

Collection, isolation and characterization of the stem cells of umbilical cord blood

TATIANA REVENCU¹⁾, VICTORIA TRIFAN²⁾, LUDMILA NACU²⁾,
 TATIANA GUTIU³⁾, L. GLOBALA⁴⁾, A. G. M. MOTOC⁵⁾, V. NACU²⁾

¹⁾Laboratory of Tissue Engineering and Cell Cultures,
 Department of Obstetrics and Gynecology

²⁾Laboratory of Tissue Engineering and Cell Cultures
 "Nicolae Testemițanu" State Medical and Pharmaceutical University,
 Kishinev, Republic of Moldova

³⁾Municipal Maternity, Kishinev, Republic of Moldova

⁴⁾Department of Human Anatomy,
 "Nicolae Testemițanu" State Medical and Pharmaceutical University,
 Kishinev, Republic of Moldova

⁵⁾Department of Human Anatomy and Embryology,
 "Victor Babeș" University of Medicine and Pharmacy, Timisoara, Romania

Abstract

The umbilical cord blood (UCB) is a rich source of hematopoietic and mesenchymal stem cells and it can be used for cellular therapy. This article presents data achieved from 50 cord blood samples (46 *in utero*, four *ex utero*). The nucleated cells were isolated from the cord blood using a dextran solution. The quantity of stem cells having the immunophenotype CD34+, CD45+ and CD90+ was determined, using flow cytometry. The nucleated cells were cultivated in a DMEM (Dulbecco's Modified Eagle Medium) and biochemical and cytogenetic tests were later performed.

Keywords: stem cells, cellular therapy, mesenchymal and hematopoietic stem cells, (umbilical) cord blood, flow cytometry.

Introduction

Pioneers of cellular therapy, the hematopoietic stem cells are still an important source of stem cells both for clinical practitioners and researchers in the field of stem cell biology. It has been noticed that the bone marrow stem cells can differentiate *in vivo* or *in vitro* as: osteoblasts [1–3], adipocytes [4], neurocytes [5–7], cardiomyocytes [8, 9], and hepatocytes [10, 11].

The number and differentiation potential of the bone marrow stem cells decrease with age, thus, the search for other sources of stem cells has a significant value.

Studies performed within the last decade have shown that the umbilical cord blood contains hematopoietic and mesenchymal stem cells and that it can be used as an alternative source of stem cells for utilization in cellular therapy.

The first successful clinical use of umbilical stem cells was in 1988. The transplant was made by Elian Gluckman, the director of the bone marrow Transplant Center of "St. Louis" Hospital (Paris), to a 6-year-old boy who was suffering from the Fanconi anemia. Several studies have proven that their use has an efficiency that is comparable to that of the use of bone marrow stem cells when treating lymphomas, leukemia and other conditions. Generally speaking, they have capacities similar to fetal stem cells, and do not raise ethical problems [12–14]. To this day, over 15 000 cord blood

cell transplants have been performed successfully on children and adults [15, 16].

Cord blood cells have a great capacity to re-build the patient's damaged hematopoietic system almost entirely. Even though cord blood contains less mesenchymal stem cells when compared to the bone marrow, the quality of the graft is superior to the bone marrow one. Thus, the amount of umbilical stem cells required for a successful transplant is 10 times less, because they are younger than the autologous cells, of bone marrow and peripheral blood cells. It has been found that the blood of a single umbilical cord, with a volume ranging from 80 to 120 mL contains the same number of hematopoietic stem cells as 1200 mL of bone marrow would [12, 17].

Umbilical blood (UB) has the following advantages:

- Fast availability – once the blood unit is taken, tested and conserved it is available for transplant [18].
- Because the stem cells of the umbilical cord are immature, the risk of graft rejection is considerably lower, thus the transplant of UB does not require full HLA compatibility between donor and recipient, this allowing samples from different donors to be used if necessity calls it [19, 20]. The explanation is that, if the blood of the newborn is immunologically immature, the immune system does not have enough time to be exposed to different immunogens and create antibodies that would counter them [21].
- Harvesting cells from the umbilical cord can be

performed in a comfortable manner, does not involve a death-risk for the donor and storage is cheaper, compared to harvesting cells from the bone marrow or peripheral blood [20, 22].

- It has been established that UB has a lower chance of cytomegalovirus infection than bone marrow, consequently reducing the complications that follow a transplant [23, 24].

