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Harnessing Cord MSC-Exosomes for Ex-Vivo Expansion of Umbilical Cord Blood CD34+ Hematopoietic Stem and Progenitor Cells

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ABSTRACT

Background & Objective: Exosomes derived from umbilical cord blood-derived mesenchymal stem cells (UCBMSC-EXOs) may enhance the proliferation of Hematopoietic Stem and Progenitor Cells (HSPCs) while preserving their undifferentiated state. This research explored the efficacy of UCBMSC-EXOs as a cell-free strategy to expand HSPCs.

Materials & Methods: In this in vitro study, UCBMSC-EXOs were isolated and characterized. Subsequently, three umbilical cord blood samples were collected, and $CD34^+$ cells were isolated using magnetic-activated cell sorting. To identify the optimal exosome concentration, $CD34^+$ cells were cultured with UCMSC-EXOs at four concentrations (1, 5, 10, and 100 ng/mL). Gene expression levels of *CXCR4*, *VLA-4*, *VLA-5, hTERT*, and *HOXA9* were assessed by quantitative RT-PCR, and CFC assays were performed to assess HSPC clonogenicity.

Results: After 7 days of co-culture, an increase in mononuclear cells was observed in all groups. Notably, the $CD34^+$ population was significantly enriched in Group III (71%) and Group IV (approximately 48%), exceeding levels seen in the other groups. Gene expression analysis revealed a significant upregulation of target genes in Group IV. Consistent with these findings, the CFC assay showed that Group IV had a significantly higher colony-forming rate than the other experimental conditions.

Conclusion: The findings indicate that UCBMSC-EXOs can effectively mimic the supportive functions of their parent cells in vitro, thereby significantly enhancing HSPC proliferation.

Keywords: *Differentiation, Extracellular vesicle, Hematopoietic stem cell, Mesenchymal stem cell, Proliferation*

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1. Introduction

Hematopoietic stem and progenitor cells (HSPCs) are considered one of the most extensively characterized cell populations and have widespread clinical applications (1). Allogeneic hematopoietic stem cell transplantation (HSCT) has emerged as a modern therapeutic approach for the management of both hematologic and non-hematologic diseases (2,3). Umbilical cord blood (UCB) has been increasingly recognized as a valuable alternative source of hematopoietic stem and progenitor cells (HSPCs) for HSCT, in comparison with bone marrow and mobilized peripheral blood progenitor cells. UCB is associated with several advantages, such as simple and non-invasive collection, rapid availability for transplantation, a lower risk and reduced severity of graft-versus-host disease (GVHD), and more relaxed human leukocyte antigen (HLA) matching criteria between donors and recipients (4,5). Nevertheless, one of the main drawbacks of UCB transplantation is the limited quantity of total nucleated cells and $CD34^+$ stem cells available for use in transplantation (6). To overcome this challenge, considerable efforts have focused on developing strategies to expand HSPCs ex vivo before infusion (7). One commonly used strategy is the application of mesenchymal stem cells (MSCs) as a feeder layer (6).

Compared with MSCs derived from adult tissues, those obtained from UCB demonstrate multiple advantages, including enhanced self-renewal ability, broader multipotent differentiation capacity, and lower immunogenicity (5,8,9).

The therapeutic benefits of MSCs in regenerative medicine are largely mediated by their paracrine actions, particularly through the release of extracellular vesicles, such as exosomes and microvesicles. Prior research has shown that MSCs produce a broad spectrum of factors that regulate hematopoiesis and can influence the functional behavior of HSPCs (10). Exosomes are enriched with selectively loaded bioactive molecules, such as proteins, lipids, mRNAs, and microRNAs, which can be delivered to recipient cells and alter their biological functions. Accordingly, exosomes may play a role in the functional reprogramming of target cells (11,12). Furthermore, MSC-based co-culture systems have been employed as feeder layers to support the ex vivo expansion of cells derived from UCB (13,14). These systems have demonstrated significantly higher expansion efficiency and improved preservation of stem cell characteristics compared with conventional liquid culture systems supplemented with growth factors (15).

Experimental evidence has indicated that exosomes derived from mesenchymal stem cells (MSC-EXOs) serve as key effectors responsible for many of the biological activities associated with MSCs (16). Multiple studies have suggested that the therapeutic efficacy of MSC-EXOs is similar to that of their parent cells, indicating that exosomes can replicate many of the functional properties of the cells from which they originate. Accordingly, the current study was designed to explore the potential contribution of UCBMSC-EXOs in promoting the ex vivo proliferation and expansion of HSPCs.

2. Materials and Methods

2.1 Cell culture

Human UCBMSCs were obtained from the Royan Institute (Reproductive Biomedicine and Stem Cell Research Center, Tehran, Iran). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin, and incubated under standard culture conditions at 37 °C in a humidified atmosphere containing 5% CO_2 (17). Cells were passaged when the adherent cells reached approximately 90% confluence.

