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ORIGINAL ARTICLE

Hypoxia Preconditioning Promotes Survival And Clonogenic Capacity of Human Umbilical Cord Blood Mesenchymal Stem Cells

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ABSTRACT

Background: In recent decade, human umbilical cord blood derived mesenchymal stem cells (hUCB-MSCs) provide enormous potential for appropriate cell therapy, but they have limited growth potential and cease to proliferate due to cellular senescence, so providing a strategy for increasing the stem cell survival is necessary.

Methods: In this investigation, MSCs characterized by flow cytometry were isolated from umbilical cord blood. Hypoxia preconditioning (HPC) was induced in a water-saturated gas mixture of 2.5% O_2 and 5% CO_2 for 15 min and then reoxygenation at 21% O_2 for 30 min at 37 °C. Subsequently, hypoxia preconditioned hUCB-MSCs were exposed to 2.5% O_2 and 5% CO_2 for 24, 48 and 72 hr (HPC + hypoxia groups). We examined the proliferation capacity of hUCB-MSCs after HPC in comparison with normoxia status, and we determined the best duration time of being under hypoxia (24, 48 or 72 hr of hypoxia). In order to assess the role of HPC on the expression of surface markers, cells were analyzed by flow cytometry. Proliferation of cells was evaluated using MTT assay, and doubling time and colony-forming unit-fibroblast (CFU-F) was calculated in each group.

Results: The MTT results showed that cell viability of HPC-UCB-MSCs significantly increased in comparison with UCB-MSCs under normoxia condition. Our study revealed that HPC reduces the doubling time of UCB-MSCs remarkably after passaging 48 hours of hypoxia. Our results proved that HPC can significantly increase the CFU-F colony numbers of hUCB-MSCs without any alteration on cell surface marker expression.

Conclusion: Our results suggested that HPC of umbilical cord blood derived mesenchymal stem cells along with induction of hypoxia can provide a suitable culture condition for rapid proliferation of mesenchymal stem cells with no effect on their immunophenotype features and it could be a potential therapeutic option for increasing the capability of MSCs.

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Introduction

Mesenchymal stem cells (MSCs) have well known advantages that make them overwhelmingly useful in the field of regenerative medicine. MSCs have properties such as regulating inflammation as a multistep event, ability to differentiate into various cell types,[1] secretion of biomolecules capable of stimulating recovery of injured cells and migration and engraftment in sites of inflammation.[2, 3]

At the first time, umbilical cord blood (UCB) transplantation was done in a child with Fanconi anemia in October 1988.[4] In recent years, researchers have begun

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to make a progress in safety and efficacy of UCB stem cells for therapeutic applications.

MSCs can be obtained from different sources. To date; bone marrow (BM) has been the most common source of MSCs,[5] but isolation of MSCs from UCB is an easier, less expensive and non-invasive procedure with less contamination risk than collecting MSCs from bone marrow aspirates. Researchers have shown that UCB-MSCs capabilities are similar to those derived from adult bone marrow.[6] In addition, these cells have some other properties such as delayed senescence, highest doubling numbers,[7] higher anti-inflammatory effect via angiopoietin 1(Ang-1)[8] and a high angiogenesis potential.[9] The high potential of proliferation of the UCB derived hematopoietic and mesenchymal stem cells in comparison with other sources provide them as an appropriate candidate for cellular therapies.[10-12]

Unfortunately, MSCs have limited growth abilities and are prone to cellular senescence.[13] Although, many problems and issues remain to be resolved about the successful transplantation of these cells as a cell-based therapy, numerous efforts have been made to increase survival and proliferation of MSCs. Since senescent MSCs are so heterogeneous and different genes and signaling pathways are responsible for MSCs aging process, different studies have used various strategies to enhance their cell viability. One of the effective methods to promote proliferation, differentiation, growth factors release and survival rate of MSCs is induction of hypoxia.[14, 15] Several studies have demonstrated that making hypoxia preconditioning conditions enhanced the expression of genes involved in prosurvival, mRNA levels of different trophic factors and migration ability of MSCs.[15, 16] It was shown that culture of MSCs in hypoxic conditions improved bone marrow MSCs yield and reduced cell's expansion time compared to normal conditions.[17]

We hypothesized that hypoxia preconditioning of UCB-MSCs can improve survival and capacity of CFU-F and decrease their doubling time. In the present study, we compared survival and proliferation abilities of HPC-UCB-MSCs under hypoxic conditions in different time periods with non-preconditioned UCB-MSCs (Norm-UCB-MSCs).

