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Hypoxia-Induced Modulation of OCT-4, SOX-2, NANOG, and KLF-4 Expression in Mesenchymal Stem Cells

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ABSTRACT

Background: Mesenchymal stem cells (MSCs) possess remarkable regenerative and immunomodulatory capabilities, largely attributed to their intrinsic stemness properties regulated by transcription factors such as OCT-4, SOX-2, NANOG, and KLF-4. However, in vitro culture under normoxia often leads to stemness decline. Hypoxic preconditioning has been proposed to preserve stemness, though the optimal duration of exposure remains unclear. This study aimed to evaluate the time-dependent effects of hypoxia on the expression of core stemness-related transcription factors in human umbilical cord Wharton's jelly-derived MSCs. **Methods:** MSCs (passage 5) obtained from the Stem Cell and Cancer Research (SCCR) Laboratory were cultured under hypoxia (5% O_2) for 0, 4, 6, 10, 12, and 24 hours. Gene expression of OCT-4, SOX-2, NANOG, and KLF-4 was quantified by qRT-PCR using GAPDH as a reference. Morphological changes were observed via phase-contrast microscopy. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. **Results:** Hypoxic exposure induced a significant, time-dependent upregulation of OCT-4, SOX-2, and NANOG, with peak expression at 12 hours (approximately 13-, 7-, and 5-fold increases, respectively; $p < 0.05$). KLF-4 expression showed a modest elevation but was not statistically significant. Prolonged hypoxia (24 hours) resulted in a marked decline in all gene expressions toward baseline levels, accompanied by cell rounding and detachment. These findings indicate that short-term hypoxia enhances MSC stemness, while extended exposure triggers stress-related responses. **Conclusion:** Controlled short-term hypoxia (approximately 12 hours at 5% O_2) optimally enhances MSC stemness gene expression without compromising morphology or viability. This duration represents an effective preconditioning window for maintaining MSC multipotency and may improve the consistency and therapeutic efficacy of MSC-based applications.

Keywords: Mesenchymal stem cells, hypoxia, stemness, OCT-4, SOX-2, NANOG, KLF-4, HIF-1α, regenerative medicine

INTRODUCTION

Mesenchymal stem cells (MSCs) are adult multipotent progenitor cells that can differentiate into a variety of cell types, including osteoblasts, adipocytes, chondrocytes, and myocytes.[1] They are typically isolated from diverse tissues such as bone marrow, adipose tissue, and umbilical cord, and possess remarkable regenerative and immunomodulatory capacities.[1] These features make MSCs one of the most extensively studied stem cell types in the field of regenerative medicine. In addition to their differentiation potential, MSCs exert therapeutic effects largely through their paracrine signaling, by secreting a broad spectrum of cytokines, growth factors, and extracellular vesicles that modulate inflammation, promote angiogenesis, and stimulate tissue repair.[2] The biological activity and clinical efficacy of MSCs, however, are closely related to their intrinsic "stemness" — the cellular ability to self-renew and maintain multipotency during proliferation and differentiation.[3]

The molecular foundation of MSC stemness is primarily regulated by a network of transcription factors that control pluripotency and lineage commitment.[4] Among these, octamer-binding transcription factor 4 (OCT-4), sex-determining region Y-box 2 (SOX-2), and homeobox protein NANOG constitute the *core pluripotency triad*, functioning cooperatively to sustain the undifferentiated state of stem cells and repress differentiation pathways.[5] Kruppel-like factor 4 (KLF-4), although not a core member, synergizes with these transcription factors to maintain the self-renewal phenotype and facilitate reprogramming of somatic cells into induced pluripotent stem cells (iPSCs). [6] The expression of these genes serves as a molecular hallmark of stemness and can be modulated by intrinsic and extrinsic cues within the cellular microenvironment. [7] Understanding the regulatory mechanisms that influence their expression is therefore critical for optimizing MSC-based therapies.

One of the most important extrinsic regulators of stem cell behavior is the microenvironmental oxygen level, or oxygen tension. [8] In vivo, MSCs naturally reside in a relatively hypoxic niche, with oxygen levels typically ranging from 1% to 7%, depending on tissue type and vascularization. [8,9] In contrast, conventional in vitro culture systems are maintained under normoxic conditions (approximately 21% O_2), which do not accurately mimic the physiological oxygen environment of stem cells. [9] This discrepancy can lead to a gradual decline in stemness, reduced proliferation, and premature differentiation during extended culture. [10] Consequently, culturing MSCs under controlled hypoxic conditions has been proposed as a strategy to maintain or enhance their stemness, proliferation, and therapeutic potency. [10] Numerous studies have shown that hypoxia promotes MSC survival, enhances colony-forming efficiency, and augments their paracrine secretion profiles. [11-13]