2.2 UCBMSC-EXOs Isolation and Characterization

UCBMSCs at passages 3–5 were used for exosome isolation. Before collecting UCBMSC-derived exosomes, the culture medium was replaced with serum-free DMEM, and the cells were incubated for an additional 24 hours. The conditioned medium was then harvested and centrifuged at 3,000×g for 10 minutes to remove cellular debris and necrotic cells. Subsequently, additional centrifugation steps at 2,000×g for 10 minutes and 10,000×g for 30 minutes were performed to further eliminate dead cells and residual debris. The supernatant was then passed through a 0.22 μm filter to exclude apoptotic bodies and microvesicles. Finally, exosomes were isolated by ultracentrifugation using a Beckman L5-65 ultracentrifuge at 100,000×g for 70 minutes (18). Following centrifugation, the supernatant was removed, and the resulting pellet was resuspended in 1 mL of cold phosphate-buffered saline (PBS). A second ultracentrifugation step at 100,000×g for 90 minutes was then performed to further purify the UCBMSC-EXOs. The final exosome pellet was resuspended in 1 mL of cold PBS and stored at −80 °C until further use. For transmission electron microscopy (TEM) analysis, exosome pellets were fixed in 3% glutaraldehyde for 2 hours and negatively stained with 1% uranyl acetate on Formvar-carbon-coated grids.

2.2.1 Dynamic Light Scattering (DLS)

DLS analysis was performed to assess the size distribution of UCBMSC-EXOs particles in suspension. For this purpose, the samples were diluted at a ratio of 1:1000 before measurement.

2.2.2 Exosomes Surface Marker Analysis with Flow Cytometry

UCBMSC-EXOs were first incubated for 30 minutes at room temperature (RT) before the addition of 5 mL aldehyde/sulfate latex beads (4% w/v, 4 μm; Molecular Probes, Leiden, The Netherlands). The mixture was then incubated for an additional 30 minutes at 37 °C and washed with PBS containing 0.5% bovine serum albumin (BSA). Subsequently, the exosome-coated beads were labeled with antibodies against CD63 and CD81, or with corresponding isotype control IgG antibodies. After further washing steps, the samples were immediately analyzed using a FACSCalibur flow cytometer (Becton Dickinson, USA).

2.3 Collection and Purification of UCB Derived Mononuclear Cells and HSPCs

UCB samples were obtained from healthy full-term deliveries following written informed consent. All samples were processed within 4 hours postpartum. Erythrocytes were depleted using hydroxyethyl starch (Sigma-Aldrich, USA), and low-density mononuclear cells (MNCs) were isolated by Ficoll density-gradient centrifugation (Lymphodex). HSPCs were then enriched from the MNC fraction using magnetic bead-conjugated anti-CD34 antibodies and a magnetic-activated cell sorting (MACS) system (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. Flow cytometry analysis confirmed that the purity of the isolated $CD34^+$ cell population exceeded 90%.

2.4 Ex-Vivo Expansion of UCB-Derived MNCs and HSPCs

Approximately 1.5×10^4 $CD34^+$-selected cells were cultured for 7 days under four distinct experimental conditions: 1. Control group: RPMI-1640 medium supplemented with 10% FBS and recombinant cytokines, including stem cell factor (SCF), thrombopoietin (TPO), and Flt3-ligand (Flt3-L), each at a concentration of 100 ng/mL (PeproTech, Rocky Hill, NJ, USA). 2. UCBMSC-EXOs group: RPMI-1640 medium containing 10% FBS,

the same cytokine cocktail, and UCBMSC-derived exosomes (10 µg/mL), without mesenchymal stem cell feeder layers. 3. UCBMSCs group: RPMI-1640 medium supplemented with 10% FBS, cytokines, and UCBMSC feeder cells at a density of 2 × 10^4 cells/mL. 4. UCBMSC + UCBMSC-EXOs group: RPMI-1640 medium containing 10% FBS, cytokines, UCBMSC-derived exosomes (10 µg/mL), and UCBMSC feeder cells (2 × 10^4 cells/mL). In all experimental groups, half of the culture medium was replaced twice per week with fresh medium and cytokines, with or without exosomes depending on the specific condition.

2.5 Flowcytometric Analysis

Flow cytometry analysis was performed to confirm the phenotype of expanded UCB-MNCs and to assess the proportion of $CD34^+$ cells among expanded $CD34^+$-selected populations.

