

The Effects of Hypoxia on U937 Cell Line in Mesenchymal Stem Cells Co-Culture System

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Abstract

Purpose: Mesenchymal Stem Cells (MSCs) are the most important members of Bone Marrow (BM) milieu. MSCs affect different kinds of cells, particularly malignant cells of hematologic malignancies, but the effects of MSCs are unclear exactly. Here we analyzed the effects of derived Umbilical Cord Blood-MSCs on proliferation, cell death and some surface markers of U937 cell line in a Co-culture system with MSCs.

Methods: Here we designed Co-culture systems as a model of BM milieu. We cultured U937 cells on UCB-MSCs and MSCs Conditioned Medium (C.M) driven and then treated U937 cells with optimum concentration of chloride cobalt (CoCl₂) as a hypoxia-mimetic agent. In addition, we applied suitable concentrations of H₂O₂ to induce cell death. Proliferation rate, cell death rate and some surface markers of hypoxic U937 cells were analyzed by MTT assay, flow cytometry and Real Time-PCR were flown respectively.

Results: UCB-MSCs showed supportive effects on U937 proliferation rate in normoxia and hypoxia. Lethal effect of H₂O₂ suppressed in the presence of UCB-MSCs in hypoxia and normoxia. Among CD11a, CD14, CD49d, CD54 and CD116 markers, CD49d was down regulated in presence of UCB-MSCs and CD116 was up regulated in hypoxia. Other markers didn't show any significant changes.

Conclusion: This work provides evidences that MSCs play critical roles in U937 cells biology. These observations shed new light on MSCs roles and demonstrated that MSCs should be regarded as an important member of BM milieu in several clinical applications such as BM transplantation prognosis and treatment of hematologic malignancies.

Introduction

As well documented, Bone marrow (BM) traditionally contains two systems: hematopoietic cells and the associated supporting stromal part.¹ One of the major sections of BM milieu is Mesenchymal Stem Cells (MSCs).^{2,3} MSCs play critical roles in biology of normal and malignant cells.⁴⁻⁸

Another important factor in BM milieu is physiologic hypoxia. The effects of hypoxia mediated by a significant master key transcription factor is called hypoxia-inducible factor (HIF). HIF, heterodimeric key transcription factor, contains HIF- α and HIF- β subunits.⁹ In hypoxia, HIF- α subunits translocate to nucleus and join to HIF- β subunits,^{10,11} so heterodimers bind to sequences of HIF target genes, which they affect different aspects of cells biology.^{12,13} In this regard, hypoxia can mediate expression of different kinds of genes in normal and malignant cells.¹⁴⁻¹⁷

Several in vitro studies have been reported that HIF is a powerful factor, which improved survival and differentiation of stem cells.^{18,19} In particular, HIF-1 caused resistance to chemotherapy and radiation approaches.²⁰

Here, we investigated the effects of Umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) on proliferation rate, cell death and some genes expression by U937 cells in hypoxia milieu.

Materials and Methods

Isolation and Culture of UCB-MSCs

UCB-MSCs were collected from umbilical cords, with informed consent, according to the Institute's human ethical committee guidelines of Tabriz University of Medical Sciences. Cells were cultured in DMEM medium (Gibco, MA, UK) with 10% fetal bovine serum (FBS) (Gibco, MA, UK) and 100 U/ml penicillin as well as 100 μ g/ml streptomycin (Pen/Strep) (Gibco, MA, UK). Cells were incubated in humidified incubator containing 5% CO₂ at 37°C. After incubation, non-adherent cells were discarded and fresh DMEM medium was added to cells. Then, fibroblastoid cells were verified by flowcytometry for MSCs markers including CD29, CD105 (Positive markers) and CD34, CD45 (Negative markers).²¹

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Cell culture

Confirmed U937 cells were purchased from the Pasture Institute of Iran. Thereafter, cells were cultured in RPMI-1640 medium (Sigma-Aldrich, USA) with 10% FBS (Gibco, MA, UK) and Pen/Strep (Gibco, UK) and were incubated. During all steps of the experiments, cell viability was checked by trypan-blue staining and it was more than 86% in all experiments.

