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Original Article

The effect of Mesenchymal Stem Cells of Amniotic Membrane on the Proliferation and Differentiation of Umbilical Cord Blood CD34+ cells

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Abstract

Introduction: Ex vivo proliferation of hematopoietic stem cells (HSCs) of umbilical cord is widely used by combination of cytokine and stromal mesenchymal stem cells (MSCs) as feeder layer due to increase the cell doses, adequately. However, numerous studies have shown that ex vivo proliferation of these cells impairs their functions, including reduced self-renewal ability, apoptosis induction, and disordered cell cycle. MSCs have different sources such as amniotic membrane with a stable karyotype and high quality because of isolation from embryonic tissues, so that they are considered as a useful source for MSCs.

Materials and Methods: In this study, isolated mesenchymal cells from the amniotic membrane were used as feeders for the HSCs proliferation. Four different cultures with various conditions were used; first one containing cytokines (stem cell factor, thrombopoietin, and FMS-related tyrosine kinase 3 ligand), second one with MSCs co-cultured with the aforementioned cytokines, third medium co-cultured with MSCs without cytokines, and finally the control medium was without co-culture condition and cytokines. Expression of mRNAs of HOXB4, GATA2, BCL2, and Survivin genes was also investigated.

Results: The findings showed that the expression of mRNAs of these genes decreased in culture with cytokine, solely; however the expression of these genes was significantly higher in co-cultured system with cytokine rather than just with cytokine.

Conclusion: : In general, the findings of this study indicate that the derived MSCs from amniotic membrane is a good source for the proliferation of umbilical cord blood CD34+ cells". Because these cells increase the UCB-CD34+ quantity and their preservation properties.

Keywords: Amniotic Membrane, Co-culture, Hematopoietic Stem Cells, Mesenchymal Stem Cells; UCB-CD34+

1. Introduction

Stem cells are primitive cells commonly found in all multicellular organisms maintaining the ability of renewing them via cell division, and they can be differentiated into various specialized cell lines [1, 2]. Modern therapeutic methods focus highly on stem cells to apply them in the fields of organ replacement and lost tissue transplantation [3]. In recent years, research on biology of embryonic stem cells and different adult tissues develops a new perspective on the origin, identity, and

therapy potency of special stem cells in each tissue of body [4, 5].

Hematopoietic stem cells (HSCs) are the early precursor cells specializing to all types of blood cells. HSCs can reconstitute the bone marrow after depletion caused by a disease or irradiation [6]. These cells contain CD34 antigens used as a marker to highly enrich the stem cells and progenitor cells in adult bone marrow, umbilical cord blood (UCB), and peripheral blood [7-9]. Among aforementioned tissues, because of high-quality proliferation and self-renewing potency, UCB is a source of hematopoietic progenitor cell (HPC). Also UCB is a source of transplant and proliferation of bone marrow in human cells [10-13].

In this study, to increase the total number of UCBs, two approaches for transplantation and ex vivo UCB proliferation before transplantation were evaluated.

Robinson et al (2006) performed co-culture unmanipulated UCB by using the bone marrow–derived mesenchymal stem cells (MSCs). MSCs, as the microenvironment cells of the bone marrow with proliferation potency, possess the secretion of a complicated network of growth factors and cytokines such as stem cell factor (SCF), thrombopoietin (TPO), FMS-related tyrosine kinase 3 (FLT3), and they are involved in HSC differentiation [14-16]. These cells can even extract from the liver, infant blood, UCB, and amniotic fluid with ability to be differentiated into a variety of mesenchymal cell types [17]. All isolated MSCs from various tissues express some embryonic markers such as OCT4, Nanog, and SSEA-4 proteins, but not HLA-II antigen, with slightly differences. In addition, these cells have a stable karyotype so that they do not form tumor tissue [18].

By using a culture system including MSCs, many harmful aspects of HSCs proliferation in ex vivo would be reduced, for example, apoptosis prevention and proliferation stimulation, potentially differentiation in terms of self-renewal. This co-culture evidence suggesting that direct cell-to-cell contact between stromal cells and HSC is essential for stem cell survival and grafting in HSCs [19]. Clinically, CD34+UCB cells ex vivo proliferation increase the total nucleated cells (TNC) and the total number of CD34+ cells [20].