2.6 RNA Extraction and Quantitative Real-time PCR (qRT-PCR) Analysis

Following co-culture, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and stored at −80 °C. RNA concentration and purity were assessed using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized using the PrimeScript First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) and stored at −20 °C until further use. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green PCR Master Mix (Applied Biosystems, USA) on a LightCycler 360 RT-PCR system (Roche, Switzerland). Gene expression levels of CXCR4, HOXA9, hTERT, VLA4, and VLA5 were analyzed using specific primers (Table 1), with GAPDH serving as the internal reference gene for normalization.

Table 1. The primers' sequences were designed for RT-qPCR.

Gene	Primer	TM	GC%	Product Length
CXCR4	F: TCCTGCCTGGTATTGTCACC	59.51	52.38	155
	R: GCTGATCCCAATGTAGTAAGGCA	60.43	47.83	
GAPHD	F: TGCACCACCAACTGCTTAGC	60	50	87
	R: GGCATGGACTGTGGTCATGAG	60	50	
HOXA9	F: GGCATTAAACCTGAACCGCTG	60.14	52.38	204
	R: GGCTGCTGGGTTATTGGGAT	60.11	55	
hTERT	F: CGGAAGAGTGTCTGGAGCAA	59.69	60	144
	R: GATGAAGCGGAGTCTGGACG	60.35	55	
VLA4	F: AATGTTGCGCATGTTCTACTGG	59.86	45.48	139
	R: AGCCAGCCTTCCACATAACATAT	59.96	43.45	
VLA5	F: TGGCCTTCGGTTTACAGTCC	59.96	55	121
	R: GGAGAGCCGAAAGGAAACCA	59.96	55	

2.7 Colony-Forming Cell (CFC) assay

Colony-forming cell (CFC) assays were performed in triplicate for all experimental conditions. After 7 days of expansion, the harvested cells were resuspended and plated in MethoCult GF H4435 methylcellulose medium (StemCell Technologies, Vancouver, Canada) following the manufacturer's instructions. Cultures were incubated for 14 days at 37 °C in a humidified atmosphere containing 5% CO_2. Colonies containing more than 50 cells were identified as CFCs and were counted using an inverted microscope (Olympus, Tokyo, Japan). The frequency of CFCs as well as the total number of colonies were determined for each experimental group.

2.8 Statistical Analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was conducted using one-way analysis of variance (ANOVA), and differences were considered statistically significant at a P-value < 0.05.

3. Result

3.1 Characterization of UCBMSC-EXOs

UCBMSC-EXOs were characterized by transmission electron microscopy (TEM). The isolated vesicles were spherical in shape, with diameters ranging from 40 to 150 nm (Figure 1-A). Also, in DLS analysis, we detected particles in our sample. To ensure homogeneity of the particles in our sample, DLS was repeated three times. The mean particle sizes in the sample were 56.8, 37.3, and 46.8 nm, respectively, consistent with the vesicle sizes observed by TEM (Figure 1-B). Flow cytometric analysis further confirmed the exosomal phenotype, demonstrating positive expression of the surface markers CD81 and CD63 (Figure 1-C).

