Original Article

Effect of acute hypoxia on *CXCR4* gene expression in C57BL/6 mouse bone marrow-derived mesenchymal stem cells

Mehdi Kadivar, Najva Alijani, Maryam Farahmandfar[1], Saman Rahmati, Nastaran Mohammadi Ghahhari, Reza Mahdian[2]

Department of Biochemistry, Pasteur Institute of Iran, [1]Department of Neuroscience, School of Advanced Medical Technologies, Tehran University of Medical Sciences, [2]Department of Molecular Medicine, Pasteur Institute of Iran, Tehran, Iran

Abstract

Background: One of the most important stimuli in stem cell biology is oxygen. Chemokine receptor 4 (CXCR4) plays a crucial role in the migration and homing of stem cells. In this study, mesenchymal stem cells (MSCs) were exposed to 1% oxygen to investigate the effect of acute hypoxia on *CXCR4* gene expression.

Materials and Methods: MSCs were isolated from C57BL/6 mouse bone marrow and were identified and expanded in normoxic culture. Cells were incubated at 37°C under 1% hypoxic conditions for periods of 4, 8, 16, 24, and 48 h. After hypoxia preconditioning, the cells were placed in normoxic condition for 8 h to achieve cellular hypoxia-reoxygenation. To assess the level of *CXCR4* gene expression, real-time quantitative reverse transcription-polymerase chain reaction was carried out for each group.

Results: Data from statistical analysis illustrated that exposure of MSCs to acute hypoxic condition down-regulates *CXCR4* expression with the maximum under-expression observed in 4 h (0.91 ± 0.107) and 8 h (50 ± 2.98) groups. Moreover, the relative gene expression of *CXCR4* was decreased after hypoxia-reoxygenation by more than 80% in 4 h (0.136 ± 0.018) and 24 h (12.77 ± 0.707) groups.

Conclusion: The results suggest that *CXCR4* expression in MSCs decreases upon acute hypoxic stress. Furthermore, hypoxia-reoxygenated MSCs showed decreased expression of *CXCR4*, compared to cells subjected to acute hypoxia. This difference could have resulted from the cells being compatible with low oxygen metabolism. In summary, before the therapeutic application of MSCs, it should be regarded as a necessity to optimize the oxygen concentration in these cells, as it is a critical factor in modulating *CXCR4* expression.

Key Words: *CXCR4*, gene expression, hypoxia, mesenchymal stem cells

Address for correspondence:
Dr. Mehdi Kadivar, Department of Biochemistry, Pasteur Institute of Iran, Tehran, Iran. E-mail: Kadivar@pasteur.ac.ir
Received: 16.03.2014, **Accepted:** 24.04.2014

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.145682

INTRODUCTION

Mesenchymal stem cells (MSCs) are adherent, fibroblast-like, and multipotent cell population with high proliferative capacity.[1] MSCs are responsible for tissue growth, and participate in regulating tissue repair and regeneration.[2] These characteristics of MSCs make them a promising therapeutic tool

How to cite this article: Kadivar M, Alijani N, Farahmandfar M, Rahmati S, Ghahhari NM, Mahdian R. Effect of acute hypoxia on *CXCR4* gene expression in C57BL/6 mouse bone marrow-derived mesenchymal stem cells. Adv Biomed Res 2014;3:222.

in clinics.[3] Chemokine receptors assist MSCs in trafficking to various tissues such as bone marrow; however, chemokine (C-X-C motif) receptor 4 (CXCR4) plays a pivotal role among them. CXCR4 is the receptor for stromal cell-derived factor-1 (SDF-1) or CXCL12 which is a CXC chemokine produced by stromal cells.[4,5]

Cell surface expression of *CXCR4* on MSCs differs from roughly insignificant to 20-40%. Such variations could be associated with differences in cell culture conditions. For instance, *CXCR4* is up-regulated when MSCs are exposed to cytokines, such as insulin-like g rowth factor-1 (IGF-1), present in the cell culture serum.[6] Additionally, to increase *CXCR4* expression in MSCs, different studies have attempted to transduce cells with a retroviral vector expressing *CXCR4*. These cells are then treated with tumor necrosis factor-α (TNF-α), interferon-β and -γ, and copaxone or insulin-like growth factor (IGF).[7]

