Scientific Research & Studies Center-Faculty of Science- Zagazig University- Egypt

Biochemistry Letters

Journal home page:

Factors affecting hematopoietic stem cells derived from umbilical cord blood

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ARTICLE INFO

Article history:
Received
Accepted
Available online

Keywords:
Serratia plymuthica, Lead Sulfide, Nanoparticle, Wastewater

ABSTRACT

Background: Stem cells from umbilical cord blood Lifetime protection umbilical cord blood contains stem cells. These stem cells were until recently received and discarded after delivery with umbilical cord and placenta. However, these cells are of great value at the moment of birth and kept for life for any use. May be necessitated by urgent medical imperatives given later. Human stem cells derived from the umbilical cord have many advantages: they are easily collected without harm to the child or mother and treatment of many diseases including blood diseases, cancer and immune system disorders**.**Because of the high cost of treatment and preservation of umbilical cord blood and the relative proportion of stem cells in which the banks of stem cells from umbilical cord blood paid great attention to the acquisition of high-quality units to ensure higher rates of success of stem cell transplantation and so I study the factors that may affect the quality of umbilical cord blood units is important.

Aim: To determine the characteristics of the laboratory hematopoietic potentiality of umbilical cord blood hematopoietic stem cells, and to study their association with maternal and neonatal factors**.** ***Materials & Methods:*** The maternal and neonatal factors that influence the total nucleated cell (TNC), and CFU yields in CB collected for the Cord Blood Bank were evaluated.***Results:*** As expected, there was a significant rise in TNC / CBU and CFU / CBU considering quality of UCB unit with increase in UCB volume**,** and also there was significant elevation of cord blood volume, TNC x 10^8 / CBU and CFU / CBU x10^5 in units collected from birth weight > 3500 gm compared to that of birth weight ≤ 3500 gm. Fetal gender affected quality of UCB units, Volume of UCB units in our study was significantly higher in male fetus compared to only in female fetus. TNC x 10^8 / CBU was elevated in preterm than collected from post term. CFU / CBU x 10^5 in preterm compared to term and post term. The increase in maternal age was associated with high volume, TNC and CFU. There is significant elevation of CBU volume, TNC , from maternal age > 20 years compared to that of maternal age ≤ 20 years.

Conclusions: Our study suggests that maternal donor characteristics significantly influence the yield of TNC and viability of UCB samples. These factors should be considered when attempting to improve the yield of potential stem cells in cord blood.

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INTRODUCTION

Umbilical cord blood (UCB) is blood that is enclosed in the placenta and the adjoined umbilical cord following delivery. UC contains hematopoietic stem cells and to a lesser extent multipotent mesenchymal cells [1]. UCB can be used as hematopoietic stem cell source for transplantation and is increasingly utilized in the treatment of malignant and nonmalignant hematologic and immunologic diseases,being, in some cases, an alternative to bone marrow transplantation[2]. When compared to hematopoietic stem cells deriving from bone marrow, UCB provides rapid availability of HLA-typed transplants that can be stored frozen until use, lower risk of graft versus-host disease regardless of HLA mismatch and no risk or pain for the donor during sample collection [3].

Shortcomings include the restricted number of stem cells and nucleated cells in UCB compared to marrow transplants, and the unfeasibility of using the donor for a second transplantation or donor lymphocyte infusion[4]. UCB can be considered as a potential source of stem cells for transplantation. Transplantation outcome is correlated with cord blood volume (CBV), number of total nucleated cells (TNC), CD34+ progenitor cells and colony forming units in UCB donations [5]. Compared with an adult peripheral blood, UCB has a larger number of progenitor cells [6]. Also, the increment of the number of colony-forming unit-granulocyte-macrophage (CFU-GM) and colony-forming unit-granulocyte was shown to be higher in samples derived from UCB compared with samples derived from adult peripheral blood[7]. Studies have found that the success rate of stem cell transplantation is correlated with the total number of formed CFU; the higher number, the higher the success rate [8,9].

In this study, we evaluated the time related variations of cord blood volume (CBV), of UCB units in relation to the obstetric, neonatal and collection factors that influence the volume and total nucleated cell cell content of UCB donations.

Materials and methods:

We collected 50 samples of HUCB from mothers undergoing caesarian section in the department of Gynecology and Obstetrics at Mansoura University Hospital. All mothers gave written informed consents

A complete data sheet was taken includes:

1) **Parental and delivery data**:

mothers name, maternal age, maternal height , maternal occupation, residence, medical history, women's smoking or non-smoking ,the cigarettes number/day, pre-pregnancy weight , gravid status, spouse's age and spouse's occupation, fetal gestational age, Delivery date, mode of delivery, indication of caesarian section, fetal gestational age, fetal heart rate and premature rapture of membranes

2)Placenta& cord data:

Cord length, placental weight, square measure of the placenta, volume of placenta, thickness of placenta, shape of placenta, region of cord adhesion, type of collection and cord blood collection volume.

3) Neonatal data:

Neonatal Apgar score, neonatal order of birth, neonatal birth weight, neonatal gender, neonatal head circumference, neonatal chest measurement and umbilical stump length.

