

## ARTICLE — CLINICAL STUDY

## Two enrichment methods to obtain CD34+ stem cells from umbilical cord blood

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### Abstract

We describe the relation between umbilical cord clamping time and two different enrichment systems of CD34+ stem cells from umbilical cord blood with the proliferative ability and bone marrow reconstitution of the stem cells obtained. After an obstetrician performed the cord blood collection, the purification of stem cells was performed either with a combination of monoclonal antibodies (negative selection) using the Stem Sep method, or with a positive cell selection based on their surface CD34 antigens using the Mini Macs system. An excellent recovery of haematopoietic progenitors (Burst Forming Unit Erythroids (BFU-E); Colony Forming Unit Granulocytes and Macrophages (CFU-GM); and Colony Forming Unit Granulocytes, Erythroids, Monocytes and Macrophages (CFU-GME)), inversely related to the increase in clamping time, was performed with the Mini Macs system (54 % of colonies, with a 90 % purity). With the Stem Sep method, haematopoietic progenitors recovery was 35 % (with a 80 % purity). By applying early clamping of umbilical cord blood we obtained a greater number of CD34+ cells and their clonogenic activity was increased with enrichment. This is a useful technique considering that the number of CD34+ stem cells usually contained from a unit of placental blood is enough for the transplant to a child, but not for an adult. Thus, using these methods, we can get a larger number of CD34+ stem cells which reduces the risk of Graft versus Host Disease also in adult patients, producing survival rates similar to those obtained with transplantation of bone marrow from unrelated donors. (Fig. 4, Ref. 18.)

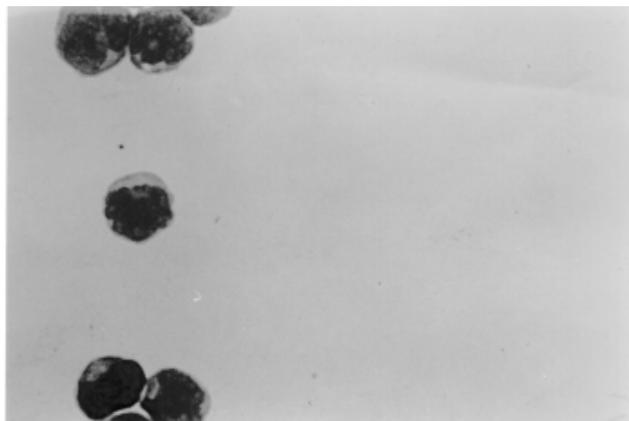
**Key words:** CD34+ stem cells, umbilical cord blood, recovery, enrichment, bone marrow reconstitution.

Haematopoietic cells are heterogeneous and belong to different lineages and are at different stages of maturity. The structural and functional integrity of the haematopoietic system is maintained by CD34+ stem cells (Fig. 1), which can self-renew, producing other stem cells or differentiate and produce a various haematopoietic lineages (1). Haematopoietic progenitor stem cell transplantation is an important therapy for certain haematological and malignant disorders (2, 3, 4, 5). Umbilical cord blood, also called placental blood, contains a high number of haematopoietic progenitor cells, which are known to possess significant potential for clinical application, and show a high proliferative capacity (6). In fact, this source of haematopoietic stem cells is characterised by a real ease of procurement, absence of risk to the donor, and a small likelihood of transmitting clinically important infections, especially Cytomegalovirus and Epstein Barr Virus (7). Recent studies have further indicated that umbilical cord blood can be given in vivo to fully and partially HLA-matched siblings or non-familial recipients for bone marrow reconstitution in genetic disorders as well as malignancies. In comparison to adult peripheral blood, umbilical cord blood has displayed decreased immune responses to alloantigens and has a low incidence of Graft

versus Host Disease, but sufficient Graft versus Leukemia activity (8). These factors are important for the risk of relapse and mortality (9). However, previous experiences show that a unit of placental blood, collected from a single donor after processing and testing, contains a relatively low number of CD34+ cells. So there may be enough haematopoietic stem cells to reconstitute the bone marrow in children, but the ability to engraft an adult might require ex vivo manipulation (10). Thus, questions arise regarding the efficiency of cord blood collection. The aim of this study was to improve obstetrician-based cord blood collection and the efficiency of CD34+ cell recovery (11). We have developed a technique which actually flushes and collects placental derived stem cells, using two systems of CD34+ cells purification: negative selection with Stem Sep method and positive selection with Mini Macs. In this study the precocity of blood collection will permit a larger

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**Fig. 1. Morphology of CD34+ cells isolated from umbilical cord blood with the Mini Macs system. May Grunwald-Giemsa stain preparation. Magn. x120.**

number of haematopoietic progenitor cells to be collected avoiding early stasis phenomena and intravascular coagulation, and reduce the transfer of red blood cells. Clamping time was calculated from the time of birth until the clamping of umbilical cord blood with clips. Umbilical cord blood volume (120 ml) increased with a plateau between 20–30 sec.

