JOURNAL OF RECENT ADVANCES IN APPLIED SCIENCES (JRAAS) 28:25-30, 2013
ISSN 0970 1990, ST CODE 08 ,www.jraas.org. E MAIL: mhcdr@rediff.com
DISPLAYED ON LINE ON MARCH 2012

COLLECTION, PROCESSING, CRYOPRESEVATION AND QUANTIFICATION OF CD34+ STEM CELLS IN UMBILICAL CORD BLOOD'

SHEEBA AFREEN[1,2], TULIKA CHANDRA[1] , ASHUTOSH KUMAR[2] , UMA SINGH[3]
[1]Department of Transfusion Medicine and Blood Bank, [2]Department of Pathology, [3]Department of Obstetrics and Gynecology, Chhattrapati Shahuji Maharaj Medical University (Earlier King Georg's Medical University), Lucknow Uttar Pradesh, India, E-Mail: tulikachandra@rediffmail.com

ABSTRACT

Umbilical cord blood (UCB) transplantation is being used as an alternative source of hematopoietic stem cells for bone marrow reconstitution. Separation, processing and cry preservation of UCB samples in large numbers for storage in cord blood banks ideally needs to be partially automated to allow large numbers of samples to be processed efficiently. Aim of the present study was to carried out for standardization of collection, processing, cry preservation and quantification of CD34+ haematopoietic stem cells in umbilical cord blood. A total of 420 umbilical cord blood units were collected. Samples were analyzed for CD34+ cells concentration and viability of mononuclear cells were done on all the samples. The mean of the CD34+ cells concentration was 1.99± 0.82 % (range 0.4-4.5) on baseline period. The mean of the CD34+ cells concentration was 1.14± 0.85 % (range 0.2-3.8) on six month. The mean of the viability of mononuclear cells was 81.80 ±3.73% (range 76-91) on baseline period. The viability of mononuclear cells count at six month was 64.24± 2.70% (range 61-70). The viability loss of mononuclear cells from baseline to six month was 20.9%. The mean of the total nucleated cells count was 11.36 ± 4.34 cells/ µl, (0.67- 16.68). In conclusion, the method demonstrates that UCB units can be routinely processed in a closed system that also achieves a significant reduction in storage needs and related costs, while maintaining quantity and quality of the haematopoitic stem cells.

KEY WORDS Umbilical cord blood, cryopreservation, CD34+ cells, viability, mononuclear cells

INTRODUCTION

Umbilical cord blood (UCB) represents an alternative source of hematopoietic stem cells (HSC) for use in allogeneic transplantation of patients affected by hematological disorders, inherited immunodeficiencies, and metabolic diseases. This source of stem cells has been successfully used to replace bone marrow and apheresis in transplants since the first transplantation using UCB, performed by Gluckman and colleagues *et al.*, in 1989[1]. Since then, the studies have progressed as to the procedures of collection, processing, characterization, quantification, cry preservation, thawing, and transportation of UCB around the world [2]. The cry preservation process is of importance for all types of stem cell collection, but is perhaps particularly critical for umbilical cord blood (UCB). The actual transplant is here harvested at the time of birth and used at a later point in time for a yet, at the time point of the harvest, often indeterminate recipient. The UCB is usually stored by either public or private cord blood banks. Public cord blood banks are usually nonprofit organizations, which offer the donor unit to matching recipients via national or international registries to potential recipients in need[3]. Cord blood banks store a donor specimen for the donor or in the case of public banks for an unknown recipient for an indeterminate time span. There are now about 170,000 frozen units in 37 cord-blood registries in 21 countries. Two thousand nine hundred units have been transplanted to date , with adults having received about one third of those units [4].