The disadvantages of using UB:

- The duration of the immune system's recovery is longer when UB stem cells are used because they are more primitive than those found in the peripheral blood and bone marrow, the patient becoming vulnerable to infections for a greater period of time [25, 26].

- The quantity of harvested UB cells may not be sufficient for a corpulent child or an adult, because the optimal volume required for a successful graft is not definitively established, this being an important limitative factor.

- Also, the viability of UB is uncertain, most of the blood units that were used until now having been cryopreserved on a 10-year period [27].

Because of these particular reasons, researches regarding the collection, testing, isolation and storage of UB are being conducted in the present.

The purpose of the research was to analyze some obstetric and the newborn factor, in concordance with the volume and the content of the nucleate cells of the umbilical blood units, to investigate the viability and the capacity to multiplication of the cells during their processing.

☒ Materials and Methods

Collecting the umbilical blood

The umbilical blood ($n=50$), has been collected from the newborns of the traveling mothers hospitalized at the No. 2 Municipal Maternity from Kishinev.

Study criteria:

- Donor's signed consent.
- Obstetric and family medical history, so that some conditions and genetic anomalies can be excluded.

- The lack of infectious diseases in the medical history, and their testing during gestation: HBsAg, viral hepatitis C, HIV, syphilis (in case of seropositive reactions, the blood were not be collected).

- On term, physiological births: 38–42 amenorrhea weeks (AW).

- Obstetric criteria: the weight of the newborn must exceed 2500 g; an Apgar score that is higher than 8; an amniotic fluid less period shorter than 12 hours; the absence of the mother's fever during labor; the lack of signs of infection on the new born; the absence of congenital anomalies of the newborn.

The cord blood units were extracted by puncturing the umbilical vein; directly after the child's birth, the tying and sectioning of the umbilical cord; respecting the aseptic and antiseptic rules, in standard 300 mL closed containers (CPDA-1 – Citrate / Phosphate / Dextrose / Adenine, Baxter Transfer Bag, C.A. de C.V., Mexico). After puncturing the umbilical vein, while the placenta is *in utero*, the blood drains into the container

under gravity's influence, avoiding contact with the exterior.

The extracted samples were subjected to the following: CBC, bacteriological anaerobic and aerobic tests, immunological tests for HBsAg, Ac-HCV (IgM + IgG), Ac-HSV₁₊₂ (IgM + IgG), Ac-anti Toxoplasmosis (IgM + IgG), Ac anti-CMV (IgM + IgG).

The following data has been recorded: the gestation period (AW), the pregnancy's number, the amniotic fluid less periods' duration, the birth's type, the weight of the newborn and the placenta and the newborn's gender.

Five mL of the mothers' blood ($n=10$) was collected in sterile tubes via puncturing of the cubital vein for immunologic testing.

Isolating the nucleated cells

For separating the nucleated cells from the basal part of the blood (erythrocytes and plasma) in the UB's fractionation, Dextrane of a 60 kDa molecular mass has been used, in a 1:1 ratio. The Dextrane was added in the large bag. After the erythrocytes' sedimentation during a 30–60 minutes period (until a visible border was created), the supernatant was transferred into the small bag of the container and separated from the other one with a clip. The liquid is placed into 15 mL tubes and then centrifuged at 100 rpm. The liquid above the sediments is removed, and the nucleated cells are placed in a sterile tube, counted and stored in cryotubes (NUNC) in a DMEM and 10% DMSO (Dimethyl sulfoxide) solution. A part of these cells was placed in a nourishing environment for cultivation.

Cultivating the cells

The cells were cultivated in a DMEM (HiMedia), 100 U/mL Gentamycin and Penicillin (Sigma) and adjuvant in a CO₂ "Binder" incubator with a 5% CO₂ concentration, at 95% humidity, up to three passages on culture 25 and 75 cm² culture plates (NUNC), and changing the nourishing environment every three days. During the course of the cultivation, the supernatant was collected on at 0, 3, 5, 7 and 9 day intervals and stored a -20°C for the performing the following biochemical research.

General analysis

The general analysis of the UB was performed on the PCE-170 analyzer, observing the cellular elements of the blood, the hematocrit value, the mean quantity of erythrocytes, the mean hemoglobin content in an erythrocyte and the mean quantity of thrombocytes.