UCB-MSCs were seeded at the density of 2×10^4 cell/well. After 24 hrs, 1×10^5 U937 cells were added to the UCB-MSCs in RPMI-1640 medium with 10% FBS and Pen/Strep for Co-culturing.

Conditioned Medium (C.M) preparation

Conditioned Medium (C.M) was prepared by adding 5 ml of RPMI-1640 without FBS to UCB-MSCs (Conflency 60%) and 24 hrs incubation.

Cells treatment

Cobalt chloride (CoCl_2) (Sigma, USA) was used to induce hypoxia. CoCl_2 dissolved in RPMI-1640 to adjusted 100 μM . Then U937 cells were treated with 100 μM of CoCl_2 .

Hydrogen peroxide (H_2O_2) (Merck, Germany) was used for cell death inducing, so H_2O_2 was diluted to 100 mM with distilled water as a stock solution and cells were treated with 0.5 mM H_2O_2 .

MTT Assay

U937 cells were co-cultured with UCB-MSCs and were incubated with 100 μM of CoCl_2 for 96 hrs. After incubation, U937 cells were collected and RPMI-1640, with 5 μl MTT solution (0.4 mg/ml), was added to cells pellet and was incubated. Then, Isopropanol/HCl (0.04M) was added and incubated overnight and optical density of solutions were measured by Picodrop (UK) in 540 and 650 nm.

Cell Death Detection with Flowcytometry

U937 cells were incubated with 0.5mM of H_2O_2 for 24 hrs. Then, live and dead cells were analyzed by forward and side scattering in flowcytometry assay (FACS Calibur, USA).^{22,23}

Real Time-PCR (RT-PCR)

Co-cultured U937 cells with UCB-MSCs (24 hrs) were harvested and RNA was extracted. Thereafter, cDNA was synthesized and expression of genes (CD11a, CD14, CD54, CD49d, and CD116) analyzed (Table 1). The threshold cycle (Ct) value for each gene was normalized Ct number of housekeeping gene (GAPDH) according to $\Delta\Delta\text{CT}$ method.

Statistical Analysis

Data are shown as means \pm SD from three separate experiments. Data were evaluated using GraphPad Prism v 5.00 (GraphPad Software, Inc., La Jolla, CA). Student's t-test (for single comparison) was used. $P < 0.05$ was regarded statistically significant.

Table 1. Primers sequences used for RT-PCR

Primers	5' 3' →
CD14	F - CTGGAACAGGTGCCTAAAGGAC R - GTCCAGTGTCAAGTTATCCACC
CD11a	F - CTGCTTTTGCCAGCCTCTCTGT R - GCTCACAGGTATCTGGCTATGG
CD49d	F - GCATACAGGTGTCCAGCAGAGA R - AGGACCAAGGTGGTAAGCAGCT
CD54	F - AGCGGCTGACGTGTGACAGTAAT R - TCTGAGACCTCTGGCTTCGTCA
CD116	F - CCTGTCAGGATTAACGTCTCGC R - CATTGCTGGGAGGGTTGAATCG

Results and Discussion

In normoxia and hypoxia proliferation of U937 was promoted by UCB-MSCs

MTT assay showed that proliferation of U937 was significantly high in co-culture with UCB-MSCs, in normoxic and hypoxic conditions ($*P < 0.05$) (Figure 1 and 2). In this regard, several studies have proved that, MSCs play noticeable roles in proliferation of malignant cells models such as U937 cells. Also, hypoxia shows suppressing effects on proliferation of leukemic cells.^{24,25} Here, we proved that hypoxia reduces proliferation of U937 cells, but in presence of UCB-MSCs, effects of hypoxia have been suppressed.