Umbilical cord blood was collected in utero after caesarian section of subjects while the placenta was still in uetro (before placental expulsion) in a special UCB collection bags (JMS, Singapore) which contains 28 ml anti-coagulant CPDA-1 (citrate-phosphate-Dextrose-Adenine) to prevent coagulation of UCB and provide nutrition to cells until processing.Then we gently mixed the blood inside the bag to ensure good mixing with anticoagulant CPDA. Blood collection unit volume and

subject name, age and gestational age were recorded. The cord blood unit was transported immediately to Mansoura Research Centre for Cord Stem Cells **(MARC-CSC)** and was prepared for mononuclear cells separation which performed manually using density gradient centrifugation method by Ficollhypaque media [10].

• **Density gradient centrifugation method (manual separation):**

The steps of this method were done in biological safety cabinet to reduce the risk of contamination.We put UCB in falcon tubes (50 ml) and then diluted cord blood 1:3 [10 ml cord blood +20 ml RPMI 1640 Medium (Stem cell technologies, Canada)]. We put 3 ml Ficollhypaque (LymphoprepTM, Fresenius Kabi Norge As, Norway) in four falcon tubes 15 ml, then put 6 ml of the diluted blood by layering over Ficoll without mixing. Tubes were centrifuged for 20 minutes at 2500 rpm /min. After centrifugation, different layers were formed:

1-Lowest layer contains red blood cells (RBCs).

2-Layer above RBCs contains Ficollhypaque solution.

3-At interface of Ficoll and plasma mononuclear cell layer (buffy coat) contains lymphocytes and monocytes. After that collection of buffy coat was done in clean 15 ml falcon tube, equal amount of RPMI was added to it then centrifuged at 2000 rpm / min for 10 minutes. Finally we removed supernatant and added RPMI to 1ml level.

▪ CBU Characterization:

1-**Total Nucleated Cell Count enumeration:**

A sample from UCB unit was used to count total nucleated cell count (TNCC) after MNCs separation using automated cell counter sysmex XS-800i cell counter (Sysmex Corporation, JAPAN).

2-**Hematopoietic Colony Forming Unit (CFU) Progenitor Cell Assay:**

A- Principle of assay:

Hematopoietic stem cells which present in bone marrow, UCB and peripheral blood divide continuously to replace old mature cells. During differentiation of HSCs into mature cells they pass throw intermediate stages including multi-potential progenitor cells and lineage-committed progenitor cells before reaching maturity.

Progenitor cells called colony forming units when cultured in specific media supplied with specific cytokines proliferate into colonies or clusters of cells that can be visualized and charectrized by inverted microscope.

Methyl cellulose media is the the standard media for colony forming unit assay as it is inert , changes in PH don't affect its quality and cells are not exposed to high tempreture as what happen with using agar based media.

Culture of CFU progenitor cells in methylcellulose media provided with suitable cytokines, each colony is derived from a single progenitor cell.

The colonies will be classified and counted based on its morphology.

B-Methocult media:

Methocult media is a methyl cellulose based media used for CFU assay. It includes many types depending on cytokines provided in media. Each type support proliferation and differentiation of specific types of colonies. These include erythroid progenitor cells (CFU - erythroid [CFU - E]) and burst-forming unit - erythroid [BFU - E]); granulocyte / macrophage progenitor cells (CFU - granulocyte, macrophage [CFU - GM]); CFU - granulocyte [CFU - G] and CFU - macrophage [CFU - M]) and multi-potential progenitor cells (CFU -

granulocyte, erythroid, macrophage, megakaryocyte [CFU - GEMM]).

In this study we used complete Methocult™ (H4035 Optimum,without EPO) media (Stem cell technologies, Canada).

It contains rh SCF, rh GM - CSF, rh IL - 3, rh G - CSF. It is used for CFU - G, CFU - M and CFU - GM assay in BM and CB.

C-Preparation of Methocult media:

1-The MethoCult™ H4035 optimum, without erythropoietin (EPO) media (Stem cell technologies, Canada) was delivered in a vial contain 100 ml of frozen culture.

2-The culture was thawed at room temperature (15 – 25 °C) and shaked well for 2 minutes. The bottle was left stand for 10 minutes to allow air bubbles to rise up.

3-A 10 ml syringe with wide pore needle (16 gauge) attached to it was used to dispense 2.5 ml of culture media in each 15 ml sterile falcon tube.

D-Procedure:

1. The TNC count of sample was counted by sysmex XS-800i cell counter.

2. In a sterile tube 1 ml of RPMI 1640 (Stem cell technologies, Canada) and 10ul of prepared antibiotic (penicillin + streptomycin + antifungal) were added.

3. We adjusted cell count to 250,000 cell / ml using formula c1xv1= c2xv2 by removing desired volume of RPMI and addition of equal amount of cell suspension.

4. The MethoCult™ media in a falcon tube were thawed in room tempreture and 250 ul of cell suspension were added to 2.5 ml of methocult media.

5. The tubes were mixed vigorously for at least 4 seconds.

6. Let stand for at least 5 minutes to allow the bubbles to rise to the top.

7. Culture media which contain cells was taken by the syringe .

8. Syringe contain media was held by one hand and by the other hand the lid of 6 well culture plate (Stem cell technologies, Canada) was removed. 1.1 ml was dispensed in first well and another 1.1 ml was dispensed in the second well. The lid was closed.

9. Medium was distributed all over the well by gently rotating the plate to make culture reach sides of the well.

10. In the remaining 4 wells 1.1 ml of distelled water were added in each well to keep humidity of plate and prevent dryness of culture.

