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The Effect of Hypoxia on the Soluble Molecules of Human Umbilical Cord-derived Mesenchymal Stem Cells (UC-MSCs)

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ABSTRACT

Background: Umbilical cord-derived stem cells (UC-MSCs) are essential cell source for cell-based therapies in regenerative medicine. Hypoxia is a key element of the stem cell niche and crucial for the preservation of UC-MSCs properties. The normal growth conditions for UC-MSCs are under atmospheric oxygen concentrations of 20–21%. However, the administration of UC-MSCs in inflammatory condition provides oxygen-deficient environments. Thus, treating UC-MSCs low oxygen exposure provides them more survival and recovery potential.
Objective: In this study, we assessed the impact of hypoxia incubation with 5% O_2 concentration for 12 h on the UC-MSCs proteome. **Methods:** UC-MSCs were isolated from UC patients regarding inform consent. At passage 5, in 80% confluent, UC-MSCs were incubated in 5% O_2 for 12 h. The morphology of UC-MSCs were assessed using microscope. The level of FGF-2, FGF-8, TNF-α and HSP-70 were analyzed using ELISA. **Results:** Hypoxic condition could change their morphology and enhance the cellular density compared to normoxic condition in vitro. The level of FGF-2, FGF-8, TNF-α and HSP-70 were significantly increased after hypoxic condition of UC-MSCs compared to normoxia.
Conclusion: Our findings suggest that hypoxic condition was able to induce survival capacity and soluble molecules secreted by UC-MSCs.

Kevwords: Secretome. Mesenchvmal Stem Cells. TNF-α. T1DM Rat.

INTRODUCTION

Umbilical cord-derived stem cells (UC-MSCs) are becoming more widely acknowledged as an essential cell source for cell-based therapies in regenerative medicine[1]. UC-MSCs have demonstrated several benefits, including ease of collection, accessibility, minimal immunogenicity, and capacity for self-renewal and multilineage differentiation, making them a possible treatment option for several disorders[2]. Various wound models have shown the use of UC-MSCs in the healing of cutaneous wounds[3]. UC-MSCs treatment does, in fact, promote healing by encouraging neovascularization in excisional wounds in both normal and diabetic rats. UC-MSCs decrease inflammation, increases re-epithelialization, and minimizes the effects of inflammation[4]. Furthermore, administration of human UC-MSCs to a murine pressure ulcer model speed up wound healing by lowering inflammation and tissue hypertrophy, promoting collagen deposition, and boosting the

expression of genes involved in healing (transforming growth factor-β (TGFβ), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), matrix metalloproteinase-9 (MMP-9) and MMP-13)[5]. Two paradigms describe the processes by which UC-MSCs influence the wound microenvironment: differentiation of UC-MSCs into distinct cell types that replace damaged cells and production of soluble substances that function in a paracrine way to guide the healing process[6]. From among them, paracrine signaling has occurred to be recognized as a crucial route underpinning UC-MSCs therapeutic/pro-healing property[7].

Since low oxygen (hypoxia) is a key element of the stem cell niche in vivo, it is crucial for the preservation of stem cell properties[8]. The normal growth conditions for UC-MSCs and other types of stem cells taken from mammalian tissues are under atmospheric oxygen concentrations of 20–21%[9]. However, the administration of UC-MSCs in the wound or inflammatory tissue provides oxygen-deficient environments which reflect actual wound microenvironments that MSCs must deal with. So, treating UC-MSCs low oxygen exposure provides them a more realistic habitat in proinflammatory condition. Several studies demonstrate that hypoxia enhances UC-MSCs abilities to survive, proliferate, migrate, and differentiate in both vitro and in vivo[8-10]. Importantly, hypoxia promotes UC-MSCs paracrine activity, especially with regard to promoting angiogenesis through up-regulation of vascular endothelial growth factor (VEGF)[10,11]. Previous study also reported that Extracellular matrix (ECM) proteins and type 2 cytokines (interleukin (IL-6), IL-13, monocyte chemoattractant protein (MCP)-1, and CD40 ligand) involved in the onset of fibrosis were shown to be secreted less when human UC-MSCs were pre-conditioned with low oxygen[12]. These findings imply that by increasing these UC-MSCs regenerative capability through hypoxia, it may be possible to speed up recovery after the transfer of cells to wounded regions.

