The Influence of Certain Obstetric Factors on the Collection, Isolation and Quality of Cord Blood Units

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Abstract — **The umbilical cord blood is a rich source of hematopoietic and mesenchymal stem cells and it can be used for cellular therapy. This article presents data taken from 50 cord blood samples (46 *in utero*, 4 *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 (DulbeccoModified Eagle Medium) and biochemical and cytogenetic research was later performed.**

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Index Terms — **stem cells, cellular therapy, mesenchymal and hematopoietic stem cells, (umbilical) cord blood, flow cytometry.**

I. 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 viro* as: Osteoblasts [1,2,3], adipocytes [4], neurocytes [5,6], cardiomiocytes [7,8] and hepatocytes [9,10].

The number and differentiation potential of the bone marrow stem cells decreases in time, 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 terapy.

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 don't raise ethical problems [11, 12]. To this day, over 15000 cord blood cell transplants have been performed successfully on children and adults [13, www.nationalcordbloodprogram.org 2009].

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 osteomedular one. Thus, tehe amount of umbilical stem cells required for a successful transplant is 10 times smaller, because they are younger than the autologic, older 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 1200ml of bone marrow would [11,14].

Umbilical Blood (UB) has the following advantages:

- Fast availability – once the blood unit is taken, tested and conserved it is available for transplant [15].
- 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[16]. The explanation is that, if the blood of the newborn is immunologically immature, the immune system doesn't have enough time to be exposed to different immunogens and create antibodies that would counter them [17].
- 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[18].
- It has been established that UB has a lower chance of cytomegalovirus infection than bone marrow, consequently reducing the complications that follow a transplant [19, 20].

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 [21,22];

- 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 cryogenized on a 10 year period [23].

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

The **aim** of this study was to optimisation the method for isolation and to assess whether certain obstetric factors influence the quality of the umbilical.

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II. MATERIALS AND METHODS

Collecting the umbilical blood

The umbilical blood (n=50), has been collected from the newborns of the travailing mothers hospitalized at the Municipal Maternity Nr.2 from Chişinău.

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 won't be collected)
- On term, physiological births (38-42 amenorrheic weeks(AW))
- Obstetric criteria: - the weight of the newborn must exceed 2500g; an Apgar score that is higher than 8; a fluidless 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 300ml 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-HSV_{1+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 fluidless period's duration, the birth's type, the weight of the newborn and the placenta and the newborn's gender.

5 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 60000 molecular mass has been used, in a 1:1 ratio. The dextrane was added in the large bag. After the the erytrhocytes' sedimentation during a 30-60 minute 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 15ml tubes and then centrifuged at 100rpm. 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 were placed in a nourishing environment for cultivation/

Cultivating the cells

The cells were cultivated in a DMEM (HIMEDIA); 100 un/ml gentamicin and penicillin (SIGMA) and adjuvant in a CO_2 "Binder" incubator with a 5% CO_2 concentration, at 95% humidity, up to 3 passages on culture 25 and 75 cm^2 culture plates (NUNC), and changing the nourishing environment every 3 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^oC for the performing the following biochemical research.

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.

The 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 Lymphochrom (Lonza, Belgia) nourishing environment, and Colchihine (HIMEDIA, India) was added 3 days later, and then they were fixed in a methanol solution and glacial acetic acid in a 3:1 ratio. The Giemsa coloration was used for visualization.

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.

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 ± 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.

Table 1 Results

	mean±DS	min-max
Cord Blood Volume (CBV),ml	71,84±25,29	42,0 – 147,0
Weight of the newborn, g	3418,3±428,56	2400,0 – 4300,0
Weight of the placenta, g	571,7±81,82	350,0 – 855,0
The mother's age, years	25,66±3,8	18 – 36
The gestation period's duration, AW	39,78±1,01	38 – 42
Number of nucleated cells (NNC) (x10^6/ml)	11,2±9,85	1,0 – 52,6
CD34+ (x10^8/ml)	7,9±0,48	
Vitality, %	93,47±0,37	
The newborn's gender - **Boy**	23 (46%)	
- **Girl**	27 (54%)	

III. RESULTS AND DISCUSSIONS

Collecting the umbilico-placental blood:

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 [25-28].

Considering the small amount of blood that can be collected from the umbilical cord (100ml 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 NCN 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 (42,0 – 147,0), an NNC of 11,2±9,85 x106/ml (1,0 – 52,6) and the quantity of CD34+ - 7,9±0,48 x108/ml with a vitality of 93,47±0,37%. The mean weight of the newborns was 3418,3±428,56g (2400,0 – 4300,0), 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: 78ml of UB with a 12,4x10^6/ml NNC was collected from boys, on average, as compared with the 66,6ml and an NNC of 10,2x10^6/ml taken from the girls.