The microenvironment of stem cells is influenced by various important physiological conditions such as oxygen tension.[8] Blood within human bone marrow consists of approximately 7% oxygen. However, the oxygen level in bone marrow differs from the maximum gradient measured near the sinuses (about 5%) to the minimum level at the inner surface of the cortical bone (about 1%).[9] One of the most frequently encountered issues in laboratory conditions to culture MSCs is that the adult cells are in bone marrow niches where the oxygen concentration is below its level in the atmosphere.[10] Hypoxia preconditioning of MSCs improves their overall *in vivo* survival rate, functional mobility to areas damaged by hypoxia, and endogenous tissue repair activation.[11-13] Also, hypoxia is one of the methods useful for increasing *CXCR4* expression.[14] Schioppa *et al*. reported that hypoxia induces overexpression of *CXCR4* in various cell types such as mononuclear phagocytes, endothelial and cancer cells.[15] Hypoxia-induced up-regulation of *CXCR4* results from the stabilization of hypoxia-inducible factor 1-α (HIF-1α) in normal and malignant B-cells.[16]

In the present study, C57BL/6 mouse bone marrow-derived MSCs were subjected to 1% oxygen to analyze the effect of acute hypoxia on the expression of chemokine receptor CXCR4.

MATERIALS AND METHODS

MSC isolation and cell culture

Bone marrow was collected from six 2-month-old C57BL/6 mice by flushing femurs and tibias using phosphate-buffered saline (PBS) (Sigma, St. Louis, MO, USA). The cells were washed with PBS and plated in a 25-cm^2 flask containing Dulbecco's modified Eagle's medium (DMEM) (Gibc o BRL, U SA) and 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). The culture was kept in a humidified CO_2/O_2 incubator (5%/21%) at 37°C. Non-adherent cells were removed by changing the medium after 3 days and adherent cells were further cultured for an additional 7 days. On day 8, the adherent cells were passaged into two new 25-cm^2 flasks. This process was continued until 12 passages under 21% O_2.

Cell surface marker characterization

The cells were cultured in an 8-well chamber slide (BD Falcon, Hamburg, Germany) and fixed with 4% paraformaldehyde for 30 min at 4°C. Then, the cells were washed with PBS, blocked, and permeabilized using 1% bovine serum albumin (BSA), 0.1% Triton X-100, 10% goat serum, and PBS for 30 min. Cells were then incubated in 1% BSA-PBS overnight at 4°C with the primary antibody [mouse anti-CD73 monoclonal antibody, unconjugated (Invitrogen, Carlsbad, CA, USA 1:100) and unconjugated mouse anti-STRO-1 monoclonal antibody (Invitrogen, Carlsbad, CA, USA 1:50)]. Subsequently, cells were incubated for 1.5 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Sigma, 1:500). After 1 h of incubation with diaminobenzidine in darkness, images of the cells were produced using inverted microscope.

Osteogenic and adipogenic differentiation of MSCs

Cells were seeded at a density of 3×10^3 cells/cm^2 and cultured for 10 days with 100 nM dexamethasone, 50 μM ascorbic acid, and 10 mM β-glycerophosphate to achieve osteogenic differentiation. Osteoblast differentiation was evaluated by alizarin red staining (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol. Adipogenic differentiation was induced in cells through culturing them in the presence of 1 μM dexamethasone, 0.2 mM indomethacin, 10 μg/ml insulin, and 0.5 mM 3-isobutyl-1-methylxanthine. After 3 weeks of culture, cells were fixed with 4% paraformaldehyde and covered with 3 mg/ml Oil Red O (Sigma, St. Louis, MO, USA). Then, the cells were dissolved in 60% isopropanol for 10 min and the excess dye was discarded using water.

Hypoxia conditions

For hypoxic exposure, tissue culture plates were placed in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA, USA) and flushed at 2 p.s.i. for 15 min with a gas mixture consisting of 1% O_2, 5% CO_2, and balanced N_2. To perform hypoxia test, the cells in passage 12 were incubated in the hypoxia chamber for periods of 4, 8, 16, 24, and 48 h. To induce hypoxia-reoxygenation, the cells were initially exposed to 1% O_2 followed by 8 h of normoxic incubation.

RNA isolation

Total cellular RNA was isolated using RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quality of RNA was assessed through 1% agarose gel electrophoresis and Nanodrop spectrophotometer (ND-1000). Only high-quality RNA samples were selected for cDNA microarray hybridization.

cDNA synthesis

cDNA synthesis was performed using AccuPower CycleScript RT PreMix (dN6) cDNA synthesis kit (Bioneer, Seoul, South Korea) according to the manufacturer's instructions. RNA was Reverse-transcribed under the following conditions: 1 μl total RNA as template, 25 mM $MgCl_2$, 10 mM dNTP mix, 10× RT buffer, 0.1 M dithiothreitol (DTT), 200 U of SuperScript™ III, 40 U of RNaseOut, and 50 μM oligo d(T) primers in a final volume of 20 μl. The reaction was performed at 15°C for 30 s, at 42°C for 4 min, and at 55°C for 30 s for 12 cycles, and then the enzyme was heat-inactivated at 95°C for 5 min.