Materials and Methods

Collection of UCB: UCB samples (n=5) were collected from placenta of full-term babies in a multiple bag system containing 63 mL of citrate phosphate dextrose adenine (Macopharma, France) and processed within 3 hr of collection. Samples of women with known histories of infectious disease, hepatitis, diabetes mellitus, abortion and genetic disease were excluded from the study. Informed consent was obtained and approval was granted by the Ethics Committee of Iranian Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran.

Isolation of MSCs from UCB: Mononuclear cell (MNC) fraction was isolated by Ficoll-Hypaque (Sigma, USA), low-density gradient (MNCs <1.077 g/mL). Gradient separation was followed by ammonium chloride (Merch, Germany) lysis of red blood cells. The collected MNCs were re-suspended in complete media [DMEM supplemented with 10% fetal bovine serum (FBS), L-glutamine, 100 U/ mL penicillin and 100 mg/ mL streptomycin (all from Sigma)]. Complete media change was performed every 3–4 days for eliminating adherent hematopoietic cells. Upon reaching cells to 70% confluence (needs approximately 3 weeks), cells were passaged with Trypsin-EDTA 0.05 % (Sigma) up to 3 times. MSCs at passage 3 were free of hematopoietic cell contamination and no further purification was needed.

Immunophenotype analysis using flow cytometry: Isolated cells from UCB were stained with phycoerythrin (PE)–conjugated antibodies against CD44, CD45, CD14, CD73, CD105 and CD106 or PerCP-Cy5.5 -conjugated antibody against CD90 (BD Pharmingen, USA). Mouse isotypic antibodies were used as the control. Cells were stained in single labels and analyzed by flow cytometry (Sysmex corporation, Japan). Analyses were performed on a Partec PAS CyFlow space (Flo Max software version 2. 4e, Germany).

Differentiation study: For further characterization of the multi-lineage differentiation capacity of isolated UCB-MSCs, the differentiation potential was evaluated *via* osteogenic, adipogenic, and chondrogenic differentiation assay.

Osteogenic Differentiation

The cultured cells were incubated in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM β-glycerol phosphate, 2 mM L-glutamine, 10 nM dexamethasone and 0.2 mM ascorbic acid. The media was changed two times per week. After three weeks, cells were fixed with 4% paraformaldehyde (Merck, Germany) for 20 minutes at room temperature (RT) and stained with 2% Alizarin Red, pH 4.1 for 20 minutes at RT to assess mineralization.

Adipogenic Differentiation

Cells were cultured in DMEM/FBS 10% in the presence of adipogenic supplements with 10 µM insulin, 0.5 µM 3-isobutyl-1-methylxanthine, 1 µM dexamethasone, and 200 µM indomethacin. On day 21, plates were stained with oil red o stains which specifically stains lipid droplets.

Chondrogenic Differentiation

Cells were incubated in serum-free medium (DMEM), 100 µg/ mL insulin, transferrin, selenium (ITS) (Gibco, Waltham, USA), 50 µg/ mL ascorbic acid, 10 µg/ mL dexamethasone and 10 ng/ mL recombinant human transforming growth factor (TGF) –β (R&D Systems Inc, Minneapolis, USA). On day 21, pellets were prepared for histology (paraffin-embedded sections) to detect sulfated glycosaminoglycan (sGAG) (Alcian Blue staining, pH 2.5). All materials were purchased from Sigma-Aldrich (Missouri, USA) unless stated otherwise.[18, 19]

Hypoxia Preconditioning Protocol

To evaluate the effect of hypoxia preconditioning, hUCB-

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MSCs cultivated in DMEM-LG with a complete growth medium containing 10% FBS. Cultured hUCB-MSCs were divided into two groups: Group 1: Normal MSCs (Norm-MSCs) were cultured in DMEM supplemented with 10% FBS in a 5% CO_2 chamber (Shell Lab, USA). Group 2: MSCs group under HPC and hypoxia in three different time periods. HPC protocol consisted of two sets of hypoxia (15 min, 2.5% O_2 and 5% CO_2) and reoxygenation (30 min, 21% O_2 and 5% CO_2), prior to hypoxia challenges (O_2:2.5%) in an airtight chamber (New Brunswick Eppendorf, Germany) that was flushed with a mixture of 2.5% O_2, 5% CO_2 and 92.5% N_2. Duration of hypoxia induction after preconditioning was different in three subsets of 24, 48 and 72 hr.