11. Name of patient and date was typed on side of culture plate .

12. Plates were incubated in 5% CO2 incubator at 37°C with 95 % humidity for 14 - 16 days.

13. The temprature of incubator, Co2 concentration and humidity were checked daily.

14. On day 7, plates were checked for growth of colnies.

15. On day 14, the number of colonies were counted using TCM 400 inverted microscope (Labomed, Inc , USA).

E-Colony counting:

1. Counting of CFU assays was done after 14 days on incubation.

2. The plates were removed from CO_2 incubator to be counted.

3. Two perpendicular lines were drawed using a permanent marker on the bottom of the dish.

4. The dishes were placed on the inverted microscope stage and adjust the focus under low power (4X objective) until the colonies are in focus.

5. The dishes were scanned using 4X objective magnification lenses.

6. All colonies in each dish were counted. We switched between 4X and 10X lenses to help in identification of colonies.

7. The total number of CFU / CBU unit is calculated according post processing TNC count of CBU.

F- Identification of colonies:

CFU - GM: Colonies of 40 cells or more with dark center (granulocytes) and clear periphery (macrophages).

CFU - G: Colonies 40 cells or more which appear dark (granulocytes only).

Colonies 40 cells or more which appear clear (macrophages only).

Statistical Methods:

The collected data was revised, coded, tabulated and introduced to a PC using Statistical package for Social Science (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). Data were presented and suitable analysis was done according to the type of data obtained for each parameter.

- **Descriptive statistics:**

1. Mean, Standard deviation (± SD), Median and range.

2. Frequency and percentage of non-numerical data.

3. Shapiro test was done to test the normality of data distribution. Significant data was considered to be nonparametric.

- Correlation analysis: To assess the strength of association between two quantitative variables. The correlation coefficient defines the strength and direction of the linear relationship between two variables.

- Regression analysis: Logistic, linear and ordinal regression analyses were used for prediction of risk factors, using generalized linear models.

  - N.B: p is significant if <0.05 at confidence interval 95%.

Results:

1. General characteristics of studied subjects

1.1 Parental & delivery data

Table (1) shows clinical characteristics of maternal donors. Median age at delivery was 24 years. Only eighteen percent had positive consanguinity. Medical history was assessed in all studied maternal donors; 2.0% had cardiac disease, 6.0 was diabetic, 2.0% had fetal congenital anomalies, 8.0% had hypertension, 2.0% had renal disease, 2.0% were rheumatic.

Median maternal height was 164.0 cm, median maternal weight was 66 kg, median maternal BMI was 25.14 kg/m2; median SBP was 120 mmHg, while median DBP was 80 mmHg; median maternal hemoglobin was 10.9 g/dL. ABO system and Rh system were assessed for all mothers, 30.0% had group A, 52.0% had group B, 18% had group O.

In table (2): Median gravidity was 2.5; median gestational age was 38.0 weeks. All cases delivered by CS. Indications of CS were previous CS in 66.0%, PROM in 6%, DM in 2%, hypertension in 2.0%, PE in 8%, post date in 10.0%, oligohydramnios in 2.0%, congenital anomaly in 2.0% and cardiac patients in 2.0%.

In table (3): Median age of spouse was 31.0 years; 60% were smokers. They had different occupations, 40.0% were employees, 24.0% were works, 10.0% were dealers, 14.0% were drivers, 6.0% were farmers, 2.0% were accountant, 2.0% were bakers, and 2.0% were unemployed.

1.2 placenta & cord data:

Table (4): Median placental weight was 541 g mean surface area of placenta was 303.12 cm2, median cord length was 45 cm. Region of cord adhesion was 70.0% central and 30.0% eccentric.

1.3 Neonatal data:

The present study was conducted on 50 cord blood units collected by trained personnel. They were processed using standard procedures.

Table (5) shows clinical characteristics of infant donors. They were 32 males (64%) and 18 females (36%). Cephalic presentation represented 92%, while breech presentation represented 8%. Median fetal heart rate was 140 (beat/minute). Median birth weight was 3.55 kg.. Median birth order was second order. Median head circumference was 34.0 cm, median chest circumference was 32.0 cm, median APGAR score at 1 minute was 6, median APGAR score at 5 minutes was 9.

1. CBU characterization: table (6)

Table (7) shows effect of UCB volume on quality of cord blood. TNC x 10^8 / CBUand CFU / CBU x 10^5 are significantly elevated in collection volume > 80 ml compared to collection volume ≤ 80 ml.

Table (8) shows effect of birth weight on quality of UCB units. There is significant elevation of TNC x 10^8 / CBU and CFU / CBU x 10^5 in units collected from birth weight > 3500 gm compared to that of birth weight ≤ 3500 gm.

Table (9) shows comparison of CBU volume / ml, TNC x 10^8 / CBU and CFU / CBU x 10^5 between male and female. The CBU volume / ml, TNC x10^8 / CBU and CFU / CBU x 10^5 are significantly elevated in units collected from males compared to females.

Table (10) shows effect of gestational age on CB quality. There is significant elevation of TNC x10^8 / CBU and CFU / CBU x 10^5 in preterm compared to term and post term. Otherwise no other significant detected.

Table (11) shows effect of maternal age on quality of UCB units. There is significant elevation of TNC x 10^8 / CBU and CFU / CBU x 10^5 in units collected

from maternal age > 20 years compared to that of maternal age ≤ 20 years

Discussion

In table (6) Characteristics of collected CBU were CBU volume / ml (80.74±27.86) ml. The mean of TNC x 10^8 / CBU was (2.04±1.84), the mean of CFU/ CBU x 10^5 was (27.98±18.87).