The beneficial effects of hypoxia are primarily mediated through the stabilization of hypoxia-inducible factor-1 alpha (HIF-1α), a transcription factor that acts as the master regulator of cellular responses to low oxygen tension. [14] Under hypoxic conditions, HIF-1α escapes proteasomal degradation, accumulates in the nucleus, and activates downstream target genes involved in metabolism, angiogenesis, and cell survival. [13,14] Importantly, HIF-1α signaling has also been implicated in the regulation of pluripotency-associated genes such as OCT-4, SOX-2, and NANOG, linking oxygen sensing directly to the control of stem cell fate. [14,15] These findings suggest that hypoxia can serve as a physiological cue to preserve or even enhance the stemness of MSCs. [13-15]

However, despite growing evidence of hypoxia's beneficial influence on MSC biology, there remains significant variability among studies regarding the optimal duration of hypoxic exposure. Some reports indicate that short-term or transient hypoxia enhances proliferation and upregulates stemness-related transcription factors, while prolonged or chronic hypoxia can trigger cellular stress, mitochondrial dysfunction, or even apoptosis.[16-18] These discrepancies highlight a critical gap in understanding how the *time-course of hypoxia* modulates the expression of stemness-associated genes. [17] Determining the precise window during which hypoxia most effectively promotes stemness is essential for improving the reproducibility and efficacy of hypoxic preconditioning strategies in MSC culture systems.

Therefore, the present study was designed to systematically evaluate the time-dependent effects of hypoxia on the expression of key stemness transcription factors — OCT-4, SOX-2, NANOG, and KLF-4 — in cultured MSCs. By assessing gene expression profiles across multiple time points of hypoxic exposure, this study aims to identify the optimal duration that maximally enhances MSC stemness while minimizing potential adverse effects of prolonged hypoxia. The findings are expected to contribute to the refinement of hypoxic preconditioning protocols, ultimately improving the therapeutic performance of MSCs in regenerative medicine and cell therapy applications.

MATERIALS AND METHODS

Cell Source and Culture Conditions

Human umbilical cord Wharton's jelly-derived mesenchymal stem cells (MSCs) passage 5 were obtained from the Stem Cell and Cancer Research (SCCR) Laboratory, where they were isolated and cultured under sterile conditions following standard protocols. The cells were characterized based on their spindle-shaped morphology and confirmed positive for typical MSC surface markers (CD73, CD90, CD105) and negative for hematopoietic markers (CD34, CD45) using flow cytometry.

Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and incubated at 37°C in 5% CO_2. Two oxygen conditions were established:

1. Normoxia (control): 21% O_2, 5% CO_2.
2. Hypoxia (experimental): 5% O_2, 5% CO_2, maintained using a hypoxic chamber.

Cells were plated at a density of 1×10^5 cells/well in 24-well plates and allowed to adhere for 24 hours before exposure to the experimental oxygen conditions.

Hypoxic Exposure and Sample Collection

After cell attachment, MSCs were exposed to hypoxia for 0, 4, 6, 10, 12, and 24 hours. Early intervals (4–6 h) were chosen to assess acute transcriptional activation, including hypoxia-inducible factor (HIF) signaling and initial changes in adhesion molecules and secretome profiles[12]. Intermediate durations (10–12 h) were selected to evaluate mid-term adaptations, such as enhanced stemness markers, proliferation, and paracrine activity[14]. The prolonged interval (24 h) allowed assessment of sustained hypoxic effects on cell viability, adhesion, and maximal HIF-mediated gene expression[5]. This design enables identification of the optimal hypoxic preconditioning window for therapeutic applications.

The 0-hour time point represented baseline (normoxic control). At each time point, cells were harvested by trypsinization and washed twice with cold phosphate-buffered saline (PBS). The cell pellets were immediately immersed in RNAlater™ Stabilization Solution (Thermo Fisher Scientific) and stored at –80°C until RNA extraction.

RNA Extraction and cDNA Synthesis

Total RNA was isolated using TRIzol reagent following the manufacturer's instructions. RNA concentration and purity were determined by spectrophotometric analysis using a Thermo Scientific™ Multiskan™ SkyHigh Microplate Spectrophotometer using the µDrop™ at absorbance ratios A260/A280 and A260/A230. Only RNA samples with ratios between 1.8–2.0 were used for further analysis. Subsequently, 1 µg of total RNA from each sample was reverse-transcribed into complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. The resulting cDNA was stored at –20°C until use.

