

# Collection, Separation, Enumeration and Cryopreservation of Umbilical Cord Blood Haematopoietic Stem Cells - An Experimental Study

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## Abstract

Cord blood as a source of haematopoietic stem cells (HSC) has several advantages as it is easily available, involves non-invasive collection procedure and is better tolerated across the HLA barrier. Since the first cord blood transplant in 1988, over 1000 cord blood HSC transplants have been done world wide. The present study was carried out for collection, separation, enumeration and cryopreservation of cord blood HSC. 30 samples of cord blood HSC were collected after delivery of infant prior to expulsion of placenta. The average cord blood volume collected was 101.33ml. Mononuclear cell count ranged from 7.36 to 25.6 X 10<sup>7</sup> ml. Viability count of mononuclear cells was 98.4%. After 6 months of cryopreservation, the viability count on revival was over 82.1%.

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Key Words : Cord blood; Cryopreservation; Haematopoietic stem cell; Viability

## Introduction

Haematopoietic Stem Cell (HSC) transplantation has come of age. Over the last four decades, this therapy has been used to correct a variety of bone marrow failure states, inborn errors of metabolism, congenital immunodeficiency states, haematologic malignancies and even solid tumours [1]. This mode of therapy generally involves the ablation of the patient's bone marrow by chemotherapy and/or irradiation and its replacement by pluripotent HSC from a voluntary donor. The major limitation of using HLA matched related sibling donors in HSC transplantation has been that only 30% to 40% of potential recipients in need of such therapy have a HLA matched related family donor [2]. Hence, the search for an alternate source of HSC has led to the development of HSC transplantation (HSCT) protocols from tissues like foetal liver, umbilical cord and mobilised peripheral blood [1]. The first cord blood transplant was performed on a child with Fanconi's anaemia in 1998 [2]. Since then over 1000 cord blood haematopoietic stem cell transplants have been done worldwide [1]. Cord blood as a source of haematopoietic stem cells has several advantages as it is easily available, involves non-invasive collection procedure and is better tolerated across the HLA barrier with lower frequency of *Cytomegalovirus* infection [1].

The present study was carried out with a view to establish a HSC Bank in the department of Transfusion Medicine, Armed Forces Medical College, Pune. The aims were firstly, to develop techniques for collection of cord blood maximising the blood volume without compromising on the sterility and quality of stem cell

yield. Secondly, to separate and enumerate the HSC in the cord blood and study the effect of cryopreservation on stem cell count and viability.

## Material and Methods

30 full term pregnant women undergoing full term vaginal deliveries were randomly selected at the time of admission for delivery. All volunteers were asked to sign informed consent forms prior to collection of cord blood. Women with known history of hepatitis, infectious diseases, diabetes mellitus, severe hypertension, abortions or bad obstetric history were excluded from the study. Umbilical cord blood (UCB) samples were obtained from normal full term vaginal deliveries as per the standard method [4,5]. The collections were made after delivery of the infant and ligation of the cord, prior to the expulsion of the placenta. The UCB was collected while the placenta was still in utero. Using strict aseptic techniques the umbilical vein was cleansed with alcohol followed by betadine. The umbilical vein was pierced and UCB collected in the standard blood collection bags containing citrate phosphate dextrose adenine-1 (CPDA-1) anticoagulant (approximately 25 ml) since total collection was approximately 100-120 ml. During collection the blood bag was shaken gently, so that the anticoagulant freely mixed with UCB. The blood bag with anticoagulant was weighed before and after collection blood to find out the volume collected. The details of the delivery and the new born were recorded and documentation carried out meticulously. UCB was transported immediately from maternity units carefully in a plastic box at 4°C without delay. UCB units were stored at 4°C and processed within 24 hours. Laminar flow cabinet was cleansed with 70% ethanol and volume of UCB was measured. A volume of 5 ml of UCB was kept in aliquots for routine testing of blood group, sterility and tests for HIV-1 and 2, HBsAg, HCV and Syphilis. Aerobic bacterial cultures

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were evaluated in BHI broth before and after cryopreservation to assess the sterility during collection and processing of samples. Removal of red blood cells was done by hydroxy ethyl starch (HES) sedimentation. Mononuclear cell (MNC) counts were done by using Turk's solution in Neubauer chamber. Viability count of MNC was done using Trypan blue dye exclusion test. UCB mononuclear cells were cryopreserved using the cryoprotectant dimethyl sulphoxide (DMSO) at a final concentration of 10%. The HSC in blood bags were stored at  $-85^{\circ}\text{C}$  [6,7,8]. Aliquot samples were taken for ABO and Rh grouping, sterility testing and revival studies.