Volume of UCB unit is an important indicator of quality of UCB. Mean volume of collected UCB units was (80.74±27.86) ml with median of 81.50 ml. As expected, there was a significant rise in all parameters considering quality of UCB unit with increase in UCB volume.

According to table (7): with increase in CBU volume TNC was significantly increased ($p < 0.0001$). Units with volume more than 80 ml had TNC of (2.60×10^8) while units with volume less than 80 ml had TNC of (0.43×10^8).

With increase in CBU volume also CFU was increased ($p < 0.001$). CBU with volume > 80 ml had higher CFU / CBU (37.50×10^5) than units with volume ≤ 80 ml (6.70×10^5).

The increases in TNC / CBU, and CFU / CBU are the same results of [11,12].

TheTNC increases with the extracted volume of cord blood.This observation further strengthens the suggestion that the collection of more umbilical cord blood during delivery provides a greater chance of success in transplantation efforts, as the volume can support a larger population of TNC [13,14].

These results are related to the fact that increase in umbilical cord blood volume means more fetal blood and more progenitor cells which positively affect quality of unit and for that umbilical cord blood banks take volume of collected unit in consideration before starting cryopreservation due to high association between volume and quality of CBU [15].

Median of birth weight in our study was 3.55 gm with. To compare effect of birth weight on quality of cord blood ,we classified birth weight into two groups; the first group birth weight ≤ 3500 gm and the second group birth weight > 3500 gm.

As regards to table (8): There was significant elevation of cord blood volume ($p < 0.001$), TNC x 10^8 / CBU ($p = 0.002$), and CFU / CBU x10^5 ($p < 0.001$) in units collected from birth weight > 3500 gm compared to that of birth weight ≤ 3500 gm.

The higher the newborn weight, the larger the total volume of UCB collected which are the same results in our study as volume of UCB collected from neonates was (93.33 ± 26.76) ml in birth weight > 3500 gm and in neonates ≤ 3500 gm was (65.95 ± 21.41) ml[16,17].

The TNC / CBU was 2.5 X 10^8 in neonates born with birth weight more than 3500 gm and 0.51 X 10^8 in neonates born with birth weight less than 3500 gm which means increase in birth weight is associated with more TNC ($p = 0.002$).

The weight of the newborn at birth presented a positive relationship with two laboratory parameters analyzed: the TNC count and blood volume. Significantly higher TNC counts and volumes were associated with birth weights above 3500 g which are the same results in our study as volume of UCB units collected from neonates with birth weight ≤ 3500 gm was (65.95 ± 21.41) ml while volume of UCB units collected from neonates with birth weight > 3500 gm was (93.33 ± 26.76) ml[18].

Colony forming unit count was another parameter was found to increase with the increase in birth weight as CFU / CBU was 6.80×10^5 in birth wt ≤ 3500 gm

while was 36.12x 10^5CFU / CBU in birth weight > 3500 gm. Same results were shown by [16].

According to table (9) : There is difference between authors on effect of fetal gender on quality of UCB unit. Volume of UCB units in our study was significantly higher (P = 0.043) in male fetus (86.68 ± 28.26) ml compared to only (70.16 ± 24.41) ml in female fetus which are results reported also by[1,19,20].

However, larger blood volume production with higher cell count among male gender can be explained by that the birth weight of males are heavier than that of fe- males [21] and in the current study there was an evidence that with heavier birth weight there was larger blood volume and parallel higher cell count.

No relation between baby's sex and UCB volume and other studies found female neonates produce larger UCB volume and higher nucleated cell content in a study[18,22].

We found that TNC / UCB from male fetus was (2.40 X 10^8) while that from female fetus was (0.525 X 10^8) which means there is significant rise (p = 0.014) in TNC / CBU in male babies compared to female ones which is also reported by another study [20].

Other studies stated that UCB units from female babies had more TNC than units taken from male babies [23,24]. Higher TNC / UCB in female babies referred to higher neutrophils level at time of delivery [25].

No significant difference in the TNC obtained from newborn male and female babie[26].

Colony forming unit count / CBU also showed significant increase (p = 0.004) in UCB collected from male babies who had (35.36 x 10^5) CFU / CBU compared to (7.85 x 10^5) CFU / CBU from female babies.

There was significant increase in CFU / CBU in male babies compared to female ones [27].

In the table (10): We classified our cases into three groups: the first group for preterm babies born less than 38 weeks (35 week to 38 weeks), the second group for term babies born between (38- 40) weeks of gestation and the third group for post term babies born more than 40 weeks (40-42 weeks) to study effect of gestational age on quality of UCB units.There is significant elevation of TNC x 10^8/ CBU in preterm compared to term and post term.

Cord blood obtained from babies delivered at 37 to 42 weeks' gestation and found that gestational age was positively correlated with TNC [28].

Cord blood obtained from babies delivered at 25 to 42 weeks' gestation and found that gestational age correlated positively with TNC[29].

a gestational age of over 40 weeks was a predictor for a larger volume of blood [24].

There was no correlation between gestational age and TNC [26].

CFU in preterm group was significantly higher than term (p = 0.001) and post term (p = 0.004) groups. It was also elevated in term group compared to post term groups (p = 0.050) , these also reported by [14,30].

As a regard to table (11): The effect of maternal age on quality of UCB units. we classified mothers into two groups, The first group for mothers ≤ 20 years and the second group for mothers > 20 years. There is significant elevation of CBU volume from maternal age > 20 years

compared to that of maternal age ≤ 20 years. Maternal age affected volume of donated UCB (P = 0.002). As volume of UCB from first group (maternal age ≤ 20 years) was (64.47 ± 18.53) ml while that collected from second group (Maternal age > 20 years) was (89.12 ± 28.34) ml.