Quantitative Real-Time PCR (qPCR) Analysis

Quantitative real-time PCR (qPCR) was conducted using a Rotor-Gene™ Q Real-Time PCR System (Qiagen) with SYBR™ Green PCR Master Mix according to the manufacturer's protocol. Gene-specific primers were used to amplify OCT-4, SOX-2, NANOG, and KLF-4, while GAPDH was employed as the endogenous reference gene for normalization. Each reaction contained 10 µL SYBR Green Mix, 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 2 µL cDNA, and 6 µL nuclease-free water, in a total volume of 20 µL. The thermal cycling conditions were: Initial denaturation 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds. The

relative expression of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method, normalizing to GAPDH and comparing each hypoxia time point to the normoxic control (0 hour).

Statistical Analysis

All experiments were conducted in triplicate (n = 3), and the results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. A p-value of <0.05 was considered statistically significant. Data processing and statistical analyses were carried out using IBM SPSS Statistics version 24.0 (IBM Corp., Armonk, NY, USA).

RESULT AND DISCUSSION

Hypoxia Modulates the Expression of Stemness-Related Genes in MSCs

Exposure of mesenchymal stem cells (MSCs) to hypoxic conditions (1–5% O_2) resulted in a time-dependent modulation of stemness-associated transcription factors, including OCT-4, SOX-2, NANOG, and KLF-4 (Figure 1). At baseline (0 h), the relative expression levels of all four genes were low and comparable. A significant upregulation was observed beginning at 4 hours of hypoxia, with OCT-4 showing the most prominent increase (~3-fold; $p < 0.05$). SOX-2 and NANOG also exhibited moderate elevation at this time point, whereas KLF-4 expression remained low. By 6–10 hours of hypoxic exposure, all genes maintained higher expression compared to normoxia, though with varying magnitudes. The peak induction was observed at 12 hours, where OCT-4 expression increased approximately 13-fold, followed by SOX-2 (~7-fold) and NANOG (~5-fold) relative to control ($p < 0.05$). KLF-4 expression also rose modestly at 12 hours but did not reach statistical significance. Interestingly, prolonged hypoxia for 24 hours resulted in a sharp decline in the expression of all four genes, returning to near-baseline levels. This suggests a transient enhancement of stemness gene expression under short-term hypoxia, with 12 hours identified as the optimal exposure duration for maintaining or enhancing MSC stemness characteristics.

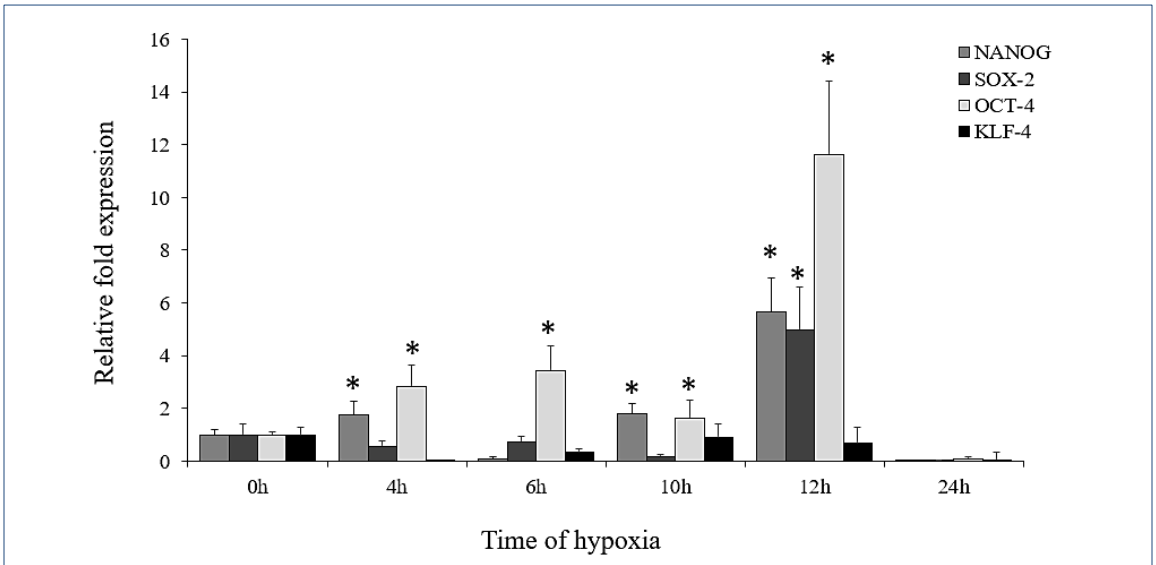

Figure 1. *Time-dependent effect of hypoxia on stemness gene expression in mesenchymal stem cells (MSCs).* Relative fold expression of OCT-4, SOX-2, NANOG, and KLF-4 was measured by quantitative real-time PCR (qPCR) after exposure to hypoxic conditions (1–5% O_2) for 0, 4, 6, 10, 12, and 24 hours. Gene expression levels were normalized to GAPDH as an internal control, and the results are presented as mean ± SD from three independent experiments (n = 3). *) indicate significant differences compared to normoxic control (0 h) ($p < 0.05$, one-way ANOVA followed by Tukey's post-hoc test).