## Results

30 samples of UCB were collected and cryopreserved. Information regarding the antenatal history, delivery, newborn, placental weight and any complications were noted (Table 1). 2 samples were HBsAg positive on testing and were discarded. 2 samples were found to be contaminated by bacteria (aerobic spore bearers) and were discarded. Contaminated samples were collected after vaginal deliveries of women reporting rupture of the membrane 24 hours before delivery. No contamination was observed on culture in the post cryopreservation samples on revival after thawing.

**Table 1**  
**Haemoglobin, weight of child, weight of placenta, age of mother and parity**

Sample No	Age years	Parity	Blood group	Hb g/dl	Child M/F	Weight Kg	Placenta gm
1.	19	G2P1	A+	10	M	2.9	400
2.	24	G2P1	B+	10	M	3.5	450
3.	20	G2P1	A+	9.5	M	2.75	350
4.	24	Primi	O+	10.4	F	2.75	500
5.	23	G2P1	A+	8	M	2	500
6.	18	Primi	B+	10.5	M	2.6	400
7.	20	Primi	B+	10	F	3.1	400
8.	24	G2P1	A+	9.5	M	2.7	400
9.	24	Primi	O+	10	M	2.85	450
10.	20	Primi	O+	12.5	M	3	400
11.	24	Primi	B+	11.4	M	2.25	500
12.	26	Primi	O+	11.5	M	2.25	500
13.	20	G2P1	A+	10.5	F	3.75	450
14.	22	Primi	O+	9	F	2.7	500
15.	22	G3P1A1	B+	10	F	2.2	500
16.	25	G2P1	A+	11.5	F	3.1	400
17.	25	Primi	B+	10.5	M	2.75	500
18.	23	G3P2	B+	11	M	2.7	600
19.	24	Primi	A+	10	M	2.75	450
20.	23	G2P1	A+	11.5	F	2.8	500
21.	31	Primi	O+	10.5	F	3.6	500
22.	24	Primi	O+	11.8	F	2.9	500
23.	22	Primi	AB+	10	F	2.45	450
24.	26	G2P1	A-	10	M	2.5	400
25.	20	G2P1	B+	9.5	F	3	450
26.	30	G2P1	O+	12.5	F	2.65	450
27.	22	G2P1	O+	9	M	2.4	500
28.	20	Primi	A+	12	F	2.2	450
29.	24	G2P1	O+	11	F	2.8	450
30.	28	G2P1	B+	11.5	M	3.2	500

The average volume of blood collected was 101.33 ml with a range of 65 to 140 ml (Table 2 and Fig. 1). The volume of cord blood collected was analyzed for correlation with the haemoglobin of mother, birth weight of child, sex of the child and placental weight (Table 1 and Fig. 2). The coefficient of correlation in this was -0.1, 0.1782, 0.12 and 0.16 respectively, showing thereby that these parameters do not effect the volume of blood collected. The effect of volume of cord blood collected on nucleated cell count was analysed using single tailed paired 't' test. The 't' was 1.373 and was found to be statistically significant;  $p < 0.10$ .

