Rev Bras Cir Cardiovasc 2008; 23(1): 29-34

Blood mesenchymal stem cell culture from the umbilical cord with and without Ficoll-Paque density gradient method

Cultivo de células mesenquimais do sangue de cordão umbilical com e sem uso do gradiente de densidade Ficoll-Paque

Rosa Sayoko KAWASAKI-OYAMA[1], Domingo Marcolino BRAILE[2], Heloisa Cristina CALDAS[3], João Carlos Ferreira LEAL[4], Eny Maria GOLONI-BERTOLLO[5], Érika Cristina PAVARINO-BERTELLI[6], Mário ABBUD FILHO[7], Izaura dos SANTOS[8]

RBCCV 44205-946

Abstract

Objectives: **Implantation of cell separation and mesenchymal stem cell culture techniques from human umbilical cord blood with and without using the Ficoll-Paque gradient density method (d=1.077g/ml).**

Methods: **Ten samples of the umbilical cord blood obtained from full-term deliveries were submitted to two different procedures of mesenchymal stem cell culture: a) Method without the Ficoll-Paque density gradient, which concentrates all nucleated cells; b) Method with the Ficoll-Paque density gradient, which selects only low-density mononuclear cells. Cells were initially plated into 25 cm^2 cultures flasks at a density of $1x10^7$ nucleated cells/cm^2 and $1x10^6$ mononuclear cells/cm^2.**

Results: **It was obtained 2-13x10^7 (median = 2.35x10^7) nucleated cells/cm^2 by the method without the Ficoll-Paque gradient density, and 3.7-15.7x10^6 (median = 7.2x10^6) mononuclear cells/cm^2 by the method with the Ficoll-Paque gradient density. In all cultures adherent cells were observed 24 hours after being cultured. Cells presented fibroblastoid and epithelioid morphology. In most of the cultures, cell proliferation occurred in the first week, but after the second week only some cultures - derived from the method without the Ficoll-Paque gradient density - maintained the growth rate reaching confluence. Those cultures were submitted to trypsinization with 0.25% trypsin/EDTA solution and cultured for two to three months.**

1. PhD; Researcher; Publications and Projects Department; Braile Biomédica.
2. Professor Livre Docente; Medical School of São José do Rio Preto (FAMERP) and UNICAMP; Director of FAMERP Post-graduate Program; Editor of Brazilian Journal of Cardiovascular Surgery.
3. Master degree; Biologist; Tranplant and Immunology Experimental Laboratory; LITEX/FAMERP.
4. Master degree; Assistant professor; FAMERP Cardiology and Cardiovascular Suergery.
5. Full Professor; Adjunct professor; FAMERP Molecular Biology Department.
6. PhD; Adjunct professor; FAMERP Molecular Biology Department.
7. PhD; adjunct professor; FAMERP Medicine I Department.
8. PhD; adjunct professor; FAMERP Obstetrics and Gynecology Department.

This study was carried out at the Medical School of São José do Rio Preto, São José do Rio Preto, SP, Brazil.

Correspondence address:
Rosa Oyama. Av Juscelino K. de Oliveira, 1505 - São José do Rio Preto - SP – Brasil -CEP 15091-450.
E-mail: rosa@braile.com.br

Article received in December 27th, 2007
Article accepted in February 1st, 2008

Conclusion: **In the samples analyzed, cell separation and mesenchymal stem cell culture techniques from human umbilical cord blood by the method without the Ficoll-Paque density gradient was more efficient than the method with the Ficoll-Paque density gradient.**

Descriptors: **Umbilical cord blood. Mesenchymal stem cells/cytology. Cells, cultured. Cell culture techniques.**

Resumo

Objetivos: **Implantação de técnicas de isolamento e cultivo de células-tronco mesenquimais do sangue de cordão umbilical humano, com e sem uso de gradiente de densidade Ficoll-Paque (d=1,077g/ml).**

Métodos: **Dez amostras de sangue de cordão umbilical humano de gestação a termo foram submetidas a dois procedimentos de cultivo de células-tronco mesenquimais: sem gradiente de densidade Ficoll-Paque e com gradiente de densidade. As células foram semeadas em frascos de 25cm^2 a uma densidade de 1x10^{7}células nucleadas/cm^2 (sem Ficoll) e 1,0x10^6 células mononucleares/cm^2 (com Ficoll). As células aderentes foram submetidas a marcação citoquímica com fosfatase ácida e reativo de Schiff.**