Apoptosis is regulated by a balance of various genes either promoting or inhibiting the programmed death of cells. Survivin and Bcl2 are members of protein families that have antiapoptotic properties and interfere with the cell cycle [21-23]. Survivin is expressed in CD34+ cells of the umbilical cord and bone marrow [24]. On the other hand, HOXB4 and GATA2 are transcription factors (TFs) that are closely related to HSC renewal. They are expressed in the primary stemness and are reduced in the differentiation phase of the cell [25].They play an essential role in gene regulation during maturation of HPCs [26-28].

This study was aimed to focus on UCB proliferation by the co-culture system on mesenchymal cells of amniotic fluid for the first time. In fact, this study was aimed to investigate the expression of four key genes in the apoptosis pathway *(BCL2)*, cell cycle (*survivn*) and the stem cell characteristics (*GATA2* and *HOXB4*) in different culture conditions.

2. Materials and Methods

2.1. Isolation of Mononuclear Cells from Umbilical Cord Blood

Initially the UCB samples were collected, and MACS-p buffer, bovine serum albumin, and Hydroxyethyl starch were used for the extraction of mononuclear cells (MNC). UCB units were thawed, and at least 10% of the MNC fraction was used to co-culture with MSC. Data obtained from these UCB units provided an examination of the numbers of TNC, $CD34^{+}$ followed by ex vivo proliferation of the UCB-MSC in co-culture system.

2.2. Isolation of CD34+Cells by MACS Technique

For each 108 volume of MNC, 100µL of FcR blocking solution was added and they were incubated for 15 min at 4°C. Subsequently, 100 µL of anti-CD34 antibody solution attached to mycroid particles was added to the cell suspension and the solution was incubated for 30 min at 4°C. Then cells were centrifuged and washed out with buffer. Then, the mixture was filtered by a 30-µM membrane filter to remove the cellular compositions out of the suspension. The LS column was used for MACS extraction. The column was rinsed with 3mL of MACS buffer, and then the cell suspension was transferred slowly into the column. The cells attached to the CD34+ antibody containing the mycroid particles stuck on the column and the other cells passed [29].

The purity of CD34+ cells was evaluated using flow cytometry (Partec) and were analyzed by using the Flow Max software.

2.3. Isolation of Amnion Mesenchymal Cells

A two-step method was used to isolate the homogenized mesenchymal cells. First, the amniotic tissue was treated with trypsin to remove the epithelial cells. Then, mesenchymal cells were released by collagenase and DNase. Amniotic mesenchymal cells were sticky and were proliferated and stored in plastic tissue culture dishes between 5 and 10 passages.

The fetal amniotic membrane was removed from the remaining of the maternal tissues and washed out with phosphate buffer saline (PBS), and then it was incubated with 2.4 U/mL dispase II at 37°C for 55 min. After that, they were incubated with 0.75 mg/mL collagenase type II for 60 min at the same temperature. The isolated cells were cultured in a 25 cm2 flask containing 6 mL of α-MEM medium with low concentration of glucose and fetal bovine serum (FBS, 10%) and then placed in a 37°C incubator and 5% CO2 pressure. The medium of culture was replaced every 3 days [30].Trypan blue 0.4% was used to determine the viability of the cells.

2.4. Ex Vivo proliferation of Mesenchymal Stem Cells

For increasing MSCs, we used the PBS for washing a mixture of trypsin-EDTA and a medium containing α-MEM-LG with 10% FBS for passage the cells. When MSCs reached a density of 70%, trypsinized cells were assayed for the presence of surface markers—CD34+ cells—by flow cytometry.

2.5. CD34+ of Umbilical Cord Blood Culture (CD34+Culture in a Medium Containing Cytokine without Mesenchymal Feeder Layer)

A total of number 30,000 cells of CD34+ were suspended in a 1-mL of Stem Span serum medium containing 100 ng/mL of SCF and TPO cytokines and 50 ng/mL of FLT3-Ligand. They were cultured in 24-well plates and incubated in humidity with 5% CO2 at 37°C for 10 days. On 3th, 7th, and tenth day after culture, the medium was replaced with a fresh one.

2.6. Co-culture of CD34+With Mesenchymal Stem Cells

First, a feeding layer of CD34+ should be prepared from MSCs. For this purpose, the feeder layer of MSCs (passage 3-6) was poured into a 24-well plate, and anα-MEM-LG medium containing 10% FBS was added and then incubated at 37°C with 5% CO2 up to a density of 70% -80%. After that, the cells were washed out with PBS. To carry out the co-culture, 3×104 newly isolated CD34+ cells were suspended in 1 mL of Stem Span without the serum containing cytokine compounds and were transferred into the 4 wells of mesenchymal feeder layer. The same number of CD34+ cells was stored in 1 mL of Stem Span without serum and any type of cytokine and was poured into 4 other wells of the other mesenchymal layer, and the plates were

stored for 14 days at the same aforementioned condition.