Rubinstein et al in 1995 address two issues that are essential for the full development of unrelated-donor PCB transplantation as a practical alternative for clinical use. The first problem is that stockpiling ("banking") a sufficiently large number of cryoprotected whole PCB units requires vast amounts of costly storage space in liquid nitrogen (LN). To establish an adequate panel, therefore, the hematopoietic cells of PCB units need to be concentrated into units of much smaller volume[5]. However, Broxmeyer et al.(1989) found unacceptably high progenitor cell losses when using techniques for red blood cell separation such as simple centrifugation, lysis with ammonium chloride, differential settling in viscous media, and filtration through density gradients[6]. Other investigators reported better recoveries[7], but their procedures required transferring the blood from collection bags to other vessels, thereby exposing the blood to the risk of bacterial and fungal contamination and increasing the possibility of identification errors. These manipulations are also time and labor intensive and do not fit easily in the routine demanded by medium- and large-scale' PCB processing and storage.

The aim of the present study was carried out standardization of procedure for collection, separation and cryopreservation of umbilical cord

blood haematopoitic stem cell as well as quantification of CD34+ cells haematopoitic stem cells were done (after thawing and washing) at six month .

MATERIALS AND METHODS

A total of 420 UCB were obtained from both vaginal and caesarian deliveries from the Department of Obstetrics and Gynecology. Processing UCB samples was done in the Department of Transfusion Medicine, Chattrapati Shahuji Maharaj Medical University, Lucknow. Written informed consent was obtained from mother. Only healthy pregnant women with known history of hepatitis, infectious disease, diabetes mellitus, severe hypertension, abortion or bad obstetrics were excluded.

Cord blood donor infants

Infants delivered at term (31-41 weeks) were included.Birth weight, baby sex, mode of delivery and gestational age of the baby were also recorded. After collection, the cord blood was sent in the transport boxes to the department of Transfusion Medicine, Chattrapati Shahuji Maharaj Medical University, Lucknow, India.

A Collection of UCB

Cord blood was collected from 310 (73.8%) normal vaginal and 110 (26.1%) cesarean deliveries after the completion of delivery before placenta expulsion in CPDA triple blood bag containing 69 ml anticoagulant citrate, phosphate, dextrose and adenine. 20 ml anticoagulant were removed before collection of umbilical cord blood. After the delivery of the baby the cord was clamped and wiped with alcohol or betadine to ensure sterility of the collection. A needle was inserted into the umbilical vein above the clamp. The blood was drained via gravity into the sterile collection bag, containing Citrate Phosphate Dextrose Adenine (CPDA) as an anticoagulant. Efforts were made to obtain maximal volumes from each collection. The umbilical cord blood units were stored at 4^0C and processed within 24 hours. Samples of 3 ml per unit were taken at this stage for nucleated cell (NC) count.

B Processing of UCB

Processing was done within 24 hours of cord blood collection. The $CD34^+$ cell concentration, total nucleated cells count and viability assay were done on all the samples. Transmissible disease testing for Human Immunodeficiency Virus (HIV-1and 2), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) and syphilis were also performed. In the processing of samples, which included the determination of cord blood volumes, the determination of initial level of total nucleated cells (TNC) and mononuclear cells (MNC) before centrifugation was done. The UCB product was mixed with HES containing solution (6% HES in 0.9 NaCl) in a 5: 1 ratio and centrifuged in a Cryofuge 6000i (Heraeus-Kendro, Hanau, Germany) at 1200 x g for 10 min. The WBC-rich plasma was expressed in a separate bag and again centrifuged at 2500 x g for 10 min. The WBC poor plasma was expressed and discarded. The remaining suspension of mononuclear cells was left whose counts were recorded. The complete process was performed in a closed system with the use of a sterile connecting device (Terumo TSCD, SC-201 AH, Leuven, Belgium).

C Assessment of Total nucleated count, $CD34^+$ and viability test

The samples were analyzed for $CD34^+$cells concentration and viability of mononuclear cell on baseline period, one month and six month. The total nucleated cells count was done before RBC depletion by automated cell counter (Sysmex KX-21, Japan). $CD34^+$ cells concentration was done by flowcytometery and viability of mononuclear cells were done by trypan blue dye exclusion test.