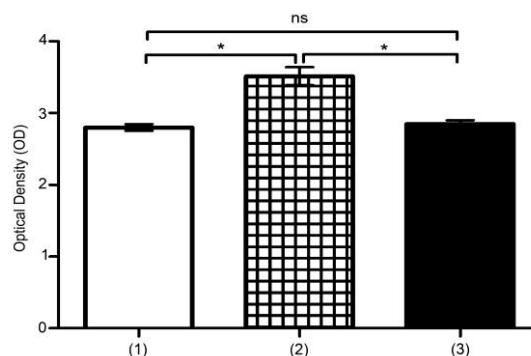


Figure 1. Assessment of U937 cells proliferation. Proliferation was analyzed by MTT assay. (1) U937 cells, (2) U937 cells+UCB-MSCs, (3) U937 cells in C.M. ns, non-significant. ($*P < 0.05$, by using student's t-test).

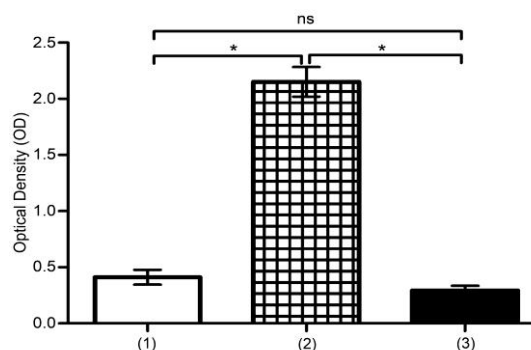


Figure 2. The evaluation of proliferation of U937 cells in hypoxia. Proliferation was analyzed by MTT assay. (1) U937 cells+100 μM CoCl_2 , (2) U937 cells+UCB-MSCs+100 μM CoCl_2 , (3) U937 cells in C.M.+100 μM CoCl_2 . ns, non-significant. ($*P < 0.05$, by using student's t-test).

UCB-MSCs supported viability of U937 in normoxia and hypoxia

U937 cells were cultured on UCB-MSCs with and without 100µM of CoCl₂, and then incubated for 96 hrs. In the next step, flowcytometry analyzing was performed on 1×10⁵ U937 cells. By comparison with C.M and control

group, the results demonstrate that UCB-MSCs support U937 cells viability in hypoxia and normoxia (*P<0.05) (Figure 3 and 4). At the present, supportive roles of MSCs on viability of normal and abnormal cells have been confirmed²⁶ and our results are the same as the others.