2.7. Bioinformatics Study/Quantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis

CD34+ cells were used to investigate the expression levels of HOXB4, GATA2, BCL2, and Survivin genes by quantitative real-time polymerase chain reaction (PCR), Total RNA was extracted from the cells by a TRizol Kit (Cinnaclone; Iran) according to the instructions provided by the manufacturer. Additionally, in co-culture conditions of HSC with MSC in the presence of cytokines, cells connected to MSC were separated by using trypsin (0.05%) and suspended with HSC for RNA extraction. The total RNA (1µg) was reverse-transcribed using Super Script III Reverse Transcriptase (Invitrogen) according to the instructions of the manufacturer. The primer sequences of each gene were designed using NCBI primer design software (Table 1). The specificity of the primer was confirmed by melting-curve analysis carried out by polymerase chain reaction (PCR) (QuntiFast SYBER Green PCR Master mix; Takara, Japan) and gel electrophoresis on 2% agarose gel with cDNA used as a template (Applied Biosystems; 7500 Sequence Detection System). β-actin gene was used as internal control. PCR amplification was as performed as follows: denaturing at 95°C for 5 min, 40 cycles for 15 seconds at 94°C, and 30 seconds at 60°C. Finally the yielded data were analyzed with (Corbett life science) REST2008 software and $2^{-\Delta\Delta Ct}$ method.

Table 1. The sequences of used primers for gene expression analysis of the *β-actin* (used as a reference gene) *HOXB4*, *GATA2*, *BCL2* and *Survivn* by Real Time PCR

Genes	Sense primer 5′ → 3′	Antisense primer 5′ → 3′	Amplicon size (bp)
HOXB4	TCCCACTCCGCGTGCAAAGA	AAGACCTGCTGGCGCGTGTA	131
GATA2	ACCCCTAAGCAGCGCAGCAA	TTGCACAGGTAGTGGCCGGT	116
BCL2	TGAGTTCGGTGGGGTCATGTGT	AAAGGCATCCCAGCCTCCGTTA	142
Survivn	GCTTCATCCACTGCCCCACTGA	AGGAAAGCGCAACCGGACGA	136
β-actin	TTCTACAATGAGCTGCGTGTGG	GTGTTGAAGGTCTCAAACATGAT	119

2.8. Statistical Analysis

All experiments were performed based on a randomized designing of complete blocks of UCB samples collected from the Milad Hospital. Three administration levels were applied to the samples in the co-culture with MSC, co-culture with MSC and cytokine, and the last medium with only cytokine, and the samples were harvested on 3, 7, and 10 days. Each treatment was performed with at least 3 independent replications. The analysis of variance (ANOVA) and one-way ANOVA and t test ($P \leq .05$) of mean comparison were performed using the SPSS software version 20. Normalization of raw data was evaluated using the Kolmogorov-Smirnov test and then was normalized by the Arcsin test.

3. Results

3.1. Reverse Transcriptase-Polymerase Chain Reaction Expression of BCL2, Survivin, GATA2, and HOXB4

Reverse Transcriptase-PCR (RT-PCR) was performed on the cDNA of fresh CD34+ cells (day 0) to determine the efficacy of primers and optimum conditions for multiplication for each gene. Figure 1 shows the results of electrophoresis of the RT-PCR products.