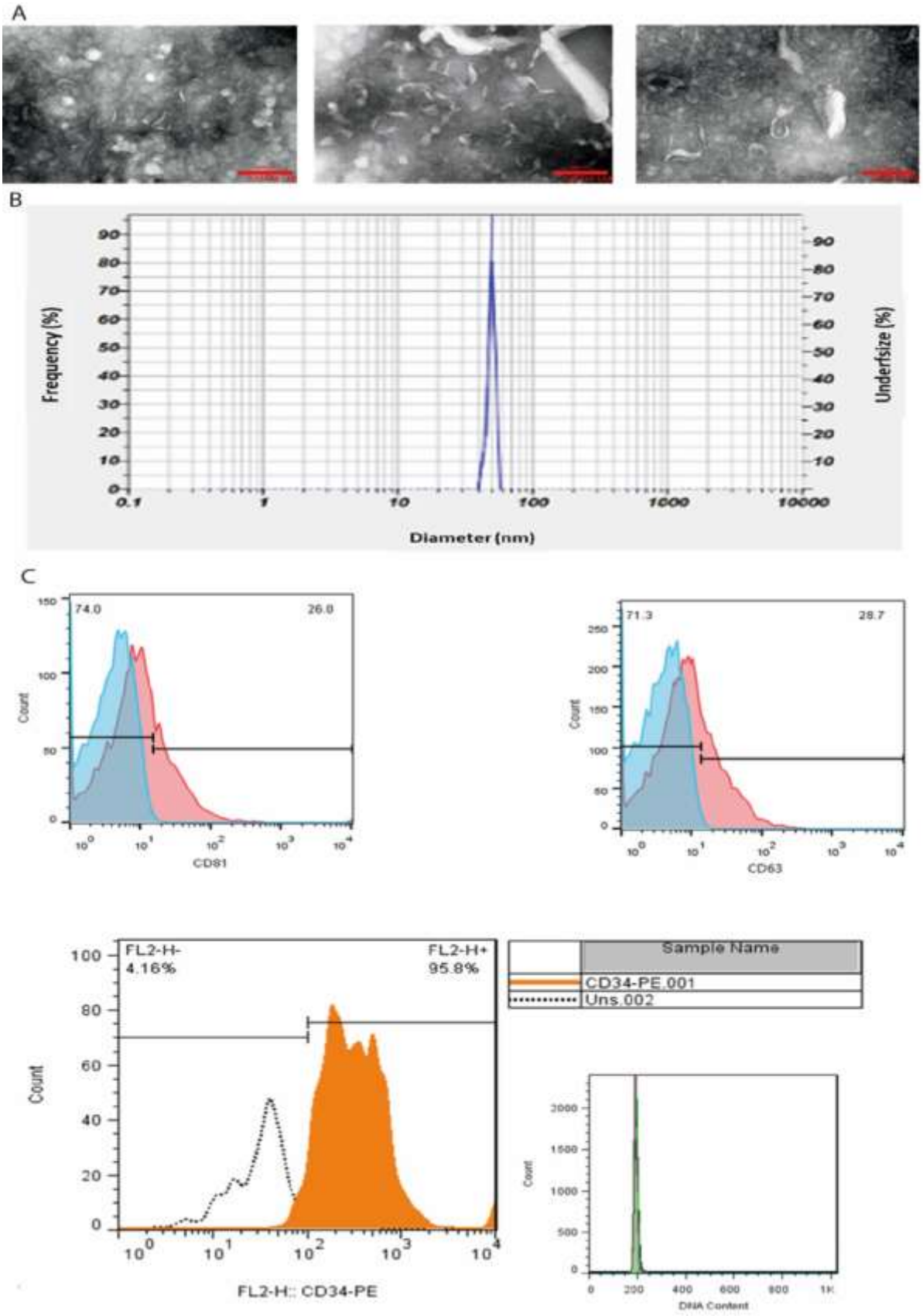


Figure 1. Characterization of MSC-EVs. (A) Representative transmission electron microscopy (TEM) images showing the typical morphology of MSC-EVs (scale bar: 200 nm). (B) Dynamic light scattering (DLS) analysis demonstrating an average particle size of approximately 57 nm. (C) Flow cytometry analysis showing the expression of EV surface markers CD81 and CD63 in MSC-EVs (upper panel), and CD34 positivity in approximately 96% of isolated cord blood total nucleated cells (TNCs), with approximately 96% of cells in the G1 phase of the cell cycle (lower panel). **(Prepared by Authors, 2026)**.

3.2 Isolation of CD34+ cells

Flow cytometric evaluation revealed a purity of 95.8% for $CD34^+$ cells isolated from UCB samples. Cell-cycle analysis of these cells showed that 95.75% were in the G1 phase at the time of division (Figure 1-C).

3.3 Effect of UCBMSC-EXOs on CD34+ cell proliferation

To determine the optimal exosome concentration for co-culture, $CD34^+$ cells isolated from UCB were cultured with UCBMSC-EXOs at concentrations of 1, 5, 10, and 100 µg/mL in the presence of 100 ng/mL FLT3-L, TPO, and SCF for 7 days. Based on the results of counting MNCs in the co-culture group of $CD34^+$ cells with exosomes, 1 µg/mL was about $1.25\pm1.4\times10^6$/mL, and in a co-culture with 5 µg/mL, it was about $1.3\pm2.3\times10^6$/mL; And in the 10 µg/mL was about $1.54\pm1.3\times10^6$/mL; Finally, at 100 µg/mL, the number of MNCs was about $1.1\pm1.1\times10^6$/mL after 7 days of co-culture. Based on the above findings, 10 µg/mL was selected as the optimal concentration for co-culture.

Finally, $CD34^+$ cells derived from UCB were allocated into four culture groups to assess cell proliferation and the effectiveness of exosomes on UCB expansion. The groups were as follows, and the co-culture period was 7 days.

The results demonstrated that MNC proliferation was highest in group IV during the culture period, followed by groups III, II, and I, respectively. The results are summarized in Table 2.

Table 2. Cell count results and flowcytometry of co-culture UCBMSC-EXOs with CD34+ cells after 7 days.

Grouping	Cell count results (TNC)	Flowcytometry results			
		CD34 expression	Actual number of CD34+ cells	Percentage of increasing CD34+ compared to before co-culture	Percentage of increasing TNC
Control group (I)	1.21±1.63×106/mL	10.8	130.680	8.712	80.67
USBMSC-EXOs (II)	1.54±1.3×106/mL	38.8	597.520	39.83	102.67
UCBMSCs (III)	1.62±1.1×106/mL	46.9	759.780	50.65	108
UCBMSC+UCBMSC-EXOs (IV)	1.83±2×106/mL	71.0	1.284500	85.63	122.3

3.4 CFC assay

The results of the colonization study are listed in Figure 2-A.