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

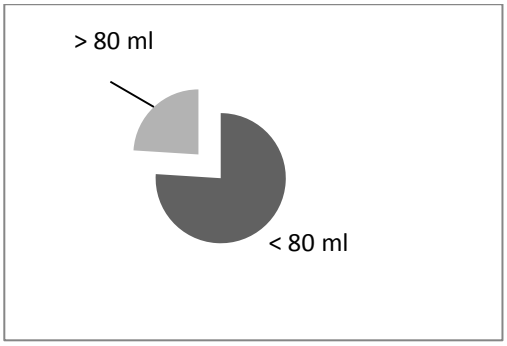

Fig.1. Volume of the collected cord blood

There are 2 main methods of UB collection: puncturing the umbilical vein while the placenta is still inside (*in utero*), or after it's expulsion (*ex utero*).

The *ex utero* collection is performed in a separate room by a well-instructed personnel, right after the placenta's ejection, in a standard collection set that contains anticoagulant (citrate-phosphate-dextrose with or without adenine). After the umbilical cord's disinfection, it is punctured [14].

The *in utero* method of extraction is performed after the child's birth, after the ligation of the cord, using the same hermetic collection system.

Randomized studies have previously shown that the *in utero* collection method is more efficient, because it allows a greater quantity of blood to be obtained, as well as a greater number of cells.

Comparing the *in utero* and *ex utero* methods, Laskey L.C. and his collaborators [29] haven't noticed a significant difference between these two methods, when it came to the number of collected cells, instead they noticed a high level of bacterial contamination or blood coagulation when the *ex utero* method was used. Sparrow R.L. and his collaborators [30] haven't noticed a difference of volume, leukocytes concentration or the total number of nucleated cells, but they noticed they remarked a greater quantity of umbilical blood obtain in case of caesarian birth, compared to normal birth. Solves P. and his cooperators [31] have noticed an increase in the volume of blood and number of nucleated cells, as well as CD34 cells obtained *in utero*, when compared to the *ex utero* method of extraction.

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 min, and the blood unit processing was performed within 24-28 hours from the time of extraction.

There are many obstetric, pre-natal and post-natal factors that have an influence on the process of blood collection, like: the birth type, the gestational age; the gender of the newborn, its weight; the placenta's weight and the time since the child's birth until the sectioning of the umbilical cord [32].

Some studies have proven the correlation between the ratio of the weight of the child/placenta and the volume of umbilical blood and NNC. A heavier newborn was associated with more UB and NNC. The weight of the placenta could be an obstetric criterion for selecting the blood units when the collection method is *ex utero*, and the weight of the newborn – when the collection method is *in utero* [31].

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

We have analyzed the difference between the

The amount of stem cells obtained from the umbilical blood is sometimes insufficient for treatment, so we cultivated them in a nourishing environment with the goal of multiplying them and excluding the cells that are not stem [33].

As a result of the cultivation, we managed to boost the number of cells from $0,25\pm0,01 \times 10^6$ to $3,1\pm0,05 \times 10^6$ 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 studied biochemical parameters were: total cholesterol, triglycerides, total proteins, albumins

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

	Spearman correlation coefficient , r	**Value, 95% ÍÎ**	**p**
Volume of cord blood,ml / weight of the newborn ,g	**0,45***	0,02 (0,01-0,03)	0,001**
NNC,x10^6/ml/ weight of the newborn,g	0,09	0,0025 (-0,002-0,01)	0,34
Volume of cord blood,ml / greutatea placentei,g	**0,47***	0,125 (0,03-0,24)	0,0005**
NNC,x10^6/ml / weight of the placenta, g	0,12	0,019 (-0,01-0,05)	0,2
Volume of cord blood,ml, / NNC,x10^6/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,x10^6/ml / gestational period, AW	0,16	1,35 (-0,5-3,8)	0,1

studied

indices, using Spearman (ρ) nonparametric 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 Thus, according to the obtained data, which correlates to those presented in literature, 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.

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

We haven't found an evident correlation between the analyze 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 doesn't depend exclusively on the obstetric factor or the volume of collected blood.

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

and Ca,

Mg and P ions and they indicate that the development of the cell cultures is ascendant and within normal limits.

Each sample is tested in order to asses the quantity of CD34+ (antigen marker of the hematopoietic cells); determining the blood group using the ABO system and the rhesus factor. Other authors [34,35] 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, represents one of the main control phases.

Studies made by Бочков Н., 2007, through the differential analysis of the colored mesenchymal stem cells (G-coloration) prove the lack of chromosomal disturbances within the first few stages of cultivation. On the 11-14 passages in some cultures, clones of a different karyotype have appeared (the monosomy of the 6th chromosome, translocation 21/22)[33].

In our study, the karyotyping the umbilical cells through cytogenetic analysis, performed on 3 passages' duration (9 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.

IV.CONCLUSION

1. 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.
2. 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.
3. 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.

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