

Artigo / Article

Hemopoietic progenitor cell identification in fetal and adult blood *Célula progenitora hematopoética - identificação em sangue fetal e de adulto*

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Hemopoietic progenitor cells give rise to all cellular elements of the blood and are of importance as a potential source of cells used for correction of various pathological conditions. The main objective of this study was to identify and quantitative hemopoietic progenitor cell in antenatal fetal blood, in cord blood at the time of delivery and in adult blood, using monoclonal antibodies to surface markers and flow cytometry. CD34+ cells, most of them probably representing progenitor cells, were detected in prenatal fetal blood as early as the 17th week of gestation. The proportion of these cells showed a tendency to decrease as the pregnancy progressed. Within the population of CD34+ cells, a relatively low proportion (less than 1%) were negative for the surface marker CD33 or HLA-Dr, indicating a population of primitive stem cells, i.e., progenitor cells no committed to a specific lineage. On the contrary, another group coexpressed CD33 or HLA-Dr, being more mature progenitor cells already committed to differentiate along a specific lineage. The percentage of CD34+ obtained in blood of adult patients after mobilization with chemotherapeutic agents and growth factors showed an average value of $2.7 \pm 3.1\%$. The percentage of CD34+ in the apheresis products of various patients varied from 0.58 to 1.48. In some cases the cells were reinfused in the patient with good results. Our findings are in agreement with previous studies suggesting that CD34+ stem cells is a heterogeneous population, with each subset having variable degree o commitment to differentiate toward a specific cell lineage. Rev. bras. hematol. hemoter. 2008; 30(Supl. 2):18-23.

Key words: Hemopoietic progenitor cell; flow cytometry; CD34+ cells; apheresis; mobilization; cord blood.

Introduction

Stem cells and progenitor cells from adult and fetal blood have enormous importance in autologous and allogeneic transplants in adults and children with malignancies and in some children with congenital deficiencies.^{1,2,3}

During the development of the human embryo and fetus, the first blood cells appear in the yolk sac by 16 to 19 days after conception; by 5-6 week, hematopoiesis is occurring mainly in the liver and by 8-19 weeks in the bone marrow of long bones.⁴

At the beginning of liver hematopoiesis (5-6 weeks of gestation), most of the cells present in hepatic sinusoids are

primitive erythroblasts. During the following 4 weeks these cells differentiate into the definitive erythroblast which produces mature erythrocytes. At this time, it is possible to identify the first macrophages, megakaryocytes, granulocytes and lymphocytes. Megakaryocytes are present during all phases of the hepatic hematopoiesis. The percentage of stem cells is high, up to 10% of all blood cells. A significant increase in granulocytes is observed beginning at 21 week of development, at the time of initiation of bone marrow hematopoietic.

At the beginning of hematopoietic (8th-9th week) in the bone marrow, the predominant cell is the primitive erythroblast, but that changes to the definitive erythroblast

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by 11th-14th week. Granulocytes appear in the bone marrow at 8-9th week of gestation. Macrophages increase in number from week 10 to week 16. Lymphocytes increase from 12% of all marrow cells by 23th week to 20%-30% in older fetuses. On the other hand, immature cells representing probably stem cells and progenitor cells decrease from 15% by 12-13th week to 1%-4% by 21-23th week. During the transition of hematopoiesis from the yolk sac to the liver and then to the bone marrow, large quantities of stem cell and progenitor cells circulate in the fetal blood.^{4,5}

With the use of monoclonal antibodies which recognize surface markers of primitive hemopoietic cells and the application of flow cytometry, it is possible to identify these cells in samples obtained from embryos and fetuses. Under normal conditions in adults the percentage of marrow cells expressing the antigen CD 34, a marker of primitive hemopoietic cells, is 1%-3%; this figure is usually ten times lower in blood (approximately 0.1%).⁶ Most of these CD 34+ cells are hemopoiesis stem cells or progenitor cells with the capacity to differentiate to all classes of mature blood cells *in vitro*.^{7,8,9}

Objectives

1. Identification and quantification of hemopoietic cells and CD34+ stem cells in prenatal fetal blood in uterus and in cord blood from newborn.

2. Identification of stem cells obtained through apheresis from patients with malignant diseases, previously mobilized with chemotherapeutic agents and the use of colony stimulating factors.