D Cryopreservation

Dimethyl sulfoxide (DMSO) (Merck Limited, Mumbai) was used at a final concentration of 10% (vol/vol). The required volume of sterile, chilled DMSO solution was added to the blood bag over the course of 15 min by using a syringe pump and an orbital mixer to assure smooth but vigorous mixing. In these experiments, UCB processed units were mixed with either 20% DMSO in saline or 50% DMSO in 5% (wt/vol) Dextran 40 (Mr 35,000-50,000) (USB Corporation Cleveland, OH,USA). Final volumes of UCB units with DMSO was a uniform 25 ml. Cryoprotectant UCB units were kept cold with wet ice throughout the addition. When the concentration of DMSO reached 10%, cell suspensions were transferred to -20^0C for 5-10 hours. Subsequently it was transferred to -40^0C for 4 hours and then stored at $-80\ ^0$C for six month.

E Thawing and Washing

Umbilical cord blood units were taken out of the -80^0C and immersed in a 37^0C water bath. After inspection, 10 % dextran 40 was slowly added followed by 5 percent human albumin and the bag was left to equilibrate for 5 minutes. The product was transferred to a transfer bag and centrifuged at 400 x *g* for 15 minutes at 4^0C. Approximately three-fourths of the wash supernatant was expressed and transferred in a second transfer bag. The wash supernatant was centrifuged at 800 x *g* for 15 minutes at 4^0C. Again, three-fourths of the wash supernatant was expressed, and the cell pellets obtained from the two centrifugation steps were combined in one bag. The combined cell pellets were resuspended in 10 percent dextran and 5 percent human albumin.

Statistical Analysis Data collected was entered in

Microsoft excel and checked for any inconsistency. Data Descriptive studies were used for means ± standard deviation. The mean and standard deviation were calculated at baseline and six months for CD34+ cells concentration and viability of mononuclear cells count. The level of statistically significance was set at 0.01 two sided for Pearson correlation ($p<0.01$).

RESULTS

Total 420 UCB samples were analyzed, concerning newborn sex distribution, 310 infants (73.8%) were males and 110 (26.1%) were females. The median gestational age was 38 wks (mean 36.57 ±1.62 wks, range 31 – 41). 290 (69.04%) of cord blood donor

TABLE 1- Characteristics of the study subjects by study site (n=420)

Characteristics	Mean± SD or N (%)
Gender	
Male	310(73.8%)
Female	110 (26.1%)
Gestational duration	36.57±1.62, (range 31-41)
Birth weight	
≤ 2500	190 (45.23%)
> 2500	230 (54.76%)
Mode of delivery	
Normal vaginal delivery	290 (69.04%)
Caesarian delivery	130 (30.9%)

were normal vaginal deliveries and 130 (30.9%) have caesarian deliveries (Table-1) .

CD34+ cells concentration-

In our study we found the mean CD34+ cells concentration was 1.99±0.82%, the minimum CD34+ cells concentration was 0.4% and the maximum CD34+ cells concentration was 4.5% (Table-2) on baseline period. After six month of cryopreservation the mean of the CD34+ cells concentration was 1.14±0.85%, the minimum CD34+ cells concentration was 0.2% and the maximum CD34+ cells concentration was 3.8% (Table-3). This loss was 40.2%.The mean of CD34+ cells concentration in normal vaginal deliveries was 1.005± 0.82%, (range 0.02-2.98) and the mean of CD34+ cells concentration in caesarian deliveries was 3.01±0.92% (range 0.12-4.08) (Table-4). The mean of CD34+ cells concentration was higher in caesarian deliveries than in normal vaginal deliveries ($p<0.01$).

Viability of mononuclear cells count-

In our study the mean of the viability of mononuclear cells was 81.80 ±3.73% (range 76-91) on baseline period (Table-2). The viability of mononuclear cells count at six month was 64.24± 2.70% (range 61-70, Table-3). The viability loss of mononuclear cells from baseline to six month was 20.9%.