There is significant elevation (p = 0.007) of TNC in unit collected from maternal age > 20 years compared to that of maternal age ≤ 20 years. Women aged ≤ 20 years had TNC / CBU of (0.46×10^8) while women aged > 20 years had TNC / CBU of (2.5×10^8).

The TNC / CBU was increased with advancement in maternal age [31].

There is significant elevation of (p < 0.001) CFU / CBU in mothers > 20 years (34.60×10^5) than mothers ≤ 20 years (6.70×10^5) which are the same results showed by [32].

The explanation of the positive effect of increased maternal age on volume and HSCs content in cord blood is that with increase in maternal age the birth weight of fetus increases which is highly correlated with quality of UCB [33].

With the increase in maternal age the complications of pregnancy are also increase so this should be taken in consider while choosing UCB donors. These results don't mean we have to collect CBU from older mothers but they mean we have not to reject units collected from older mothers as it may of good quality [34].

Acknowledgement

I acknowledge Mansoura Research Centre for Cord Stem Cells (MARC-CSC) and Science and technology Devlopment fund for their support.

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TABLES

Table (1): Clinical characteristics of maternal donors.

			N=50	
Mother age at delivery;	**Median**	**Min - Max**	24.0	17-40
Consanguinity; N, %			9	18.0
Medical history; N, %	**No**		39	78.0
	Cardiac		1	2.0
	DM		3	6.0
	Fetal congenital anomalies		1	2.0
	Hypertension		4	8.0
	Renal		1	2.0
	Rheumatic		1	2.0
Maternal height (cm)	**Median**	**Min - Max**	164	150-170
Maternal weight (kg)	**Median**	**Min - Max**	66	50-98
Maternal BMI (kg/cm2)	**Median**	**Min - Max**	25.14	21.0-35.7
Maternal SBP (mmHg)	**Median**	**Min - Max**	120.0	90-150
Maternal DBP (mmHg)	**Median**	**Min - Max**	80.0	60-110
Maternal hemoglobin (g/dL)	**Median**	**Min - Max**	10.9	9.3-13.7
ABO; N, %	**A**		15	30.0
	B		26	52.0
	O		9	18.0
	AB		0	0.0

Table (2): Obstetric history of all maternal donors.

			N=50	
Gravidity; N, %	**Median**	**Min - Max**	2.5	1-6
Gestational age (weeks)	**Median**	**Min - Max**	38.0	35-42
Delivery method; N, %	**CS**		50	100.0
Indications of CS; N, %	**Previous CS**		33	66.0
	PROM		3	6.0

	DM	1	2.0
	Hypertension	1	2.0
	PE	4	8.0
	post date	5	10.0
	oligohydraminos	1	2.0
	congenital anomaly	1	2.0
	cardiac patient	1	2.0

Table (3): Clinical characteristics of spouse donors.

		N=50	
Spouse age	**Median, range**	31	25-45
Spouse Occupation; N, %	**Employee**	20	40.0
	Worker	12	24.0
	Dealer	5	10.0
	Farmer	3	6.0
	Accountant	1	2.0
	Baker	1	2.0
	Driver	7	14.0
	Un employed	1	2.0
Smoking; N, %		30	60.0

Table (4): Characteristics of placenta and cord.

		N=50	
Placental weight (g)	**Median, range**	541	310-780
Surface area of placenta (cm^2)	**Mean ± SD**	303.12 ± 58.51	
Cord length (cm)	**Median, range**	45	32-55
Region of cord adhesion; N, %	**Central**	35	70.0
	Eccentric	15	30.0
Number of cord punctures; N, %	**1**	40	80.0
	2	9	18.0
	5	1	2.0

Table (5): Clinical characteristics of infant donors.

			N=50	
Infant gender; N, %	**Male**		32	64.0
	Female		18	36.0
Fetal presentation; N, %	**Cephalic**		46	92.0
	Breech		4	8.0
Fetal Heart Rate (beat/minute)	**Median**	**Min -Max**	140	130-145
Birth weight (Kg)	**Median**	**Min -Max**	3.55	1.50-4.50
Birth order	**Median**	**Min -Max**	2.0	1-5
Head circumference (cm)	**Median**	**Min -Max**	34	28-38
Chest circumference (cm)	**Median**	**Min -Max**	32	26-36
APGAR 1	**Median**	**Min -Max**	6	3-9
APGAR 5	**Median**	**Min -Max**	9	7-10

Table (6):Cord blood characteristic

	Mean ± SD	**Median**	**Range**
CBU Volume / ml	**80.74 ± 27.86**	**81.50**	**30.0- 165.0**
TNC x 10^8 / CBU	**2.04 ± 1.84**	**1.65**	**0.3 – 6.10**
CFU/ CBU x 10^5	**27.98-18.87**	**33.89**	**0.6-60.6**

Table (7): Comparison of TNC x 10^8 / CBU, and CFU / CBU x 10^5 between CBU with volume ≤ 80 ml and volume > 80 ml.

Parameter	**Volume ≤ 80 ml (n = 25)**	**Volume > 80 ml (n = 25)**	***P***

TNC x 10^8 / CBU	**Median**	**Min - Max**	0.43	0.03-3.70	2.60	0.37-6.10	<0.001
CFU / CBU x 10^5	**Median**	**Min - Max**	6.70	0.6-42.11	37.50	22.6-60.60	<0.001

Mann-whitney tests*. **P** between two groups.