Resultados: **No procedimento sem gradiente de densidade Ficoll, foram obtidas 2,0-13,0x10^7 células nucleadas (mediana=2,35x10^7) e, no procedimento com gradiente de densidade Ficoll, foram obtidas 3,7-15,7x10^6 células mononucleares (mediana=7,2x10^6). Em todas as culturas foram observadas células aderentes 24 horas após o início de cultivo. As células apresentaram morfologias fibroblastóides ou epitelióides. Na maioria das culturas houve proliferação celular nas primeiras semanas de cultivo, mas após a segunda semana, somente três culturas provenientes do método sem gradiente de densidade Ficoll-Paque mantiveram crescimento celular, formando focos confluentes de células. Essas culturas foram submetidas a várias etapas de tripsinização para espalhamento ou subdivisão e permaneceram em cultivo por períodos que variaram de dois a três meses.**

Conclusão: **Nas amostras estudadas, o isolamento e cultivo de células-tronco mesenquimais do sangue de cordão umbilical humano pelo método sem gradiente de densidade Ficoll-Paque foi mais eficiente do que o método com gradiente de densidade Ficoll-Paque.**

Descritores: **Sangue de cordão umbilical. Células-tronco mesenquimais/citologia. Células cultivadas. Técnicas de cultura de células.**

INTRODUCTION

In the last decade, a considerable number of studies have proven that human umbilical cord blood (UCB) has hematopoietic stem cells (SCH) and a pool of mesenchymal stem cells (MSC). MSCs are capable of multilineage proliferation and differentiation, similarly to those observed in bone marrow cells.

These characteristics create expectations of using cellular therapies to regenerate tissues and organs affected by the so called incurable diseases, such as neurologic, cardiac, and kidney diseases, etc. Nevertheless, as these cells correspond to only a small portion of mononuclear cells present in each sample, it is necessary to isolate and multiply them *in vitro.*

Mesenchymal stem cells different isolation and culture protocols of umbilical cord blood have already been employed successfully, all involving one early stage of mononuclear cell separation using Ficoll-Paque density gradient [1-4]. Despite the positive outcomes observed in these groups, many studies have reported great difficulty to culture and maintain these cells *in vitro*, and others report a total failure in isolating and culture of these same cells [5-8].

All mesenchymal stem cell protocols of umbilical cord blood start with an isolation phase of mononuclear cells with Ficoll-Paque density gradient, which requires several manipulation and centrifugation phases of umbilical cord blood. This procedure increases the risk of contamination of UCB. In attempting to speed up the process making the mononuclear cell isolation processes faster, several methods have already been tested such as the use of poligeline, hydroxyethyl starch gel (HES) and gelatin to deplete red blood cells (RBC). Despite the efficacy of these procedures, the release of these products for clinical practice depends on the approval of the centers for disease control in several countries [9].

Moreover, these procedures also involve several phases of the manipulation process that increase the risk of contamination. Also, easy to handle and closely sealed filtration systems to concentrate mononuclear cells, the so-called SCF SYSTEM (the stem cell collection filter system), have already been tested. This system has proven to be more efficient and faster in separating mononuclear cells when in comparison to the conventional technique which uses Ficoll-Paque density gradient; however, the release of this system for clinical practice yet requires further studies [10].

In face of these difficulties, our group has developed a mesenchymal stem cell culture of UCB from the isolation of nucleated cells present in the buffy coat collected through centrifugation without Ficoll-Paque density gradient. These cells correspond to all nucleated cells present on UCB and not only to the low-density mononuclear cells isolated by

the Ficoll-Paque density gradient. This protocol was compared to the conventional procedure using Ficoll-Paque density gradient centrifugation in order to isolate mononuclear cells.