The average nucleated cell count/ml of cord blood was  $13.97 \times 10^7$  with a range of 4.8 to  $27.2 \times 10^7$  and an average viability count of 98.4% prior to preservation of the sample (Table 2). One ml of each sample was revived after a variable period of time but within 6 months of preserving the sample. The average nucleated cell count/ml of the revived sample was  $0.7116 \times 10^7$  (i.e 71.16%) with a range of 58% to 80% and average viability count of 82.1% (Table 3 and 4). This indicated an average loss of 28.84% of progenitor cells. The cell

**Table 2**  
**Volume of cord blood collected total cell count, nucleated cell count (NCC), differential cell count and viability count**

Sample No.	Volume collected (ml)	Total cell count $\times 10^7$	NCC/ml $\times 10^7$	Viability count %	Differential count %
1.	80	79	17.6	95	84
2.	140	66	11	100	80
3.	110	44	8.96	98	78
4.	110	37	12.48	98	88
5.	80	40	7.96	99	80
6.	65	16	8.8	96	70
7.	110	90	17.6	99	90
8.	120	75	14.72	99	88
9.	110	91	19.2	99	88
10.	85	24	7.36	98	80
11.	100	55	12.8	98	82
12.	90	35	8	99	76
13.	80	62	13.12	98	84
14.	120	115	24	98	95
15.	95	14	12.8	99	90
16.	105	44	8.8	99	78
17.	120	58	8.4	99	80
18.	100	54.4	27.2	100	96
19.	85	64	10.4	99	80
20.	105	68	13.6	98	86
21.	110	115.2	19.2	99	92
22.	85	61.7	13.72	99	90
23.	120	128	25.6	98	94
24.	85	33.6	4.8	97	80
25.	100	70.4	8.8	99	82
26.	100	113.6	22.72	99	95
27.	100	31.68	5.76	98	76
28.	110	84	14.6	99	82
29.	120	68.4	16.7	98	84
30.	100	112.4	22.4	99	90
Average	101.33	65.01	13.97	98.43	

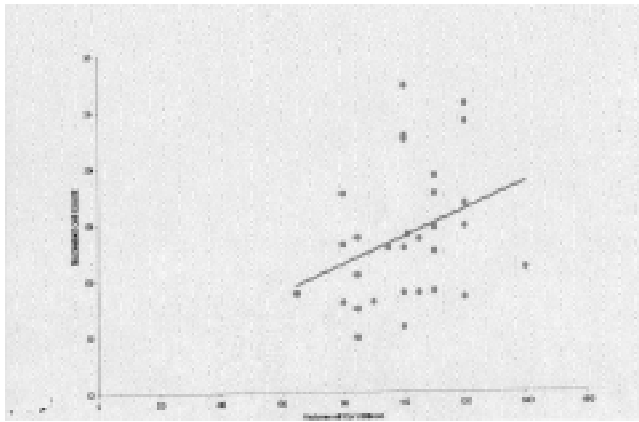


Fig. 1 : Scatter diagram depicting the nucleated cell count against the volume of cord blood

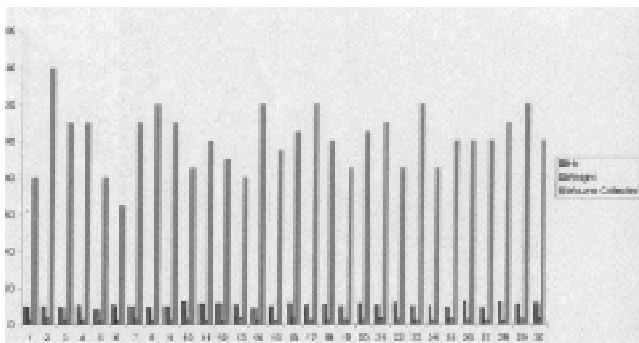


Fig. 2 : Histogram depicting the haemoglobin, weight of the child and volume of cord blood collected for the 30 samples

recovery after cryopreservation was evaluated for any effect by the number of days of freezing. The correlation co-efficient in this study was found to be  $-0.24817$ , thereby implying a very insignificant negative correlation. Similarly, the effect on cell viability showed a negative correlation co-efficient of  $0.13948$ .

## 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 [9]. To alleviate a shortage of suitable donors and reduce the length of the bone marrow donor search process, Rubinstein et al (New York), Bertolini et al (Milano) and Wernet et al (Dusseldorf) initiated Placental Blood Banking Programs almost simultaneously in 1993 [10].