Cytogenetic analysis

Karyotyping by G-banding was used for finding out the karyotype of the UB in the beginning, as well as at the end of the cultivation, in order to establish whether the cultivation process caused changes in the structure of the chromosomal complex. For performing the test, the cells were cultivated in a LymphoChrome (Lonza, Belgium) nourishing environment and Colchicine (HiMedia, India) was added three days later, and then they were fixed in a methanol solution and glacial acetic

acid in a 3:1 ratio. The Giemsa staining was used for visualization.

Biochemical analysis

Through biochemical analysis of the supernatant, the dynamic of the proteins, lipids, carbohydrates, cholesterol and mineral substances (the concentration of Na, Mg, Ca, P, K) from the environment was evaluated at different periods of the cultivation process.

Immunologic investigations

The immunologic investigations of the UB have included determining IgA, IgM, IgG through the radial method of immunodiffusion in gel (after Mancini G *et al.*) [28] and the cells of the CD34⁺ phenotype, utilizing CD34 monoclonal antibodies (France). The immune circulating complexes from the mother's blood were identified through the precipitation reaction with a 3.75% Polyethylene glycol (PEG); the T (CD3⁺, CD45⁺) and B (CD19⁺) groups lymphocytes were identified through flow cytometry at the Partec PAS (France) machine with CD3, CD45 and CD19 (France) monoclonal antibodies. The research was performed in cooperation with the Immunologic Depart of the "Toma Ciorbă" Infectious Diseases Hospital from Kishinev.

Statistical analysis

The statistical processing of the obtained results was performed using the Microsoft Excel and Stats Direct (the 1.9.58u8, 2001 version) software. The arithmetic mean was calculated \pm the standard deviation ($M \pm \sigma$). For testing the significant difference between the studied indices, the nonparametric statistical test of analysis, of the Spearman (ρ) correlation was applied.

Results

Considering the small amount of blood that can be collected from the umbilical cord (100 mL on average), the first priority is obtaining the greatest possible quantity of blood, as well as reducing the risk of bacterial contamination. To optimize the methods of cord blood collection, we have analyzed several obstetric factors and factors regarding the newborn, in conformity with the volume and the NNC content of the blood units.

The collected cord blood samples ($n=50$) using the *in utero* method had a mean volume of 71.84 ± 25.29 mL

(42–147), an NNC of $11.2 \pm 9.85 \times 10^6/\text{mL}$ (1–52.6) and the quantity of CD34⁺ – $7.9 \pm 0.48 \times 10^8/\text{mL}$ with a vitality of $93.47 \pm 0.37\%$. The mean weight of the newborns was 3418.3 ± 428.56 g (2400–4300 g), 23 (46%) of them being boys and 27 (54%) girls.

From the analyzed data, we have determined that the CBV/NNC was greater for boys than for girls: 78 mL of UB with a $12.4 \times 10^6/\text{mL}$ NNC was collected from boys, on average, as compared with the 66.6 mL and an NNC of $10.2 \times 10^6/\text{mL}$ taken from the girls (Table 1).

Table 1 – The results of study

	Mean \pm SD	Min.–Max.
Cord blood volume (CBV) [mL]	71.84 \pm 25.29	42–147
Weight of the newborn [g]	3418.3 \pm 428.56	2400–4300
Weight of the placenta [g]	571.7 \pm 81.82	350–855
The mother's age [years]	25.66 \pm 3.8	18–36
The gestation period's duration [AW]	39.78 \pm 1.01	38–42
Number of nucleated cells (NNC) [$\times 10^6/\text{mL}$]	11.2 \pm 9.85	1–52.6
CD34 ⁺ [$\times 10^8/\text{mL}$]	7.9 \pm 0.48	
Vitality [%]	93.47 \pm 0.37	
The newborn's gender:		
▪ Boys		23 (46%)
▪ Girls		27 (54%)

In 24% of the cases, the volume of collected UB blood exceeded 80 mL ($n=12$), and in 76% of the cases it ranged between 40 and 80 mL (Figure 1).

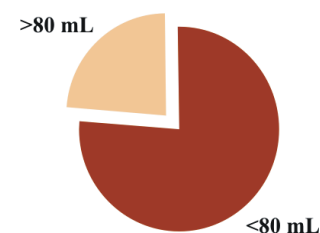


Figure 1 – Volume of the collected cord blood.

Our study shows that the units of UB were collected through the gravitational method, in a closed system, following physiological births, *per vias naturalis*; and that the placenta was *in utero*. The duration of the extraction was 5–8 minutes and the blood unit processing was performed within 24–28 hours from the time of extraction.