3. Use of stem cells obtained through apheresis for performing autologous transplants in adult patient.

Materials

120 blood samples were processed for the identification of progenitor cells.

A. Twenty three (23) samples of prenatal fetal blood were obtained from the umbilical cord of pregnancies (17 to 32 weeks of gestation), attended at the Perinatology Unit of the University Hospital of Caracas.

B. Twenty five (25) samples of cord blood of newborns (37 to 41 weeks of gestation). The patients were attended at the Obstetrics Service of the University Hospital of Caracas.

C. Eight (8) samples of peripheral blood of patients with various types of malignancies, taken before being treated for mobilization.

D. Twenty five (25) samples of peripheral blood of 12 patients with various malignancies after mobilization with cyclophosphamide y GM-CSF o G-CSF.

E. Thirty nine (39) samples of the apheresis product of 12 patients with various malignancies after mobilization. The diagnosis were:

- Infiltrating mammary ductal carcinoma (3)
- Lymphoma no Hodgkin (6)
- Multiple Myeloma of the IgG type (1)
- Acute myeloid leukemia in complete remission (1)
- Chronic myeloid leukemia (1)

Methods

Prenatal Blood

Twenty three prenatal fetal blood samples were obtained from umbilical cord blood by cordocentesis or intrahepatic vein puncture guided by fetal ultrasound.

The indication of cordocentesis (16 patients) or intrahepatic puncture (6 patients) or cardiocentesis (1 patient) were congenital malformation suspicion due to previous pregnancies with congenital malformation, abnormal fetal ultrasound, elevated maternal levels of alpha-fetoprotein or acetylcholine in serum or amniotic liquid, maternal age more than 35 years old, Rh incompatibility, maternal rubella

Stem cells collection

1. Cells collection was done in 12 patients with malignancies using the apheresis machine Baxter Fenwal CS3000 and Haemonetics MCS Plus at the Clinical El Avila Blood Bank.

2. A hemodialysis 126A x 1.5 rigid catheter or Hickman catheter was placed in the cava vein through the subclavia vein.

3. Hematological parameters were determined with a Coulter S Plus IV pre mobilization.

4. Cyclophosphamide (4g/m² intravenously) was used in 5 patients with malignant diseases (breast carcinoma and Non Hodgkin lymphoma) and G-CSF (16µg/Kg/day subcutaneously) and in 7 patients with NHL, AML, MM the GM-CSF was replaced by G-CSF.

Hematological parameters and CD34+ were done, this were obtained using monoclonal antibodies by flow cytometry in blood postmobilization.

1. 15 liters of blood were processed by apheresis to each patient.

2. The cryopreservation of the cells was done as follows: 45 ml of Hespan (Hidroxiethylstarch from Fresenius Laboratories) + 14,4 ml of albumin + 6,6 ml de DMSO (dimetilsulfoxido de los Laboratories Sigma).

3. Number of cells obtained inside of Baxter freezing bag 3x10⁷/ml a 8 x 10⁸/ml.

4. CD34+, HLA-Dr and others markers were determined in a sample from the apheresis product.

5. The apheresis product bag with the preservation solution was placed between two metal plates in order to form a thin layer of cells and kept in a Blood Bank freezer at -80°C.

Prenatal fetal blood processing

To each fetal blood the following studies were done:

1. Hemoglobin, hematocrit, MCV.
2. Electrophoresis of hemoglobin in cellulose acetate membranes from Helena Laboratories with buffer Tris-HCl-EDTA- Borato Ph: 8. Control blood were used such as Fetal Hb, Hb AA, Hb AS, Hb A₂

Immunophenotypic studies in prenatal fetal blood, cord blood, adult blood and apheresis product

Hematological parameters from blood samples were obtained by using a Coulter S Plus IV Counter. The number of cells was set at a concentration of 10.000/ μ l in phosphate buffer saline (PBS); 50 μ l of the cell suspension was incubated with the appropriate FITC-labeled monoclonal antibody (CD34, CD19, CD20, CD10, CD2, CD4, CD8, HLA-Dr, CD45). The samples of newborn cord blood were also stained simultaneously with monoclonal antibodies labeled with FITC or PE to allow the further separation of CD34+ cells (CD33-, CD34+/HLA-Dr+ y CD34+/HLADr-).