#### Materials and methods

Obstetricians at the Institute of Obstetrics and Gynaecology — University of Catania — S. Bambino Hospital obtained, after informed consent and according to institutional guidelines, 150 samples of umbilical cord blood.

Placental blood units were collected from freshly delivered placentas in preservative containing 21 ml of CPD anticoagulant (Monohydrate citric acid 3.27 g, Citrate sodium 26.30 g, Dihydrogenophosphate 2.51 g, Monohydrate dextrose 25.50 g in 1000 ml of injectable water). Abstracted data were obtained from the mother's interviews and the mother's and infant's medical records. For purpose of consent and analysis, the mother was considered to be the donor. The collection (12) was effected after the delivery of an infant (keeping the baby under the level of his mother's perineal region, to increase the blood flow from placenta), after the double ligation of the cord (by applying two hemostatic clamps a few cm from the umbilicus), the cord cutting (in the space between the two clamps), and before the placental detachment. The distal portion of the umbilical cord was cleaned with iodated alcohol (Betadine solution and alcohol), the umbilical vein was cannulated and the sample was collected by gravity into sterile set for placental collection. A higher blood quantity was collected when the sampling was performed before placental detachment, both in vaginal deliveries and in caesarean sections. Residual materno-placental circulation represented a "Vis a tergo" that permitted blood to flow from the maternal section of placenta. In addition there were uterine contractions during the placental stage. The blood quantity collected depended on: the period of gestation, placental weight, placental infarction, umbilical cord features, fetal respiratory distress, placental weight/neonate

weight ratio, the timing of bleeding after delivery, the clamping time and the kind of placental detachment (central or limbic). A greater blood quantity was obtained when the pregnancy was at term and was significantly and directly related to the placental weight and to the absence of infarction. Early blood collection, when other conditions were equal, permitted a collection of a greater blood quantity, despite early stasis phenomena and intravascular coagulation.

Of the 150 sampling collected, 131 were analysed. The remaining were not useful for the study, being less than 60 ml, (and then too much diluted with the anticoagulant), or showing precocious coagulation phenomena.

Once collected, the blood was separated over a Ficoll — Hypaque density gradient (d-1.077) at 400 g for 40 minutes at 20 °C. Interface mononuclear cells were collected, twice washed, and resuspended in Iscove's modified Dulbecco's medium. Cell vitality was assessed by the trypan blue exclusion test, and cell mortality never exceeded 10 %. Cord blood mononuclear cells were resuspended in Iscove's modified Dulbecco's medium containing 20 % heat-inactivated fetal calf serum at a cellular density of  $2 \times 10^6$  cells/ml and then treated with overnight plastic adherence cycles. Cells were washed three times, resuspended in PBS and prepared for either Mini Macs or Stem Sep separation. CD34+ cells were then resuspended in a small volume of medium, counted, and plated in semisolid medium for clonogenic assays and liquid culture as indicated below. Purification of CD34+ cells from total cord blood was obtained either with a combination of monoclonal antibodies, glycoforina A, CD3, CD2, CD24, CD19, CD14, CD16, CD66 negative selections, using the Stem Sep method or positive selection of CD34-expressing cells using a Mini Macs system. An excellent recovery of haematopoietic progenitors (with a CD34+ purity of 99 %) and haematopoietic colony forming cells (Burst Forming Unit Erythroids; and Colony Forming Unit Granulocyte and Macrophages) were obtained with the Mini Macs system. Purified cells, with Mini Macs or Stem Sep system, were plated in methylcellulose culture at a density of  $1 \times 10^3$  cells (two plates per point), in 0.9 % methylcellulose, and 3.0 IU/ml erythropoietin, Granulocyte and Macrophage Cell Stem Factor (10 ng/ml), and Interleukine 3 (100 U/ml) in Iscove's modified Dulbecco's medium supplemented with A-thioglycerolo (10-4 mol/ml) at 37 °C in a 5 % CO<sub>2</sub>/O<sub>2</sub> humidified atmosphere. Colonies were categorised after 14 days.

