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

B. H. Mutlak, I. M. Mnati
Departement of Biology,College of Education Ibn Al-Haitham, University of Baghdad

Abstract

Umbilical cord blood (UCB) contains hematopoietic and mesenchymal stem cells(HSCs,MSCs) that have proven useful clinically to reconstitute the hematopoietic system in children and some adults .

Fifteen cord blood samples were collected from placenta of newly delivered women in Al-Kadhemia hospital in Baghdad for normal vaginal delivery.

Mono nucleated cells (MNCs) were isolated by using density gradient centrifugation and the MNCs count and viability were determinated by trypan blue.MNCs were cryopreserved using the cryoprotectant solution of 10% concentration of dimethyl sulfoxid (DMSO)using slow cooling and rapid thaw.

The aim of the present study is to collect, separate and enumerate the stem cells in cord blood(CB) and study the effect of cryopreservation on stem cells count and viability.

The results of the present study showed that the average cord blood volume collected was 81.66 ml. Mononuclear cell count ranged from 5.6-14.6 $x10^6$.Viability count of mononuclear cells was 93.9%.After three months of cryopreservation , the viability count on revival was 73.9%.This study represents an attempt to isolate the stem cells from UCB. The results of the present study confirmed that UCB provides a great source of stem cells for using in medical applications.

Introduction

Transplantation of UCB was successfully performed for the first time in 1989[1], the potential of UCB as a source of HSCs and MSCs for transplantation rapidly became an area of intense clinical and scientific interest [2].

In order to effectively utilize cord blood(CB) clinically ,it must be frozen and banked .The protocols used for this have largely been adapted from those originally designed for bone marrow (BM)stem cells ,and there is no consensus on optimal procedures for CB cells [3] .

Cryopreservation processing that yields consistent recovery of functionally viable cells is crucial for the further successful use of this important cell resource [4] .

To develop a successful cell cryopreservation strategy,the key events inherent to freezing a biological cell must be considered and each potential source of damage should be mitigated if at all possible.A simple way to organize these steps includes:-

A:-Determining the cryoprotectant (CPA)type and concentration .

B:-Determining the cooling and warming rats.

C:-Preparation of cells for use including any washing procedures [4,5] .

The closest consensus considering CB stem cells is presented as 5-10% concentration of dimethyl sulfoxide (DMSO) using slow cooling and rapid thaw[5].

Previous studies have examined factors that influence the viability and recovery of cells obtained from UCB after storage .Shlebak and colleagues [6] observed that liquid storage of CB for 9 h resulted a decline in post thaw recovery of colony forming unit-granulocytes monocytes (CFU-GM).Rogers and colleagues [7] observed that the post thaw viability of CB

decreased when it was stored for 24 h of storage prior to cryopreservation.In contrast ,Beaujean and colleagues observed that peripheral blood (PB)stem cells could be effectively stored for 24 h prior to cryopreservation without significant loss of post –thaw viability [8].The recent series of studies have examined optimal liquid storage conditions and duration for PB, BM and CB. In general ,these studies showed that dilution the product with storage solution improved recovery of mononuclear cells .Furthermore, favorable recovery was observed using storage solutions appropriate for human use [4 ,5].

Thus ,the aim of the present study were to develop technique for collection of CB and to separate ,enumerate the stem cells in the cord blood and study the effect of cryopreservation on stem cells count and viability .This information would be essential in establishing processing guidelines for the processing of CB that ensures optimum recovery and reduces costs associated with the operation of CB processing centers.

Material and Methods

Fifteen UCB samples were obtained freshly from pregnant women undergoing full term vaginal deliveries were randomly selected at the time of admission for delivery from Al-Kadhmia hospital in Baghdad .All volunteers were asked to sign informed consent forms perior to collection of CB .Women with known history of hepatitis , diabetes mellitus ,severe hypertension ,abortions or bad obstetric history were excluded from the study. Umbilical cord blood samples were obtained from normal full term vaginal deliveries as per the standard method [9].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 ,the umbilical vein was pierced and UCB collected in the standard anticoagulant treated blood collection bags .Since total collection was approximately(65-100) ml. The details of the delivery and the new born were recorded ,UCB was transported immediately from maternity units and processed within 6 h .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, Rh grouping and revival studies .

