Review Article
Insight in Hypoxia-Mimetic Agents as Potential Tools for Mesenchymal Stem Cell Priming in Regenerative Medicine

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Hypoxia-mimetic agents are new potential tools in MSC priming instead of hypoxia incubators or chambers. Several pharmaceutical/chemical hypoxia-mimetic agents can be used to induce hypoxia in the tissues: deferoxamine (DFO), dimethyloxaloylglycine (DMOG), 2,4-dinitrophenol (DNP), cobalt chloride (CoCl2), and isoflurane (ISO). Hypoxia-mimetic agents can increase cell proliferation, preserve or enhance differentiation potential, increase migration potential, and induce neovascularization in a concentration- and stem cell source-dependent manner. Moreover, hypoxia-mimetic agents may increase HIF-1α, changing the metabolism and enhancing glycolysis like hypoxia. So, there is clear evidence that treatment with hypoxia-mimetic agents is beneficial in regenerative medicine, preserving stem cell capacities. These agents are not studied so wildly as hypoxia but, considering the low cost and ease of use, are believed to find application as pretreatment of many diseases such as ischemic heart disease and myocardial fibrosis and promote cardiac and cartilage regeneration. The knowledge of MSC priming is critical in evaluating safety procedures and use in clinics. In this review, similarities and differences between hypoxia and hypoxia-mimetic agents in terms of their therapeutic efficiency are considered in detail. The advantages, challenges, and future perspectives in MSC priming with hypoxia mimetic agents are also discussed.

1. Introduction

The proper functioning of human tissues and organs depends on natural regeneration processes. The regenerative potential is primarily maintained by the stem and progenitor cells whose progeny replace aged or injured cells when needed [1–3]. Mesenchymal stem cells (MSCs) are stromal cells that self-renew and display multipotency, together with unique immunomodulatory properties. Numerous studies are currently carried out on MSCs to treat neurodegenerative or immune-derived inflammatory diseases [1, 4, 5]. MSCs can be isolated from adult tissues (e.g., bone marrow (BM), adipose tissue (AD), skeletal muscle (SM), and dental pulp (DP)) [6–9] or fetal tissues (e.g., placenta, amniotic fluid (AF), Wharton jelly (WJ), and umbilical cord (UC)) [10, 11]. Epidermal stem cells, multipotent skin-derived precursors, and other stem cells can also be efficiently isolated from human skin [12].

By July 2020, 1,138 clinical trials have been registered at clinicaltrials.gov [13], mostly in traumatology, pneumology, neurology, cardiology, and immunology [14–19]. Most registered cases were in phases 1 (Ph1) and 2 (Ph2) of clinical trials. The percentage of particular phases of clinical trials in the fields mentioned above is as follows: in traumatology (total 234 cases), 30.7% in Ph1, 58.5% in Ph2, 9.8% in Ph3, and 0.8% in Ph4; in pneumology (total 99 cases), 43.4% in Ph1, 53.5% in Ph2, 3.0% in Ph3, and 0% in Ph4; in neurology (total 97 cases), 31.9% in Ph1, 62.8% in Ph2, 4.1% in Ph3, and 1.0% in Ph4; in cardiology (total 83 cases), 25.5% in Ph1, 60.2% in Ph2, 14.4% in Ph3, and 0% in Ph4; and in immunology (total 78 cases), 17.9% in Ph1, 64.1% in Ph2, 17.9% in Ph3, and 0% in Ph4 [13, 16]. The outcomes
of 18 clinical tests have already been described [16], and bone marrow was the most common source of isolated cells. MSCs have found potential applications in the treatment of multiple sclerosis (MS), Crohn’s disease (CD), diabetes mellitus (DM), graft-versus-host disease (GVHD), rejection after liver transplant, liver disorders [5], and acute and chronic wounds [20, 21].

Despite such notable progress, there are still numerous challenges. Clinical applications demand systemic administration of the high number of stem cells (50–200 million per patient) [16, 22]. The number of stem cells in human tissues is usually small [23], and their efficient proliferation in vitro is challenging [24–26]. Both MSC aging and spontaneous differentiation are factors that may occur in vitro. The isolated stem cells are usually grown in vitro under ambient conditions where oxygen concentration is four to ten times higher than in a stem cell niche [27–30]. Thus, high oxygen concentration upon MSC culture results in early senescence and nuclear damage and may increase the doubling time [31–33]. Poor MSC engraftment after transplantation was also revealed [34].

Over the last few years, numerous low oxygen priming approaches have been explored for MSC clinical application [35, 36]. MSCs growing under hypoxia [37] demonstrate enhanced proliferation, immunomodulatory properties [38–43], efficient survival, and neovascularization after grafting.