METHODS

Collection of umbilical cord blood

Ten samples of UCB were collected from full-term placenta of healthy women, nonsmokers, nondrinkers, age ranging from 20 to 40 years, regardless ethnic group at the Obstetric Service of Hospital de Base Regional Medical School Foundation (FUNFARME) from the Medical School of São José do Rio Preto. Free written informed consent was obtained from the mothers or the next of kin following the guidelines of the local Institutional Review Board and Ethics Committee, according to certificate Nº 168/2005. Blood was collected in sterile syringes containing heparin sodium (1000 IU).

Processing of umbilical cord blood

UCB samples were diluted in a proportion of 1:1 in a phosphate-buffered saline solution (PBS) and submitted to two different mesenchymal culture cell procedures: a) Method without Ficoll-Paque density gradient (*d*=1.077 g/mL) (Amershan Pharmacia); b) Method with Ficoll-Paque density gradient. These procedures are described as follows:

a) Method without Ficoll-Paque density gradient: UCB samples were transferred to centrifuge tubes (15 mL) and submitted to centrifugation at 1000 rpm for 10 minutes to obtain buffy coat. The buffy coat containing all nucleated cells present in UCB was transferred to a new centrifuge tube, which was washed out twice with culture medium through centrifugation at 1000 rpm for 8 minutes. The number of nucleated cells was estimated after counting into Neubauer chamber and cellular viability was determined by the Tripan Blue exclusion method.

b) Method with Ficoll-Paque density gradient: UCB samples were transferred to centrifuge tubes (15 mL) containing Ficoll-Paque solution and submitted to centrifugation at 2000 rpm for 30 minutes in order to isolate low-density mononuclear cells. Mononuclear cells were transferred to a new tube and washed twice with culture medium through centrifugation at 2000 rpm for 10 minutes, according the protocol described by Erices et al. [2]. Cell number estimate and viability followed the same procedure described for the nucleated cells.

Culture of mesenchymal cells

Cultures of mesenchymal stem cells were initiated from nucleated and mononuclear cells. Mesenchymal stem cells isolation was possible due to its capacity of adhesion to the flasks, differently from the nonadherent hematopoietic mesenchymal stem cells, which are eliminated from the culture during the procedures of medium change. The culture procedures are described below:

a) Culture of Mesenchymal Stem Cell from nucleated cells: cultures were initiated in cell culture flasks of 25 cm^2 at a density of $1x10^7$ nucleated cells/cm^2. Cells were nurtured with culture medium á-MEM supplemented with 20% fetal bovine serum, 1% antibiotic/antimycotic, and 1% glutamine. Cultures were incubated at 37ºC, humidified atmosphere containing 5% CO2. The first change of culture medium was performed 24-48 hours after initial plating to eliminate nonadherent cells. Posteriorly, the culture medium was changed at every four days and cellular growth assessed daily under an inverted microscope. When the cells reached 50-60% confluence, they were subdivided after trypsin/EDTA (0.025%) (Gibco-BRL).

b) Culture of mesenchymal stem cell from mononuclear cells: cultures were initiated in culture flasks of 25 cm^2 at a density of $1.0x10^6$ mononuclear cells/cm^2, following the same procedures described for nucleated cells

Cytochemistry characterization of mesenchymal cells

Adherent cells were submitted to cytochemistry labeling with acid phosphatase (AP) and periodic acid-Schiff reactive (PAS), according to the protocol (with modifications) described by Erices et al. [3].

RESULTS

Ten samples were submitted to isolation protocols of nucleated and mononuclear cells. In isolation procedures of nucleated cells (without Ficoll) were obtained 2.0-13.0$x10^7$ (median of $2.35x10^7$) cells/cm^2. In isolation procedures of mononuclear cells (with Ficoll) were obtained 3.7-15.7$x10^6$ (median of $7.2x10^7$) cells/cm^2. In all cultured samples were observed adherent cells 24 hours after initial plating. After the second week of plating, the growth rate of the cultures from isolation with Ficoll decreased until complete stagnation. In the cultures from isolation without Ficoll only three samples kept their growth, forming confluent focuses of cells. These cultures were submitted to several phases of trypsinization for dissemination or subdivision and were kept in culture for period varying from two to three months.