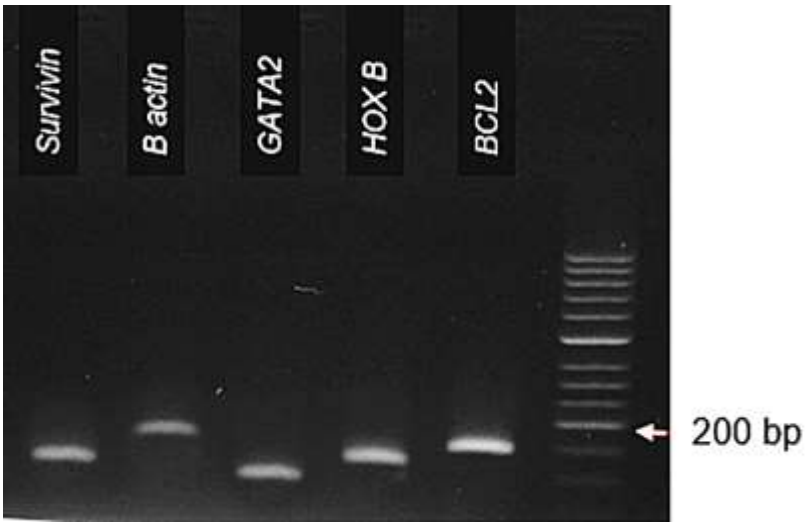

Figure 1. Results of the electrophoresis of reverse transcriptase-polymerase chain reaction products on 2% agarose gel.

3.2. Quantitative Real-Time-Polymerase Chain Reaction of HOXB4 and GATA2Genes in CD34+Cells under Different Culture Conditions

Expression of HOXB4 and GATA2 genes was performed using cDNA of CD34 cells on 3th, 7th, and 10th days with cytokine culture similar to day 0. The findings showed that in the medium containing cytokines without mesenchymal stromal layers, the expression of the mRNA expression of HOXB4 and GATA2 genes were slowly decreased till to the lowest level on day 10.

On the other hand, in the coexistence culture containing stromal mesenchymal cells and cytokine, the expression of HOXB4 on days 3 and 7 did not show reduction significantly compared with day 0, and only on 10thday of culture, the expression of HOXB4 was decreased significantly (Figure 2A). In addition, expression of GATA2 in the co-culture medium with stromal mesenchymal cells and cytokine did not change significantly on days 3, 7, and 10, compared with the day 0 (Figure 2B).