Flow cytometry

For identification and quantification of stem cells and other blood cell types were used a Beckton & Dickinson Flow Cytometer.

Lymphoid cells of the B lineage were identified as those staining with CD19, CD20, and CD10 and also with the HLA-Dr, anti-CD45 reagents, recognizing leukocyte antigens. Lymphoid cells of the T lineage were identified as those staining with CD2, CD7, CD4, and CD8 and also with the HLA-Dr, anti-CD45 reagents, recognizing leukocyte antigens. Cell of myeloid lineage were those staining with CD33, CD13, CD14.

Results

Hemoglobin, hematocrit, MCV, and Hb class were determined in all samples of prenatal blood. The values of Hb and crit increased in fetal blood with gestational age. The average HB at 17-20 weeks of gestation was 11,6 \pm 1 g/dl (crit 37 \pm 4, MCV 130 \pm 4), at 21-26 weeks was 12 \pm 1 g/dl (crit 38,7 \pm 3, MCV 125 \pm 8), at 27-32 weeks 13 \pm 1 g/dl (crit 40,9 \pm 4,1, MCV 117,9 \pm 7,6).

Hb electrophoresis was performed to assure that the blood samples were of fetal origin, and not from maternal origin. All blood samples contained fetal Hb (Hb $\alpha_2 \gamma_2$); no samples were found with Hb A (Hb $\alpha_2 \beta_2$), the normal adult Hb.

The immunophenotypic analysis of prenatal blood cells showed the presence of leukocytes positive for the

markers CD45 and HLA-Dr in samples of 17 to 32 weeks of gestation.

It was detected at 17-20 weeks CD45: 16,25 \pm 20,86, HLA-Dr: 9,5 \pm 10,61, at 21-25 weeks CD45: 65 \pm 48,8, HLA-Dr: 7,24 \pm 6,71 at 26-32 weeks CD45: 70 \pm 10, HLA-Dr: 21,5 \pm 19,09.

Lymphocytes T expressing markers such as CD2, CD7, CD4 and CD8 were also detected. CD2 and CD7 are markers of most T lymphocytes, while CD-4 and CD-8 identify subsets of T lymphocytes: CD4 helper subsets and CD8 the cytolytic subsets.

It was detected at 17-20 weeks CD2: 7,75 \pm 11,5, CD7: 10,9 \pm 13,8, CD4: 17,1 \pm 13, CD8: 7,38 \pm 5,87, at 21-25 weeks CD2: 1,3 \pm 0,4, CD7: 2,35 \pm 0,92, CD4: 13 \pm 2,83, CD8: 2,5 \pm 0,71, at 26-32 weeks CD2: 25 \pm 0,6, CD7: 34 \pm 0,7, CD4: 29 \pm 17, CD8: 12,5 \pm 4,95.

Similarly, markers for B lymphocytes were detected: CD10 (pre-B lymphocytes), CD19 and CD20 (immature B lymphocytes). It was detected in fetal blood at 17-20 weeks CD19: 2,5 \pm 0,2, CD20: 2,5 \pm 0,3, CD10: 3,3 \pm 2,1, at 21-25 weeks CD19: 6,5 \pm 4,95, CD20: 2,2 \pm 0, CD10: 3 \pm 2,83 at 26-32 weeks CD19: 16 \pm 7,07, CD20: 7 \pm 0,5, CD10: 1,25 \pm 1,06.

The number of CD34+ stem cells showed a tendency to diminish as the gestational age increased. CD34+ in fetal blood at 17-20 weeks, 3,23 \pm 4,33; at 21-25 weeks 2,03 \pm 0,87, and at 26-32 weeks: 2,56 \pm 0,67.

Fetal blood cells also expressed myeloid markers: CD13, CD14 y CD33.

CD13 is usually found in monocytes and polymorphonuclears, while CD14 is expressed only in monocytes, and CD33 in cells of the myeloid lineage. CD11b+ cells were also detected. This marker is usually found in monocytes, myelocytes, NK cells and in subsets of T lymphocytes.

It was detected in fetal blood at 17-20 weeks CD13: 7,7 \pm 7,4, CD14: 1,9 \pm 1,2, CD33: 2,6 \pm 0,6, at 21-25 weeks CD13: 7,7 \pm 1,4, CD14: 0,8 \pm 0, CD33: 4,8 \pm 1,7, at t 26-32 weeks CD13: 9,7 \pm 7,4, CD14: 1,9 \pm 1,2, CD33: 8 \pm 4,2, CD11b: 39 \pm 0.