An interest in identifying the UC-MSCs hypoxia responsive properties at the proteome level has been sparked by the theory that pre-conditioning UC-MSCs with hypoxia improves their functional properties and increases their appeal for clinical translational research. This approach might offer a tool for forecasting the efficacy, viability, and safety of UC-MSCs. In this study, we assessed the impact of hypoxia (5% O_2) incubation for 12 h on the UC-MSCs proteome. To assess the effect of hypoxia on UC-MSCs characteristics related to inflammatory and wound healing capacity, in vitro functional investigations were carried out.

MATERIAL AND METHODS

Isolation and preparation of MSCs

According to the informed consent, pregnant patients' umbilical cords (UCs) were taken. UC-MSCs isolation was carried out according to our previous report[10]. The UC-MSCs were incubated at 37 °C and 5% CO_2 in a culture flask with Dubbelco's Modified Eagle Medium (DMEM; Sigma-Aldrich, MI, USA), 10% fetal bovine serum (FBS; Gibco Invitrogen, NY, USA, 1.5% penicillin (100 U/mL)/streptomycin (100 g/mL) (Gibco), and 0.25% amphotericin B (Gibco). Every three days, a different culture media was used. Once MSC confluence had reached 80%, the cells were passage into a new flask. UC-MSCs from the fifth passage were used.

Induction of hypoxia in MSCs

After 80% confluence, UC-MSCs were subjected to a hypoxic environment using a hypoxia chamber for 12 hours at 5% oxygen concentration (Stem Cell Technologies). The oxygen partial pressure (pO2) value was checked using an oxygen controller (BioSpherix, Lacona, NY, USA). The medium (H-MSC-CM) was collected after the experiment.

Enzyme linked immunosorbent assay

According to the manufacturer's instructions, the number of soluble molecules in H-MSC-CM was analyzed (Invitrogen, CA, USA). Using ELISA, the levels of fibroblast growth factor-2 (FGF-2),

FGF-9, tumor necrosis factor-α (TNF-α) and heat shock protein-70 (HSP-700 were examined. Using a microplate reader with a wavelength of 450, the data were examined (Bio-Rad, CA, USA).

Data analysis

All statistical calculations were measured with SPSS ver. 24 (IBM, NY, USA). Data with normal distribution and homogeneity were measured using one-way analysis of variance (ANOVA), followed by a Tukey's post-hoc test. The mean ± standard error of mean (SEM) of each number is displayed. A statistically significant value was defined as $p < 0.05$.

RESULTS

The characteristic and hypoxia effect in UC-MSCs

We examined cell morphology in the fifth passage to carry out the characteristics of UC-MSCs. The cells displayed a fibroblast-like spindle-shaped cell characteristic (Figure. 1A). Our previous study also reported that UC-MSCs showed a positive expression of CD90, CD73, CD105 and negative expression of CD45, CD34, CD11c, CD19, CD16 and HLA-DR. UC-MSCs also expressed the adipogenic and osteogenic differentiation capability through alizarin red and oil red O staining followed by osteogenic and adipogenic induction[2,10]. After 12 h 5% hypoxia condition, UC-MSCs showed more spindle-shaped with higher cell density (Figure. 1B).

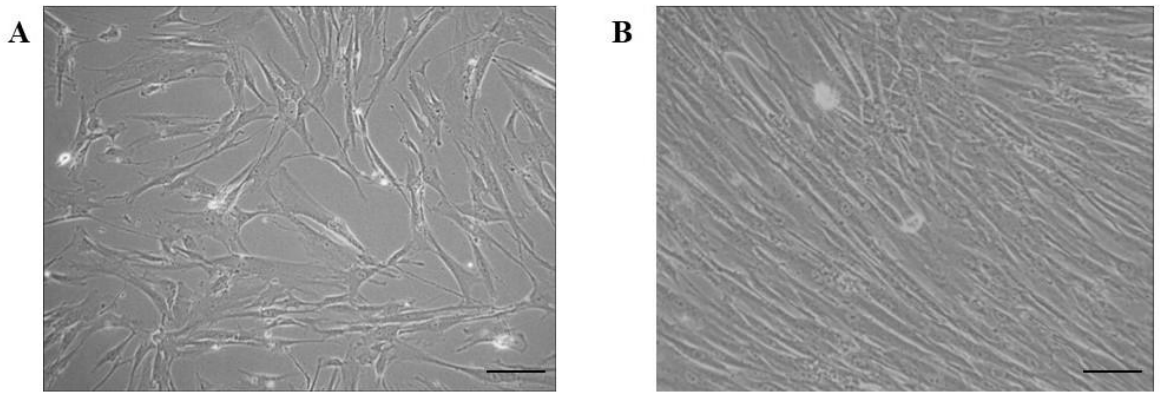

Figure. 1 The morphological characteristic of UC-MSCs under **a** normoxia and **b** hypoxia condition. Scale bars: 100 µm.