Cell Viability Assay by 4, 5-Dimethylthiazol-2, 5-Diphenyl-Tetrazoliumbromide (MTT) Assay: The MSCs were seeded in 96-well plates at a density of 5×10^3 cells/well. They were kept in CO_2 chamber for 24 hours to make sure that cells are stuck to the plates. Thereafter, cells were divided into two groups; HPC-MSCs and Normal-MSCs. HPC protocol was implemented in HPC group, as was mentioned above. After 24, 48 and 72 hours, cell growth media was replaced with 500 µg/ mL 4,5-dimethylthiazol-2,5-diphenyl-tetrazoliumbromide (MTT)(Sigma, USA) per well in each group for 4 hr, and then it was replaced with 100 µl isopropanol (2-propanol) (Merck, Germany). The intensity was measured with a microplate reader at an absorption wavelength of 570 nm by Elisa reader (EPSON LQ 2180, Japan) after dissolution of purple formazan. On the next day, two other plates from each group were trypsinized and MTT test was performed. In order to confirm the accuracy of the results, the experiment was repeated further for two times.

Population Doubling Time (PDT) Assessment: UCB-MSCs at a concentration of 2×10^3 cells/well were plated in six well culture plates. They were incubated for 24 hours in normoxic chamber (21% O_2 and 5% CO_2). After 24 hours, HPC protocol was applied in half of the plates (were incubation in the hypoxia chamber (2.5% O_2 and 5% CO_2) for 24 hours). At this time, the cells were trypsinized and counted with a hemocytometer in each group (HPC along with hypoxia and Normoxic MSCs). The PDT was calculated for either group of MSCs according to the following formulae: PDT=culture time (CT)/population doubling number (PDN). To determine PDN, the equation PDN=log Ni/Nf×3.31 was used; "Ni" was the number of cells when harvested and "Nf" was the number of seeded cells.[20] The experiment was repeated similarly in next two consecutive days. In order to confirm the authenticity of the results, the test was repeated three times.

Immunophenotype Change Assessment Using Flow Cytometry: Flow cytometry was performed for evaluating the effect of HPC on expression of cell surface markers. Flasks were trypsinized, washed and incubated for 20 min at 4°C with specific abovementioned antihuman antibodies. Analyses were performed on a Partec PAS CyFlow space (Flo Max software version 2.4e, Germany).

Colony-Forming Unit-Fibroblast (CFU-F) Assay: CFU-F assays were performed to determine frequency of MSC. UCB-MSCs were cultured in 10 cm^2 culture plates in limited dilutions (500, 1000 cells) to verify the colony forming ability. Cultures were maintained for 14 days at 37 °C, 5% CO_2 in expansion medium. At day 14, medium was removed and the resultant colonies were washed twice with PBS (Sigma), fixed and stained with giemsa (Sigma, USA) for 10 minutes at room temperature. Colonies with more than 50 cells were counted under a microscope by two independent observers. CFU-F assays were done in both groups of MSCs either under normoxic or HPC plus 72 hours hypoxia conditions.