Cord blood was diluted 1:1 with phosphate buffer saline (PBS) then carefully over laid on Ficoll-paque at a ratio of 3:1 in 10 ml sterile conical tubes .The specimens were centrifuged on a cooling centrifuge for 20 min at 2000 rpm at $4C^0$.After density gradient centrifugation ,the resulting mononucleated cells (MNCs) were retrieved from buffy coat layer by pipeting and washing [1-2]times with PBS .Mononucleated cells counts and viability were done by using trypan blue dye exclusion test .UCB mononuclear cells were cryopreserved using the cryoprotectant solution which composed of (10% dimethyl sulfoxide (DMSO) +40% minimum essential medium (MEM)+50% fetal calf serum (FCS).The cells dispensed into sterile freezing ampoules (plastic cryo vials)with 1ml per ampoules.

The ampoules (which had been labeled with the name of the cells and the date of freezing) were sealed and placed in $+4C^0$ for 12 h then $-4C^0$ for 12 h . The frozen vials were transferred into $-20\ C^0$ for 24h then $-70\ C^0$ for 24 h and in $-196C^0$ in liquid nitrogen tank for long –term storage [10]. After a minimum of three months of storage in liquid nitrogen , one ampoule of each sample was thawed in a $37\ C^0$ water path for viability assessment .The resulting solutions containing the thawed cells were centrifuged in a cooling centrifuge .After removal of the supernatant ,cells were re-diluted for analysis.

Results

15 samples of UCB were collected and cryopreserved. Information regarding the antenatal history ,delivery ,newborn and any complications were noted in Table (1).

The average volume of CB collected was 81.66 ml with arrange of 65-100 ml Table(2).

The volume of CB collected was analyzed for correlation with the haemoglobin of mother ,birth weight of child ,sex of the child Table (1) and Figure(1).The coefficient of correlation

in this study was -0.78,-0.51 and 0.57 respectively ,showing thereby that these parameters do not effect the volume of CB collected .The effect of volume of CB collected on nucleated cell count Figure (2) was analyzed using single tailed paired (t)test. The (t) was 0.786 and was found to be statistically significant; $p<0.10$.

The average nucleated cell count /ml of CB was $9.88x10^6$ and with a range of 5.6-14.6 $x10^6$ and an average viability count of 93.9% prior to preservation of the sample Table (2).

One ml of each sample was revived after a variable period of time but within three months of preserving the sample. The average nucleated cell count /ml of the revived sample was 0.68 $x10^6$ (i.e 68%) with a range of 42% to 99% and average viability count of 73.9% of progenitor cells Table (3). This indicated an average loss of 20% of progenitor cells Table (4) and Figure (3).

Discussion

Until recently ,blood that remained in the umbilical cord and placenta after delivary was routinely discarded .Human UCB is now considered a valuable source for stem cells ,this blood is known to contain both HSCs and MSCs [11] .Since the first cord blood transplant performed in 1989 [1],there has been a substantial increase in the clinical use and research investigation of UCB in haemopoietic transplantation and regenerative medicine [12].

This study was prompted by the demonstration that UCB can be used as a source of stem cell for transplantation .UCB is abundantly available and easy to collect ,and frozen ,CB is immediately available for transplantation .UCB is normally discarded ,it can be readily collected without danger to the mother or infant and technical feasibility of using UCB for transplantation has been established [13].