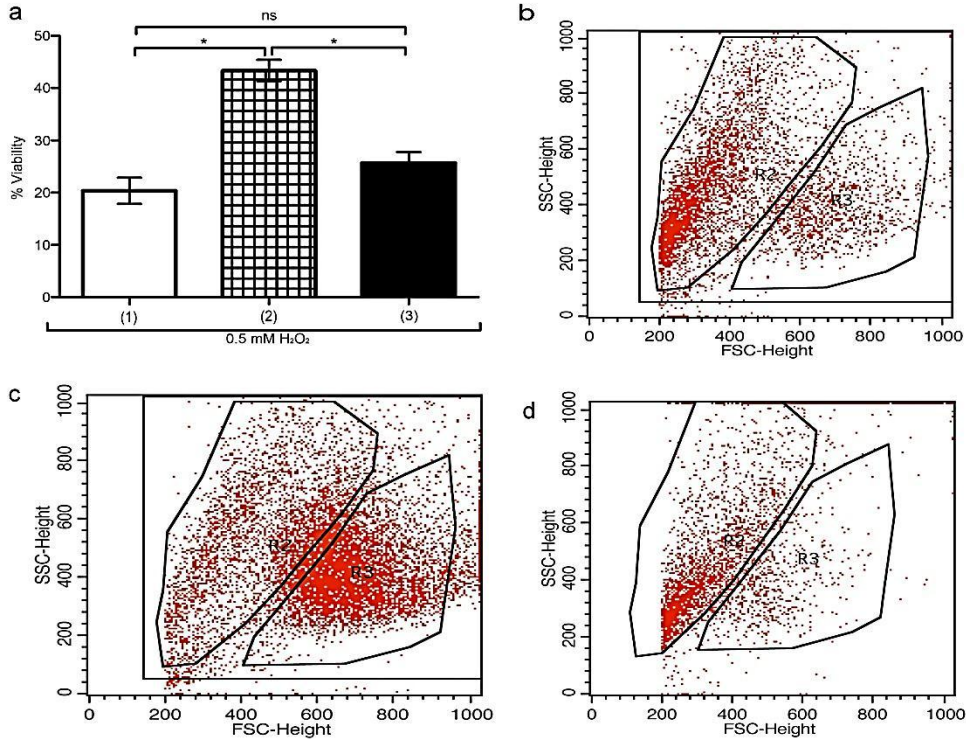


Figure 3. Assessment of U937 cells viability in presence of 0.5mM H₂O₂. (a) Diagram shows the viability of U937 cells in presence of 0.5mM H₂O₂. Viability was analyzed by Flowcytometry. (1) U937 cells, (2) U937 cells+UCB-MSCs, (3) U937 cells in C.M. ns, non-significant. (b) Viability of treated U937 cells+0.5mM H₂O₂ are shown using dot plot. (c) Viability of cultured U937 cells+UCB-MSCs+0.5mM H₂O₂ are shown using dot plot. (d) Viability of cultured U937 cells in C.M+0.5mM H₂O₂ are shown using dot plot. ns, non-significant. (*P<0.05, by using student's t-test).

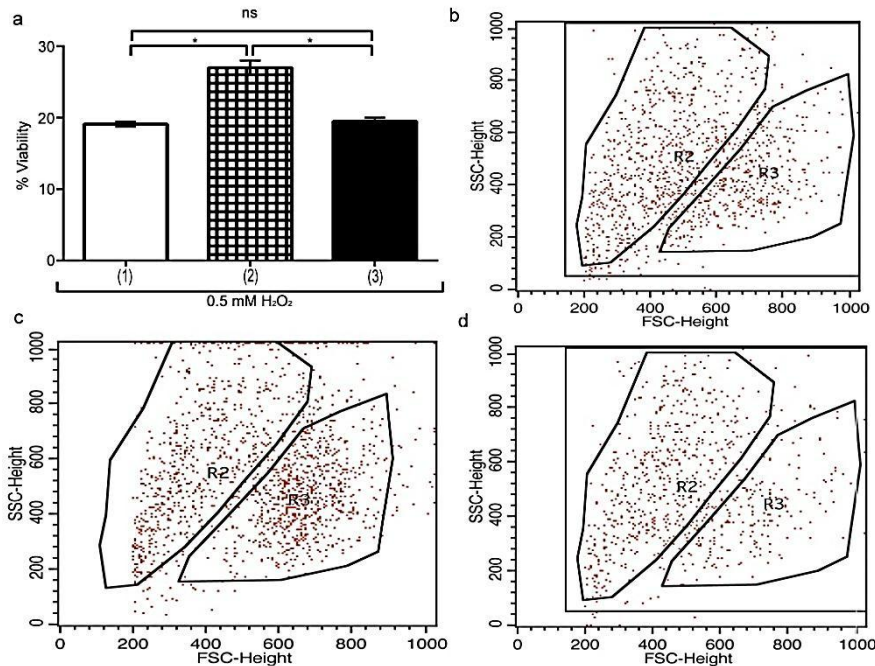


Figure 4. Viability of U937 cells in presence of 0.5 mM H₂O₂ and hypoxia was analyzed by flowcytometry. (a) Diagram shows the viability of U937 cells. (1) U937 cells+100µM CoCl₂, (2) co-cultured U937 cells+UCB-MSCs+100µM CoCl₂, (3) cultured U937 cells in C.M+100µM CoCl₂. (b) Viability of treated U937 cells+100µM CoCl₂ are shown using dot plot. (c) Viability of cultured U937 cells+UCB-MSCs+100µM CoCl₂ are shown using dot plot. (d) Viability of cultured U937 cells in C.M+100µM CoCl₂ are shown using dot plot. ns, non-significant. (*P<0.05, by using student's t-test).

