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The effect of amniotic membrane extract on umbilical cord blood mesenchymal stem cell expansion: is there any need to save the amniotic membrane besides the umbilical cord blood?

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ABSTRACT

Objective(s): Umbilical cord blood is a good source of the mesenchymal stem cells that can be banked, expanded and used in regenerative medicine. The objective of this study was to test whether amniotic membrane extract, as a rich source of growth factors such as basic-fibroblast growth factor, can promote the proliferation potential of the umbilical cord mesenchymal stem cells.

Materials and Methods: The study design was interventional. Umbilical cord mesenchymal stem cells were isolated from voluntary healthy infants from hospitals in Shiraz, Iran, cultured in the presence of basic-fibroblast growth factor and amniotic membrane extracts (from pooled - samples), and compared with control cultures. Proliferation assay was performed and duplication number and time were calculated. The expression of stem cell's specific markers and the differentiation capacity toward osteogenic and adipogenic lineages were evaluated.

Results: Amniotic membrane extract led to a significant increase in the proliferation rate and duplication number and a decrease in the duplication time without any change in the cell morphology. Both amniotic membrane extract and basic-fibroblast growth factor altered the expressing of CD44 and CD105 in cell population. Treating basic-fibroblast growth factor but not the amniotic membrane extract favored the differentiation potential of the stem cells toward osteogenic lineage.

Conclusion: The amniotic membrane extract administration accelerated cell proliferation and modified the CD marker characteristics which may be due to the induction of differentiation toward a specific lineage. Amniotic membrane extract may enhance the proliferation rate and duplication number of the stem cell through changing the duplication time.

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Introduction

Friedenstein and colleagues reported the isolation of mesenchymal stem cells (MSC) from guinea pig bone marrow in 1970. They named the isolated cells as fibroblast-colony forming unit because the cells were adherent to the plate and showed a fibroblast-like morphology (1). The MSCs can proliferate *in vitro* and differentiate in an appropriate environment to mesodermal lineages such as osteoblast, chonroblast and adipocyte (2). Further research showed that mesenchymal stem cells can be differentiated into the non-mesodermal tissues such as hepatocyte (3), neuron (4), and insulin producing cells (5).

An alternative source of mesenchymal stem cells is umbilical cord blood. Umbilical cord blood is discarded as a medical waste after parturition. It is a good source for therapeutic purposes because they are non-immunogenic, can be prepared by a non-invasive procedure, and are free from ethical issues (6). The cord blood contains a rich source of stem cells including hematopoietic cells (7) as well as MSCs (8). The cells derived from the cord blood are more immature and, therefore, their differentiation potential is more than bone marrow-derived MSCs (BMMSC). Human umbilical cord blood mesenchymal stem cells (HUCBMSC) have a longer telomere length (8) and express a lower level of CD106 compared to the BMMSCs. It has been shown that the mesenchymal stem cells derived from the umbilical cord have less chance to be contaminated with viral infectious agents (9). In

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spite of all advantages, HUCBMSC has less capacity to form colony than BMMSC and Wharton's jelly-derived MSC (10); therefore, supplying sufficient numbers of the cells is a critical hindrance for the clinical cell therapy approaches. To increase the proliferation capacity of the MSCs, it has been suggested that culture media should be supplemented with basic-fibroblast growth factor (bFGF) (10). In fact, bFGF is the most common growth factor added to MSC culture media to accelerate cell proliferation (11) and reduce the population doubling time (12). However, bFGF can modify the differentiation capacity of MSC in favor of the osteogenic lineage and limits its neurogenic capacity (11).

There is a controversy regarding the effects of bFGF on immunophenotype characteristic of the stem cells. Basic fibroblast growth factor has been reported to reduce the expression of some surface CD markers such as CD44 (13); meanwhile, others reported no modification in immunophenotype characteristic (12). CD44 is a transmembrane glycoprotein that has significant roles in cell growth, survival, differentiation (14), cell adhesion, motility, matrix degradation and proliferation (15). Down-regulation of CD105 in HUCBMSCs was reported after the beginning of the differentiation process (16). CD105 or endoglin is another transmembrane glycoprotein (17) and it has been shown that its overexpression leads to an enhancement in cell proliferation (18). Changes in the expression of the CD markers involved in cell division can alter cell proliferation rate.