Table 2 shows the data obtained in the study, by analyzing the relationship between some factors impacting the volume of UB and the number of NNC.

Table 2 – The analysis of the factors influencing the collected CBV/NNC

	Spearman correlation coefficient (r)	Value (95% CI)	P
Volume of cord blood [mL] / weight of the newborn [g]	0.45*	0.02 (0.01–0.03)	0.001**
NNC [$\times 10^6/\text{mL}$] / weight of the newborn [g]	0.09	0.0025 (-0.002–0.01)	0.34
Volume of cord blood [mL] / weight of the placenta [g]	0.47*	0.125 (0.03–0.24)	0.0005**
NNC [$\times 10^6/\text{mL}$] / weight of the placenta [g]	0.12	0.019 (-0.01–0.05)	0.2
Volume of cord blood [mL] / NNC [$\times 10^6/\text{mL}$]	0.25	0.64 (0–1.7)	0.07
Volume of cord blood [mL] / gestational period [AW]	0.33*	6 (2.5–12.5)	0.003**
NNC [$\times 10^6/\text{mL}$] / gestational period [AW]	0.16	1.35 (-0.5–3.8)	0.1

*Presence of a moderate correlation; **The correlation coefficient is statistically insignificant ($p < 0.05$).

We have analyzed the difference between the studied indices, using Spearman (ρ) non-parametric correlation test, and have determined significant statistic differences

between the quantity of collected UB and the newborn's weight, the placenta's weight, the gestational period (Figure 2). Thus, according to the obtained data, which

correlates to those presented in bibliography, the estimation of the fetus's weight could serve as a criterion for collecting UB *in utero*, and the weight of the placenta

– when the collecting is performed *ex utero*. The volume of cord blood was greater in prolonged pregnancies, when compared to on term pregnancies (Figure 2).

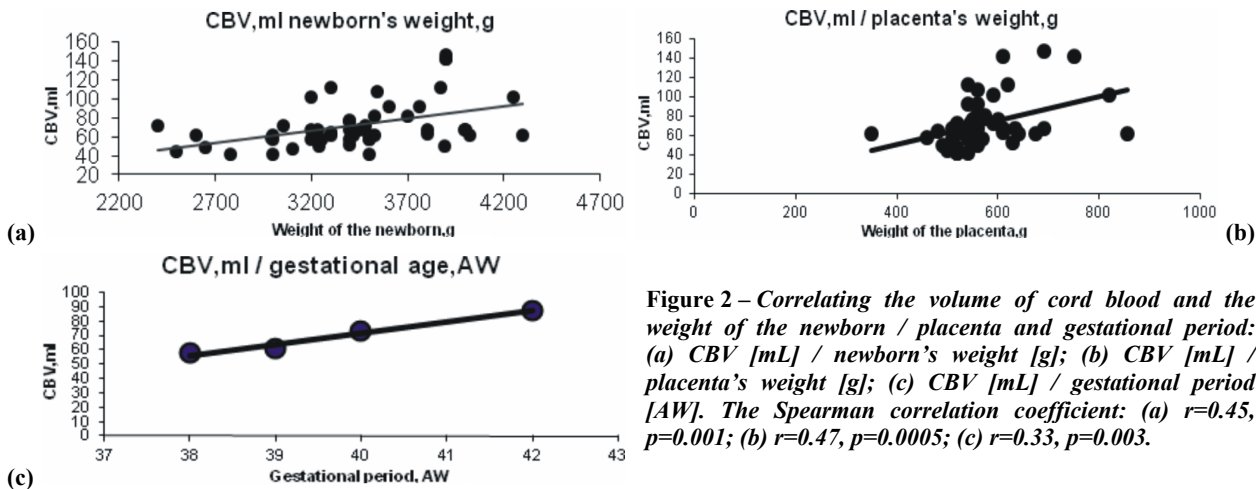


Figure 2 – Correlating the volume of cord blood and the weight of the newborn / placenta and gestational period: (a) CBV [mL] / newborn's weight [g]; (b) CBV [mL] / placenta's weight [g]; (c) CBV [mL] / gestational period [AW]. The Spearman correlation coefficient: (a) $r=0.45$, $p=0.001$; (b) $r=0.47$, $p=0.0005$; (c) $r=0.33$, $p=0.003$.

The thing that matters the most for the success of a transplant is the number of nucleated cells. The higher the NNC involved in the graft is, the more accurate the prognosis and the higher the chance of success of the transplant.