Hypoxic condition affects the soluble molecule profile of UC-MSCs

To further understand the effects of using different percentages of oxygen (i.e., normoxic and hypoxic conditions) on the H-MSC-CM, ELISA assay was performed. From the results, it was observed that the use of different oxygen percentages modulated the UC-MSCs to produce a different pattern of soluble molecule expression (Figure. 2A-D), in which the hypoxic preconditioning led to an increased secretion profile of UC-MSCs when compared to the normoxic preconditioning. FGF2 and FGF8 (both $p < 0.001$) were found to be significantly upregulated in the hypoxic condition when compared to the normoxic-related levels (Figure. 2A, B). Moreover, we also found that the level of TNF-α and HSP70 were significantly enhanced (both $p < 0.001$) in hypoxic condition compared to normoxia (Figure. 2C, D).

DISCUSSION

According to reports, the stem cell niche reflects and has a significant influence on stem cell biology and destiny, in which oxygen content is a key factor and regulator[13,14]. In fact, it has been

proposed that low oxygen levels, or hypoxia, are crucial for the preservation of stem cells' flexibility and ability to multiply[14]. Several studies reported that the Normoxia environment, as ambient oxygen tension of 21%, is frequently used for traditional in vitro cultivation[15,16]. Although it varies from tissue to tissue and ranges from 1 to 13%[13], the in vivo physiologic oxygen concentration is lower than this. This shows that the oxygen concentration utilized during routine in vitro cultivation of primary human cells, like MSCs, does not reflect the in vivo environment since it is higher than the usual pressure seen in the majority of mammalian organs[14,16]. Additionally, it has been proposed that cultivating MSCs in Normoxia circumstances may result in a decrease in their therapeutic potential[15]. The major goal in this study was to examine the effect of hypoxic circumstance on UC-MSCs soluble molecules with particular attention to protein associated inflammatory and wound healing condition.