**significant (P value < 0.05)

Table (8): Comparison of CBU volume / ml, TNC x 10^8 / CBU and CFU / CBU x 10^5 between birth weight ≤ 3500 gm. and birth weight > 3500 gm.

Parameter			**Birth wt. ≤ 3500 gm. (n = 23)**		**Birth wt. > 3500 gm (n = 27)**		***P***
CBU volume / ml*	**Mean ± SD**		**65.95 ± 21.41**		**93.33 ± 26.76**		<0.001
TNC x 10^8 / CBU	**Median**	**Min - Max**	0.51	0.03-4.90	2.5	0.05-6.10	0.002
CFU / CBU x 10^5	**Median**	**Min - Max**	6.80	0.60-55.9	36.12	0.9-60.6	<0.001

Independent samples T test*, Mann-whitney tests. **P** between two groups.

**significant (P value < 0.05)

Table (9) : Comparison of CBU volume / ml, TNC x 10^8 / CBU and CFU / CBU x 10^5 between male and female.

Parameter			**Male (n = 32)**		**Female (n = 18)**		***P***
CBU volume / ml	**Mean ± SD**		**86.68 ± 28.26**		**70.16 ± 24.41**		0.043
TNC x 10^8 / CBU	**Median**	**Min - Max**	2.40	0.14-6.1	0.525	0.03-5.80	0.014

CFU / CBU x 10^5	**Median**	**Min - Max**	35.36	1.18-60.60	7.85	0.6-59.70	0.004

Independent samples T test*, Mann-whitney tests. **P** between two groups.

**significant (P value < 0.05)

Table (10): Comparison of CBU volume / ml, TNC x 10^8 / CBU and CFU / CBU x 10^5 between preterm, term and post term.

Parameter			**Preterm (n=17)**		**Term (n=27)**		**Post term (n=6)**		***P*^{1}**	***P*^{2}**	***P*^{3}**	***P*^{4}**
TNC x 10^8 / CBU	**Median**	**Min - Max**	2.80	0.3-6.10	1.20	0.03-5.30	0.44	0.14-1.60	0.010	0.005	0.272	0.005
CFU / CBU x 10^5	**Median**	**Min - Max**	42.06	5.80-60.60	30.10	0.60-57.50	3.20	0.60-38.90	0.001	0.004	0.050	<0.001

kruskal wallis, Mann-whitney tests*. **P**^{1} between preterm and term group. **P**^{2} between preterm and post term group. **P**^{3} between term and post term group. **P**^{4} between 3groups.

**significant (P value < 0.05)

P^1, significance between preterm versus term; **P**^{2}, significance between preterm versus post term; **P**^{3}, significance between term versus post term; **P**^{4}, significance between 4 groups.

Table (11): Comparison of CBU volume / ml, TNC x 10^8 / CBU and CFU / CBU x 10^5 between maternal age ≤ 20 and Maternal age > 20 years.

Parameter			**Maternal age ≤ 20 gm. (n = 17)**		**Maternal age > 20gm (n = 33)**		***P***
CBU volume / ml*	**Mean ± SD**		**64.47 ± 18.53**		**89.12 ± 28.34**		0.002
TNC x 10^8 / CBU	**Median**	**Min - Max**	0.46	0.05-3.60	2.5	0.03-6.10	0.007

CFU / CBU x 10^5	**Median**	**Min - Max**	6.70	0.60-36.40	34.60	0.6-60.6	<0.001

Independent samples T test*, Mann-whitney tests. **P** between two groups.

**significant (P value < 0.05)

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FIGURES

Colony forming unit assay

Case (1)

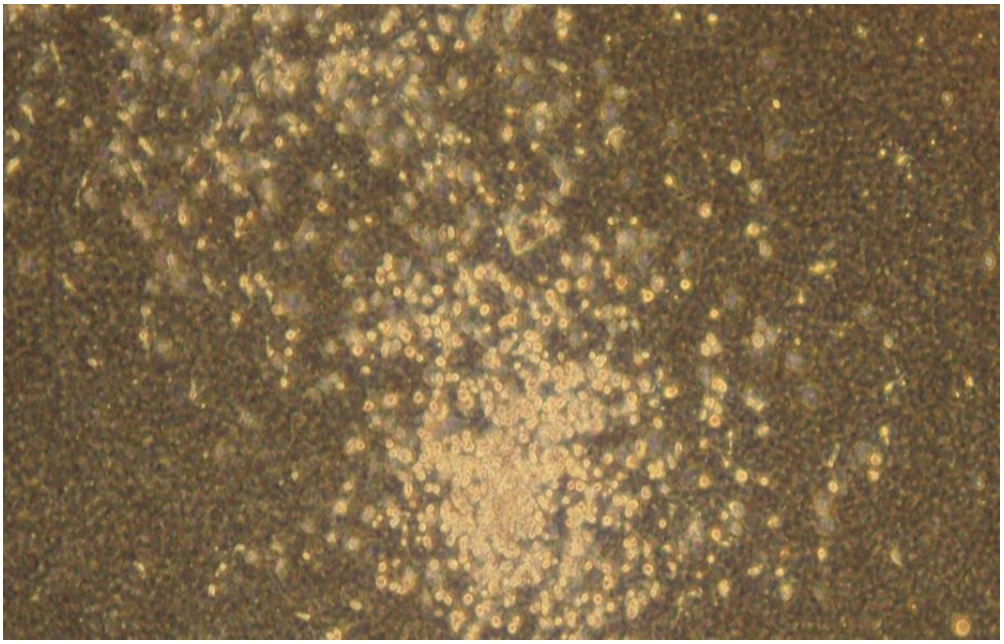

Figure (1): Colony forming unit – macrophage (CFU - M) 4X.