Aminiotic membrane (AM) is another waste product of delivery process. It composes of 3 layers: the epithelial layer, basement membrane and underlying connective tissue (19). Amniotic membrane can produce a verity of growth enhancing substances such as epidermal growth factor, transforming growth factor (TGF)-alpha, keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), bFGF, TGF-beta1, -beta2, -beta3 (20), proteinase inhibitors (21) and heparin sulfate proteoglycan (22). The production of growth factors by AM promotes wound healing (21) and accelerates epithelialization (23).

Amniotic membrane extract (AME) was shown to cure the chemical corneal burn because of its anti-inflammatory effects (23). Moreover, it has been also reported that the growth factor content of the amnion led to endothelial cell proliferation (24). Soluble factors in the AM stroma have been demonstrated to modify the differentiation of the mesenchymal cell (25). AME was reported to increase the limbal cell viability (26) and contain essential substances for treatment of corneal defect (27).

The cell yield of the isolated blood samples was low and amniotic membrane extract as a rich source of growth factors such as bFGF (20) can potentially induce HUCBMSC proliferation. However, the administration of bFGF as a proliferative factor could change the immunophenotyping (13) and differentiation potential of the cells toward osteogenic linage (11). Besides, b-FGF is an expensive growth factor that causes a higher cost for cell therapy and also changes the stem cell features. Therefore, this study was designed to find a reliable substitute for bFGF. To do this, we investigated the effects of human AME on the proliferation capacity of HUCBMSCs.

Material and Methods

Umbilical cord blood mesenchymal stem cell isolation

The experimental procedures were in accordance to the university ethical committee guidelines. Mesenchymal stem cells were isolated from the cord blood of 100 full-term healthy infants delivered by cesarean sections in Hafez hospital, Shiraz, Iran with informed parental consents. The blood samples were transferred into the falcon tubes containing 500 U of heparin (Rotexmedica, Germany). The mononuclear cells (MNC) were separated through density gradient centrifugation over ficoll (Biosera, UK). The MNC was removed and washed with DMEM (biosera, UK). The cells were re-suspended in DMEM containing 10% FCS, 1% penicillin/streptomycin and 1%L-Glutamin, plated in T25 flasks, and kept at 37 °C and 5% CO_2 for 3 days. The culture media were replaced every 3 days.

Preparation of amniotic membrane extract

The amniotic membrane was separated from the chorionic membrane and washed in cold phosphate buffer saline (PBS) containing 1% penicillin/streptomycin. A pool of ten amniotic membranes were minced into small pieces on ice and treated with trypsin for 10 min at 37 °C and 5% CO_2. The amniotic membrane pieces were centrifuged at 800 RPM for 5 min and the supernatant was discarded. The membranes were snap-frozen in liquid nitrogen. The membranes were thawed on ice in the presence of PBS containing protease inhibitor (Sigma, USA), and thereafter, homogenized at the amplitude 80-90% in a total time of 5 min and pulse 30/10. The cell lysate was then centrifuged at 3000 RPM at 4 °C for 10 min. The supernatant was centrifuged once more at 12 000 RPM at 4 °C for 20 min. The supernatant was aliquoted at 100 µl and snap-frozen in liquid nitrogen and transferred to -80 °C until use. The protein yield of the extract was assessed by Bradford protein assay.

Cytotoxicity of the extract was assessed by culturing 50 000 cells in the presence of the serial dilutions of the extract. The cell survival was evaluated with Neutral Red assay.

Experimental design

The cells were divided into 3 parts and cultured in the following conditions: DMEM containing 10% FCS (as control culture), the same culture medium containing the AME at a concentration of 480 µg/ml (21) (as experimental culture) or bFGF (Sigma, USA) and heparin at a concentration of 5 ng/ml and 5 µg/ml, respectively (as a positive control culture).