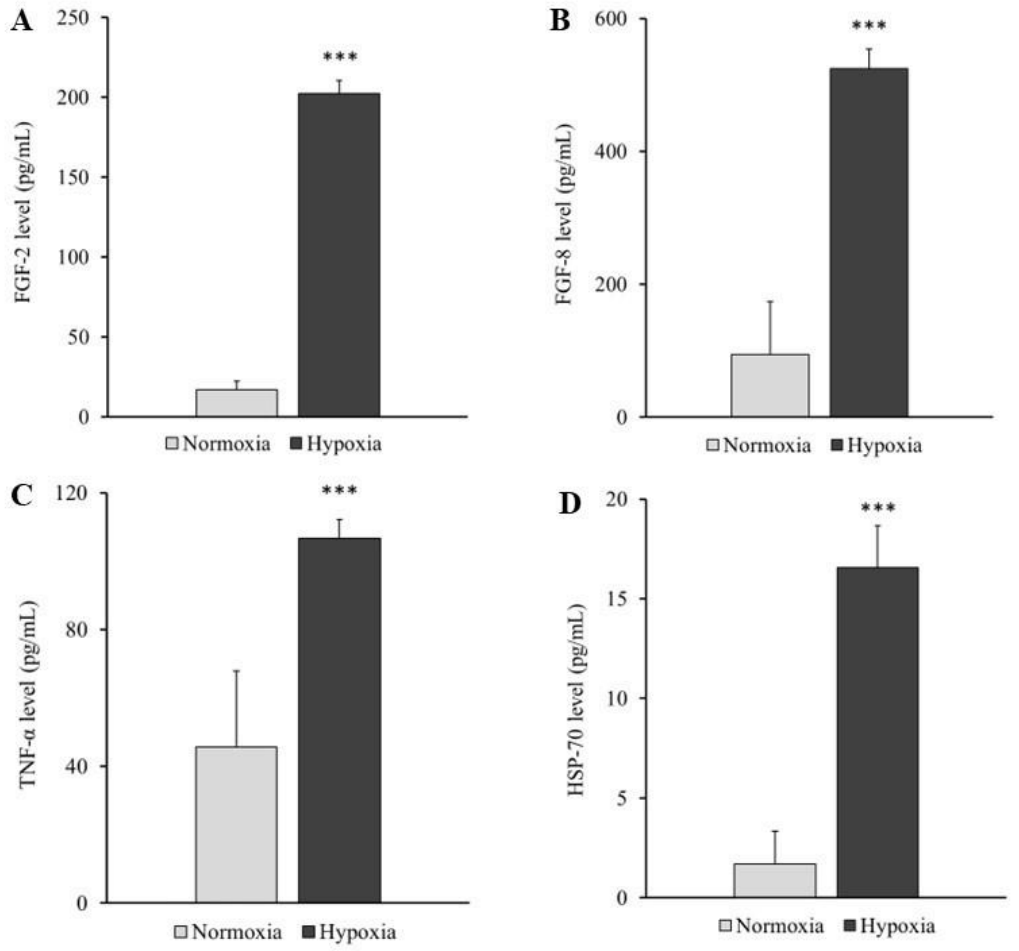

Figure. 2 Specific UC-MSCs soluble molecules (**a-** FGF-2; **b-** FGF-8; **c-** TNF-α and **d-** HSP-70) after hypoxia and Normoxia incubation. Values are shown as mean ± SEM, n = 3; ***$p < 0.001$.

We demonstrated that UC-MSCs under hypoxic circumstances could change their morphology and enhance the cellular density compared to Normoxia condition in vitro (Figure. 1a, b). These suggest that UC-MSCs could maintain their survival capability through hypoxia stress condition. The effects of hypoxic on UC-MSCs survival have already been discussed in previous study[14-16]. Hypoxic milieu (24 h in 0.5% O_2) promoted the survival of MSCs in vitro in a near anoxic environment[17], and another study revealed that 1% O_2 is an optimal hypoxic level to favor cell survival. These data were supported by our study that reported hypoxic condition increased the level of HSP-70 compared to normoxia (Figure 2d). HSP-70 is a potent survival protein whose depletion triggers massive caspase-independent cell death and inhibiting lysosomal membrane permebilization[18-20].

The proteomic analysis performed in the present work revealed that UC-MSCs were able to produce molecules with regeneration potential other than those commonly described in the literature, particularly under hypoxic conditions. Of these, FGF-2 and FGF-8 were found to be significantly upregulated under hypoxic conditions when compared to the normoxic conditions (Figure. 2a, b). These proteins have been reported to have important roles in fibroblast cell growth and differentiation, inhibition of apoptosis, and angiogenic effects[21-23]. On the other hand, TNF-α were also found to be significantly increased under hypoxic condition, compared to normoxia (Figure. 2c). TNF-α is already known as a potent proinflammatory mediator[8,24,] and their expression in hypoxia condition was related with the damage received during hypoxic stress[25]. However, our previous studies reported that UC-MSCs under hypoxia condition produced a higher level of IL-10 as anti-inflammatory cytokine compared to other proinflammatory mediators[9,10]. These suggest that hypoxic condition could enhance the survivability of UC-MSCs and provide more robust anti-inflammatory cytokines and growth factors in their culture medium, indicating the promising potential of H-MSC soluble molecules in inhibiting inflammation and promoting wound healing.

Taking all of this into account, future standardized studies should be designed to analyze the use of various hypoxic oxygen concentrations (for example, below 5%) and compare them to the normoxic state, to gain insight into the true impact of low oxygen levels on the UC-MSCs soluble molecules physiology.

CONCLUSION

In present study, we have demonstrated that the hypoxic condition was able to induce survival and soluble molecules secreted by UC-MSCs compared to normoxic condition. Hypoxic condition enhanced FGF-2, FGF-8 by UC-MSCs which is important for wound healing. Although TNF-α was increased, the upregulation of HSP-70 suggests the survival capacity of UC-MSCs in hypoxic microenvironment.

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AUTHORS' CONTRIBUTIONS

AW: Conceptualization, Methodology, Investigation, Data analysis, Formal analysis, Writing – original draft, Preparation. **IA**: Supervision, Conceptualization, Review & editing, Project administration, Resources Funding acquisition. **FEH**: Methodology, Investigation, Data interpretation. **RCSI**: Methodology, Investigation, Data interpretation. **HAS**: Methodology, Investigation, Data interpretation.

COMPETING INTERESTS

The authors declare that there is no conflict of interest.

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