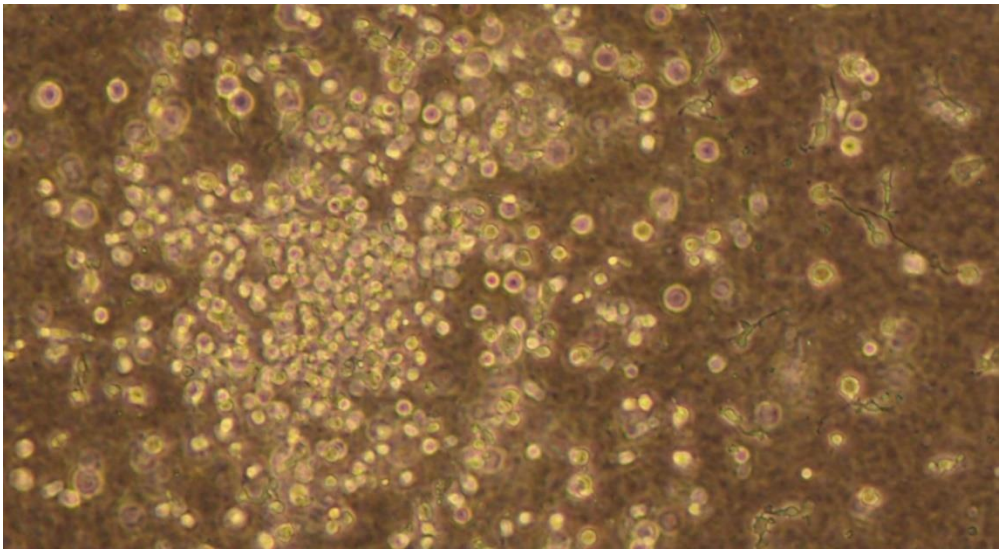

Figure (1): Colony forming unit – macrophage (CFU - M) 10X.

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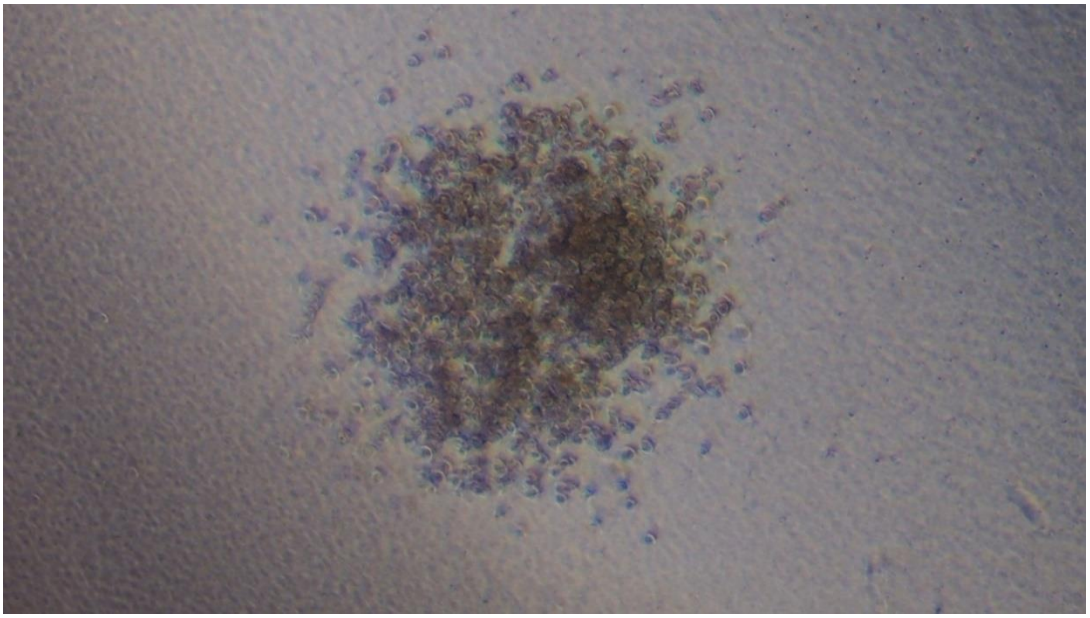
Case (18)



Figure(2): Colony forming unit – granulocyte (CFU - G) 4X.

