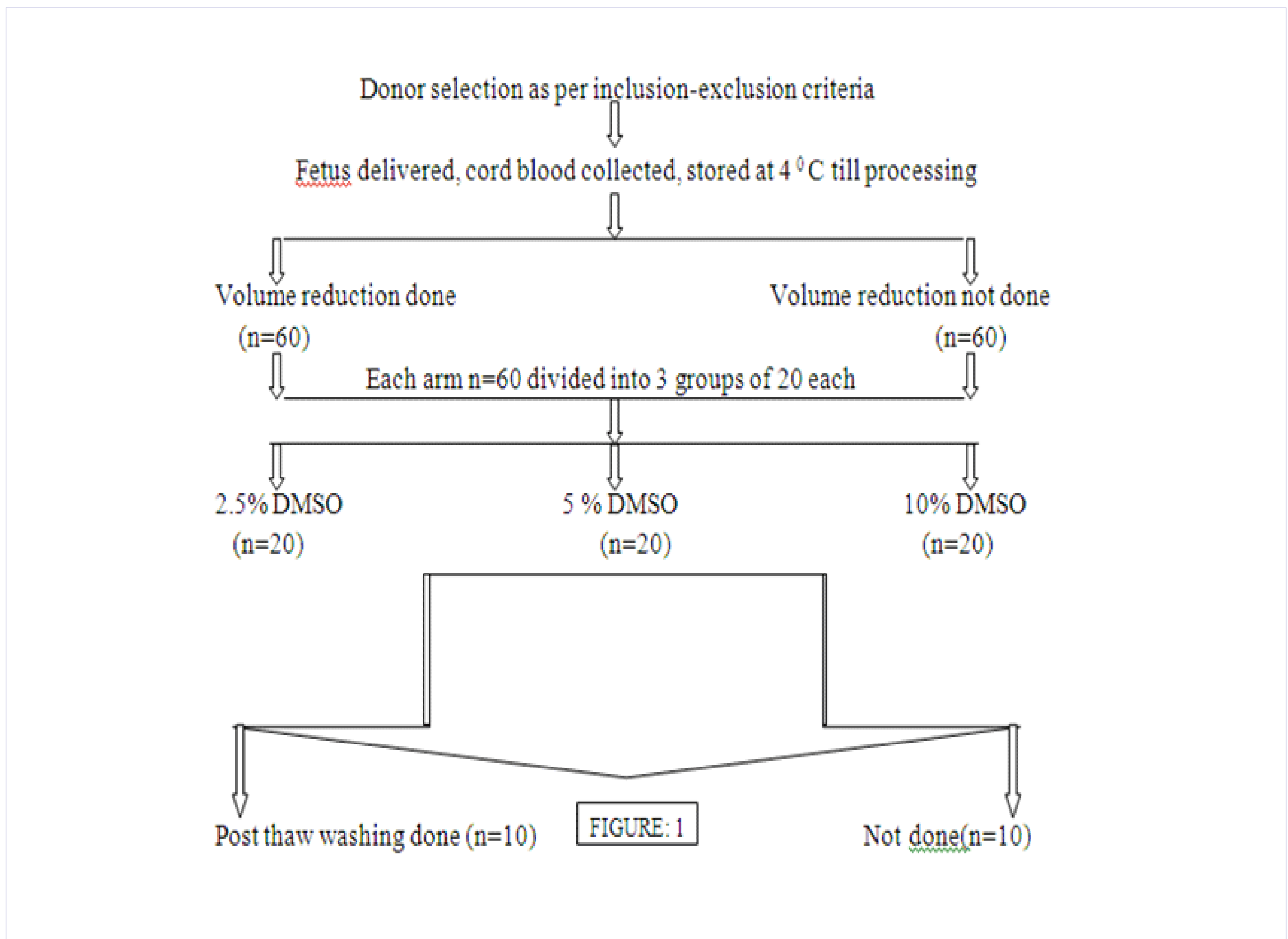

Figure 1: Outline for Cord Blood Processing [14]