3.5 RNA extraction

After RNA extraction, RNA quantity and purity were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific), which measures the optical density of samples at wavelengths corresponding to nucleic acids. The results of the different groups are presented in Table 3. Electrophoresis on a 1% gel was also used to assess consistency (Figure 2-B). RNA integrity was additionally assessed by 1% agarose gel electrophoresis (Figure 2-B); the 28S band was visible in the 1500–2000 bp range and the 18S band at approximately 700 bp.

Table 3. Nanodrop results for every 4 groups.

Groups	Concentration(ng/uL)	OD260/280	OD260/230
Pre coculture	138	2.0	2.3
I	204	1.81	2.2
II	128	1.80	1.95
III	168	1.86	2.0
IV	200	1.90	2.1

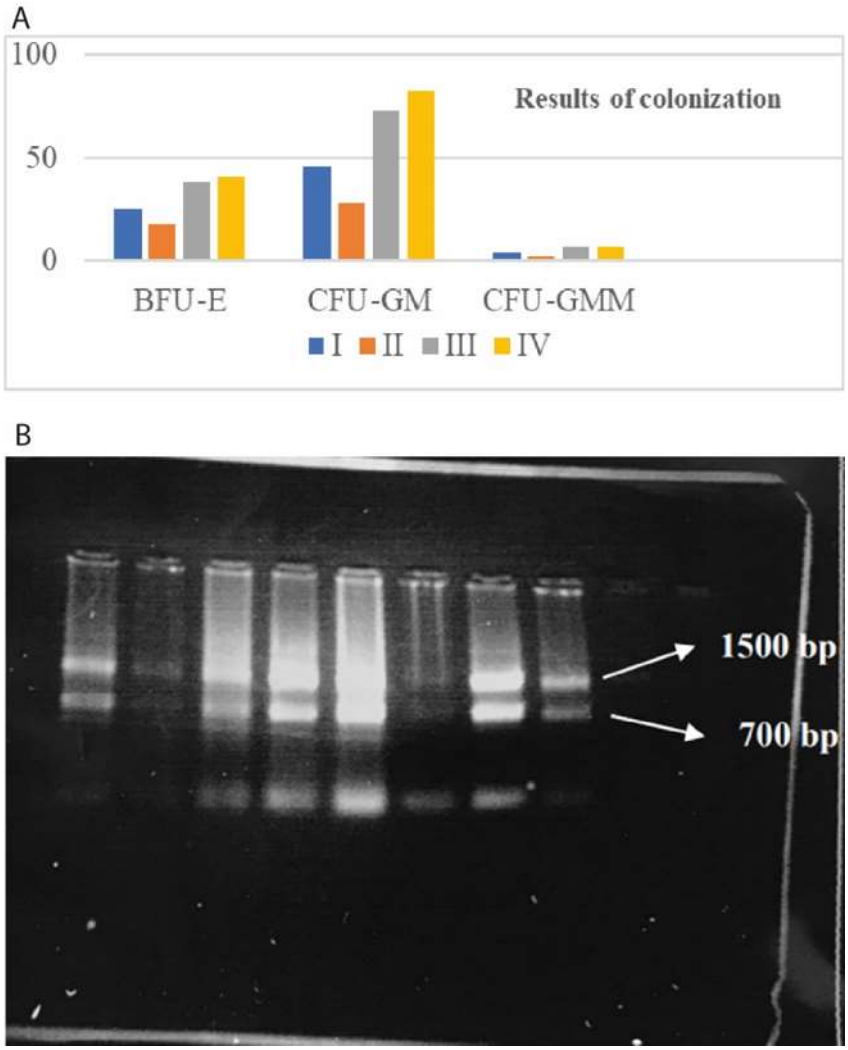


Figure 2. (A) Results of the methylcellulose (Methocult) culture assay demonstrating enhanced cell colonization in the group co-cultured with MSC-EVs and MSCs compared with the other three experimental groups. (B) Representative results of RNA extraction from co-cultured MSCs. **(Prepared by Authors, 2026)**.

3.6 Gene expression analysis (qRT-PCR)

3.6.1 Specificity qRT-PCR products

In a qRT-PCR, a melting curve analysis is used to confirm the product specificity. Melting curves for the GAPDH housekeeping gene are presented.

3.6.2 Amplification plot

QRT-PCR was run for 40 cycles. Ct for each sample, obtained using the multiplication diagrams, was determined for all samples.