Cells from the beginning of culture presented epithelioid and fibroblastoid morphologies (Figure 1A). After the subdivisions, there has been predominance of fibroblastoid cells (Figure 1B) and, in some cases, the presence of a large rounded multinucleated cell (Figure 1C). During trypsinization, fibroblastoid cells detached rapidly from the

flask, whereas the rounded cells needed a longer period of exposition to trypsin to be detached from the flask. The cytochemistry characterization highlighted fibroblastoid cells PAS positive and AP negative and rounded cells AP positive and PAS negative (Figure 2).

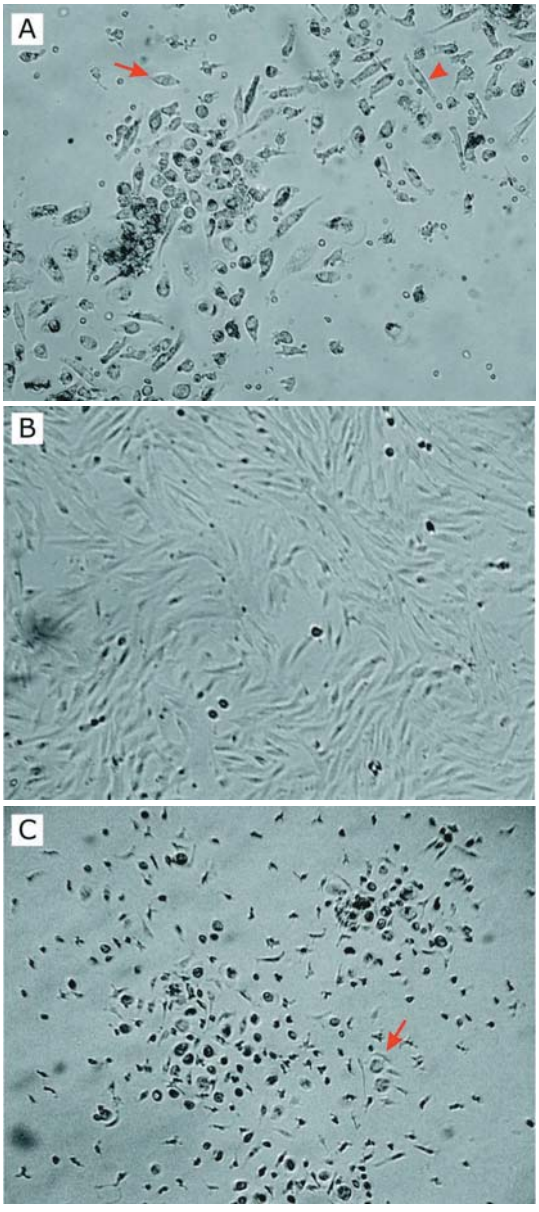

Fig. 1 – Microphotograph showing adherent cells from umbilical. A. Fibroblastoid (arrow) and epithelioid cells (arrow head) observed in initial plating. B. Confluent fibroblastoid cells. C. Rounded cells (arrow). Magnification x200.

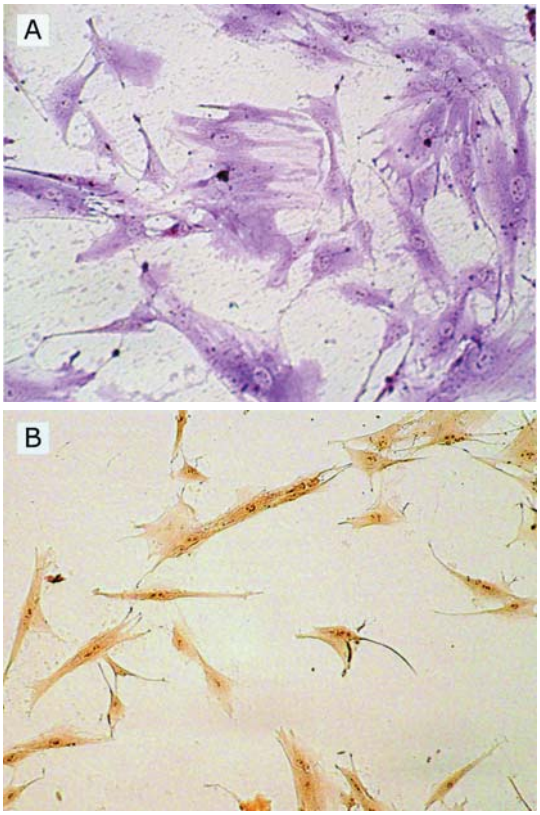

Fig. 2 – Microphotograph showing adherent cells from umbilical Cord blood. A. Cytochemistry analysis showing mesenchymal cells labeled positively by periodic acid-Schiff reactive (PAS). B. Cytochemistry analysis showing mesenchymal cells labeled negatively by acid phosphatase. Magnification x200.