Approximately 10,000 HLA-A,B, and DR typed UCB HSC have been collected, tested and cryopreserved for

Table 3

Volume of cord blood cryopreserved, nucleated cell count, differential cell count and viability count

Sample No	Volume preserved ml	NCC/ml x 10 <sup>7</sup>	Viability count %	Differential count %
1.	79	0.8	94	90
2.	66	0.75	90	84
3.	44	0.7	80	84
4.	37	0.7	84	90
5.	40	0.66	76	86
6.	16	0.66	74	82
7.	90	0.74	82	94
8.	75	0.68	78	90
9.	91	0.68	76	92
10.	24	0.6	72	86
11.	55	0.64	74	88
12.	35	0.58	70	86
13.	62	0.74	90	90
14.	115	0.76	92	96
15.	13.8	0.74	90	92
16.	44	0.7	88	86
17.	58	0.68	78	88
18.	54	0.78	80	96
19.	40	0.68	75	88
20.	60	0.68	80	92
21.	76	0.76	80	94
22.	60	0.72	76	94
23.	128	0.78	78	96
24.	32	0.68	74	88
25.	70	0.7	90	86
26.	110	0.78	94	96
27.	30	0.68	80	84
28.	84	0.74	90	86
29.	68.4	0.76	88	90
30.	112.4	0.8	90	94

Table 4

Comparative pre-cryo preservation and post-cryo preservation TNCC, mononuclear cell count, and viability

	Cell counts before and after preservation	
	Pre-preservation	Post-preservation
Nucleated cell count (per ml)	13.97 x 10 <sup>7</sup> (range 4.8 to 27.2 x 10 <sup>7</sup> )	0.7116 x 10 <sup>7</sup> (71.16% range 58% to 80%)
Mononuclear cell count (per ml)	12.51 x 10 <sup>7</sup>	0.63 x 10 <sup>7</sup>
Viability	98.4%	82.1%

clinical use in transplantation worldwide. The present study was carried out to establish a HSC Bank in the Dept of Transfusion Medicine, Armed Forces Medical College, Pune. 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. Thus, UCB is a novel and unique source of transplantable stem cells that can be used for treatment of diseases that normally require bone marrow transplantation. UCB, which is normally discarded, can be readily collected without danger to the mother or infant and the technical feasibility of using umbilical cord blood for transplantation has been established. UCB from a single birth contains a number of stem cells within the range required for autologous and HLA-compatible allogeneic transplantation in both infants and adults and provides comparable number of stem cells to that found in bone marrow.

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. Data collected in the present study indicate that the closed system allows an average collection of 101.33 ml of cord blood (Median range 99 ml) [11].

As our experiments have demonstrated, collection of increased volumes of cord blood results in increased number of recovered cells, the ability to routinely harvest large amounts of cord blood should result in more cells available for use in transplantation. To make the banking of cord blood possible on a large scale, it would be preferable to store separated cord blood samples rather than large blood bags. It was demonstrated that optimum number of mononuclear cells could be routinely obtained by HES sedimentation [11].

In the present study, the haemoglobin of the mother, birth weight of child, sex of child and placental weight did not affect the volume of cord blood sample collected or the nucleated cell counts of the sample which compares well with other studies. Increased volume of cord blood sample resulted in increased recovery of nucleated cell counts, an important surrogate marker of HSC and in turn of the transplant potential of the cord blood sample [11,4].

It appeared that cell losses did not occur in frozen samples but were due to variables in the freezing and thawing procedures that were not under direct control. However, it should be noted that it was routinely possible to obtain cell recoveries of more than 82% viability and cell recoveries of 71.16% on an average, which though slightly lower than those in the previous studies, were comparable [1].

Since the first cord blood transplant performed in 1988 [1], cord blood transplantation is increasingly used as source of HSC. The number of cord blood transplants

reported to Eurocord Registry is 500. More than 500 patients have received cord blood transplants via the New York Cord Blood Bank [1].

Placental-UCB is now considered to be a useful source of HSC for transplantation. Multiple reports clearly document that donor-derived multilineage haematopoiesis after UCB transplantation is sustained with the longest follow-up of nearly 10 years. With this new source of HSC, we may be able to identify a donor for any given patient who needs a transplant. However, carefully planned and controlled long term studies are still needed for donor selection strategies, collection procedures, processing and banking methodologies

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