#### Results

We studied the effect of clamping time on the number of CD34+ cells isolated. The modification of umbilical cord blood depending on clamping time was investigated. Clamping time was calculated from a fetus birth till the clamping of umbilical cord blood with clips. The umbilical cord blood volume collected increased when clamping was performed earlier, with a "plateau" between 20 and 30 seconds (Figs 2, 3). The volume of detected umbilical cord blood was equal when clamping time was increased over 30 seconds. White blood cells, red blood cells and the haematocrit of the newborn increased as clamping time increased. A bigger blood quantity was obtained when bleeding was performed before placental detachment, both in vaginal deliveries and in Caesarean sections under ultrasonic guidance. Residual materno-placental circulation represented a "Vis a tergo" that permitted blood to flow from the fetal sections of the placenta to the umbilical cord; in addition there

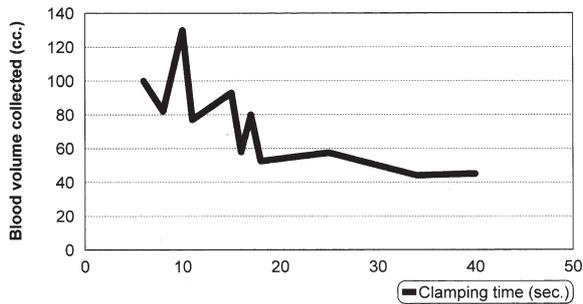


Fig. 2. Relation between umbilical cord clamping time and fetal blood volume collected.

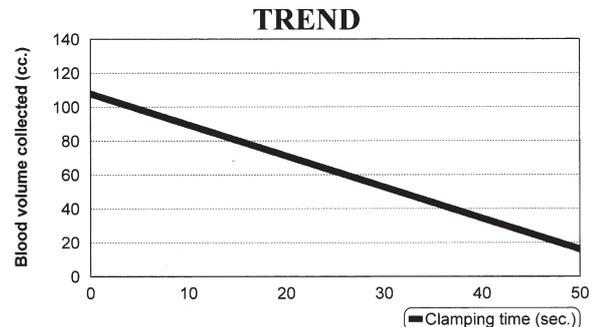


Fig. 3. Relation between umbilical cord clamping time and fetal blood volume collected. (Trend)

were uterine contractions during the placental stage. Anyway early blood collection permitted a larger quantity collection, avoiding the early stasis phenomena and intravascular coagulation.

Haematopoietic progenitors were purified from cord blood using two methods: Mini Macs separation (the final index of purification was 99 %); and Stem Sep method (obtaining a purity of 80 %).

The effect of clamping time on the clonogenic capacity was related to how early the blood was collected. The purified umbilical cord blood progenitors included a large majority of Burst Forming Unit Erythroid and Colony Forming Unit Granulocytes and Macrophage colonies and a minority of multipotent progenitors (Colony Forming Unit Granulocytes, Erythroids, Monocytes and Macrophages). These progenitors gave rise to large colonies in clonogenic culture. The number of colonies obtained with Mini Macs system was larger (54 %) than the those obtained with the selection to the negative using the Stem Sep system (35 %) (Fig. 4). In both cases each colony contained  $10^5$  cells.

## Discussion

Haematopoietic stem cells are increasingly used for treatment of both malignant and non-malignant disorders (13, 14, 15). Various attempts have been made in recent years to expand and manipulate these cells in order to increase their therapeutic potential. In particular, cord blood is an attractive alternative to bone marrow or to peripheral blood cells mobilised by growth factors as a source of transplantable haematopoietic tissue (16, 17). This study was directed to improve the feasibility of obstetrician-based cord blood collection system and enrichment of CD34+ progenitor cord blood to obtain the optimal haematopoietic progenitor recovery (18). It has been reported that the earlier collection permits collection of a larger quantity, (avoiding early stasis phenomena and intravascular coagulation). This method has several clinical advantages: a) if a newborn is underweight (preterm) is necessary to immediately cut the umbilical cord to avoid risks related to abrupt circulatory overload and to permit reanimation as soon as possible, b) immediate umbilical cord clamping is needed in red cell materno-fetal isoimmunization to reduce the transfer of fetal blood containing red cells with superficial antigens to his mother, c) in addition, if the mother is Rh negative, it is helpful not to occlude the umbilical cord near the placenta and to reduce the possi-