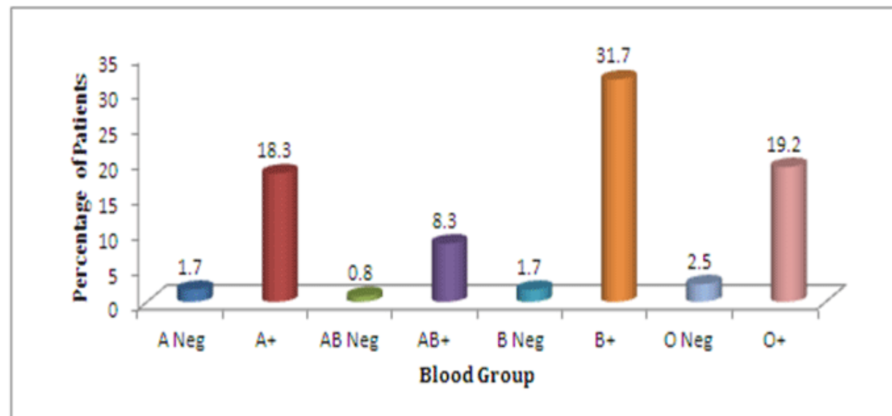

Figure 2: Showing Distribution of ABO Blood Group in Study Population

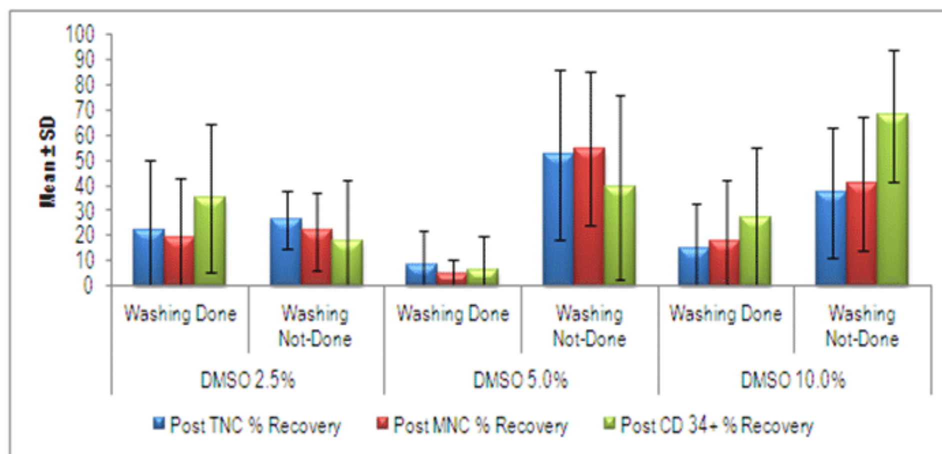

Figure 3: The role of DMSO and post thaw washing on cellular profiles have been summarised

Table 4

DMSO %	Washing done (n=60)	Washing not done (n=60)	P value (Washing vs No Wash)
2.5% DMSO (n=40)	22.33 ± 27.52	26.31 ± 11.44	0.079
5% DMSO (n=40)	8.11 ± 13.79	52.36 ± 33.65	<0.001
10%DMSO (n=40)	14.63 ± 18.31	36.97 ± 25.73	0.001

Role of DMSO Concentration and Washing on Post Thaw Mean Mnc % Recovery

Washing led to significant MNC loss at 5% and 10% DMSO concentration. Mean MNC % recovery was found best in No wash arm with 5% DMSO followed by 10% DMSO. Post thaw mean viable MNC count in 2.5% arm was much less compared to 5% and 10% DMSO arms. Results shown in (Table 5).

Role of DMSO Concentration and Washing on Post Thaw Mean Cd34+ % Recovery

Washing led to significant CD34+ cell loss at all DMSO concentrations. Mean CD 34+ % recovery was found best in No wash arm with 10% DMSO followed by 5% DMSO. Post thaw mean viable CD 34+ cell count in 2.5% arm was significantly much better compared to 5% and 10% DMSO arms when washing was considered. Results shown in (Table 6).

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Table 5			
DMSO%	Washing done (n=60)	Washing not done (n=60)	P value (Washing vs NoWash)
2.5% DMSO (n=40)	18.97 ± 23.79	21.80 ± 15.62	0.083
5% DMSO (n=40)	4.61 ± 5.87	54.53 ± 30.64	<0.001
10% DMSO (n=40)	17.81 ± 24.34	40.51 ± 26.59	0.003

Table 6			
DMSO%	Washing done (n=60)	Washing not done (n=60)	P value (Washing vs No Wash)
2.5% DMSO (n=40)	35.02 ± 29.19	17.88 ± 24.46	0.033
5% DMSO (n=40)	6.48 ± 13.73	39.49 ± 36.57	0.006
10% DMSO (n=40)	27.23 ± 28.06	67.94 ± 26.20	<0.001

Discussion

In our study, cryopreservation related variables significantly affected the final post thaw final cellular yield. Five percent DMSO [28,29] with no-wash was found to be the best protocol for optimum final TNC (52.36 ± 33.65 %) and MNC (54.53 ± 30.64%) percentage recovery, whereas 10% DMSO without washing was found to give the best (67.94 ± 26.20 %) CD 34+ cell percentage recovery.[35] Since 10% DMSO has potential cytotoxicity[30], its feasibility in maximum CD 34+ cell recovery is to be reconsidered with the aid of further experimental studies and CFU assays and recent studies [28] have been done with lesser concentration of DMSO for stem cell cryopreservation. Overall, 5% DMSO seems promising and optimal for direct post thaw infusion (without washing) in terms of better progenitor cell recovery after cryopreservation – a fact to be evaluated by further infusion based clinical studies with follow up in post transplant phase. Cell loss in tubes during volume reduction was significantly more owing to less separation and settling of red cell sludge due to less cross sectional surface area in falcon tubes in comparison to blood collection bags. Perhaps the use of automation for volume reduction might reduce the appreciable loss of cells of interest at an initial stage of processing for better cellular recovery after thawing. In terms of the source, donor selection remains critical in cord blood banking as many more source related factors may attribute to initial volume and cell number collected in each unit that is critical in determining cellular dosage after cryopreservation. Source related factors if assessed in a larger sample size could probably add more to the attributability of Gravida status, gestational age and placental weight as maternal factors regulating initial cellular yield. Maternal age(>25 years), foetal birth weight> 3 kg, cord-clamp-time interval <1 minute and In utero collection method all positively correlate to better initial TNC and MNC yields. . However in better set ups with better sterility and with good rapport with the obsteretician, even Ex Utero method can actually give adequate cellular yield. Hold over time [27] of >12 hours did not significantly reduce the TNC and MNC counts when kept at 40 C overnight after collection. This is helpful specially in resource constrained set ups where laboratory facilities are not available round the clock. Thus further studies are required to optimize the collection and cryopreservation related variables so as to obtain an effective therapeutic dose of stem cells for prospective transplant recipients.

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Declarations

This manuscript describes novel work and is not under consideration for publication/published by any other journal. All author(s) have approved the manuscript and this submission.

The author(s) certify that there is no conflict of interest with any financial/research/academic organization, with regards to the content/research work discussed in the manuscript.

Prior permission from the Institute Ethical Committee (Reference number - NK/1725/MD/11529-30) was taken for allowing conducting the study in the premises on the enrolled subjects.

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