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Effect of hypoxia on cardiomyocyte differentiation potential of bone marrow-derived mesenchymal stem cells: *In vitro* experimental study

Sadia Nazir[1]*, Tahir Maqbool[2]

[1*2]Institute of Molecular Biology and Biotechnology/Center for Research in Molecular Medicine, The University of Lahore, Lahore, Pakistan

***Corresponding Author:** Dr. Sadia Nazir,
Institute of Molecular Biology and Biotechnology

Abstract
Background: BM-MSCs are potential candidates for the treatment of MI. *In vitro* hypoxia pretreatment of BM-MSCs improves survival, proliferation, and homing of these cells. The present study aims to investigate the effect of hypoxia on the growth, survival, and differentiation potential of these cells.
Methods: Ten male SD rats were obtained from the animal house of the university. Bone marrow was collected, cells were isolated, and cultured. Cells were divided into hypoxic (1%) and normoxic (21%) groups. Hypoxia was given for 0h, 12h, 24h, 36h, and 48h. MTT and crystal violet assays were performed for cell viability. The 24h hypoxic and normoxic groups were treated with 5-AZA for cardiac differentiation, and morphology was observed.
Results: Improved cellular growth, proliferation, and survival in the hypoxic group. MTT and crystal violet results showed the highest proliferation at 24 h of hypoxia. A decline in growth was observed at 36-48 h of hypoxia. Cardiomyocyte–like morphology was observed with better growth in 24h hypoxia as compared to normoxia.
Conclusion: *In vitro* hypoxia treatment caused improved growth, survival, and differentiation potential of BM-MSC.

Keywords: BM-MSC, hypoxia, pretreatment, cardiac differentiation, myocardial infarction

Introduction:

Stem cell therapy represents a novel therapeutic strategy for cardiovascular disease, especially after myocardial infarction (MI) (1). Stem cells can be obtained from various sources, but bone marrow-derived mesenchymal stem cells (BM-MSCs) have shown effects that are more promising in the treatment of MI. Numerous preclinical studies and clinical trials have been conducted to date, showing that MSC transplantation improves cardiac function, including reduced infarct size, improved left ventricular function, and reduced remodeling (2). However, poor homing and survival of these cells in the harsh post-MI myocardial environment. It is crucial to explore novel techniques to improve the homing, survival, and differentiation potential of BM-MSC to enhance their cardiac repair functions (3).

BM-MSCs are sourced from bone marrow, where oxygen concentration is low. By creating a hypoxic environment, this actually mimics the physiological niche of these cells, thus promoting the survival and differentiation potential of bone marrow-derived stem cells (4). Previous studies have shown that low oxygen levels (hypoxia) enhance the chondrogenic and osteogenic differentiation potential of these cells. Hypoxia improves survival and cardiomyogenic differentiation of MSC, embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs) (5). Hypoxia-induced stress leads to modifications in genes and proteins associated with cardiomyocyte differentiation. Hypoxia-inducible factor-1α (HIF-1α) upregulation was observed in MSCs preconditioned under hypoxia (6). A recent study suggested that hypoxia reduces levels of reactive oxygen species (ROS), thereby enhancing heart regeneration after MI (7).

The aim of the current study is to investigate the *in vitro* effect of hypoxia on the growth, proliferation, and differentiation potential of BM-MSC into cardiomyocyte-like cells.

Material and Methods

The review and ethical board of the University of Lahore approved the study (Ref-IMBB/BBBC/24/906-A). Sprague-Dawley (SD) male rats (n=10), weighing 120-180g, were obtained from the animal house facility of the university. Handling, experiment and care of the animals were in accordance with ethical guidelines laid by the University of Lahore.

Bone marrow was obtained from the tibia and femur of SD rats, following aseptic techniques. Cells were isolated and cultured in culture media composed of fetal bovine serum (10%), penicillin, streptomycin, and phenol red. Cells were cultured until they reached 80% confluence. Adherent cells were trypsinized and divided into groups. Subculturing of cells was performed under two conditions: normoxia (20% oxygen) and hypoxia (1% oxygen). 10 µM 5-Azacytidine (5-AZA) (Sigma–Aldrich) was added to the culture media for cardiomyocyte differentiation, and cells were incubated for 24 hours. The media was replaced every 3 days for 14 days. Cardiomyocyte-like differentiation was achieved. Cell viability was assessed by MTT and crystal violet analysis by the method delineated by Maqbool et al. (8). Morphological analysis of cultured cells was done by the Floid cell imaging station.