A number of different procedures have been proposed for UCB collections ,including open systems in which CB is collected by gravity in bottles or closed systems in which modified blood collections are used [14] .Data collected in the present study indicated that the closed system allows an average collection of 81.66 ml of CB. As our experiments have demonstrated that collection of increased volume of CB results in increased number of recovered cells ,the ability to routinely harvest large amounts of CB should result in more cells available for use in transplantation [13].

In the present study ,the haemoglobin of the mother ,birth weight of child ,six of children did not affect the volume of CB sample collected or the NCCs of the sample which compares well with other studies . Increased volume of CB sample resulted in increased recovery of NCCs ,an important surrogate marker of HSCs and in turn of the transplant potential of the CB sample [14,15].

It appeared that cell losses did not occur in frozen samples but were due to variables in 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 73.9% viability and cell recoveries of 68% on an average ,which though slightly similar to those in the previous studies ,were comparable [16].

References

1- Broxmeyer,H.E.;Douglas ,G.W. ;Hangoc,G.and Cooper,S .(1989) . Proc .Natl.Acad.Sci.USA,86:3828-3832.

2-Hows,J.M.(2001).J.Clin.Pathol.,54:428-434.

3-Hubel,A.;Carlquist,D.;Clay,M.and Cullough,J.(2003). Cytother. 5 (5) :370-376.

4-Berz,D.;Cormack,M.and Peter ,J.(2007).Am.J.Hematol.,110:645-650.

5-Woods,E.J.;Pollok,K.E.;Byers,M.and Perry,B.C.(2007). Transfus .Med. Hemother. 34:276-285.

6-Shlebak,A.A.;Marley,S.B.and Roberts,I.A.(1999).Bone Marrow Transplant . 23:131-136(abstract).

7-Rogers,I.;Sutherland,D.and Holt,D. (2001).Cytother. 3:269-276.

8-Beaujean,F.;Pico,J.and Norol,F.(1996).j.Hematother. 5:681-686.
9-Rubinstein,P.;Dorila,L.and Rosenfield,R.E.(1995).Proc .Acad.Natl . Sci.USA,1995:10119-10122.
10-Al-Azawi,I.N.(2003).Experimental study on culture of mammalian embryonic stem cells .Ph.D.,thesis ,College of Science ,Al- Mustensiriya Univ.:70-72.
11-Mutlak,B.H.(2007).*In vitro* study of UCB-derived stem cells and their neurogenic differentiation.Ph.D.thesis,College of Education /Ibn –Al- Haitham,Baghdad Univ.:62-88.
12-Moise,K.J.(2005).Obst.Gyne. 106(6):1393-1407.
13-Dhot,C.P.;Sirohi,M.D.and Swamy,B.G.(2003).MJAFI,59:298-301.
14-Cairo,M.S.and Wagner,J.E.(1997).Blood,90(12):4665-4675.
15- Rubinstein,P.(1994).Blood Cells,20:587-600.
16-Shpall,E.;Lima,M.;Jones,R.and Champlin,R.(2007).Blood 110(8):3064-3066.

Table(1):- Age of mother, blood group, haemoglobin , weight of child and sex of child.

Sample No.	Age years	Blood group	Hb g/dl	Child M/F	Weight Kg
1	18	A^+	10	M	3.5
2	27	AB^+	9.5	M	2.6
3	22	O^+	10	F	3.1
4	19	O^+	12.5	M	2.25
5	21	B^+	10.5	F	2.8
6	25	A^+	11.5	M	2.2
7	20	AB^+	10	M	3.2
8	19	O^+	12.5	M	3
9	23	A+	8	F	2.4
10	22	A^+	12	M	2.8
11	25	B^+	11.5	M	2.75
12	26	B^+	10.5	M	2.9
13	18	B^+	12	M	3.2
14	21	A^+	12.5	F	2.7
15	23	O^+	9	F	2.3