Statistical Analysis

Data are presented as mean±standard deviation. The

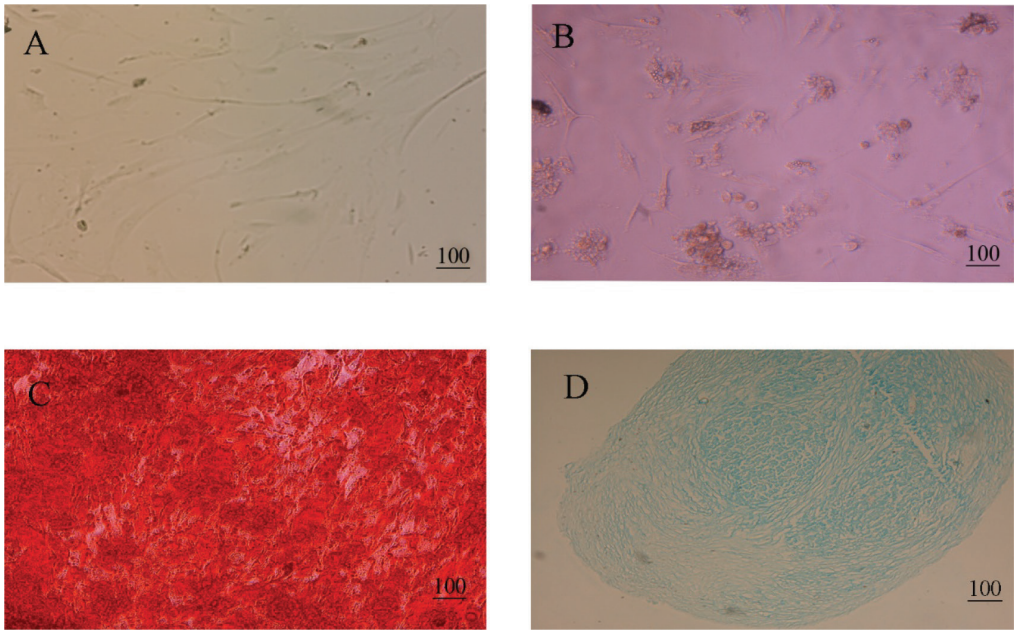


Figure 1: Multilineage differentiation ability of hUCB-MSCs. (A) Fibroblastic-like morphology of non-differentiated hUCB-MSCs which were cultured after 5 days at third passage (negative control). (B) Adipogenic differentiated hUCB-MSCs stained with Oil Red-O. (C) Osteogenic differentiated hUCB-MSCs stained with Alizarin Red. (D) Chondrogenic differentiated hUCB-MSCs stained with Alcian Blue. (magnification :100).

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independent sample t-test was used (Equal variances not assumed). Differences were considered significant at P≤0.05. The SPSS software package (version 20; IBM Inc., USA) was used for statistical analysis.

Results

UCB-MSCs Morphology and Phenotype

MSCs were isolated according to standard techniques for the isolation of mononuclear cells from UCB. MSCs from UCB exhibited identical morphological and phenotypic characteristics as well as similar plastic adherent growth and fibroblastic morphology. Mineral calcium sediments in differentiated cells were marked by Alizarin Red-S staining in MSCs which were cultured in osteogenic medium. When MSCs were cultured in plates with adipogenic medium, fat vesicles were marked by Oil Red-O staining in differentiated cells. Alcian blue staining confirmed chondrogenic differentiation by deposition of a hyline matrix rich in proteoglycans. Results from Alcian blue staining showed that isolated hUCB-MSCs preserved their potential for differentiation to chondrocytes (Figure 1).

FACS Analysis of Typical CD Markers Present on UCB-MSCs

Regardless of culture condition, MSCs in each group were strongly positive for MSC markers (CD44, CD73, CD90 and CD105), while lacking of CD14, CD45 and CD106 markers were observed (Figure 2).

Cell Viability Assay by MTT Test After HPC

MTT results demonstrated that there is a significant difference in cell expansion rate between normoxia (0.14±0.01) and HPC with 24 hours hypoxia (0.17±0.04) (P≤0.05). Again, it was revealed that in the group of UCB-MSCs which were under HPC and 48 hours of hypoxia, there was an increase in the amount of cellular metabolic activity *Versus* normoxic MSCs (0.17±0.02 *vs.* 0.21±0.04). Our results showed that HPC plus 72 hours hypoxia remarkably increased cell proliferation in comparison with normoxic conditions (0.21±0.04 *vs.* 0.18±0.04) (Figure 3).