Immunophenotyping

The cultured cells were harvested at the second passage and washed with PBS containing 2% FCS. Aliquots of 1×10^6 per 100 µl cells were separately stained with combinations of antibodies against CD44, CD90, CD144 (FITC conjugated), CD105 (PerCP conjugated), CD106 (PE-conjugated) and CD34 (PE-conjugated) (all from Abcam, UK, Cambridge). Isotype matched antibodies were used to exclude non-specific bindings. A four color FACS caliber flowcytometry machine with CellQuest pro software for acquisition of data was used to analyze the stained cells. The WinMDI-free software was used for graphical presentation of the results.

Osteogenic differentiation

Osteogenic differentiation of the HUCBMSCs was stimulated by culturing the cells at the passage 2 in the presence of NH-Osteo Diff Medium (MACS, Germany) with or without supplementation with bFGF or AM extract. The cells were cultured for 4 weeks and stained with Alizarin Red S.

Adipogenic differentiation

Adipogenic differentiation of the HUCBMSC was induced by culturing the cells at the passage 2 in the DMEM supplemented with human adipogenic stimulatory supplements (Stem Cell Technologies Inc, Canada). Amniotic membrane extract or bFGF was also added to the media to evaluate their impact on adipogenic differentiation as well. The cells were cultured for 3 weeks, and then stained with Oil Red O.

Measuring duplication time and cell number expansion

To calculate the duplication time for the first passage, the cells were cultured at a density of 1×10^6 in the presence of media, AME or bFGF. The cells were harvested and the number of viable cells was counted by trypan blue dye exclusion and hemocytometer. The following formula was used for calculation.

$$T=\Delta t.\ln 2/\ln((\Delta N/N_0)+1)$$

Where T is the generation or duplication time, Δt is the culture duration, ΔN is the difference between the cell number during Δt, and N_0 is the number of cells at the beginning of the culture (28).

The duplication number was calculated by the following formula:

Population doubling number = $(\log_{10}(N_H) - \log_{10}(N_i))/\log_{10}$ (3).

Where N_i is the inoculation number and N_H is the harvested cell number (29).

Proliferation assay

All the cultures were exposed to the 50 ng/ml of BrdU (Sigma, USA) for 12 hr before they were harvested. The harvested cells were washed with PBS, re-suspended at. 5×10^5 - 1×10^6 cells/ml, fixed in 70% ethanol for 5 min at room temperature, and then washed again. Afterward, the fixed cells were incubated in 1.5 M HCl at 37 °C for 30 min, washed with PBS containing 0.5% BSA, and kept in the same buffer for 10 min at room temperature. Anti-BrdU rat monoclonal antibody (1:40) (Abcam, UK, Cambridge) was added to the cells and incubated for 1 hr at room temperature. Lastly, anti-rat FITC-conjugated secondary antibody (1:100) (Abcam, UK, Cambridge) was added and incubated for 30 min at room temperature. After washing with PBS, the cells were post-fixed in 1% paraformaldehyde. The percentages of the positive cells were analyzed by flowcytometery. All graphs were depicted by WinMDI software.

Statistical analyses

All the experiments were performed in at least triplicate. The data were analyzed by Analyze of Variances (ANOVA) and Least Standard Deviation (LSD). The analysis was done using SPSS 15.0 for windows (IBM, USA) and graphically presented by Microsoft Excel. A *P value* less than 0.05 was considered as statistically significant.

Results

Umbilical cord blood culture

In the primary cultures of HUCBMSCs, two types of the cells were observed; fibroblast-like cells with spindle or satellite shape and osteoclast-like cells with polygonal to flat-shape morphology where the osteoclast-like cells were tightly attached to the plate and could not be harvested easily. In this way, we were able to harvest and separate most of the fibroblast-like cells from osteoclast-like cells by different cell attachment property at the first passage of the cultures (Figure 1). In most cases, osteoclast-like cells could not be seen at the second passages of MSCs. The exposure of the UCBMSCs to the AME or bFGF did not change the MSCs morphology. The cell yield of the blood samples was 30% and the protein yield of the extract was determined as 7.2 mg/ml by Bradford assay.