Statistical analysis

Data was entered and analyzed by using GraphPad Prism version 8. Data were expressed in mean ± SD, and p <0.05 and p <0.01 were considered statistically significant.

Results

Cell viability analysis

The MTT and crystal violet assays were performed to assess the effect of hypoxia on cell viability (Figure 1a-d). Better growth and viability of cells were observed in the hypoxic group as compared to the normoxic group. Viability at 12 and 24 hours of hypoxia was more rapid than in normoxia (p < 0.001). A decline in viability was observed at 36 and 48 hours of hypoxia preconditioning. These results suggested that *in vitro* pretreatment of BM-MSCs with hypoxia for 24 hours could effectively boost the proliferation potential of these cells.

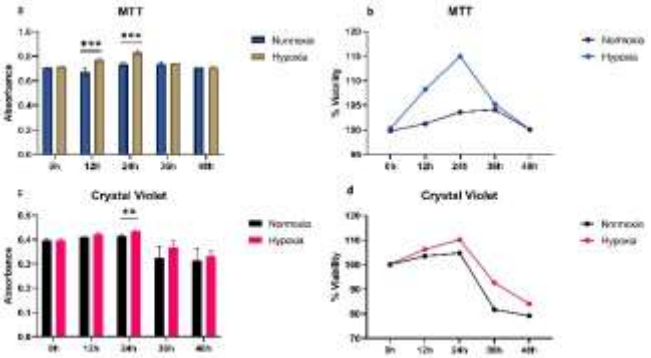

Figure 1(a-d). MTT and Crystal violet assays for cell viability, showing higher absorbance and % viability in the hypoxic group, peaking at 24h compared to the normoxic group. Data expressed in mean± SD. ** p=0.02, ***p<0.01

Morphological analysis

Figure 2a-j showed the stem cells' morphology at 0h, 12h, 24h, 36h, and 48h, respectively. Round, floating cells at 0h and characteristic BM-MSC features in cells at 12h, 24h, 36h, and 48h were observed. Cells were typically spindle-shaped with processes, and growth was greater in the hypoxic group than in the normoxic group. After a peak in growth at 24h, a decline was observed at 36-48 hours of hypoxia. Figures 3a and b showed cardiac myocyte-like differentiation of BM-MSC in both the normoxic and the 24 h hypoxic groups. Cells showed cardiomyocyte-like morphology with a typical branching network. These results suggested that hypoxia could promote the proliferation, growth, and cardiac differentiation potential of BM-MSC.

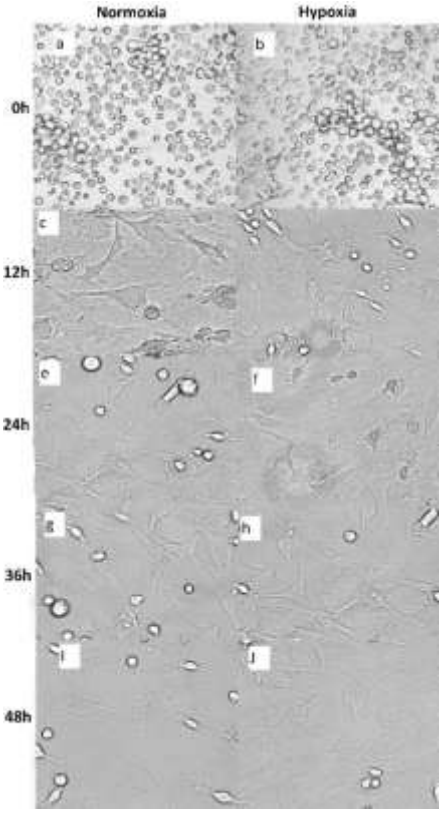

Figure 2 (a-j). Morphology analysis of BM-MSC on 0h, 12h, 24h, 36h, and 48h, respectively, between hypoxic and normoxic groups. Cell proliferation is enhanced in the 24h hypoxia group as compared to normoxia. Decline in growth on 36 and 48h of hypoxia.