Studies in cord blood

Hematological parameters were also determined in all samples of cord blood taken at the time of delivery. The mean SD values of Hb and hematocrit were 14,1 \pm 0,07 g/dl and 42,8 \pm 1,6%, respectively, for pregnancies of 37-41 weeks. The MCV showed macrocytosis up to 112 fl.

Most white cells in cord blood stained positive for the marker CD45 (95,8 \pm 2,53%), a marker of leukocytes, while only 0,86 \pm 0,33% of the cells stained positive for CD34.

Similarly, the expression of lymphoid surface markers in cord blood were: T lymphocytes (CD2: 72,1 \pm 11 %), pre-B lymphocytes (CD10: 0,4 \pm 0,51 %), B lymphocytes (CD19: 15,7 \pm 3,7 % and CD20: 13,9 \pm 5,5 %). Cells positive for CD 33, a marker of myeloid cells and CD 14, a marker of monocytes were 0,83 \pm 0,3 % and 0,54 \pm 0,2%, respectively.

To differentiate subsets of progenitor cells CD 34+, we used simultaneous staining with CD34 PE/ CD33-FITC y CD34 PE / HLA-Dr-FITC monoclonal antibodies. Cells CD34+ / CD33 - represented $0.8 \pm 0.5\%$ of the total population CD34+ / CD33 + represented only 0.3 ± 0.1 .

Apheresis of stem cells in adults

The majority of the patients showed pre-mobilization pancytopenia due to previous multiple chemotherapy and radiotherapy cycles as treatment of their malignant disease.

In general our patients post mobilization had anemia Hb 11.1 ± 0.9 , crit: $34.8 \pm 2.7\%$, WBC count showed values of $3580 \pm 2145/\mu\text{l}$ (range: 1300- 8800/ μl), monocytosis $24.7 \pm 13.6\%$ with light eosinophilia de $7.5 \pm 4.7\%$. Platelet count was $67200 \pm 96200/\text{l}$.

The CD34+ cells were determined daily and the apheresis was done when the CD34+ began to increased and then 8 units of platelets concentrates were infused when the patient had less than $50.000/\mu\text{l}$ platelets since it was noticed that each apheresis decreases the platelet count in approximately $15\,000/\mu\text{l}$.

The percentage of CD34+ obtained in blood of adult patients postmobilization showed average values of $2.7 \pm 3.1\%$ and leukocytes, lymphocytes B and T, subsets of T lymphocytes (helper and cytolytic), monocytes and myelocytes were also present in the phenotypic study.

The percentage of CD34+ postmobilization with G-CSF vs. leukocytes count in blood in a patient with AML in complete remission is showed in the table IV. It could be seen that the patient had a leucocytosis of $20.600/\mu\text{l}$ at 4th day postmobilization that increased to $58.500/\mu\text{l}$ at 70 day and the CD34+ percentage increased from 0.03% to 0.20% and then it dropped to 0.012% .

A CBC also was done to each apheresis product and the leukocyte count increased up to $767 \times 10^3/\mu\text{l}$ (168 ± 23.9) and the platelet count up to $5.620 \times 10^3/\mu\text{l}$ ($2.579 \times 10^3/\mu\text{l} \pm 2640$) while the hemoglobin decreased to $6 \text{ g/dl} \pm 2.6$ and the hematocrit to $24\% \pm 10.8$ (Table VII). When the mobilization was done with G-CSF the amount of WBC in the apheresis product was bigger.

The percentage of CD34+ in the apheresis products of various patients was 0.50 to 6.33 . The viability of the apheresis cells with try pan blue was 78% to 97% depending of the time of the collection.

The cell collected by apheresis showed surface markers stained with monoclonal antibodies anti-HLA-Dr and anti-CD45. Some cells stained with anti CD33 (myeloid marker), anti-CD13 and anti-CD14 (monocytic markers).

The percentage of cells staining with monoclonal antibodies recognizing B lymphocyte markers were: CD19: 1.15 ± 0.93 ; CD20: 1.15 ± 0.93 ; and CD10: 1.33 ± 1.62 .