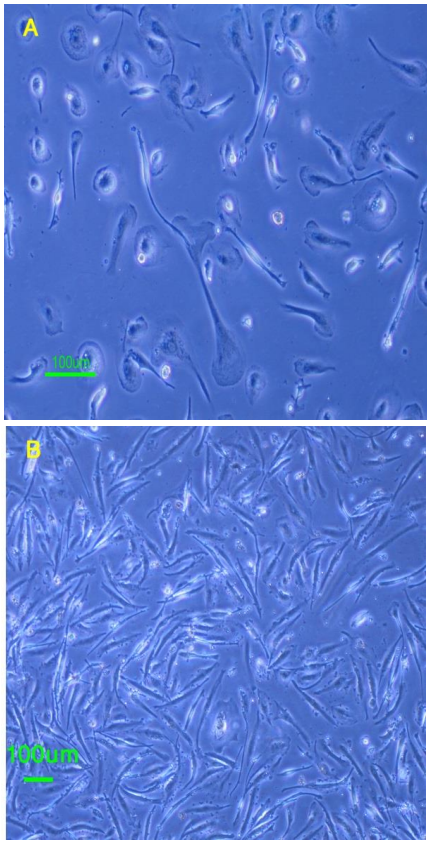

Figure 1. Mesenchymal stem cells isolated from the cord blood contain two types of cells, fibroblast-like and osteoclast-like cells. The primary culture contains a large number of osteoclast-like cells (A) while they are rare in the first passage (B)

The cytotoxicity assay was performed to find the best extract dilution and the results revealed that the extract dilution at 1:15 was not toxic as compared with bFGF and control cultures.

CD Marker expression

The fibroblast-like cells in the control cultures (with base culture media without bFGF or AM extract) were negative for the expression of CD34, CD144 and CD90 (0.22% ± 0.32, 0.25% ± 0.26 and 0.08% ± 0.04; respectively). Most, but not all, fibroblast-like cells were $CD105^{-}$ $CD106^{-}$. The frequencies of positive cells for CD105 and CD106 were 22.61 ± 5.6 and 7.3 ± 4.6, respectively. However, they expressed CD44 (73.4% ± 4.6). The administration of the AME or bFGF led to a significant decrease in the percentage of the cells with CD105 (4.1% ± 1.5 and 4.6% ± 1.9; respectively) and CD44 (25.8% ± 4.0 and 23.3% ± 7.6; respectively) expression (Figure 2). The percentages of different cell populations in the second passage are summarized in Table 1.

The Proliferation rate of the cells

The flowcytometry data showed that the percentages of the BrdU-positive cells significantly increased in the AME-treated cultures compared to