Morphological alterations of MSCs under hypoxic exposure

Mesenchymal stem cells (MSCs) exhibited distinct morphological responses to varying durations of hypoxia (Figure 2). Under normoxic conditions (0 h, Figure Xa), MSCs showed a typical fibroblast-like, spindle-shaped morphology with strong adherence and homogeneous distribution. After 4 and 6 hours of hypoxia (Figure 2b–c), cells remained elongated and attached, with no obvious morphological deterioration, indicating early tolerance to low oxygen tension. At 10 hours of hypoxic exposure (Figure 2d), MSCs still maintained their characteristic spindle-like shape, although a slight reduction in cell density was observed. By 12 hours (Figure 2e), several cells began to exhibit morphological stress features, such as partial rounding and decreased adherence. Prolonged hypoxia for 24 hours (Figure 2f) resulted in marked cellular shrinkage and detachment, suggesting decreased cell viability and tolerance at extended exposure durations. These observations suggest that short-term hypoxia (≤10 h) is well tolerated by MSCs and may preserve their characteristic morphology, while prolonged hypoxia (>12 h) induces visible morphological changes consistent with stress or early apoptosis.

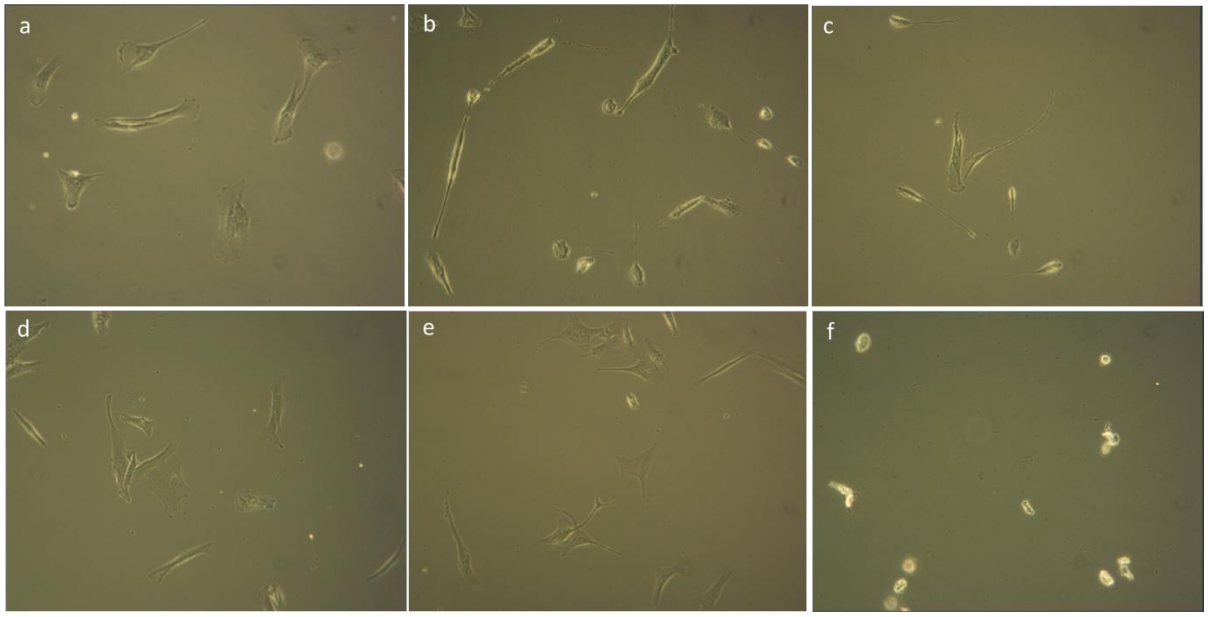

Figure 2. Morphological changes of mesenchymal stem cells (MSCs) under different durations of hypoxic exposure. Representative phase-contrast micrographs show MSCs exposed to hypoxia for (a) 0 h, (b) 4 h, (c) 6 h, (d) 10 h, (e) 12 h, and (f) 24 h. Cells maintained their typical spindle-shaped morphology at shorter exposures (0–10 h), while prolonged hypoxia (≥12 h) led to reduced cell density and signs of stress or detachment. Scale bar = 100 µm.