3.6.3 Calculation and comparison of results related to gene expression in pre- and post-co-culture

After obtaining the Ct for the target gene in the post-culture and control samples, gene expression changes between the study and control groups were calculated using the formula $2^{-\Delta\Delta Ct}$.

One-way ANOVA was used to compare gene expression among the experimental groups, including CD34+ cells before co-culture (control group), after co-culture with exosomes, and after co-culture with exosomes combined with mesenchymal stem cells.

3.6.4 Comparison of samples pre- and post-co-culture for CXCR4 gene expression

CXCR4 expression increased in all groups; however, the increases observed in Groups I, II, and III were not statistically significant. Group II showed a 4.43-fold increase in *CXCR4* expression, which did not reach statistical significance ($P<0.1$). Group IV demonstrated a significant increase ($P=0.0008$), corresponding to a 17.4-fold elevation relative to pre-co-culture levels (Figure 3a).

3.6.5 Comparison of samples pre- and post-co-culture for VLA4 gene expression

According to the analysis, VLA4 gene expression revealed fold increases of 4.36, 7.53, 4.44, and 10.99 in Groups I, II, III, and IV, respectively. Statistically significant upregulation was observed in Group IV ($P<0.001$) and Group II ($P<0.05$) (Figure 3b).

3.6.6 Comparison of pre-and post-co-culture samples for VLA5 gene expression

VLA5 expression was significantly increased in three groups: I with $P<0.001$, IV with $P<0.0001$, and group II with $P<0.01$. Also, in group IV, gene expression increased

15.2-fold compared with before co-culture; group I increased 8.5-fold, and group II increased 5.6-fold (Figure 3c).

3.6.7 Comparison of pre-and post-co-culture samples for hTERT gene expression

Among all experimental groups, significant upregulation of *hTERT* expression was observed only in Group IV ($P<0.05$), corresponding to a 2.15-fold increase relative to pre-co-culture levels. Increases in the other groups were not significant (Figure 3d).

3.6.8 Comparison of pre-and post-co-culture samples for HOXA9 gene expression

HOXA9 expression was significantly increased in all groups: Group I ($P < 0.05$), Group II ($P < 0.01$), Group III ($P < 0.001$), and Group IV ($P < 0.0001$), with fold increases of 1.8, 1.51, 1.81, and 2.52, respectively (Figure 3e).

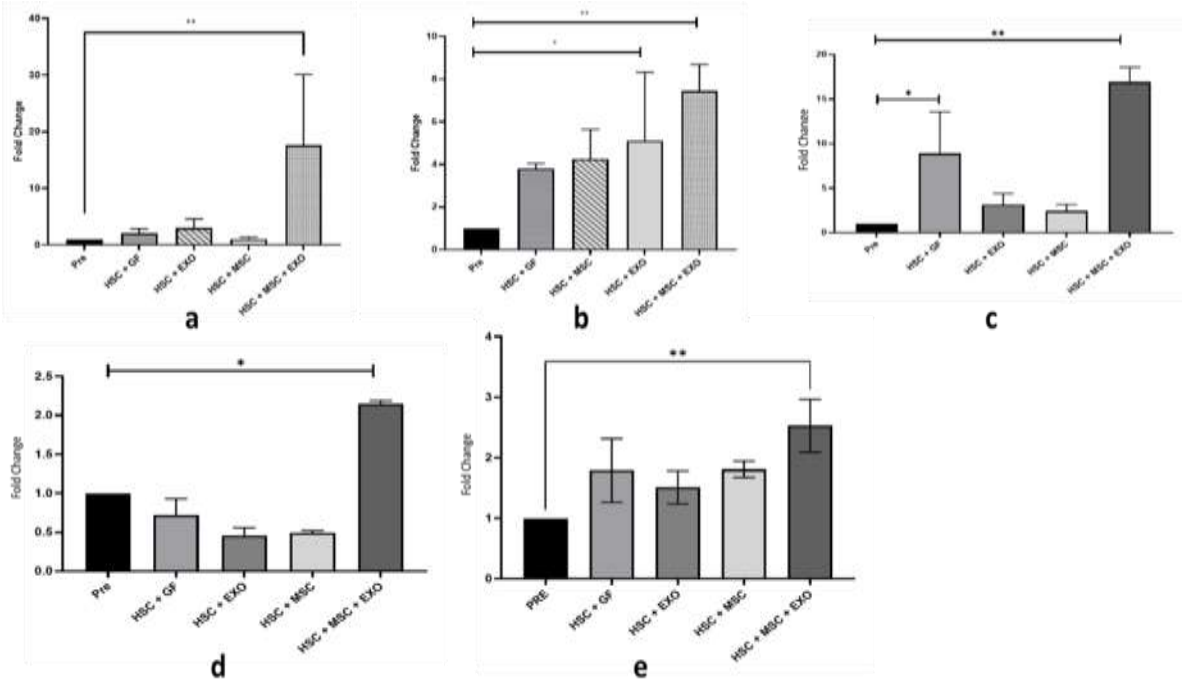


Figure 3. Comparison of gene expression in different study groups: CXCR4 (a), VLA4 (b), VLA5 (c), hTERT (d). **(Prepared by Authors, 2026)**.