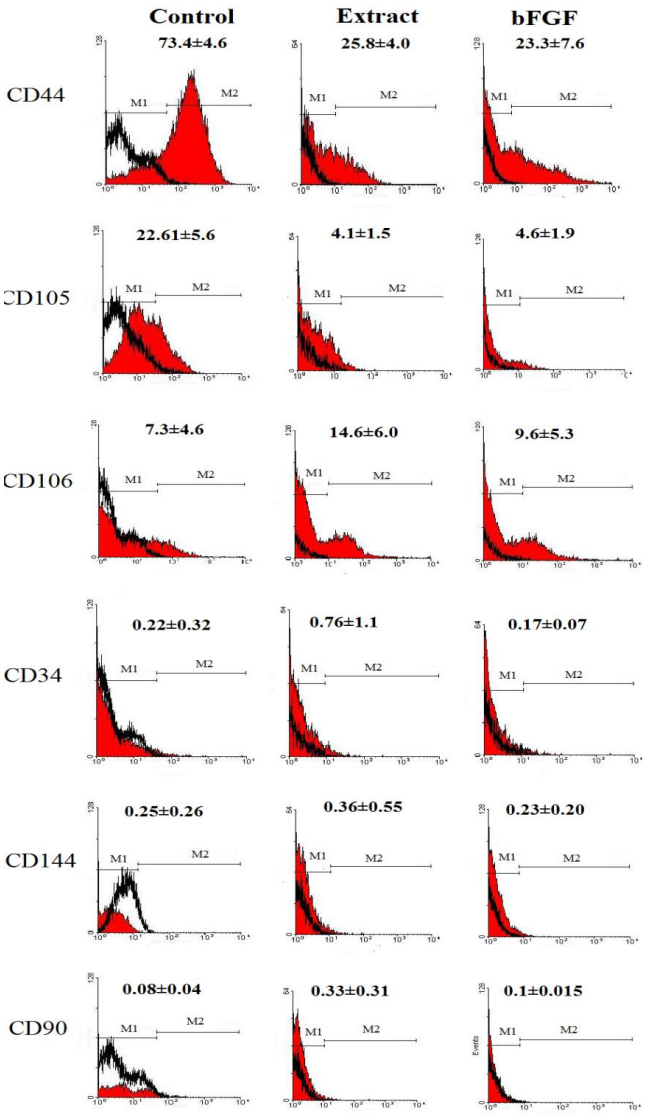

Figure 2. Cell surface markers' expression of amniotic membrane extract- and bFGF-treated umbilical cord blood mesenchymal stem cells compared with control culture. Black-open histograms indicate matched fluorochrome conjugated-isotypes and red-filled histograms indicated CD marker expression.

the control cultures (71.48% ± 17.28 and 46.25% ± 17.18; respectively) (P=0.035) (Figure 3). On the other hand, although the percentage of the BrdU-positive cells in the cultures exposed to bFGF (67.88 ± 12.89) also increased, the statistical analyses showed no significant difference with control cultures (P= 0.091) (Figure 4).

Cell number duplication and doubling time

The data showed a significant reduction in the duplication time after the cells were exposed to the AME or bFGF compared to the control cultures (P=0.002 and 0.003, respectively). The duplication number also increased significantly in the extract- and bFGF-treated cells compared to the control (P=0.004) (Table 2).

Table 1. The percentages of umbilical cord blood mesenchymal stem cells expressed the CD marker in extract-or bFGF-treated cultures compared to the negative control

CD markers	Negative control (mean ±SD)	Extract-treated (mean ±SD)	bFGF-treated (mean ±SD)	*P* value
CD44	73.4±4.6	25.8±4.0*	23.3±7.6*	0.000
CD105	22.61± 5.6	4.1±1.5*	4.6±1.9*	0.000
CD106	7.3±4.6	14.6±6.0	9.6±5.3	0.305
CD90	0.08±0.04	0.33± 0.31	0.1±0.015	0.342
CD34	0.22±0.32	0.76±1.1	0.17±0.07	0.521
CD144	0.25±0.26	0.36±0.55	0.23±0.20	0.568

* Significant difference with negative control (P<0.05)

Differentiation potential of the HUCBMSC

The HUCBMSCs differentiated into adipocyte and osteocyte as indicated by Oil Red O and Alizarin Red S stains, respectively. The administration of the bFGF could increase the osteogenic potential of the HUCBMSCs. However, the osteogenic differentiation potential of the cells did not change by exposure to the extract as compared with the control cultures (Figure 5). The adipogenic capacity was reduced by bFGF treatments compared with the controls (Figure 6). The administration of the extract to the culture medium did not modify the adipogenic capacity of the HUCBMSC compared with the control culture.

Discussion

Sufficient number of the cells is essential for a successful cell therapy strategy and, therefore, it is important to reduce the duplication time and enhance the generation number to have a high yield of cells per culture volume. Basic Fibroblast Growth Factor as a mitogen can reduce the generation time; researchers have added it to the cultures of HUCBMSCs (12) or adipocyte derived-MSC (11) to enhance the cell proliferation. Our data also showed bFGF was able to reduce the duplication time and accelerated the proliferation rate of the HUCBMSCs. Basic fibroblast growth factor supplementation at a concentration higher than 5 ng/ml has been reported to alter cell morphology of BMMSCs (11). However, in our study 5 ng/ml of bFGF did not change the HUCBMSCs' appearance. We also tested the AME to find if it can support or enhance the growth of HUCBMSCs as bFGF does. In fact, we showed that AME could accelerate the proliferation rate of the cells without changing the cell morphology. It has been shown that the supplementation of the media with a combination of growth factors containing bFGF, transforming growth factor and platelet-derived growth factor reduces the generation time