Effect of HPC on Population Doubling Time (PDT)

The effect of different periods of hypoxia following HPC protocol on doubling time of UCB-MSCs population was calculated. It was shown that 24 hours hypoxia does not create a significant difference in the doubling time in comparison with normoxia condition (0.92 *vs.* 0.97, P=0.45). However, PDT remarkably decreased in the HPC group after passaging 48 hours of hypoxia (1 *vs.* 1.20, P=0.014). It was also indicated that 72 hours hypoxia significantly reduced PDT of UCB-MSCs in comparison with normoxia (0.95 *vs.* 1.38, P=0.008). In summary, it can be said that 48 and 72 hours hypoxia immediately after HPC can improve proliferation of UCB-MSCs, significantly (Figure 4).

The Impact of Hypoxia Preconditioning on Colony Forming Ability of UCB-MSCs (CFU-F Assay)

CFU-F colony numbers was determined by limit dilution assay (CFU-F assay). As expected, number of CFUs was increased after HPC plus hypoxia. Our results disclosed that HPC along with hypoxia can significantly increase CFU-F colony numbers derived from hUCB-

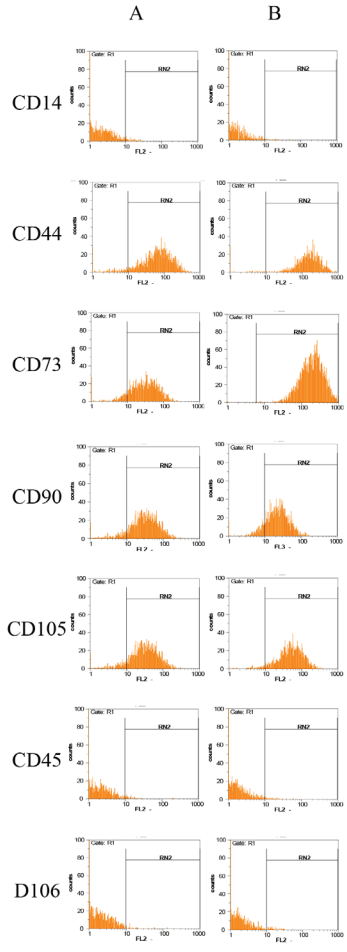


Figure 2: Immunophenotype analysis of UCB-MSCs. Column A shows hUCB-MSCs after hypoxia preconditioning and 72 hours hypoxia and Column B shows hUCB-MSCs under normoxia (Normal-MSCs). Cells were trypsinized, labeled with antibodies against indicated antigens and analysed by flow cytometry. No significant difference was found between two groups (P>0.05).

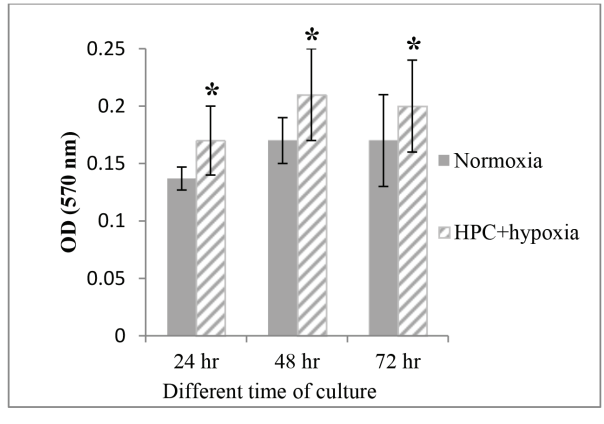


Figure 3: Quantitative analysis of cell viability by MTT test in UCB-MSCs cultured in normoxia condition and UCB-MSCs preconditioned with hypoxia (HPC+hypoxia). Data are mean±SD, representative of 3 independent experiments. *P≤0.05.