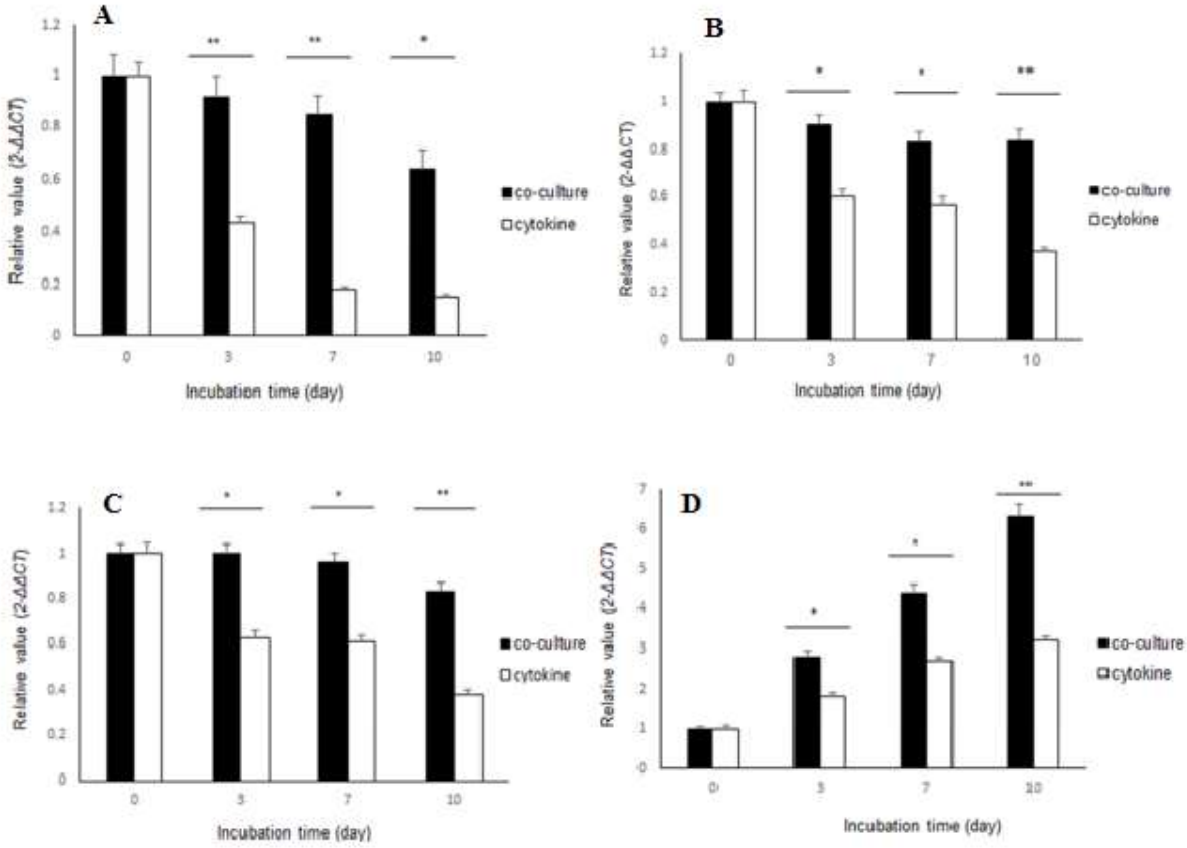

Figure 2. Relative expression of HOXB4, GATA2, BCL2 and Survivin genes in CD34 cells on days 3, 7, and 10 under different culture conditions. HOXB4 gene (A), GATA2 gene (B), BCL2 gene (C) and survivn gene (D)
(Coculture with cytokine: Cytokine/Culture with cytokine: cytokine).

3.3. Quantitative Real Time-Polymerase Chain Reaction of Survivin and BCL2 Genes in CD34+Cells under Different Culture Conditions

Expression of Survivin and BCL2 genes was performed by using cDNA of CD34 cells on 3th, 7th, and 10th days with cytokine culture similar to day 0. The findings showed that in the medium containing cytokines without the mesenchymal stromal layer, the BCL2 expression gradually was reduced to the lowest level on the 10th day. However, in the medium containing cytokine and the mesenchymal stromal layer, BCL2 expression in CD34 cells did not show significantly reduction on days 3 and 7 similar to day 0 (Figure 2C).

The amount of mRNA expression of the Survivin gene in the culture containing the mesenchymal layer was increased until the highest level on day 10. In addition, the expression of the Survivin gene in CD34 cells in coexistence with the cytokine-mediated stromal mesenchymal layer slowly was increased the highest level on the tenth day (Figure 2D).

3.4. Proliferation of CD34 Cells Isolated From Umbilical Cord Blood under Different Culture Conditions (Cytokine and Mesenchymal Stromal Layer)

The highest rate of CD34 cell proliferation was observed on day 7 in all three conditions. The multiplicity of CD34 cells in combination with cytokine and the mesenchymal stromal layer was also higher rather than the cytokine medium, alone (Figure 3).

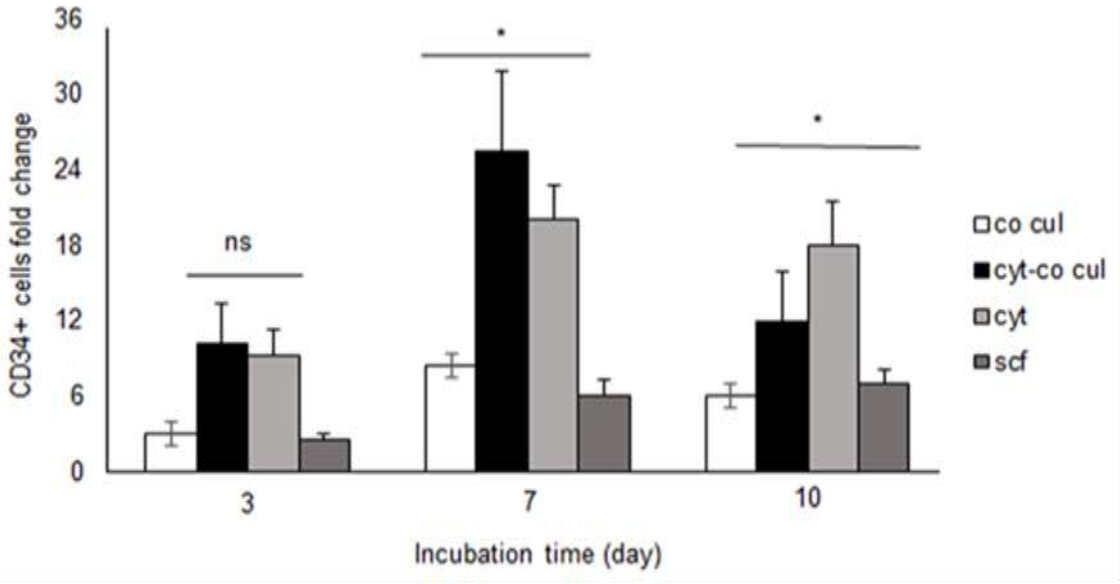

Figure 3. Comparison of the proliferation of CD34 cells isolated from umbilical cord blood in different culture conditions and on different days of culture (3, 7, and 10).