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

Sample No.	Volume collected (ml)	NCC\ml $x10^6$	Viability count %
1	80	10.1	94
2	65	8.2	91
3	85	7.4	91
4	90	12.8	95
5	80	8	90
6	70	8.7	95
7	95	10.4	96
8	80	8.8	94
9	90	13.75	96
10	95	14.6	93
11	100	13.8	94
12	75	8.6	93
13	65	5.6	95
14	75	9	96
15	80	8.5	95
Mean	81.66	9.88	93.9

Table(3): Volume of cord blood cryopreserved, nucleated cell count (NCC) ,and viability count

Sample No.	Volume preserved	NCC/ml $x10^6$	Viability count %
1	59	0.99	85
2	44	0.91	82
3	37	0.60	77
4	44	0.57	65
5	60	0.58	72
6	36	0.45	52
7	37	0.54	78
8	40	0.42	67
9	42	0.9	85
10	55	o.8	80
11	53	0.74	78
12	58	0.73	80
13	25	0.4 9	69
14	40	0.69	59
15	50	0.80	79
Mean	45.3	0.68	73.9

Table (4): Comparative pre-cryo preservation and post –cryo preservation nucleated cell count and viability.

	Cell counts before and after preservation	
	Pre-cryopreservation	Post-cryopreservation
Nucleated cell count/ml	$9.88x10^6$(range 5.6 to $14.6x\ 10^6$	$0.68x10^6$ (range 0.42 to $0.99\ x10^6$
Viability	93.9%	73.9%

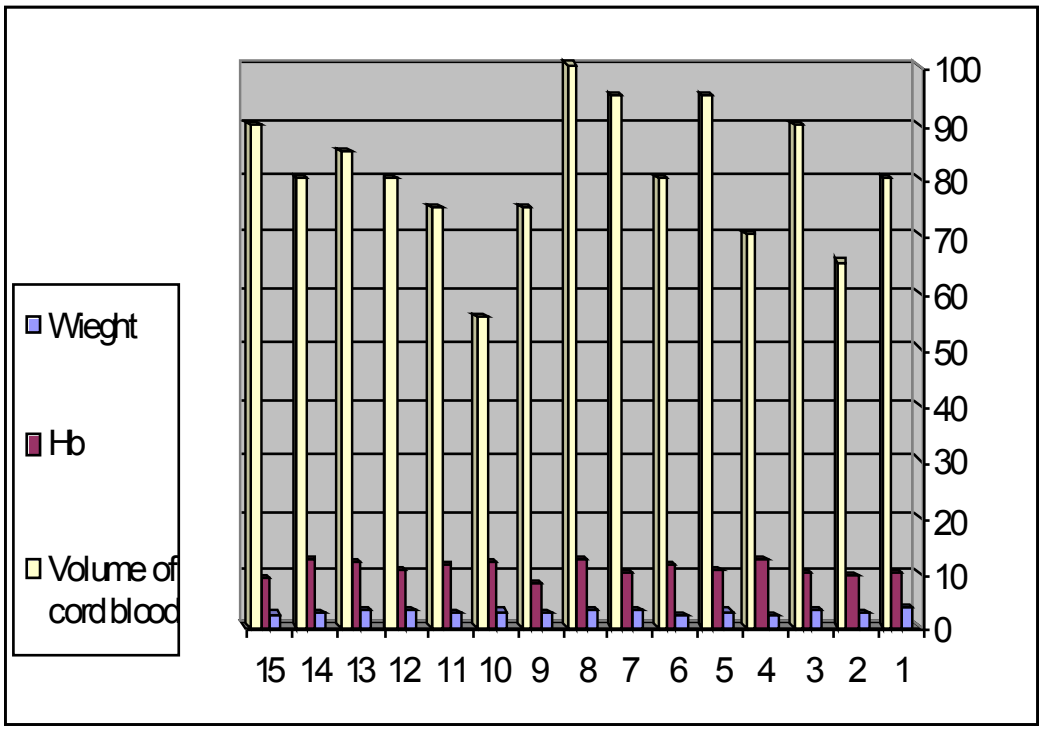

Fig.(1): Histogram depiciting the haemoglobin ,weight of the child and volume of the cord blood collected for the 15 samples