DISCUSSION

Our study examined comparatively the isolation and culture procedure of mesenchymnal cells of umbilical cord blood without Ficoll-Paque density gradient. This procedure, which enables to separate all nucleated cells present in the umbilical cord blood, was compared to the standard technique using Ficoll-Paque density gradient, which allows the selective isolation of mononuclear cells. The results show that both mononuclear cells and nucleated cells isolated from UCB, when cultured in vitro, were able to produce adherent cells with different morphologies. Among the cell types observed, we could see fibroblastoid-shaped elongated cells (*spindle-shaped cells*), egg-shaped cells similar to epithelioid cells, and rounded cells similar to osteoclasts (*osteoclast-like cells*).

Different studies with umbilical cord blood have identified similar cells, whose nature was determined by

means of immunocytochemistry and cytochemistry labeling. Fibroblastoid cells (elongated) proved to be positive for mesenchymal cell markers and the rounded cells were positive for osteoclast markers. The confirmation that fibroblastoid cells corresponded to mesenchymal stem cells was established by their cellular differentiation capacity induced by growth factors [2,4,11-14].

In the present study, adherent cells were characterized cytochemistrically with PAS and acid phosphatase. PAS labels mesenchymal cells (fibroblastoids) positively and the osteoclasts (rounded cells) negatively, whereas acid phosphatase labels the osteoclasts (rounded cells) positively and the mesenchymal cells (fibroblastoids) negatively. The results obtained associated to morphological analysis indicate that the fibroblastoid cells correspond to the mesenchymal cells, thus demonstrating that it is possible isolate and culture these cells from UCB without using Ficoll-Paque density gradient.

Although in the present study specific mesenchymal stem cell markers have not been used and the induction of cellular differentiation to confirm the presence of stem cells, the intense cellular proliferation observed in the three samples is a predictor of the presence of stem cells. That's because, the differentiated cells or senescent cells have a limited life span, characterized by loss of proliferation capacity and morphology alteration, leading to culture stagnation [15].

The success rate in isolating and plating mesenchymal stem cells from UCB observed in our study was of 30% (n=3/10). These data are in accordance with the literature findings [2,4,11-14]. The major difficulty in plating mesenchymal stem cells from UCB results from the small number of mesenchymal stem cells present in each sample. According to Goodwin et al. [1] only about $0.05\text{-}2.8x10^6$ mononuclear cells planted correspond to a mesenchymal stem cell. Moreover, survival or death of these cells can be strongly affected by changes in sample storage time until the beginning of the plating, quantity of mononuclear cells obtained, the presence of clot, hemolysis, as well as by the own conditions of plating of each laboratory [11.15].

Another factor that hampers the detection of mesenchymal stem cells in vitro is that these cells are normally detected after 2-4 weeks of plating, differently of what is observed in the mesenchymal stem cell cultures from bone marrow or from fatty tissue, in which these cells are identified after 4-5 days of plating [16]. However, once established, the mesenchymal stem cell cultures from UCB are capable of generating much more mother cells than those from bone marrow [15]. This is resultant from the immaturity of the newborn cells when compared to the adult cells. Aging is associated to the reduction of mesenchymal stem cell life span and to its differentiation capacity [17].

These difficulties are responsible for the scepticism of some researchers as to the presence of mesenchymal stem cells in the UCB [5-8]. However, a number of studies have proved the presence of mesenchymal stem cells in the UCB, their potential of cellular differentiation and proliferation, showing that the umbilical cord blood can be an important source of cells for cellular therapeutics for treatment of diseases the so called incurable diseases [1-4,11-20].