4. Discussion

The dissemination of HSCs in vivo was demonstrated by HSCT **(19)**. The bone marrow niche tightly regulates their expansion and activity in vivo **(20)**; consequently, efforts in this field have focused on optimizing $CD34^+$ cell proliferation ex vivo. HSCs can be expanded in liquid culture media supplemented with growth factors, or on MSCs used as a supportive layer that recreates the bone marrow niche **(21)**.

In 2021, Nurhayati et al. cultured UCB mononuclear cells with human and animal feeder cells within a microcapsule and reported up to a 197-fold increase in cell proliferation **(22)**. Walenda et al. demonstrated that simultaneous culture of UCB-HSCs on an MSC feeder layer enhanced proliferation compared with standard culture medium **(23)**. Protein cocktails containing G-CSF, TPO, IL6, FLT3L, and SCF are commonly used for HSCs expansion in liquid culture media, and we used FLT3L, TPO, and SCF factors to improve the culture conditions. Current strategies for UCB expansion primarily aim to recreate the physiological conditions of the bone marrow niche. Most studies now use molecules, vesicles, and cells in the bone marrow niche to provide these conditions **(24)**. EXOs released into the extracellular space are among the most critical mediators of MSC paracrine activity within the bone marrow niche. On this basis, numerous studies have examined the therapeutic and immunomodulatory effects of EXOs **(14,25–27)**. Xie et al. used MSC-derived microvesicles in a feeder layer to support HSCs, increasing proliferation and reducing differentiation **(28)**. Accordingly, the present study aimed to investigate the effects of UCBMSC-EXOs on HSPC proliferation.

Homing of HSPCs to the bone marrow is probably initiated by cellular adhesion to endothelial surfaces, a process in which VLA-4 plays a key role. SDF-1 induces the function of VLA-4 and LFA-1 in human UCB cells. β1-integrins are essential mediators of HSC–niche interactions; specifically, VLA-4 (α4β1) contributes to HSC retention within the bone marrow microenvironment, whereas VLA-5 (α5β1) facilitates HSC binding to SPP1. In addition, these integrins mediate SDF-1 function. VLA-4, VLA-5, and CXCR4 play important roles in the adhesion interactions involved in

HSC homing and migration (29).

In the present study, the expression of VLA-4, VLA-5, and CXCR4 was examined across four co-culture groups. CXCR4 expression was significantly increased only in Group IV ($P = 0.0008$; 17.4-fold). VLA-4 expression was significantly upregulated in Group IV ($P < 0.001$; 10.99-fold) and Group II ($P < 0.05$; 7.53-fold). VLA-5 expression was significantly elevated in Group I ($P < 0.001$; 8.5-fold), Group II ($P < 0.01$; 5.6-fold), and Group IV ($P < 0.0001$; 15.2-fold). These findings suggest that UCBMSC-EXOs, particularly in combination with an MSC feeder layer, may enhance the homing potential of expanded HSPCs.

Telomerase is a large ribonucleoproteins complex consisting of the reverse transcriptase protein (hTERT) and a template (hTR) RNA. Both components are essential for telomerase activity. Most HSPCs in the cell cycle have telomerase activity. Exposure to HSPCs and precursor cells with cytokines increases *hTERT* activity, but this effect is reduced with subsequent proliferation and differentiation. The level of telomerase activity may be related to cell self-renewal. This association suggests that high *hTERT* expression, or even transient telomerase activity, may increase primary hematopoietic cell self-regeneration (30). In the current study, *hTERT* expression was evaluated across all groups, and it differed significantly only in group IV ($P<0.05$). Homeobox transcription factors were first identified as a conserved gene family essential for embryonic development and have since emerged as another class of critical evolutionary genes that shape early hematopoietic evolution. Several members of the HOX family are expressed in most populations with high levels of primary HSPCs, whereas their expression is permanently regulated. In $CD34^+$ cells, the final differentiation stage is undetectable. This observation suggests that Hox genes are involved in self-renewal and functional growth mechanisms in the early stages of hematopoiesis. Accordingly, it has been shown that increasing engineered expression of several members of the Hox gene family affects the replication and differentiation programs of primary human and mouse HSPCs. *HOXA9* is expressed in areas rich in primary cells with long-term culture or myeloid precursor cells (31). In the present study, *HOXA9* expression was significantly increased in all experimental groups, further supporting the proliferative and stemness-promoting effects of the culture conditions used.