DISCUSSION

Allogenic HSC transplantation derived either from bone marrow or UCB has been successfully used in the treatment of thousands of patients with high risk haematological disorders. The principal limitations of allogenic HSC transplantation are the lack of suitable HLA matched donors and complication of graft versus host disease. Although there are currently more than 1.5 million HLA - A, B and DR typed marrow donors registered in marrow donor registries worldwide, 50% of all patients requiring transplant therapy are still unable to find a suitably matched donor[8]. To alleviate a shortage of suitable donors and reduce the length of the bone marrow donor search process, Rubinstein *et al.* (New York) initiated Placental Blood Banking Programs almost simultaneously in 1993[9].Approximately 10,000 HLA-A,B, and DR typed UCB HSC have been collected, tested and cryopreserved for clinical use in transplantation worldwide. This study was prompted by the demonstration that UCB can be used as a source of HSC for allogenic transplantation. UCB is abundantly available and easy to collect, and frozen cord blood is immediately available for transplantation. When establishing large cord blood banks, it seems possible to balance common and uncommon HLA types, thus including minorities who are poorly represented within registries of bone marrow donors in adults[10]. In the unrelated cord blood transplant setting, despite the higher HLA permissiveness, a large number of stored units are required and, consequently, the development of cord blood banks is necessary. The New York Blood Center has been a pioneer in this field. In 1994 Kurtzberg *et al.* reported the first two unrelated cord blood transplants, and to date more than 700 related and unrelated cord blood transplants have been performed[11]. Successfully hematopoietic and immunological engraftment can occur when UCB is the source of stem cells, even in cases of HLA antigen disparity between donor and recipient[12]. As a result of these transplants more cord blood banks are being set up in Europe (London, Milan, Düsseldorf etc) as part of the European Cord Blood Bank Project (Eurocord), with more than 40000 units currently stored all over the world[13]. A number of different procedures have been proposed for UCB collections, including open systems in which cord blood is collected by gravity in bottles or plastic bottles or closed systems in which modified blood collections are used.

In our study that we have used closed system that allows an average volume of cord blood collection was 126.72 ± 27.68 ml (range 60-165) and the mean of the total nucleated cells count was 11.36± 4.34 cells/µl, (range 0.67-16.68). Another study shows

TABLE 2- CD34+ cells concentration and viability of mononuclear cells at one month

Characteristics	Mean±SD	Median	Range	Minimum	Maximum
CD34+ Cells concentration(%)	1.14 ± 0.85	1.13	3.6	0.2	3.8
Viability of mononuclear cells (%)	64.24± 2.70	65.00	9	61	70

TABLE 3- CD34+ cells concentration and viability of mononuclear cells at six months

Characteristics	Mean±SD	Median	Range	Minimum	Maximum
CD34+ Cells concentration(%)	1.99 ± 0.82	1.98	4.1	0.4	4.5
Viability of mononuclear cells (%)	81.80 ± 3.73	81.00	15	76	91

TABLE 4- Descriptive Statistics of the cord blood derived CD34+ cells concentration in different group

Characteristics	CD34+ cells concentration (%)				
Mode of delivery	Mean ± SD	Median	Range	Minimum	Maximum
Normal vaginal delivery	1.005 ±0.82	1.035	2.96	0.02	2.98
Caesarian delivery	3.01± 0.92	3.15	3.96	0.12	4.08