Real-time reverse transcription-polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify cDNA for 18S rRNA (as an internal standard) and CXCR4 using the following primers: 18S rRNA forward primer, 5′-CACGGACAGGATTGACAGATTG-3′; 18S rRNA reverse primer, 5′-GATGGCTACGTA CATGGCTG-3′; CXCR4 forward primer, 5′-CATCGTCATCCTCTCCTGTTACTG-3′; and CXCR4 reverse primer, 5′-GATGAGGATG ACTGTCGTCTTGAG-3′. The PCR was performed in a total volume of 25 μl consisting of 10 pmol of each of the reverse and forward primers, 12 μl SYBR Premix Ex Taq (2X), and 5 μl cDNA (10 ng/μl). Thermal cycling was initiated with a first denaturation step of 95°C for 15 s, followed by 40 cycles of 95°C for 5 s, 60°C for 25 s, and 95°C for 15 s in a Corbett 6000 real-time system.

Data analysis

A suitable threshold was applied on amplification plots and the resulting C_t (threshold cycles) values were used for relative quantification. The C_t values of CXCR4 were normalized against 18S rRNA plot and the resulting values were compared with the respective normoxic control group using the $2^{-\Delta\Delta Ct}$ method. Triplicate experiments were performed separately for cells from five flasks. Student's paired *t*-test and one-way analysis of variance followed by Bonferroni's correction were used to analyze the results and determine the level of significance. Statistical analyses were conducted with SPSS statistical software (version 16; SPSS, Chicago, IL, USA).

RESULTS

Isolation, culture, and characterization of bone marrow-derived cells

MSCs isolated from C57BL/6 mice bone marrow were cultured in appropriate medium as described and reached 80% confluence by day 14. Cells at the second passage stained strongly for CD73 and STRO-1. Positive staining for these markers confirmed that the isolated cells were MSCs [Figure 1]. To verify that immunodepleted MSCs were multipotent, their ability to differentiate into osteocytes and adipocytes was analyzed. After 10 days in osteogenic medium or 21 days in adipogenic medium, MSCs differentiated into adipocytes [Figure 2] or osteocytes [Figure 3] as indicated by increase of Oil Red O or alizarin red staining, respectively.

CXCR4 gene expression analysis

As shown in Figure 4a, after exposure of cells to short-term hypoxia (1% O_2) for 4, 8, 16, 24, and 48 h, *CXCR4* relative gene expression was decreased in comparison to normoxic cells as control samples. The maximum decrease was observed in 4 h (0.91 ± 0.107) group, while the minimum decrease was in 8 h (50 ± 2.98) group. Figure 4b shows that hypoxia-reoxygenation treatment leads to *CXCR4* gene under-expression, with the maximum decrease measured in 4 h (0.136 ± 0.018) group and the minimum decrease in 24 h (12.77 ± 0.707) group. Figure 4c presents a comparison between different hypoxia groups and their related hypoxia-reoxygenation groups. The differences between hypoxia and normoxia groups, hypoxia-reoxygenation and normoxia groups were significant as calculated by One-Way Anova. Also differences between hypoxia and hypoxia-reoxygenation groups were significant, as calculated by Student's *t*-test ($P < 0.001$).

DISCUSSION

Metabolic flexibility is one of the features represented by MSCs, helping them to survive under ischemic

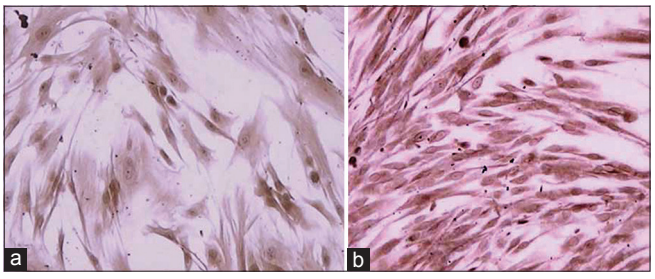

Figure 1: Cell surface epitope characterization of bone marrow-derived cells. (a) Staining for CD73, (b) staining for STRO-1. Cell surface staining on passage 2 bone marrow-derived cells was performed with (a) CD73 and (b) STRO-1 antibodies and diaminobenzidine (DAB) solution. Results showed strong staining for these markers