We have not found an evident correlation between the analysis of obstetric factors and the NNC, and the correlation between the volume of collected blood and NNC is statistically insignificant. This allows us to conclude that the quality of the cell graft does not depend exclusively on the obstetric factor or the volume of collected blood (Figure 3).

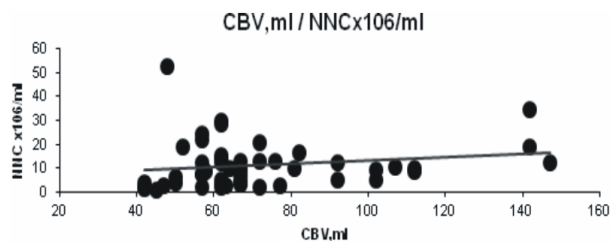


Figure 3 – The correlation between the quantity of cord blood and the number of nucleated cells. The Spearman correlation coefficient, $r=0.25$ ($p=0.07$).

We also analyzed the cellular composition of the cord blood. The results are within the normal parameters of peripheral blood (Table 3).

Table 3 – Cellular composition of umbilical cord blood

Parameters	Mean±SD (n=50)	Normal values
Erythrocytes [$\times 10^6/\mu\text{L}$]	2.35±0.46	2.35–2.95
Hemoglobin [g/L]	81.1±15.3	150–240
Hematocrit value [%]	23.85±5.04	44–70
Mean erythrocyte volume [fL]	102.45±5.2	96–108
Mean quantity of hemoglobin in erythrocyte [pg]	34.51±2.19	32–34
Leukocytes [$10^3/\mu\text{L}$]	8.99±4.05	9–30
Lymphocytes [$10^3/\mu\text{L}$]	3.14±2.07	1–2
Lymphocytes [%]	33.55±6.58	20–40
Monocytes [$10^3/\mu\text{L}$]	0.52±0.37	0.1–0.7
Monocytes [%]	6.1±2.92	2–8

Parameters	Mean±SD (n=50)	Normal values
Granulocytes [$10^3/\mu\text{L}$]	5.72±2.71	–
Granulocytes [%]	60.2±7.03	–
Platelets [$10^3/\mu\text{L}$]	176.96±81.5	150–300
Platelets [%]	0.11±0.06	–
Mean platelets volume [fL]	6.29±0.62	7.4–10.4

Because of the cultivation, we managed to boost the number of cells from $0.25\pm 0.01 \times 10^6$ to $3.1\pm 0.05 \times 10^6$ on the 5th day of cultivation, which constitutes a growth of 12.4 ($p<0.05$) times.

The collected supernatant was studied at 0, 3, 5, 7 and 9 days. The evaluated biochemical parameters were: total cholesterol, triglycerides, total proteins, albumins and Ca, Mg and P ions (Table 4).

Table 4 – Biochemical parameters of the cultivation environment

	Day 3	Day 5	Day 7	Day 9
Total cholesterol [g/L]	0.55 ±0.22	0.47 ±0.14	0.46 ±0.27	0.51 ±0.15
Triglycerides [g/L]	26.25 ±3.14	26.44 ±4.12	28.33 ±1.89	28.57 ±2.5
Total proteins [g/L]	153.1 ±33.22	141.2 ±16.04	148.88 ±18.69	164.47 ±22.5
Albumins [g/L]	10.28 ±3.94	10.52 ±1.97	9.97 ±3.19	9.56 ±1.21
Ca [mM/L]	4.46 ±1.66	3.32 ±1.38	4 ±1.18	5.06 ±2.44
Mg [mM/L]	0.55 ±0.23	0.65 ±0.24	0.66 ±0.3	0.73 ±0.19
P [mM/L]	0.66 ±0.17	0.74 ±0.38	0.81 ±0.43	0.54 ±0.22

The biochemical findings indicate that the development of the cell cultures is ascendant and within normal limits.

In our study, the karyotyping the umbilical cells through cytogenetic analysis, performed on three passages' duration (nine days) has established that, in the umbilical blood in 356 metaphases, there was found a normal karyotype in all of the cases (100%) 46 XX or 46 XY (Figure 4).