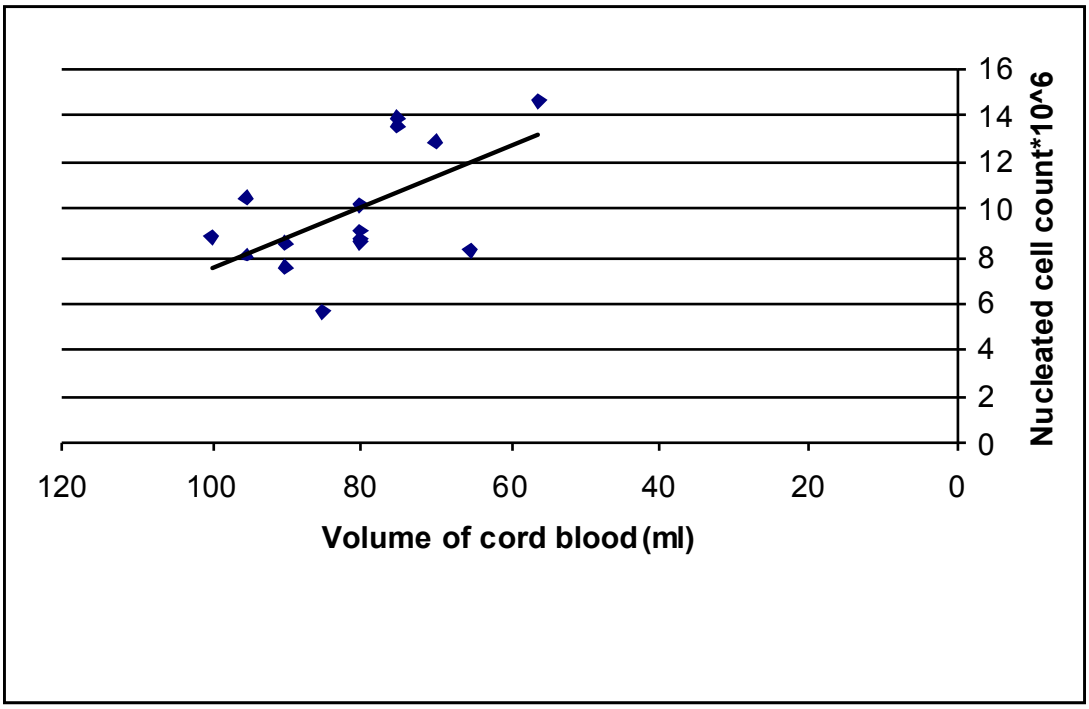

Fig.(2): Scatter diagram depiciting the nucleated cell count against the volume of cord blood