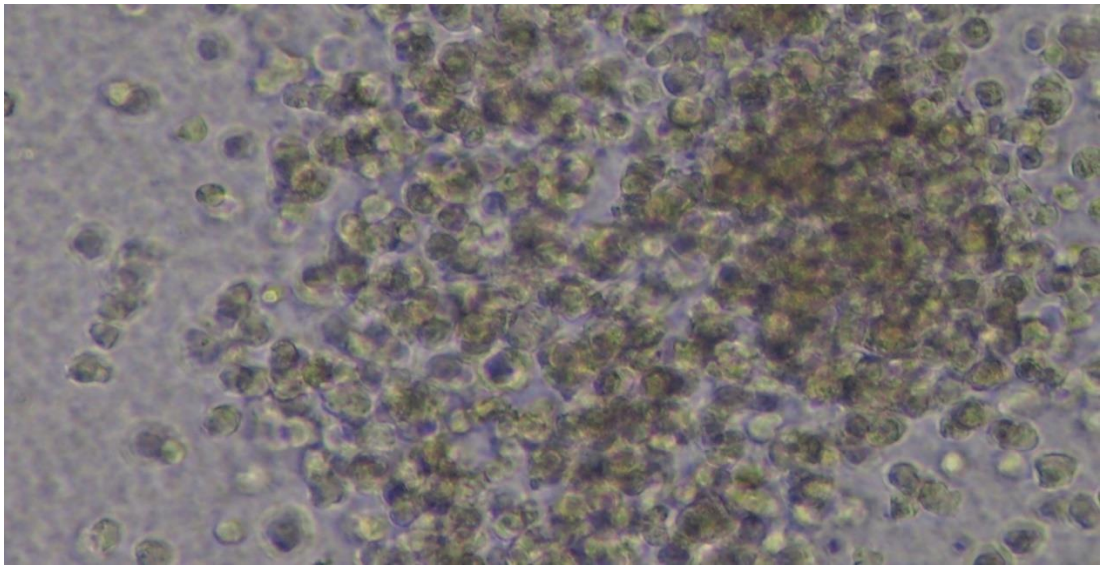


Figure (2): Colony forming unit – granulocyte (CFU - G) 10X.

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Case (34)

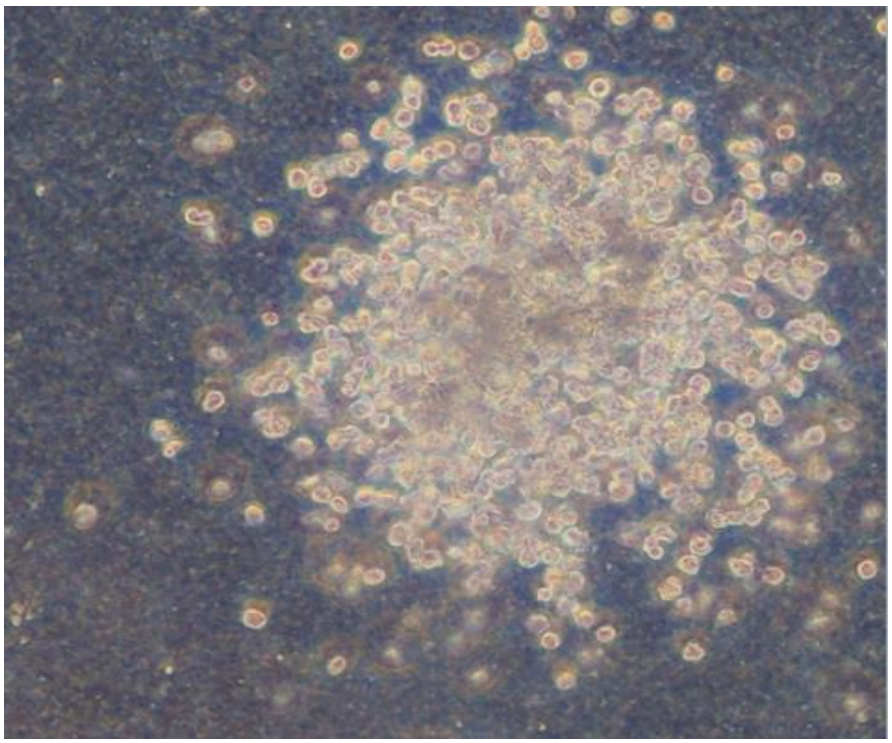

Figure (3): Colony forming unit – granulocyte macrophage (CFU - GM) 4X.

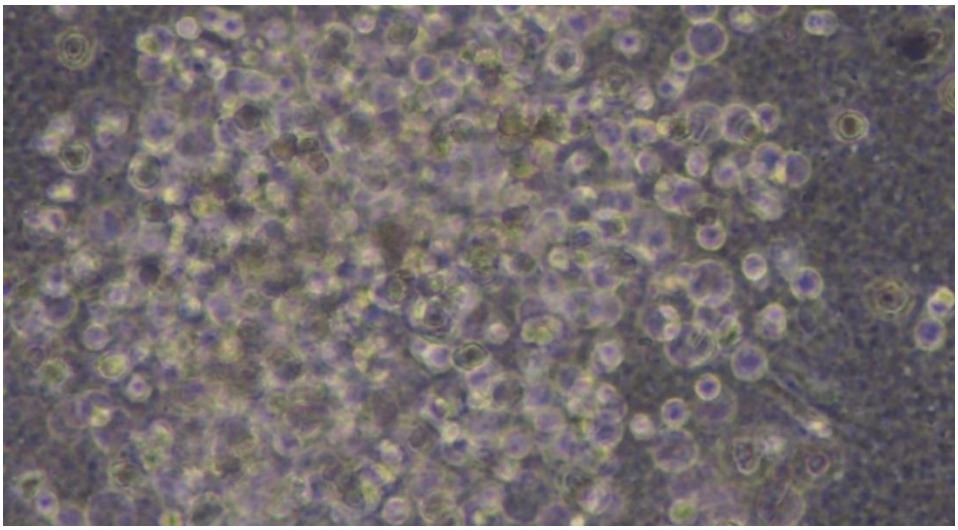

Figure (3): Colony forming unit – granulocyte macrophage (CFU - GM) 10X.