that the closed system allows an average collection of 101.33 ml (range, 65-140ml) of cord blood[10] and they also found that average nucleated cells count/ml of cord blood was 13.97x10 [7] with a range of 4.8 to 27.2x10[7]. The amount of fetal blood remaining in the placenta and the umbilical cord after clamping and dissection depends on several factors. The technique of umbilical cord blood collection varies between different cord blood banks[14]. In our study umbilical cord blood was collected before placental delivery. While Reboredo *et al.* reported that the cord blood was collected before and after the placenta delivery. The collection from the placenta in utero is easy and does not disturb the natural course of birth or the postpartum period[15]. Several centers have used separation methods for umbilical cord blood prior to cryopreservation, and good recoveries have been obtained following HES sedimentation[16], 3% gelatin sedimentation[17]. Apart from the methods described by Sousa *et al.* (1997) the manipulations are performed in open systems, incompatible with normal blood banking procedures[18]. When the method does not involve a closed bag system of separate ion, the risk of microbial contamination is increased. In the present study we have used separation methods for UCB prior to cryopresevation and RBC depletion was done by using HES sedimentation. In comparison with other methods of sedimentation, HES 6% in NaCl does not require any laboratory preparation since it is commercially available and it can be used in a closed system, thus impeding possible microbial contamination during handling. It is frequently used in surgery for volume replacement[19] and is licensed in many countries for RBC removal from bone marrow to be used for transplantation[20]. The success of UCB cells transplantation is largely related to the number of TNC and CD34+ cells. The quality of Cord blood unit depends on its content in total nucleated cells (TNC), colony forming cells (CFC) and CD34+ cells[21]. Some authors published a study in which they found that $CD34^+$ cell concentration decreased after thawing and increased after washing, although the differences we observed were not significant. The recovery of $CD34^+$ cells was 97 percent (95% CI, 50.1%-143.1%) post thaw and 148.9 percent (95% CI, 112.8%-185%) post wash [22]. In our study the mean of the CD34+ cells concentration was 1.99 ± 0.82%, (range 0.4-4.5) before cryopresevation. After thawing and washing at six month the mean of the CD34+ cells concentration was 1.14 ± 0.85 % (range 0.02-3.8). The loss of CD34+ cells from baseline to one month was 40.2%. Some authors shows that viability, when assessed by AO/PI staining, decreased after thawing, from 97 percent (95% CI, 95%-99%) pre freeze to 62 percent (95% CI, 54%-69%) post thaw ($p < 0.001$). There was no significant impact of washing on viability[23].In our study viability of mononuclear cell was assessed by Trypan blue

dye exclusion test and we found that the mean viability of mononuclear cell on baseline period was 81.80 ± 3.73% (range76-91). After crayopreservation of six month the mean viability of mononuclear cells was 64.24± 2.70 The loss from baseline to six month was found to be 20.9%.Furthermore, a number of factors have been described that may influence the total volume collected, quantification of UCB CD34+ cells, and TNC and that may account for the variations in the reported results. Some reports have shown that cesarean delivery provides collection of a higher volume of blood increasing the absolute value of CD34+ cells[24]. Previous studies reported that the mode of delivery has no impact on CB yield[25]. Our finding shows that a significantly higher cord blood derived CD34+ cells concentration was found in caesarian delivery than in vaginal delivery ($p<0.01$). UCB cryopreservation as unmanipulated blood results in high costs for large-scale UCB banking. Depleting cord blood stem cells of RBC avoids exposure to incompatible red cell antigens, minimizes the infusion of free hemoglobin and limits the volume and the amount of DMSO needed for cryo preservation.

CONCLUSION

We describe a simple and effective system for UCB processing in a triple bag, which removes 85.8 □ 5.8% of RBC and allows the storage of UCB units in small volumes, thus reducing the cost of large-scale UCB banking. In this study we have used separation methods for UCB prior to cryopreservation, and good recoveries have been obtained following HES sedimentation (6% HES in 0.9 NaCl). The method demonstrates that UCB units can be routinely processed in a closed system that also achieves a significant reduction in storage needs and related costs, while maintaining quantity and quality of the haematopoietic stem cells.

ACKNOWLEDGEMENT

I would like to thanks all the junior resident and staff nurses of the obstetrics and gynaecology department for collection of umbilical cord blood. This research work was supported by a Grant from the Council of Science and Technology, Uttar Pradesh, Lucknow, India.