The percentage of cell expressing T lymphocyte markers were: CD2: 51.60 ± 36.9 ; CD7: 26.1 ± 26.1 ; CD4 (helper T

lymphocytes): 17.2 ± 10.7 ; CD8 (cytolytic T lymphocytes): 21.7 ± 24.1 .

The average of collected cells in 33 apheresis in cancer patients after mobilization was $2.32 \pm 1.62 \times 10^5$. The median number of MNC harvested per apheresis was $1.75 \pm 1.25 \times 10^8$ /Kg. The total number of CD34+ cells per kg of body weight was $2.25 \pm 2.57 \times 10^6$.

When it was compared the percentage of CD34+ cells in blood postmobilization and in the apheresis products, the CD34+ cell increased in the product in relation to the blood.

The number of CD34+ reinfused was 3.6 ± 14.7 cells $\times 10^6$ /Kg. The range was big and it depended of the amount of therapies that the patient received.

Discussion

The prenatal cord blood samples used in this study were taken in the course of diagnostic procedures in patients suspected of having congenital malformations. We assumed that the existence of defects such as neural, gut, renal malformation did not influence the results since the hematopoietic cells have their embryological origin completely separated from other fetal tissue as it has been demonstrated in the last century for several investigators doing microscopic analysis of section of human embryos have described the sequence of hematopoietic development that occur in embryos and fetus that it is completely different to others tissue. Thus, it has been described that during human development in uterus the blood cells show up in the yolk sac for first time around days 16-19, follow by fetal hepatic hematopoiesis between the 5th and 6th week and then between the weeks 8-19 migration and expansion of hematopoietic cells to the long bones. This expansion occur mainly between the 11-12th week of fetal life, so, most of the bones become producers of hematopoietic cells by this time.¹⁰ The liver become the primary site of hematopoiesis from week 6 to 22 of gestation; from here on the bone marrow becomes the principal production site of blood cells.^{11,12} These findings already described for other authors support our hypothesis that prenatal blood cells from fetuses with congenital malformation are appropriate for the study of cellular surface antigens since most fetuses' malformations do not affect the development of fetal hematopoiesis.

Various hematological parameters studied indicate that the blood samples obtained were actually fetal blood, not contaminated with maternal blood. For example, in all samples the MCV was high indicating the macrocytosis typical of fetal blood, and the electrophoresis of Hb showed the absence of Hb AA, the normal adult hemoglobin an the presence of fetal hemoglobin in all samples of pregnancies below 32 weeks o gestation.¹³

In the samples from 17-20th week of gestation the hemoglobin concentration was $11.6 \pm 1 \text{ g/dl}$; it increased with

gestational age reaching the value of 13 ± 1 g/dl by the 27-32 week. Similarly, in the same period the hematocrit increased from 37% to 40.9%. The MCV was 130 fl (higher than the normal adult value) decreasing to 117 fl by the end of the pregnancy. This macrocytosis is characteristic of fetal red cells; maternal erythrocytes show lower values of MCV, unless the mother suffers from severe macrocytic anemia due to folic acid or vitamin B12 deficiency.¹⁴ These results are in agreement with those reported in the literature.

CD34+ cells, most of them probably representing stem cells, were detected in prenatal fetal blood as early as the 17th week of gestation. The proportion of these cells showed a tendency to decrease as the pregnancy progressed. It has been reported previously the presence of the CD34 molecule on hemopoietic cells from human fetal liver; the number of molecules in the surface of these cells is greater than on hemopoietic cells of the adult.^{15,16}

Hemopoietic progenitor cells have been identified as early as the 23th day of gestation in the yolk sac and in embryonic tissues. Hemopoietic islands are observed in the lumen of the primitive vessels in the yolk sac wall and CD34+ cells can be detected also in the ventral region of the dorsal aorta around the pre umbilical region.¹⁷ Huyhn *et al.* isolated CD34+ cells from the yolk sac, liver and embryo proper of 35-40 days embryos and cultured them in methylcellulose; they reported that hemopoietic progenitors were more numerous in the embryo proper than in liver or yolk sac. They interpreted these results as evidence of the presence of hemopoietic precursors in the embryo proper before initiation of liver hemopoiesis. These results confirm earlier results indicating that definitive liver hemopoiesis follows after migration of progenitors from embryonic issues.¹⁸