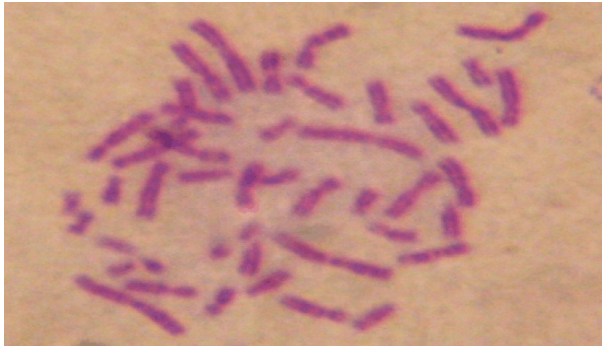


Figure 4 – Normal karyotype of the predecessor cells, 14 days from cultivation.

Determining cells of the CD34+ phenotype is consi-

dered to be more important, because their quantity is measured for determining the quality of the graft meant for transplant. In the samples that we have collected, the number of CD34+ phenotyped cells was $0.36 \pm 0.034 \times 10^7$.

Ten UB samples were immunologically investigated, and T (CD3+, CD45+) and B (CD19+) group lymphocytes were found. Concurrently the Ig A, M and G were determined in the cord blood and maternal blood.

The Ig were present in small, practically undetectable quantities within the cord blood, whilst within the mother's blood they had normal values, which allows us to confirm the previously performed researches that say that for finding the infectious diseases, the maternal blood, not the UB must be investigated (Figure 5).

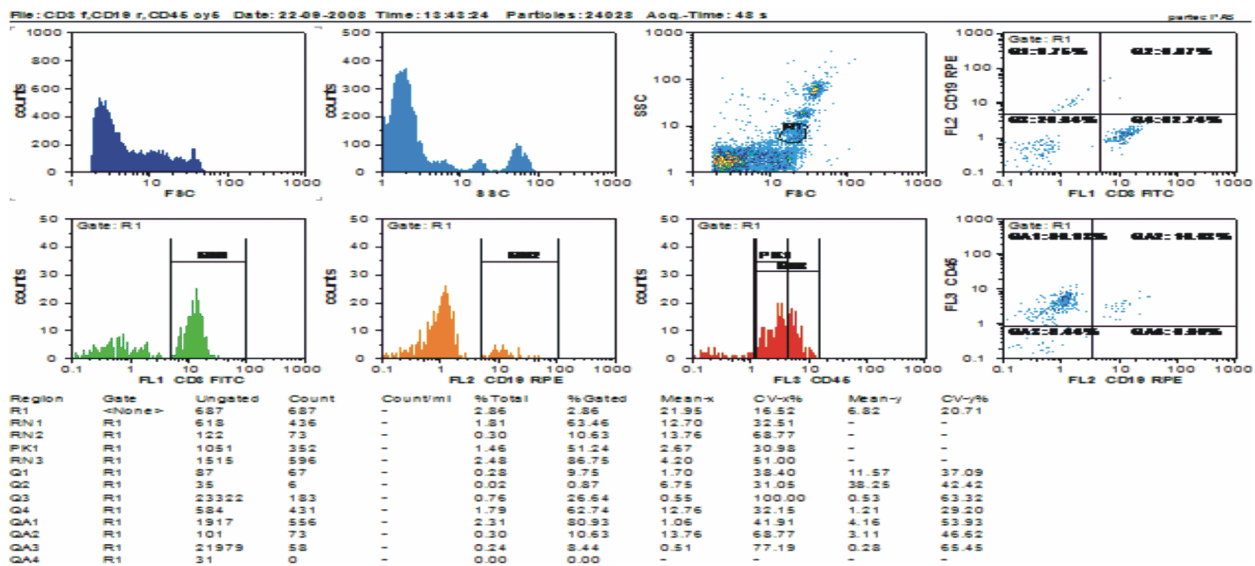


Figure 5 – Flow cytometry: T- and B-lymphocytes evaluation.

Discussion

Cellular therapy continues to be one of the most exciting and rapidly evolving areas in medicine with promising results in cancer treatment and regenerative medicine. The alternative to a bone marrow transplant is cord blood transplant.

The stem cells are attributed to the nucleated cells (NNC) that is why an important criterion in establishing the quality of the graft in the umbilical blood is appreciating the number of these said cells [11, 17, 29, 30].

The collection strategy is the first key step in collecting good quality UCB units, with a proper volume of blood, suitable for banking and donation. According to the literature, factors that minimize the problems of low cellularity and small volume of blood collected include: (1) placing the neonate on the mother's abdomen immediately after birth; (2) the correct timing of clamping the cord [31]; (3) the position of the placenta at the time of blood collection [32–36].