Our findings demonstrated that UCBMSC-EXOs can partially mimic the biological effects of their parent MSCs and enhance the proliferation of UCB-derived HSPCs. Co-culturing USBMSC-EXOs with UCBMSCs can enhance EXOs' impact, including increased cell numbers, colonization, and expression of target genes. On the other hand, we observed that hTERT and HOXA9 are important for self-renewal, and we also observed increased expression of VLA4, CXCR4, and VLA5 as homing genes in these cells. As a result, it can be said that UCBMSC-EXOs have effectively promoted HSPCs' growth. TEM and DLS findings indicate the presence of particles between 30 and 150 nm, which confirms that the particles in our samples were UCBMSC-EXOs. The images of UCBMSC-EXOs in the present study were similar to those of Lyu TS et al., and the homogenization of the particles in our extract sample showed the purity of the particles (32).

After exosomes isolating, co-culture in four groups was performed to evaluate $CD34^+$ cell proliferation. In the first group, co-culture of $CD34^+$ cells with FLT3, TPO, and SCF cytokines increased the mean numbers of TNC, $CD34^+$, and CFU-C cells, and *CXCR4* expression also increased. However, increased expression of *HOXA9*, an essential gene in self-renewal, and of *VLA5*, a homing gene in this group, was observed. To further assess the role of UCBMSCs in HSPCs expansion, $CD34^+$ cells were cultured on an MSC feeder layer in the presence of cytokines (Group III). This condition resulted in increased cellular proliferation and higher $CD34^+$ expression compared with the previous groups. Although *CXCR4* expression did not significantly differ from pre-co-culture levels, *HOXA9* expression and colony formation were enhanced. Consistent with the findings of Van der Garde et al., BFU-E colony formation was lower than CFU-GM and CFU-GMM formation in co-culture systems using UCBMSCs compared with BM-MSCs (33).

To further demonstrate the boosting effect of UCBMSC-EXOs and MSCs in the last group, $CD34^+$ cells were cultured with the optimal EXOs concentration and UCBMSCs as a feeder layer. The goal was to show the enhancing effects of UCBMSC-EXOs on $CD34^+$ cell proliferation. In this group, we observed increased CD34+ cell-surface expression and significantly increased expression of the *VLA4*, *VLA5*, *hTERT*, *HOXA9*, and *CXCR4* genes. So, both the potential for self-renewal and homing in these cells multiplied. These findings indicate enhanced self-renewal and homing capacities, highlighting the supportive role of UCBMSC-EXOs in HSPCs proliferation. Notably, UCB cell proliferation was markedly greater when UCBMSC-EXOs were combined with MSC feeder layers. In the present study, we used the MACS method to isolate HSPCs (with 95% purity) by positive selection. In addition to examining the CD34 marker in this study, we also assessed CXCR4 as a homing-related marker, which demonstrated significant upregulation ($P<0.01$).

One limitation of our study was the omission of a 14-day cultivation period for UCBHSCs in the cell culture facility. This decision was based on our concern regarding the elevated risk of culture medium contamination. To mitigate this risk, we aimed to reach the target number of $CD34^+$ cells as quickly as possible, and we achieved it within 7 days.

5. Conclusion

In conclusion, UCBMSC-EXOs enhanced the ex-vivo proliferation of UCB $CD34^+$ cells while promoting characteristics associated with HSPCs. Our findings showed that UCBMSC-EXOs can both mimic and potentiate the biological effects of their parent MSCs. These results provide further insight into the potential application of UCBMSC-EXOs as supportive agents for ex-vivo UCB expansion and stem cell engineering strategies.

6. Declarations

6.1 Acknowledgments

The authors gratefully acknowledge the financial support provided by the Department of Hematology and Blood Transfusion Sciences, Tehran University of Medical Sciences.

6.2 Ethical Considerations

The study protocol was reviewed and approved by the Medical Ethics Committee of Tehran University of Medical Sciences (Approval No. IR.TUMS.SPH.REC.1397.319).

6.3 Authors' Contributions

Asma Javadi Rad, Mohammad Khani-Eshratabadi, Morteza Zarrabi, Narjes Seddighi, and Seyed Hadi Mousavi contributed to the research and preparation of the manuscript. All authors reviewed and approved the final version of the manuscript.

6.4 Conflict of Interest

The authors declare that they have no conflicts of interest related to this work.

6.5 Fund or Financial Support

This study was financially supported by Tehran University of Medical Sciences.

6.6 Using Artificial Intelligence Tools (AI Tools)

The authors did not utilize any AI tools in conducting this research or preparing the manuscript.

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