stress and maintain their multipotency.[17,18] HIF is a transcription factor and is one of the master regulators controlling the cellular response to the tension caused by low oxygen level.[19] The HIF complex binds to DNA regulatory sequences called hypoxia response elements (HREs) that are present in the promoter or enhancer regions of HIF target genes.[20] Based on the literature, HIF proteins induce CXCR4 expression in cooperation with nuclear factor-kb (NF-kb).[21,22] HIF-1 is also involved indirectly in CXCR4 expression. This is induced by activation of HREs in Ets1 promoter, a transcription factor of CXCR4.[23] Changes in oxygen level are an important regulator of CXCR4 expression. Hypoxia stabilizes CXCR4 transcripts, contributing to increased *CXCR4* gene expression. This suggests that hypoxia-regulated RNA binding factors could interact with CXCR4 and stabilize its mRNA at the post-transcriptional level.[15] Hung *et al*. showed the positive effect of short-term exposure of human bone marrow-derived MSCs to 1% oxygen on chemokine receptors, including CX3CR1 and CXCR4, at the mRNA and protein levels.[2]

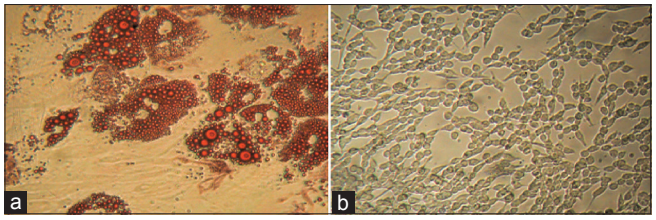

Figure 2: Adipogenic differentiation of bone marrow-derived mesenchymal stem cells. Oil Red O staining of cultures that were treated (a) and not treated (b) with adipogenic media for 3 weeks

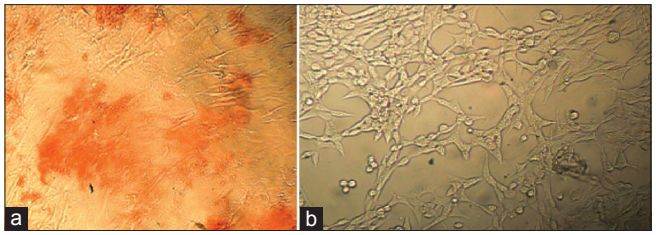

Figure 3: Osteogenic differentiation of bone marrow-derived mesenchymal stem cells. Alizarin red staining of cultures that were treated (a) and not treated (b) with osteogenic media for 2 weeks

Hypoxia activates HIF in almost all cell types; however, HIF alone is not responsible for the cell-type specific gene expression. In fact, functional interactions of HIF with other transcription factors determine the cell-type specific gene expression induced by hypoxia.[19] Furthermore, functional transcription mediated by HIF-1 is accomplished through building a complex between HIF-1 and co-activators like CREB binding protein (CBP)/p300, and steroid receptor co-activator (SRC)/p160 family. More co-factors are also required to join the HIF-1/co-activator complex, leading to hypoxia-induced transcription activation in a tissue-specific manner.[20] Tissue-specific expression of HIF-1α isoforms and cooperation of one of the isoforms with specific transcription factors, co-activators, or co-repressors is associated with substantial variations observed in the level and specificity of target genes' activation.[19]