Several studies have shown that the total blood volume collected and the cellularity of UCB units could be influenced by several obstetric factors: placental weight (volume and cellularity improved when the placenta was >600 g), the neonate's birth weight (>3390 g), gestational age (>39 weeks), fetal blood pH,

duration of labor, the length of the umbilical cord (>55 cm), the experience of the person collecting the blood, placing the neonate on the mother's abdomen just after birth and the correct timing of clamping the cord (>30 seconds, <1 minute) [31, 32, 37–39].

As already described, there are two main techniques for collecting UCB: *in utero* and *ex utero*. Surbek DV *et al.* and Selves P *et al.* compared both collection strategies after vaginal deliveries, and concluded that *in utero* collection was associated with a significantly higher volume and TNC number. Selves found that the proportion of UCB units excluded, before processing, was 33% for the *ex utero* technique and 25% for the *in utero* technique [33, 34]. With regards to the *ex utero* technique, the presence of hemorrhagic foci in the delivered placenta and of blood clots in the placental fetal vessels could explain the lower levels of hematopoietic progenitors in UCB collected using this technique. Selves P *et al.* and Tamburini A *et al.* compared the *in utero* and *ex utero* techniques after delivery by Caesarean section and found that there was no significant difference in cell content between UCB units collected with the two techniques [35, 36].

In the present study, the gestational period, the weight and the gender of the newborn and the placenta's weight correlated with the volume of the collected samples, but not with their quality.

The amount of stem cells obtained from the umbilical blood is sometimes insufficient for treatment. The procedure of the cord blood transplant is confined to a single cord blood unit and used on children aged 12 and below. As a single unit of cord blood may not contain sufficient stem cells needed for an adult, a novel procedure would be to combine two cord blood units to increase the stem cell dose. However, this initially raised the issue on whether the two separate units would react with one another or against the patient. Dr. Koh said, "A double cord blood unit transplant is an innovative concept which enables us to use cord blood in adults. The best thing about cord blood transplants is that differences in blood group between the recipient and donors do not matter as much. The two units of cord blood can also be of different blood groups." [40].

We may also use and cultivation of stem cells in a nourishing environment with the goal of multiplying them and excluding the cells that are not stem [41].

Each sample is tested in order to assess the quantity of CD34+ (antigen marker of the hematopoietic cells); determining the blood group using the ABO system and the *Rhesus* factor. Other authors [42, 43] recommend the karyotyping for tracing any genetic maladies.

Cultivating stem cells could lead to the creation of anomalous karyotyped cells, which would contribute to "spoiling" the cellular graft. Appreciating the cytogenetic factors of the stem cells quality like: karyotyping, determining the ploidy and the chromosomal anomalies, and represents one of the main control phases.

Studies made by Bocicov NP (2007) [44] through the differential analysis of the colored mesenchymal stem cells (G-staining) prove the lack of chromosomal disturbances within the first few stages of cultivation. On the 11–14 passages some cultures, clones of a different karyotype have appeared (the monosomy of the 6th chromosome, translocation 21/22) [41].

In this study, we have demonstrated that the development of the cells during the cultivations periods is ascending and within normal limits, no pathological modifications being found, by biochemical, morphologic and karyotypic research.

☒ Conclusions

The gestational period, the weight and the gender of the newborn and the placenta's weight correlated with the volume of the collected samples, but not with their quality. Separating the cells using macromolecular liquids is an efficient, more cost effective way, the quantity of mononuclear obtained cells being about 85% of the total number. Biochemical, morphologic and karyotypic research indicates that the development of the cells during the cultivations periods is ascending and within normal limits, no pathological modifications being found.

Acknowledgments

These investigations were made possible thanks to the Institutional project No. 06.420.049A "Stem cells in the process of skeletal tissue regeneration" and of the Independent project No. 08.819.09.01F "Optimizing the separation and cryoconservation methods of umbilical blood – hematopoietic cell source".

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Corresponding author

Tatiana Revencu, PhD student, Laboratory of Tissue Engineering and Cell Cultures, Department of Obstetrics and Gynecology, “Nicolae Testemițanu” State Medical and Pharmaceutical University, 165 Ștefan cel Mare și Sfânt Avenue, MD-2004 Kishinev, Republic of Moldova; Phone +373 22 227782, e-mail: revencut@yahoo.com

Received: September 14th, 2012

Accepted: March 30th, 2013