REFERENCES

1. Gluckman E, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, Devergie A, et al. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical- cord blood from an HLA-identical sibling, N Engl J Med1989;21:1174-1178
2. Canabarro R., Sporleder H., Gomes T., Zanatta G., Scribel L., Freitas F., Neumann J., Pranker P. Immunophenotypic evaluation, and physiological and laboratory correlations of hematopoietic stem cells from umbilical cord blood,BIOCELL 2007;31(3): 397-403
3. Valeri CR, Pivacek LE. Effects of the temperature, the duration of frozen storage, and the freezing container on in vitro measurements in human peripheral blood mononuclear cells, Transfusion 1996;36:303–308
4. Sanz MA. Cord-blood transplantation in patients with leukemia—a real alternative for adults, N Engl J Med 2004;351:2328–2330.
5. Rubinstein P, Dobrila L, . Rosenfied R.E., Adamson J. W., Migllaccio G., Migliaccio A. R., Taylor P. E. and Stevens C. E. Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution, Medical Sciences1995;92:10119-10122.
6. Broxmeyer, H. E., Douglas, G. W., Hangoc, G., Cooper, S., Bard, J., English, D., Arny, M., Thomas, L. and Boyse, E. A. Proc. Natl. Acad. Sci1989;86:3828-3832.
7. Harris, D. T., Schumacher, M. J., Rychlik, S., Booth, A., Acevedo, A., Rubinstein, P., Bard, J. and Boyse, E. A. Bone Marrow Transplant1994;13,135-143
8. Confer DL. Unrelated marrow donor registries. Curr Opin Hematol 1997;4:408-13
9. Rubinstein P, Adamson JW and Stevens C. The placental umbilical cord blood program of the New York Blood Center. A progress report, Ann NY Acad Sci 1999;30:328-336
10. Col PS Dhot, Maj D Sirohi and Brig GLN Swamy. Collection, Separation, Enumeration and Cryopreservation of Umbilical Cord Blood Haematopoietic Stem Cells - An Experimental Study, MJAFI 2003;59:298-301
11. Kurtzberg J, Graham ML, Casey J et al. The use of umbilical cord blood in mismatched related and unrelated hemopoietic stem cell transplantation, Blood Cells 1994;20: 275-284
12. Rubinstein P, Carrier C, Scaradavou A et al. Initial results of the placental umbilical cord blood program for unrelated bone marrow reconstitution, New Engl J Med 1998;339:1565-1577
13. Rebulla P, Lecchi L, Porretti L et al. Practical placental blood banking, Transfus Med Rev 1999;13:205–226
14. Bertolini F, Battaglia M, Zibera C et al. A new method for the placental/cord blood processing in the collection bag. I. Analysis of factors involved in red blood cell removal, Bone Marrow Transplant 1996;18:783–786
15. N M-Reboredo, A Dıaz, A Castro and RG Villaescusa. Collection, processing and cryopreservation of umbilical cord blood for

unrelated transplantation, Bone Marrow Transplantation 2000;26:1263–1270.
16. Bertolini F, Battaglia M, Zibera C et al. A new method for the placental/cord blood processing in the collection bag. I.Analysis of factors involved in red blood cell removal, Bone Marrow Transplant1996;18:783–78
17. Nagler A, Peacock M, Tantoco M et al. Separation of hematopoietic progenitor cells from human umbilical cord blood, J Hematother1993; 2:243–245.
18. Sousa T, de Sousa ME, Godinho MI et al. Umbilical cord blood processing: volume reduction and recovery of CD34 cells, Bone Marrow Transplant 1997;19:311–313
19. Stehling LC. Volume replacement in the surgical patient. In: Rossi EC, Simon TL, Moss GS (eds). Principles of Transfusion Medicine. Williams and Wilkins: Baltimore, 1991, pp 429–34
20. Walker RH. Technical Manual of the American Association of Blood Banks. AABB: Arlington 1990, pp 646–647
21. Wagner JE, Kurtzberg J. Cord blood stem cells, CurrOpin Hematol1997;4:413–418
22. Vincent Laroche, David H. McKenna, Gary Moroff, Therese Schierman, Diane Kadidlo, and Jeffrey McCullough. Cell loss and recovery in umbilical cord blood processing: a comparison of postthaw and postwash samples, Transfusion 2005;45:190
23. Raquel Canabarro, Heloísa Sporleder, Thaís Gomes, Geancarlo Zanatta, Letícia Scribe and Fernando Freitas. Immunophenotypic evaluation, and physiological and laboratory correlations of hematopoietic stem cells from umbilical cord blood, BIOCELL 2007;31(3):397-03
24. Yamada T, Okamoto Y, Kasamatsu H, Horie Y, Yamashita N, Matsumoto K. Factors affecting the volume of umbilical cord blood collections, Acta Obstet Gynecol Scand2000;79, 830-833
25. Lim FT, van Winsen L, Willemze R, Kanhai HH and Falkenburg JH. Influence of delivery on numbers of leukocytes, leukocyte subpopulations, and hematopoietic progenitor cells in human umbilical cord blood, Blood Cells1994;20: 547–559.