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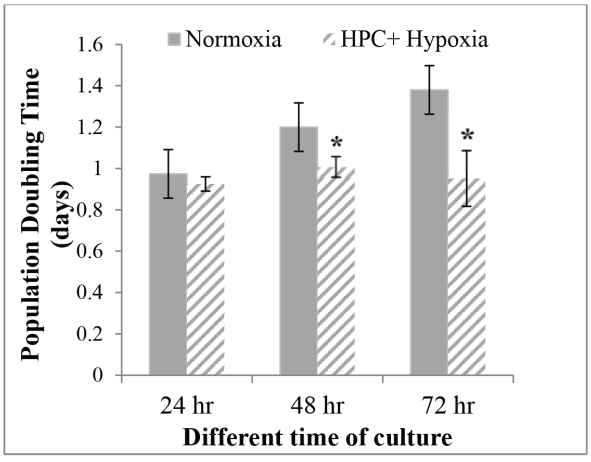


Figure 4: Population doubling time of UCB-MSCs in different culture conditions (HPC+hypoxia in three time periods of 24, 48 and 72 hours versus normoxia) Data shown are mean±SD. *P≤0.05.

MSCs in comparison with normoxia group in both subsets of primary cultured cell counts. In order to confirm the authenticity of the results, the test was performed three times for each group (n=3. P<0.05). Mean frequency of CFU in 500 cell concentration in HPC plus 72 hours hypoxia and Normoxic-MSCs groups were 32±2.5 and 7.5±2.5, respectively. Assay of colonies in 1000 cells concentration indicated that there was also a large variation in the number of colonies obtained in the CFU-F assay between HPC treated MSCs and normoxic-MSCs, respectively. (54±1 *vs*. 23.5±8.5) (Figure 5).

Discussion

UCB-MSCs are a favorable cell source for cell therapy because of their accessibility, immunosuppression potential, multipotent property without requiring ethical considerations. In recent years, these cells have been regarded as an alternative and promising source for stem cell therapy.

Although, some studies have shown that UCB-MSCs do not have adipogenic differentiation capacity;[7] in this study, we demonstrated three differentiating capabilities of the UCB-MSCs.

Monitoring the apoptosis of transplanted stem cells has become a major challenge in regenerative medicine. Numerous hard work have been made in order to increase survival of MSCs. The oxygen concentration in the microenvironment of the stem cells plays an important role in controlling stem cell potency and its proliferation and differentiation ability. It is proved that a lot of pathways such as 78-kDa glucose-regulated protein (GRP78/BIP) explains the enhanced MSCs bioactivity and survival under hypoxia preconditioning.[21]

Under hypoxic conditions, cells develop a compatibility program that leads to the induction of transcription factor genes, which are regulated by hypoxia-inducible factor 1-alpha (HIF-1α). One of these factors is vascular endothelial growth factor (VEGF) that increases cell survival.[22] It was shown that octamer binding transcription factor 4 (Oct-4) expression and telomerase activity increase under hypoxic conditions in MSCs.[23, 24]

The partial pressure oxygen (PO_2) of inspired air decreases after it enters the lungs and as it transfers in the blood vessels. By the time it reaches tissues, PO_2 levels have reduced to 2%–9% (14–65 mm Hg).[25] A lot of studies have been done about optimum oxygen concentration of the tissues. It has reported that oxygen tensions as low as 1% decrease cell proliferation and maintain embryonic stem cell pluripotent potential, while higher oxygen tensions appear to maintain pluripotent features with no effect on proliferation.[26]

Some studies have proved that prolonged hypoxia has a deleterious effect on some cells, but the application of transient periods of non-lethal hypoxia before a longer episode of hypoxia significantly delays the development