Expression of CD49d down regulated in co-cultured U937 cells

U937 cells were seeded on UCB-MSCs and were treated with 100 μ M CoCl₂ for 24 hrs. Then, RNAs of U937 cells were extracted, cDNAs were synthesized and RT-PCR was performed.

CD49d is a part of VLA-4 and expresses on monocytes. Naturally, CD49d interacts with VCAM-1 to help migration of monocytes.²⁷ Hypoxia lead to CD49d up-regulation, which lead to transmigration of monocytes.^{28,29} Contrary, our findings showed, CD49d expression was down-regulated in presence of UCB-MSCs (Figure 5) (**P*<0.05).

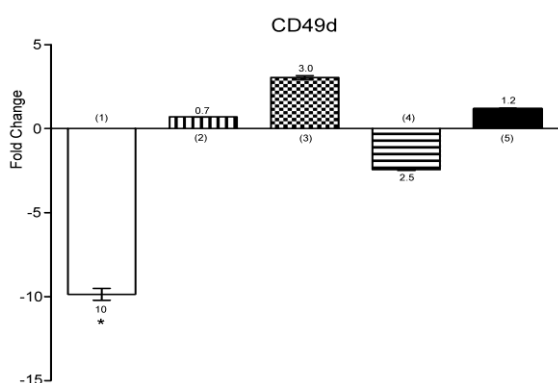


Figure 5. Histogram shows the expression of CD49d by U937 cells. Expression of CD49d was analyzed by using RT-PCR. (1) U937 cells+UCB-MSCs, (2) U937 cells in C.M., (3) U937 cells+100 μ M CoCl₂, (4) U937 cells+UCB-MSCs+100 μ M CoCl₂, (5) U937 cells in C.M.+100 μ M CoCl₂. ns, non-significant. (**P*<0.05, by using student's t-test).

Hypoxia up-regulated CD116 expression

We seeded U937 cells on UCB-MSCs. In the next step, the U937 cells were treated with 100 μ M CoCl₂ and incubated for 24 hrs. Then, we performed RT-PCR on co-cultured U937 cells.

CD116 is receptor of GM-CSF and expresses on various myeloid cells.³⁰ Furthermore, GM-CSF/CD116 has a direct role in proliferation and survival of monocyte lineage.^{31,32} As shown in Figure 6, CD116 expression shows meaningful up-regulation in hypoxia (**P*<0.05).

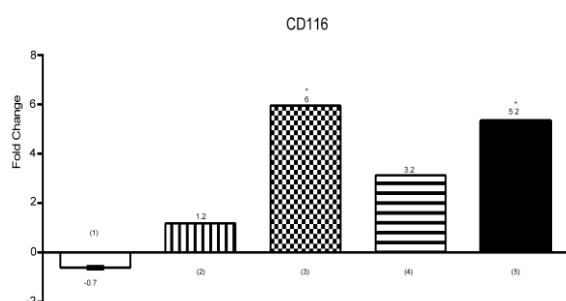


Figure 6. Expression of CD116 by U937 cells is shown on the diagram. Expression was analyzed by using RT-PCR. (1) U937 cells+UCB-MSCs, (2) U937 cells in C.M., (3) U937 cells+100 μ M CoCl₂, (4) U937 cells+UCB-MSCs+100 μ M CoCl₂, (5) U937 cells in C.M.+100 μ M CoCl₂. ns, non-significant. (**P*<0.05, by using student's t-test).