DISCUSSION

This study demonstrated that hypoxic exposure induces time-dependent morphological alterations in mesenchymal stem cells (MSCs), reflecting progressive cellular adaptation and stress responses at the molecular level. During the early phase of hypoxia (0–10 h), MSCs maintained their characteristic fibroblast-like, spindle-shaped morphology, indicating that moderate hypoxic stress may enhance their physiological stemness. However, prolonged hypoxic exposure (≥12 h) resulted in cell shrinkage, rounding, and detachment, suggestive of cytoskeletal reorganization, impaired adhesion, and possible initiation of apoptosis.

At the molecular level, MSCs respond to decreased oxygen tension primarily through the activation of hypoxia-inducible factor-1α (HIF-1α). [18-19] Under hypoxia, HIF-1α escapes proteasomal degradation and translocate to the nucleus, where it activates transcription of genes involved in angiogenesis (e.g., *VEGF*), glycolytic metabolism (e.g., *GLUT1, LDHA*), and cell survival (e.g., *BCL2*). [20] This adaptive response supports cell survival during short-term hypoxia by enhancing anaerobic ATP production and reducing oxidative stress. [18] These mechanisms explain why MSCs retained normal morphology and adhesion within the first 6–10 hours of hypoxic incubation. [21] Beyond 12 hours, however, the accumulation of reactive oxygen species (ROS) and metabolic byproducts likely surpasses the antioxidant capacity of the cells. [17,21] Persistent activations of HIF-1α may also shift toward a maladaptive state, promoting pro-apoptotic signalling via *BNIP3*, *BAX*, and *Caspase-3* pathways. [19,22] Concurrently, cytoskeletal components such as actin and tubulin become destabilized, leading to the observed morphological rounding and detachment. [23] Prolonged hypoxias may also suppress the expression of adhesion molecules (e.g., *integrins*, *VCAM-1*) and extracellular matrix regulators, thereby reducing cell–substrate interactions. [24]

Interestingly, mild hypoxia (typically 1–5% O_2 for 6–12 hours) has been widely reported to enhance MSC proliferation, paracrine secretion, and stemness gene expression (*OCT4, SOX2, NANOG*). [20,22,25] These effects are mediated by transient HIF-1α activation that upregulates autocrine factors like *FGF2* and *IGF-1*, contributing to cell renewal and repair mechanisms. In contrast, excessive or prolonged hypoxia (>12–24 h) triggers a metabolic shift toward sustained glycolysis, mitochondrial dysfunction, and energy depletion, leading to loss of viability and morphological integrity. [26] The morphological evidence in this study aligns with these molecular events: spindle-shaped cells at ≤10 h represent cytoprotective adaptation, while the rounded, detached cells at ≥12 h reflect energy failure and onset of hypoxia-induced apoptosis. [27] These findings support the concept that controlled, short-term hypoxia serves as a preconditioning strategy to enhance MSC resilience and secretory function, whereas prolonged oxygen deprivation is detrimental.[28]

Overall, these results highlight the biphasic role of hypoxia in MSC biology—protective and stemness-promoting under mild, transient conditions, but cytotoxic when sustained. Understanding the molecular thresholds between adaptive and detrimental hypoxia is crucial for optimizing hypoxic preconditioning protocols in regenerative medicine, particularly in enhancing the therapeutic potency of MSCs and their secretome.

CONCLUSION

This study demonstrates that hypoxia exerts a biphasic effect on mesenchymal stem cells (MSCs), enhancing stemness-related gene expression during short-term exposure while inducing stress responses with prolonged treatment. Specifically, a 12-hour hypoxic exposure (5% O_2) significantly upregulated OCT-4, SOX-2, and NANOG expression, indicating optimal activation of the pluripotency network. However, beyond this duration, sustained hypoxia led to morphological alterations and decreased gene expression, suggesting the onset of cellular stress and reduced viability. These findings underscore the importance of precisely controlling hypoxic duration to harness its beneficial effects while avoiding cytotoxic consequences. Establishing an optimal hypoxic preconditioning window can improve MSC-based therapeutic strategies by preserving stemness, enhancing paracrine activity, and promoting overall regenerative potential.

Competing interests

The authors report no conflicts of interest.

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Authors' contributions

All authors equally participated in the study, including conceptualization, data collection and analysis, literature review, manuscript drafting, and editing.

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