4. Discussion

UCB has been confirmed as an advantageous alternative source of HSCs for the past two decades. HSCs are now developed to a combination of cytokines [31]. HSCs rely on cytokines *in vivo* and a variety of components from their niche, such as MSCs, endothelial cells, osteoblasts, as well as the extracellular matrix [32]. The need for an optimized culture system resembling *in vivo* niche and supporting HSCs' growth *in vitro seems necessary*. Among the various cell types used for this purpose, MSCs were more efficient [33].

In this study, mesenchymal cells isolated from the amniotic membrane were used as feeders for the development of HSCs for the first time. Four different cultures were used, including medium containing cytokines (SCF, TPO, and FLT3L), medium with MSCs with the aforementioned cytokines, and culture medium with MSCs without cytotoxic compounds.

Expression of HOXB4, GATA2, BCL2, and Survivin genes in different culture conditions was investigated. Present study showed that expression of HOXB4, GATA2, BCL2, and Survivin genes was decreased only in culture with cytokines.

Kadekar et al evaluated an equal number of CD34 cells from a single UCB unit on 2 feeders; CD34 cells that were propagated on a separator feeder were doubled in TNCs and more than 2-fold in CD34 cells were larger than those of proliferated cells on a UCB. AsP-MSCs have higher engraftment potential, they are better feeders for HSCs'ex vivo maintenance than C-MSCs. C-MSCs and P-MSCs are morphologically and phenotypically similar but exhibit different abilities for*ex vivo* hematopoiesis [34].HSCs are expanded to suspension cultures. MSCs are the most appropriate feeders among the different cells [35]. According to Celebi et al, irradiated MSCs can imitate the niche *ex vivo* and support the HSCs' expansion.

One of the initially requirements for the successful reproduction of HSCs is that the conditions of ex vivo culture trigger symmetric divisions of stem cells, so that they can maintain the self-renewal [36]. HOXB4 and GATA2 are TFs that are closely related to the self-renewal of HSCs. Also, Bcl2 and Survivin are responsible for apoptotic process inhibition [28].

Present study revealed a reduction in mRNA expression of HOXB4 and GATA2 genes in proliferated CD34 cells in a cytokine culture without MSCs. Correspondingly, McGuckn used two different cytokines for CD34 proliferation in a medium without a mesenchymal nutrient layer and demonstrated a reduction in the expression of HOXB4 after four weeks of proliferation in the presence of both cytokines [37]. In another research, in the cytokine composition to proliferate cord blood CD34 cells, it was observed that the expression of HOXB4 mRNA had gradually reduced during the proliferation [38].

Overexpressed BCL2 in HSCs was due to excess in quantity of HSC during ex vivo proliferation and enhanced renewal and repopulating ability of these cells among irradiated mice [21].Fukuda et al revealed that Survivin is the only antiapoptotic protein s inducing the cytokine stimulation in the CD34 cells [24].

In another study, reduced expression of the number of genes associated with stem cell status, including GATA2 and BMP8B, was observed in proliferated CD34 cells in cytokine fluid medium [39]. Reduced expressed of mRNA of HOXB4 and GATA2 genes in proliferated CD34 cells in the cytokine culture may be attributed to conditions in cell proliferation environment and thereby lack of specific signals to stimulate the HSCs to maintain their inherent properties [40]. Another possible reason may be related to preserve the primary phenotype of proliferated cells in coexisting conditions, as a results of this study.

5. Conclusion

Cord blood hematopoietic cells are a very good source for transplantation in a wide range of diseases, but due to the low dose of these cells in the cord blood they cannot be used in adults. One method of ex vivo proliferation of these cells is the use of different cytokine treatments and co-culture medium. Our focus was on alternative source of MSCs, which it didn't have any ethical problems and also could be access easily.

In the present study, we successfully isolated a population of stem cells they were easily separated and were the only cells that didn't have any fibroblast-like cells (unlike cord blood and placenta). We have reported that hAM-MSCs are a good feeder for UCB-HSC proliferation, and given that cell therapy protocols nowadays would rather embryonic tissues to adult tissues, our data suggest that the amniotic membrane is a suitable source for Mesenchymal stem cells and could be used in co-culture systems.

Conflict of interest

The authors declare no conflict of interest.

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