CD11a, CD54 and CD14 status in hypoxia

U937 cells were cultured with UCB-MSCs, C.M and 100 μ M CoCl₂ for 24 hrs. RT-PCR performed and didn't show any significant changes in CD11a, CD54 and CD14 markers expression (*P*>0.05) (Figure 7). CD14 has a role in the phagocytic activity of monocytes³³ and CD11a is classical marker of myeloid differentiation that involves in cell adhesion. Additionally, CD11a and CD14 related to differentiation of U937 cells to monocyte/macrophage.^{34,35} CD54 up-regulation in high pressure of oxygen in endothelial cells documented by recent studies,³⁶ while it should be pointed out that hypoxia enhanced CD54 expression, presumably through NF- κ B pathway activation,³⁷ but in our finding CD54 didn't show any trends in all conditions. Combined with previous findings this discrepancy can be due to differences in cell lines and incubation times.

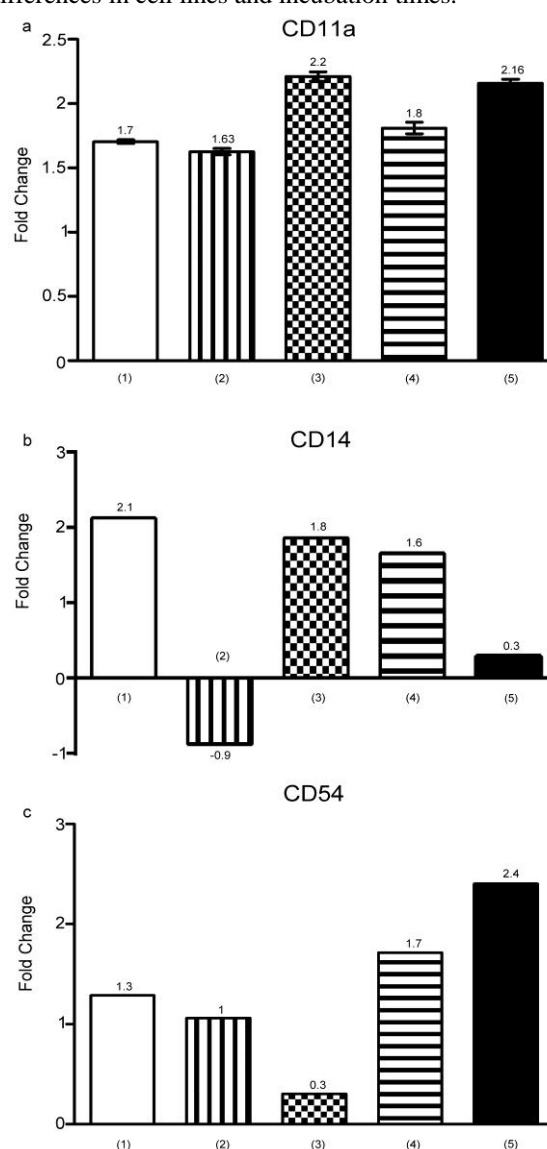


Figure 7. Diagrams show expression of (a) CD11a, (b) CD14 and (c) CD54 by U937 cells. RT-PCR performed for assessment of markers expression. (1) U937 cells+UCB-MSCs, (2) cells in C.M., (3) U937 cells+100 μ M CoCl₂, (4) U937 cells+UCB-MSCs+100 μ M CoCl₂, (5) U937 cells in C.M.+100 μ M CoCl₂. ns, non-significant. (**P*<0.05, by using student's t-test).

Conclusion

In orchestrate with previous studies, hypoxia suppresses the U937 proliferation in comparison to normoxia. Reciprocally, when U937 co-cultured with the UCB-MSCs, proliferation of U937 was increased. Apoptotic effects of H₂O₂ on U937 cells were reduced in presence of the UCB-MSCs. Also, CD49d expression was down-regulated and CD116 was up-regulated in the presence of UCB-MSCs and hypoxia respectively. Our findings can be useful in clinical applications and provide a new sight into the roles of MSCs in bone marrow co-transplantation efficacy. We suggest that other experiments should be performing on bone marrow-derived MSCs or animal models.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors declare no conflict of interests.

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