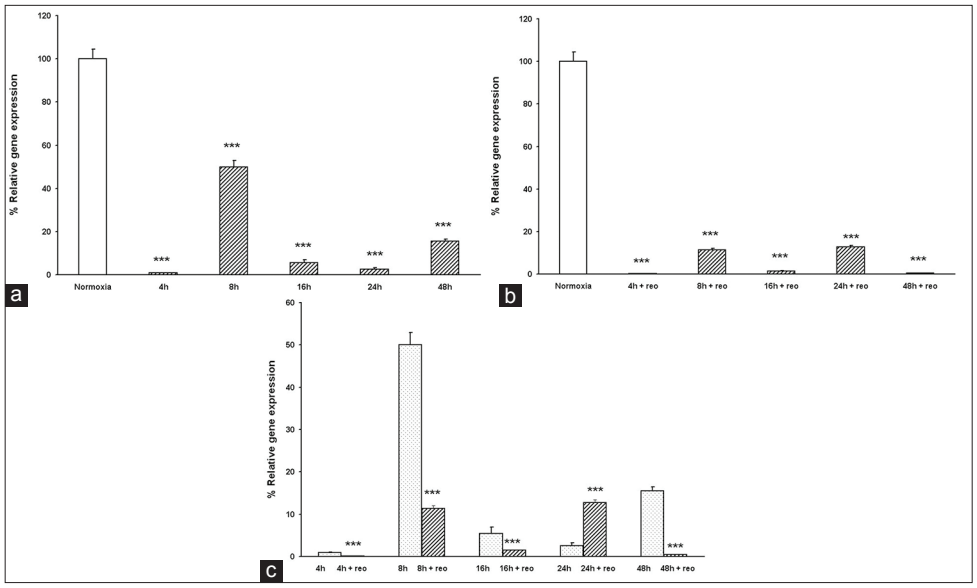

Figure 4: Real-time reverse transcription-polymerase chain reaction analysis of the mRNA expression of CXCR4 receptor gene: (a) comparison between hypoxia group and normoxia group; (b) comparison between hypoxia-reoxygenation group and normoxia group; (c) comparison between hypoxia group and hypoxia-reoxygenation groups. Control groups were treated under normoxia condition. Data show relative mRNA expression of CXCR4 normalized against 18S rRNA. Values are mean ± standard error of mean ***$P < 0.001$

More importantly, the expression level of a single gene induced by hypoxia in the same cells depends on other elements such as factors affecting HIF-1α activity, active or inactive metabolic pathways, real oxygen concentration in the cells' microenvironment, and the length of culture period.

In the present study, the effect of short-term hypoxia with 1% O_2 on *CXCR4* gene expression in C57BL/6 murine bone marrow-derived MSCs was analyzed. The results suggest that the exposure of cells of interest to acute hypoxia for periods of 4, 8, 16, 24, and 48 h decreases *CXCR4* gene expression compared to cells cultured under normoxia condition. Moreover, hypoxia-reoxygenation of MSCs led to decreased *CXCR4* receptor gene expression. The first stage reduction of *CXCR4* expression could have resulted from long-term culture of cells in normoxic conditions followed by acute hypoxia shock. Compatibility of cells with normoxic condition can also be responsible for this decrease. The second stage decrease of *CXCR4* receptor gene expression could have occurred as an outcome of compatibility of cells with new oxygen concentration (hypoxia) and suppressive effect of normoxia on CXCR4 promoter.

Despite numerous studies performed on the effect of hypoxia on gene expression, there is still a discrepancy in this field. The contradiction observed in data could have resulted from diversity of species, cell lines versus primary cells used, the ambient oxygen level, cell culture design, and the time points evaluated. Differences in cell isolation methods, experimental parameters, growth factors, oxygen tension, and specific evaluation techniques highlight the challenges in determining the role of oxygen in stem cell differentiation. In conclusion, as there are many factors affecting *CXCR4* receptor gene expression, it is necessary to optimize the factors including oxygen concentration, cell type, cell passage numbers, cell density, and the type of culture design before using these cells in therapeutics. As the function of *CXCR4* is critical in the systemic injection of stem cells, it is important to optimize mentioned factors before these kinds of stem cells administrations.

ACKNOWLEDGMENT

This project was supported by the Pasteur Institute of Iran (Project No. 510). The authors would like to acknowledge people working in the biochemistry and molecular medicine departments of Pasteur Institute of Iran.