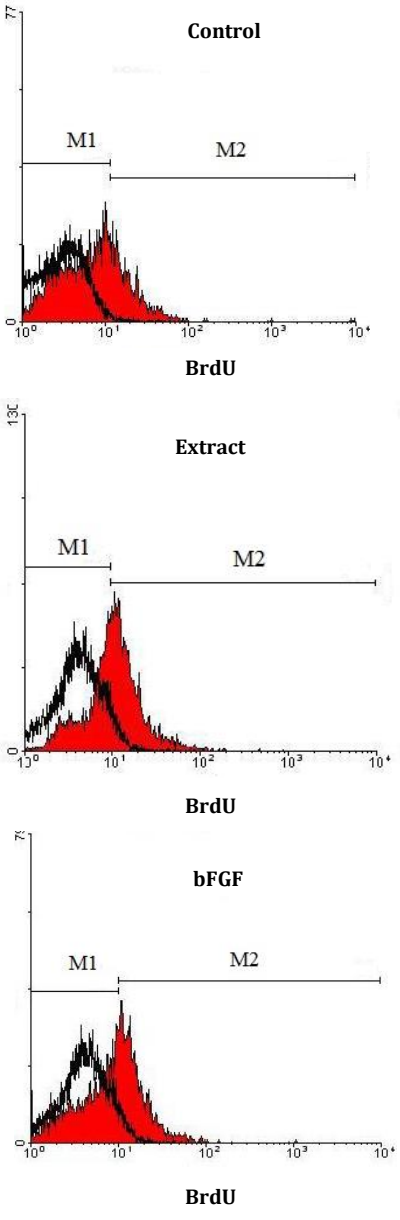

Figure 3. The percentages of the BrdU-positive cells in cultures treated with media (control), amniotic membrane extract or bFGF

Table 2. The comparison of the duplication time and number in the extract- or bFGF-treated cultures with negative control (mean ± SD)

	Negative control	Extract-treated culture	bFGF-treated culture
Duplication time [min]	14190.00± 1770.2	9057.6±10.59.4*	9470±415.1*
Duplication number	0.4099±.048	0.6417±.076*	0.6086±.026*

* Significant difference with negative control (P<0.05)

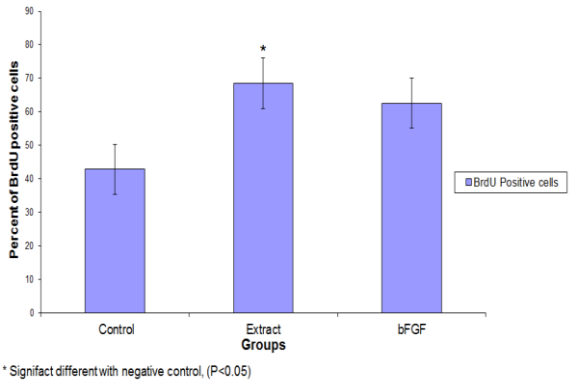

Figure 4. The percentage of BrdU positive cells in cultures treated with ordinary medium (control), amniotic membrane extract or bFGF