In the prenatal blood samples we could identify leukocytes as cell staining with the anti-HLA-Dr and anti-CD-45 monoclonal antibodies. Within this population, we also detected granulocytic leukocytes as indicated by staining with anti-CD33 monoclonal antibodies. Neutrophilic granulocytes have been detected in bone marrow of 10-13th week fetuses. By 21th week of gestation Neutrophilic granulocytes are the most numerous leukocytes in the blood, and around 60% of the cells in the bone marrow were proliferating Neutrophilic granulocytes or mature cells. The eosinophils are also present in bone marrow of 10 week embryos (1%), gradually increasing to 5% of all marrow cells by the 21th week. On the other hand, the basophiles are scanty by 10th week of gestation and remain in low amounts (0.3%) even by the 28th week.

Mature and immature neutrophils were identified in the spleen only by the 16th week of gestation.^{4,19} Unfortunately, the anti-CD33 monoclonal antibody used in our study does not allow differentiating among the classes of granulocytes.

T lymphocytes, identified by the CD-2 and CD-7 surface molecules, subsets of T lymphocytes, identified by the CD-4

or CD-8 surface markers, and B lymphocytes bearing the CD19 or CD20 surface marker were also detected in prenatal fetal blood from the 17th week of gestation. Morphologically, lymphocytes have been identified in fetal liver as early as the 5th week of gestation;^{4,19} however in these early studies no subsets of lymphocytes were differentiated.

Within the population of CD34+ cells, a relatively low proportion (less than 1%) were negative for the surface marker CD33 or HLA-Dr, indicating a population of primitive stem cells, i.e., progenitor cells not committed to a specific lineage. On the contrary, another group co expressed CD33 or HLA-Dr, more mature progenitor cells already committed to differentiate along a specific lineage. This pattern is similar to that observed in hemopoietic stem cells of adult bone marrow, where the more primitive cells are CD34+, CD38- and HLA-Dr- (can differentiate to hemopoietic progenitors or stromal cells), and the less primitive cells, CD34+, CD38-, HLA Dr+, which differentiate only to hemopoietic progenitors.²⁰ Waller *et al.* have also reported results suggesting the existence of separate populations of stem cells, the more primitive expressing also variable amounts of CD13, CD49, CD50 and CD54 but not more mature markers such as CD13, CD15, CD3 and CD20.²⁰

Our findings are in agreement with previous studies suggesting that CD34+ stem cells is a heterogeneous population, with each subset having variable degree of commitment to differentiate toward a specific cell lineage.

Resumo

As células progenitoras hematopoéticas são as responsáveis pela produção de todos os elementos do sangue e são as potenciais fontes de células usadas para o tratamento de várias condições patológicas. O principal objetivo deste trabalho foi identificar e quantificar as células progenitoras hematopoéticas no sangue fetal do período pré-natal, no sangue de cordão umbilical no momento do parto e no sangue do adulto, usando anticorpos monoclonais para marcadores de superfície e citometria de fluxo. As células CD34+ na maioria das vezes representam células progenitoras e foram detectadas no sangue fetal pré-natal tão precoce como na 17ª semana de gestação. A proporção destas células mostrou a tendência de diminuir durante a progressão da gestação. Dentro da população de células CD34+, uma proporção relativamente pequena (menos de 1%) foi negativa para os marcadores de superfície CD33 ou HLA-Dr, indicando uma população de células primitivas, isto é, células progenitoras não comissionadas com uma linhagem específica. Ao contrário, outro grupo co-expressa CD33 ou HLA-Dr, sendo progenitores celulares mais maduros já comprometidos com linhagens específicas. A porcentagem de CD34+ obtida no sangue de adultos após mobilização com agentes quimioterápicos e fator de crescimento mostrou uma média de 2.7+/-3.1%. 0 % de CD34+ no produto aferético de vários pacientes variou de 0.58 a 1.48. Em alguns casos as células foram infundidas nos pacientes com bons resultados. Nossos achados estão de acordo com estudos prévios sugerindo que células CD34+ sejam uma população heterogênea

com cada subgrupo apresentando graus de comprometimento com diferentes linhagens específicas. *Rev. bras. hematol. hemoter.* 2008; 30(Supl. 2):18-23.

Palavras-chave: Célula progenitora hematopoietica; citometria de fluxo; células CD34+; aferese; mobilização; sangue de cordão.

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