CONCLUSION

The procedure to obtain nucleated cells without using Ficoll-Plaque density gradient has shown to be more efficient for the culture of mesenchymal stem cell from UCB when compared to the procedure using Ficoll-Paque density gradient. The possibility of isolating and plating mesenchymal cells without using Ficoll, which is clearly known to be toxic, has become the safest and fastest procedure. These results are preliminary and need further studies to be validated.

ACKNOWLEDGEMENTS

This study was carried out with the financial support of BAP (Researcher and Grant-holders) and Braile Biomédica Indústria, Comércio e Representações SA.

REFERENCES

1. Goodwin HS, Bicknese AR, Chien SN, Bogucki BD, Quinn CO, Wall DA. Multilineage differention activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers. Biol Blood Marrow Transplant. 2001;7(11):581-8.

2. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol. 2000;109(1):235-42.

3. McGuckin CP, Forraz N, Allouard Q, Pettengell R. Umbilical cord blood stem cells can expand hematopoietic and neuroglial progenitors in vitro. Exp Cell Res. 2004;295(2):350-9.

4. Park KS, Lee YS, Kang KS. In vitro neuronal and osteogenic differentiation of mesenchymal stem cells from human umbilical cord blood. J Vet Sci. 2006;7(4):343-8.

5. Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B, Hows JM. Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. Br J Haematol. 2003;121(2):368-74.

6. Mareschi K, Biasin E, Piacibello W, Aglietta M, Madon E, Fagioli F. Isolation of human mesenchymal stem cells: bone marrow versus umbilical cord blood. Haematologica. 2001;86(10):1099-100.

7. Romanov YA, Svintsitskaya VA, Smirnov VN. Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. Stem Cells. 2003;21(1):105-10.

8. Yu M, Xiao Z, Shen L, Li L. Mid-trimester fetal blood-derived adherent cells share characteristics similar to mesenchymal stem cells but full-term umbilical cord blood does not. Br J Haematol. 2004;124(5):666-75.

9. Perutelli P, Catellani S, Scarso L, Cornaglia-Ferraris P, Dini G. Processing of human cord blood by three different procedures for red blood cell depletion and mononuclear cell recovery. Vox Sang. 1999;76(4):237-40.

10. Yasutake M, Sumita S, Terashima S, Tokushima Y, Nitadori Y, Takahashi TA. Stem cell collection filter system for human placental/umbilical cord blood processing. Vox Sang. 2000;80(2):101-5.

11. Gang EJ, Hong SH, Jeong JA, Hwang SH, Kim SW, Yang IH, et al. In vitro mesengenic potential of human umbilical cord blood-derived mesenchymal stem cells. Biochem Biophys Res Commun. 2004;321(1):102-8.

12. Hong SH, Gang EJ, Jeong JA, Ahn C, Hwang SH, Yang IH, et al. In vitro differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocyte-like cells. Biochem Biophys Res Commun. 2005;330(4):1153-61.

13. Chang YJ, Tseng CP, Hsu LF, Hsieh TB, Hwang SM. Characterization of two populations of mesenchymal progenitor cells in umbilical cord blood. Cell Biol Int. 2006;30(6):495-9.

14. Kang XQ, Zang WJ, Bao LJ, Li DL, Xu XL, Yu XJ. Differentiating characterization of human umbilical cord blood-derived mesenchymal stem cells in vitro. Cell Biol Int. 2006;30(7):569-75.

15. Bieback K, Kern S, Klüter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells. 2004;22(4):625-34.

16. Kern S, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells. 2006;24(5):1294-301.

17. Stenderup K, Justesen J, Clausen C, Kassem M. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. Bone. 2003;33(6):919-26.

18. Gang EJ, Jeong JA, Hong SH, Hwang SH, Kim SW, Yang IH, et al. Skeletal myogenic differentiation of mesenchymal stem cells isolated from human umbilical cord blood. Stem Cells. 2004;22(4):617-24.

19. Wagner W, Wein F, Seckinger A, Frankhauser M, Wirkner U, Krause U. Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. Exp Hematol. 2005;33(11):1402-16.

20. Lee MW, Choi J, Yang MS, Moon YJ, Park JS, Kim HC, et al. Mesenchymal stem cells from cryopreserved human umbilical cord blood. Biochem Biophys Res Commun. 2004;320(1): 273-8.