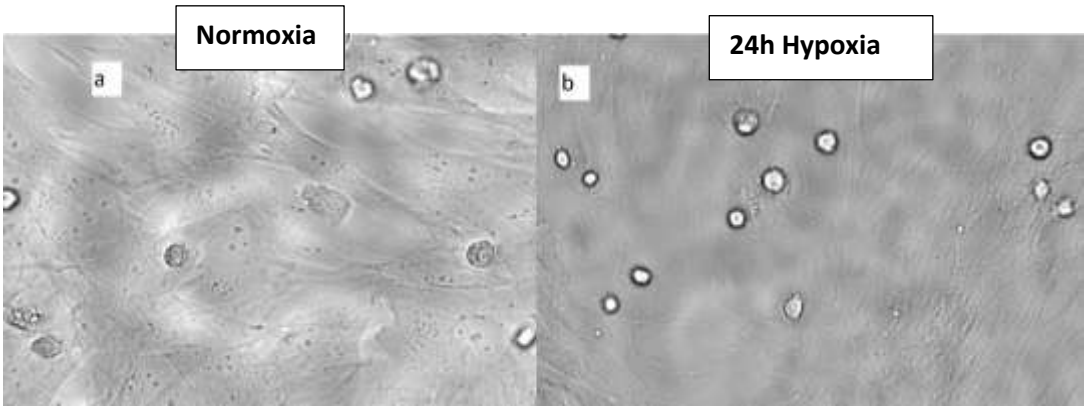

Figure 3 (a-b). Cardiomyocyte-like morphology of BM-MSC in normoxic and 24h hypoxic group. Images taken from the floid cell imaging station on day 14. Cell growth is higher in the 24h hypoxic group than in the normoxic group.

Discussion

Mesenchymal stem cells are an appealing source for reparative and regenerative medicine. MSCs can be obtained from various sources, but BM-MSCs show promising potential in cardiovascular disease treatment (9). Previous studies have demonstrated the beneficial role of BM-MSCs in improving cardiac function and reducing infarct size after transplantation. BM-MSC can be successfully differentiated in cardiomyocytes, especially after MI (10,11). This also signifies the importance of hypoxia and BM-MSC differentiation and cardiac repair after a stressful, harsh hypoxic environment of the heart (12).

Microenvironment and culture conditions directly affect the therapeutic and differentiation potential of these cells (13). Previous research has shown the roles of oxygen tension and MSC differentiation potential (14,15). A study by Jackson et al. concludes that oxygen acts as a key factor in stem cell biology (16).

Standard culture practices are carried out under normoxic conditions with 21% oxygen concentration. Nevertheless, under physiological conditions, oxygen tension and levels differ from tissue to tissue, ranging from 1-13%. BM-MSCs are commonly cultured under normoxic conditions, although the oxygen concentration in the bone marrow is very low, ranging from 1% to 7%. This implies that, for BM-MSCs, exposure to in vitro hypoxic conditions mimics the physiological niche of these cells (17,18). Studies concluded that high oxygen concentration can be lethal and leads to the production of reactive oxygen species (19). ROS-associated damage to the myocardium is the leading pathophysiological mechanism in MI. Hypoxia reduces ROS production and accumulation, thereby improving cardiac repair (20). Moreover, *in vitro* hypoxic pretreatment of BM-MSC closely resembles the physiological microenvironment of these cells and thus improves their proliferation, differentiation potential, engraftment, and survival (21).

A study conducted by Kahrizi et al. showed that hypoxia improved in vitro chondrogenic, adipogenic, and osteogenic differentiation potential of MSC under low oxygen tension, especially 3% O2 (22). In the present study, we investigate the effect of hypoxia on the proliferation, survival, growth, and differentiation potential of BM-MSC. The current study revealed that 12 and 24 hours of hypoxia enhance the proliferation rate and viability of BM-MSCs compared to normoxia, peaking at 24 hours. Morphology of BM-MSCs in the current study revealed changes over time, with cells exhibiting typical BM-MSC-like features. Cardiomyocyte-like differentiation was also observed afterward, with a higher proportion of cells in the hypoxia group than in the normoxic group.

Conclusion

This study concluded that hypoxia affects the bio-effectiveness of *in vitro* BM-MSCs. Hypoxia promotes the *in vitro* survival and differentiation potential of BM-MSCs. Further studies are required to explore the mechanistic insight for improving the homing and engraftment of BM-MSCs in the treatment of MI.

Conflict of interest

No conflict of interest.

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