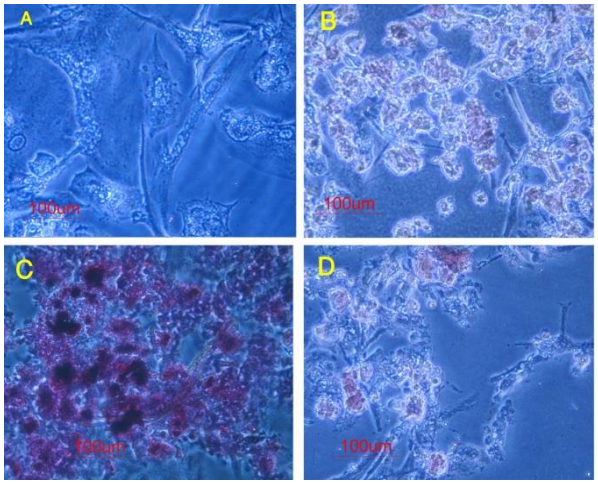

Figure 5. Osteogenic capacity of umbilical cord blood mesenchymal stem cells: A, the cells cultured in ordinary medium; B, the cells cultured in the presence of osteogenic medium; C, osteogenic capacity of the cells in the presence of bFGF; and D, amniotic membrane extract. Alizarin red S staining

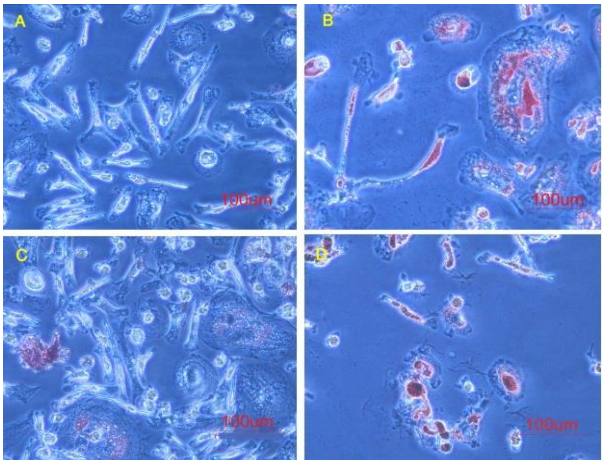

Figure 6. Adipogenic capacity of the umbilical cord blood mesenchymal stem cells: A, the cells cultured in ordinary medium; B, the cells cultured in the presence of adipogenic medium; C, adipogenic capacity the cells cultured in the presence of bFGF: and D, and amniotic membrane extract. Oil red O staining

of the cells (30). Literature shows that the crude AME contain arrays of these cell growth factors (20). Increase in the ulcer fibroblast proliferation was shown by administration of AME (31). It has been also reported that the AME accelerate the cell proliferation and cell viability in the human limbal and corneal epithelium (26). The present study also revealed that AME supplementation could accelerate the cell proliferation rate even better than the proliferation rate in bFGF-treated cultures.

Similar to bFGF, AME could alter the expression of CD105 and CD44 markers in the growing HUCBMSCs. Literature showed controversial reports about the effect of bFGF on CD44 expression pattern. It has been reported that the proliferation of $CD44^+$ cancer cell population is decreased by administration of bFGF in a dose dependent manner (13). Some researchers reported a decrease in the frequency of CD44 expressing cells after administration of bFGF to the culture medium of human BMMSCs (11, 32). However, the others demonstrated an increase in the CD44 positive cells after exposing the cells isolated from mouse periodontal ligament to bFGF (33). Our data confirmed the findings of Sotiropoulou *et al* (11) and Nakagawa *et al (*32) that isolated the cells from the same species as we did.

Adding bFGF to the culture media alters the differentiation potential of BMMSCs toward osteogenic and at a lower extent toward adipogenic lineages (10). Our data showed similar results as the differentiation potential toward osteogenic lineage of the HUCBMSCs increased by supplementation of the culture media with bFGF. However, we found a reduction in adipogenic differentiation potential of HUCBMSCs after administration of bFGF. On the other hand, the AME supplementation did not alter the differentiation capacity of the HUCBMSCs in comparison with the effect of bFGF. Therefore, changing the differentiation capacity of MSCs in the presence of bFGF may interfere with treatment in some cell therapy approaches.

Conclusion

AME could enhance the proliferation rate and duplication number of the HUCBMSC by reducing the generation time. The AME content may exert their effects by promoting the reduction of expression of some surface markers like CD44. Besides, AME led to an increase in proliferation rate without altering cell morphology or differentiation capacity toward osteogenic or adipogenic lineage. Therefore, the AME can be used to increase cell proliferation rate and to prepare a sufficient number of the HUCBMSCs for cell therapy approaches.

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