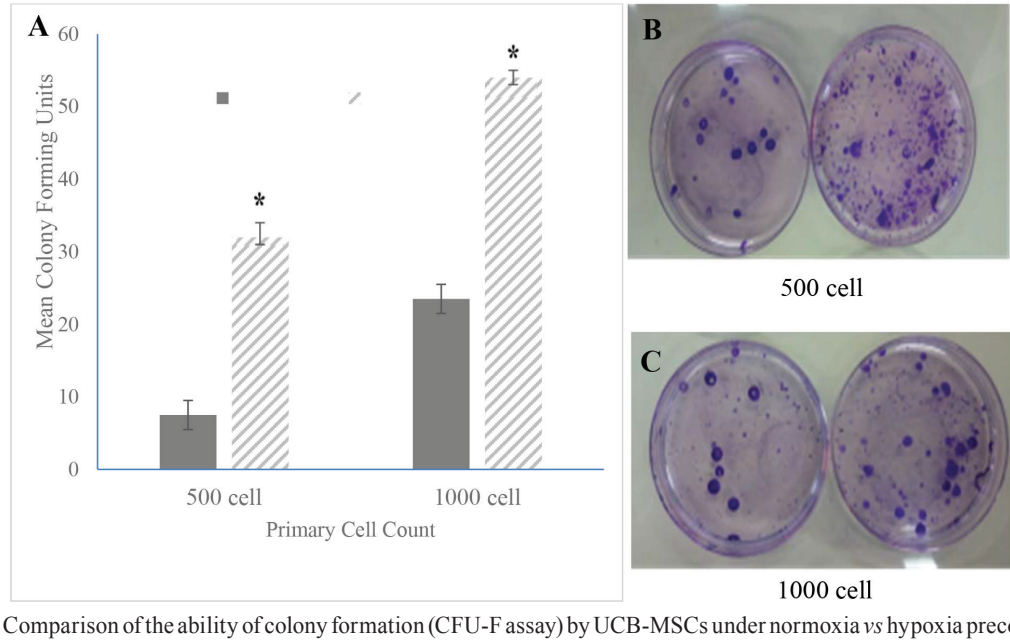


Figure 5: (A) Comparison of the ability of colony formation (CFU-F assay) by UCB-MSCs under normoxia *vs* hypoxia preconditioning+72 hours of hypoxia (HPC+hypoxia) in different concentrations (500 and 1000 cells). (B) CFU-F assay in 500 cells per well. (C) CFU-F assay in 1000 cells per well. In each picture left plate belongs to Normoxic-UCB-MSCs and right one belongs to HPC-UCB-MSCs. Values are mean±SD (n=3). *P<0.05.

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of programmed cell death.[27]

The time of hypoxia after preconditioning is an important factor. In this study, we tried to evaluate the different time periods of being under hypoxia condition.

Our results demonstrated that hypoxia preconditioning of UCB-MSCs does not have destructive effects on proliferation and survival of these cells. Interestingly, we showed that HPC along with hypoxia increases viability of UCB-MSCs. For investigating the effect of HPC plus hypoxia on cell proliferation, we compared doubling time of cells exposed to different periods of hypoxia (24, 48 and 72 hours). We demonstrated that HPC and more than 24 hours hypoxia leads to an increase in cell proliferation.

The results of a comparative study on the effects of different oxygen concentrations (between 1.5% and 5% O_2) on cell growth analysis of UCB- MSC populations revealed a marked increase in cell proliferation at 2.5% O_2 in 72 hours compared to normoxic conditions. A significant level of HIF-1α in hypoxic MSCs cultured at 2.5% or 5% O_2 can be observed.[28] In this study, we used 2.5% O_2 and found that 24 hours of hypoxia does not affect proliferation of UCB-MSCs, but 48 and 72 hours hypoxia could increase cell proliferation. Previously, it was established that hypoxia can improve proliferation and clonogenic capacities of BM-MSCs and UCB-CD133+cell population without affecting their differentiation potential. Microarray experiments indicated that 183 genes in UCB-CD133+ cells and 45 genes in BM-MSCs were differentially regulated by hypoxia.[29]

Recently, it was shown that the effect of hypoxia on the expansion rate of MSCs is dependent on cell source and only prenatal sources of MSCs (amniotic fluid MSCs and UCB-MSCs) proliferated significantly faster under hypoxia than normoxia.[30] This discrepancy might be happened due to unalike O_2 concentrations.

Considering our results in parallel to other existing data of the literature, it can be said that hypoxia preconditioning is a physiologic maneuver with a wide range of beneficial effects.

The present study demonstrated that HPC pretreatment prevents the MSCs from apoptosis, which could be an effective method to promote cell transplantation efficiency. These findings could be of value for prospective regenerative strategies.

An obvious limitation of the present study was the kind of tests we used, so further *in vitro* studies are suggested.

Conclusion

It showed that more than 48 hours of hypoxia following hypoxia preconditioning leads to a considerable increase in cell proliferation and inhibition of cell death. Basically, preconditioning of MSCs ex vivo by hypoxia prior to their use in therapy is an adaptive way that prepares them to survive in lethal environments and to enhance their function.

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Conflict of Interest: None declared.

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