REFERENCES

1. Fan J, Varshney RR, Ren L, Cai D, Wang DA. Synovium-derived mesenchymal stem cells: A new cell source for musculoskeletal regeneration. Tissue Eng Part B Rev 2009;15:75-86.
2. Hung SC, Pochampally RR, Hsu SC, Sanchez C, Chen SC, Spees J, *et al*. Short-term exposure of multipotent stromal cells to low oxygen increases their expression of CX3CR1 and CXCR4 and their engraftment *in vivo*. PLoS One 2007;2:e416.
3. Ohnishi S, Yasuda T, Kitamura S, Nagaya N. Effect of hypoxia on gene expression of bone marrow-derived mesenchymal stem cells and mononuclear cells. Stem Cells 2007;25:1166-77.
4. Sordi V, Malosio ML, Marchesi F, Mercalli A, Melzi R, Giordano T, *et al*. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. Blood 2005;106:419-27.
5. Bobis S, Jarocha D, Majka M. Mesenchymal stem cells: Characteristics and clinical applications. Folia Histochem Cytobiol 2006;44:215-30.
6. Kyriakou C, Rabin N, Pizzey A, Nathwani A, Yong K. Factors that influence short-term homing of human bone marrow-derived mesenchymal stem cells in a xenogeneic animal model. Haematologica 2008;93:1457-65.
7. Potapova IA, Brink PR, Cohen IS, Doronin SV. Culturing of human mesenchymal stem cells as three-dimensional aggregates induces functional expression of CXCR4 that regulates adhesion to endothelial cells. J Biol Chem 2008;283:13100-7.
8. Ma T, Grayson WL, Fröhlich M, Vunjak-Novakovic G. Hypoxia and stem cell-based engineering of mesenchymal tissues. Biotechnol Prog 2009;25:32-42.
9. Fehrer C, Brunauer R, Laschober G, Unterluggauer H, Reitinger S, Kloss F, *et al*. Reduced oxygen tension attenuates differentiation capacity of human mesenchymal stem cells and prolongs their lifespan. Aging Cell 2007;6:745-57.
10. Fink T, Abildtrup L, Fogd K, Abdallah BM, Kassem M, Ebbesen P,*et al*. Induction of adipocyte-like phenotype in human mesenchymal stem cells by hypoxia. Stem Cells 2004;22:1346-55.
11. Nekanti U, Dastidar S, Venugopal P, Totey S, Ta M. Increased proliferation and analysis of differential gene expression in human Wharton's jelly-derived mesenchymal stromal cells under hypoxia. Int J Biol Sci 2010;6:499-512.
12. Valorani MG, Germani A, Otto WR, Harper L, Biddle A, Khoo CP, *et al*. Hypoxia increases Sca-1/CD44 co-expression in murine mesenchymal stem cells and enhances their adipogenic differentiation potential. Cell Tissue Res 2010;341:111-20.
13. Rosová I, Dao M, Capoccia B, Link D, Nolta JA. Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. Stem Cells 2008;26:2173-82.
14. Tang YL, Zhu W, Cheng M, Chen L, Zhang J, Sun T, *et al*. Hypoxic preconditioning enhances the benefit of cardiac progenitor cell therapy for treatment of myocardial infarction by inducing CXCR4 expression. Circ Res 2009;104:1209-16.
15. Schioppa T, Uranchimeg B, Saccani A, Biswas SK, Doni A, Rapisarda A, *et al*. Regulation of the chemokine receptor CXCR4 by hypoxia. J Exp Med 2003;198:1391-402.
16. Povan E, Tosello V, Indraccolo S, Masiero M, Persano L, Esposito G, *et al*. Differential regulation of hypoxia-induced CXCR4 triggering during B-cell development and lymphomagenesis. Cancer Res 2007;67:8605-14.
17. Lavrentieva A, Majore I, Kasper C, Hass R. Effects of hypoxic culture conditions on umbilical cord-derived human mesenchymal stem cells. Cell Commun Signal 2010;8:18.
18. Mylotte LA, Duffy AM, Murphy M, O'Brien T, Samali A, Barry F, *et al*. Metabolic flexibility permits mesenchymal stem cell survival in an ischemic environment. Stem Cells 2008;26:1325-36.
19. Kaluz S, Kaluzová M, Stanbridge EJ. Regulation of gene expression by hypoxia: Integration of the HIF-transduced hypoxic signal at the hypoxia-responsive element. Clin Chim Acta 2008;395:6-13.
20. Berchner-Pfannschmidt U, Frede S, Wotzlaw C, Fandrey J. Imaging of the hypoxia-inducible factor pathway: Insights into oxygen sensing. Eur Respir J 2008;32:210-7.
21. Ratajczak MZ, Zuba-Surma E, Kucia M, Reca R, Wojakowski W, Ratajczak J.

The pleiotropic effects of the SDF-1-CXCR4 axis in organogenesis, regeneration and tumorigenesis. Leukemia 2006;20:1915-24.
22. Zhang Y, Wittner M, Bouamar H, Jarrier P, Vainchenker W, Louache F. Identification of CXCR4 as a new nitric oxide-regulated gene in human CD34+cells. Stem Cells 2007;25:211-9.
23. Maroni P, Bendinelli P, Matteucci E, Desiderio MA. HGF induces CXCR4 and CXCL12-mediated tumor invasion through Ets1 and NF-kappaB. Carcinogenesis 2007;28:267-79.

Source of Support: This project was supported by the Pasteur Institute of Iran (Project No. 510), **Conflict of Interest:** None declared.