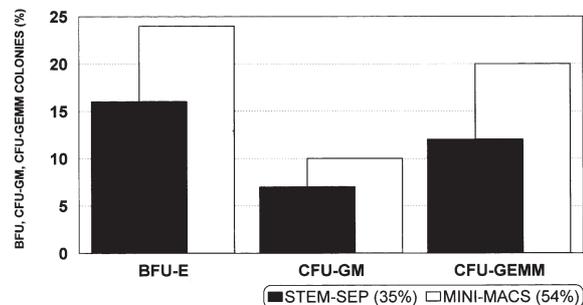


Fig. 4. CD34+ recovery performed using Stem Sep Method (35 % of colonies) and Mini Macs Method (54 % of colonies). BFU-E — Burst Forming Unit Erythroid, CFU-GM — Colony Forming Unit Granulocyte and Macrophage, CFU-GEMM — Colony Forming Unit Granulocyte, Erythroid, Monocyte and Macrophage.

le passage of fetal red cells to the maternal circulation. With this study, we found that a more efficient recovery of CD34+ cells from UCB was obtained by early clamping. The blood volume collected was related to the decrease in clamping time, with a “plateau” between 20 and 30 seconds. Using the magnetic cell sorting Mini Macs or Stem Sep system to isolate CD34+ cells we demonstrated an excellent recovery of haematopoietic progenitors and haematopoietic colony forming cells when an umbilical cord blood cut was made early. We can state that by applying early clamping of the umbilical cord, we obtained a greater number of CD34+ cells; than their clonogenic activity increased with enrichment and, finally, that by using a positive selection (Mini Macs method) we got better results (54 % of colonies) than by using a negative selection (Stem Sep system — 35 % of colonies), obtaining a yield of 99 % instead of 80 %. This is particularly useful considering that the number of CD34+ stem cells contained in one unit of placental blood is enough for transplanting a child, but not for an adult. Thus, by using these methods, we increased the possibility of engraftment in adult patients, and the possibility of producing survival rates similar to those obtained with the transplantation of bone marrow from unrelated donors. Umbilical cord blood may be considered a useful source of haematopoietic stem cells for patients who do not have an HLA-related donor.

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## Abstrakt

Pafumi C., Zizza G., Pernicone G., Bandiera S., Farina M., Russo A., Iraci M., Cianci A.:  
Dve obohacujúce metódy získavania CD34+ kmeňových buniek z pupočníkovej krvi  
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Opisujeme vzťah medzi časom zasvorkovania a získaným krvným objemom a dve obohacujúce metódy získavania CD34+ kmeňových buniek z pupočníkovej krvi za účelom určenia vynikajúcej izolácie s vysokou schopnosťou proliferácie a rekonštitúcie kostnej drene. Pôrodník odoberie krv z pupočnej šnúry a následne vykoná purifikáciu kmeňových buniek, a to buď kombináciou monoklonálnych protilátok (negatívna selekcia) použitím metódy Stem Sep, buď pozitívnu bunkovú selekciu využitím povrchových antigénov CD34 použitím systému Mini Macs. Bola urobená vynikajúca izolácia krvotvorných progenitorov; Burst Forming Unit Erythroid (BFU-E); Colony Forming Unit Granulocyte and Macrophage (CFU-GM); a Colony Forming Unit Granulocyte, Erythroid, Monocyte and Macrophage (CFU-GME); nepriamo úmerná vzostupu času svorkovania. Pri použití systému Mini Macs sa dosiahla izolácia 54 % kolónií s 90 % čistotou, kým pri použití metódy Stem Sep sa dosiahla 35 % izolácia krvotvorných progenitorov s 80 % čistotou. Skorým zasvorkovaním krvi pupočnej šnúry sme dosiahli vyšší počet CD34+ buniek a ich klonogénna aktivita sa obohatením zvýšila. Tento fakt je osobitne dôležitý, keď zväzíme, že počet CD34+ kmeňových buniek obsiahnutých v jednotke placentárnej krvi je dostatočný na transplantáciu u dieťaťa, no na transplantáciu u dospelého človeka nestačí. Preto pri použití týchto metód môžeme získať vyšší počet CD34+ kmeňových buniek, ktoré zvyšujú možnosť redukcie reakcie transplantát versus recipient u dospelých pacientov s mierou prežitia, ktorá je podobná ako pri transplantáciách kostnej drene od nepríbuzných donorov. (*Obr. 4, lit. 18.*)

Kľúčové slová: CD34+ kmeňové bunky, izolácia, obohatenie, rekonštitúcia kostnej drene.