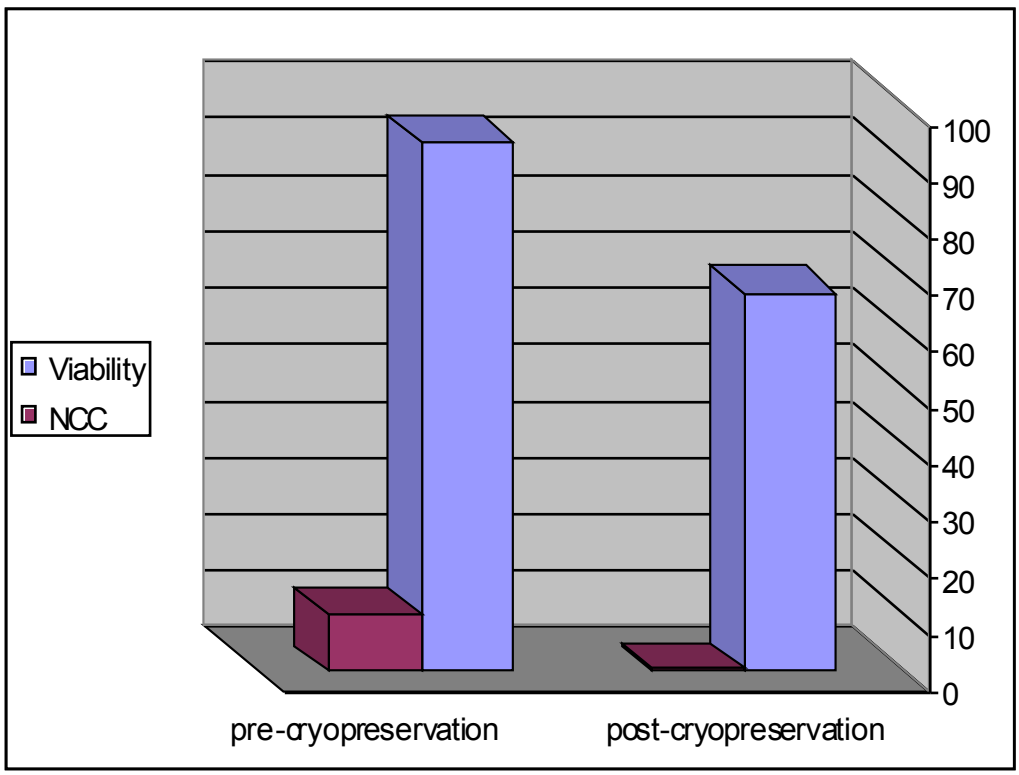

Fig.(3): Histogram depiciting the comparative pre-cryo preservation and post-cryo-preservation nucleated cell count and viability

دراسة تجريبية لجمع وفصل وتعداد والحفظ بالتجميد للخلايا الجذعية المشتقة من دم الحبل السري

بيداء حسين مطلك وانتظار محمد مناتي
قسم علوم الحياة ،كلية التربية –ابن الهيثم،جامعة بغداد

الخلاصة

يحوي دم الحبل السري على الخلايا الجذعية المكونة للدم والخلايا الجذعية اللحُمية التي اثبتت فائدتها من الناحية السريرية لأعادة تكوين النظام المكون للدم لدى الأطفال وبعض البالغين .

تم جمع 15 أنموذجا" لدم الحبل السري من مشيمة الولادات الحديثة للأمهات اللواتي ولدن طبيعياً في مستشفى الكاظمية في بغداد .

عزلت الخلايا الأحادية الأنوية بأستخدام النبذ المركزي المتدرج الكثافي، وتم حددت حيوية واعداد الخلايا بأستخدام صبغة المثيل الأزرق . حفظت الخلايا بأستعمال محلول الحفظ بالتجميد والمكون من 10% (DMSO) وبأستعمال التجميد البطيء ، والأذابة السريعة.

ان هدف الدراسة الحالية هو جمع وفصل الخلايا الجذعية وتعدادها المشتقة من دم الحبل السري ودراسة تأثير الحفظ بالتجميد في حيوية واعداد الخلايا الجذعية .

بينت نتائج الدراسة الحالية إن معدل حجم الدم الذي جمع هو 66.81 مل ،اما اعداد الخلايا الأحادية النواة فقد تراوحت بين $5.6 - 14.6 \times 10^6$ ،في حين كانت حيوية الخلايا الأحادية النواة هي 93.9%.وبعد مرور ثلاثة اشهر من الحفظ بالتجميد فأن حيوية الخلايا التي تم اعادة انعاشها هي73.9%.

تعد الدراسة الحالية محاولةً لفصل الخلايا الجذعية من دم الحبل السري واكدت نتائج الدراسة الحالية ان دم الحبل السري يعد مصدراً سهل المنال للحصول على الخلايا الجذعية التي يمكن حفظها واستعملالات في كثير من الأستخدامات الطبية.