As such, several hypoxia-mimetic agents can be used to induce hypoxia in tissues, e.g., deferoxamine (DFO), dimethyl-oxaloylglycine (DMOG), 2,4-dinitrophenol (DNP), cobalt chloride (CoCl2), and isoﬂurane (ISO). Could they effectively replace the hypoxia chambers/incubators in the MSC priming? This review looks for an answer to this question and discusses similarities and differences between the effects of hypoxia and hypoxia-mimetic agents. The oxygen concentration, incubation time, and MSC therapeutic efﬁciency are described in detail. Since there are still some ambiguities in the literature regarding hypoxia as a standard approach in MSC production, this issue will be extensively discussed.

To summarize the recent ﬁndings on hypoxia in this review, we searched PubMed, Scopus, Science Direct, and Web of Science databases from 2006 to September 2021 for potentially relevant studies published in English. Original papers, systematic reviews, and book chapters were reviewed. The search strategy ﬁrst has focused on critical terms: hypoxia, hypoxia mimetic agents, mesenchymal stem cells, and clinical applications of MSC. These criteria have been extended with the more detailed terms: application in regenerative medicine, cell treatment, cell-based therapies, mesenchymal cells’ source (Warton jelly, umbilical cord, bone marrow, umbilical cord blood, adipose, and dental pulp originated from human, rat, and mouse), and chemicals: deferoxamine, cobalt chloride, isoﬂurane, dimethyl-oxaloylglycine, and 2,4-dinitrophenol. We excluded studies enrolling hypoxia/hypoxia-mimetic agents together with specific adjuvants such as immunomodulators.

2. Role of Hypoxia in a Stem Cell Niche

The stem cell niche is a microenvironment, which governs stem cell’s functions and fate [44]. Morphogens, growth factors, cytokines, oxygen tension, extracellular matrix, and shear stress could affect stem cells within the niche [5, 45].

MSCs can be found in the niches close to blood capillaries throughout the body [46]. The oxygen concentration in the tissues where MSCs reside is low despite their efﬁcient vascularization [47, 48]. The oxygen concentration is much lower in human tissues than in inhaled air (21%). It happens because the oxygen concentration of the inhaled air constantly drops, entering the lungs, and when it reaches organs and tissues, its concentration ranges from 2% to 9% [49, 50]. Since the concentration of O2 in blastocysts and stem cells niches is very low, oxygen tensions tend to be critical in their metabolic milieu. Hypoxia sustains the phenotype of hematopoietic, embryonic, neural, and mesenchymal stem cells and inﬂuences stem cells’ function and fate. Furthermore, hypoxia acts on stem cells via different molecular pathways, including signaling of homolog translocation-associated (Drosophila) (Notch) and octamer-binding transcription factor 4 (Oct4), the stemness controllers [25].

Stem cells are physiologically adapted to hypoxia. Therefore, hypoxic priming should maintain MSCs in an undifferentiated state and preserve their functions and plasticity.

3. Hypoxia versus Hypoxia-Mimetic Agents for MSC Priming

Injured tissues have poor vascularity (especially in ischemic injuries) and cannot maintain the metabolism of implanted not-primed MSC at an appropriate rate; therefore, most cells undergo apoptosis soon after transplantation. It is due to stem cells grown in normoxia not adapting quickly to the conditions of hypoxia. Hence, to survive after transplantation, stem cells must be trained ex vivo to sustain hypoxia conditions [51].

The simplest solution is to cultivate MSCs under low oxygen conditions. Various hypoxia incubators and chambers were used for MSC culture. However, both have limitations in their use [52]. They suggest that pharmaceutical/chemical agents are more valuable because they provide higher oxygen tension stability than hypoxic chambers and are not expensive [53].

Now the question arises whether pharmacological or chemical hypoxia-mimetic agents act similarly on stem cells. Before answering this question, we intend to discuss the influence of hypoxia on the crucial MSC features.

3.1. Cell Surface Markers and Morphology

The most important MSC feature is their immunophenotype that defines their stemness according to the International Society for Cellular Therapy (ISCT). MSCs express CD90, CD105, and CD73 antigens and do not express CD11b, CD14, CD19, CD45, CD34, and CD79a antigens, nor human leukocyte antigen-DR isotype (HLA-DR). The proteins SRY-box transcription factor 2 (SOX2) and Oct4 occur in embryonic stem cell- (ESC-) like [54]. The expression of other surface markers depends on the MSC tissue source. Homeobox transcription factor NANOG, reduced expression-1 (REX-1), T cell receptor alpha locus 1-60 (TRA-1-60), TRA-1-81, stage-specific mouse embryonic antigen (SSEA-3), and
SSEA-4 markers have been found on MSCs isolated from human liver and fetal blood but not on the cells derived from adult bone marrow [55, 56].

The influence of the hypoxia priming on MSC's surface markers is summarized in Table 1. Hypoxic conditions in the oxygen range of 2-5% preserve the expression of surface markers on MSCs. Only in low oxygen concentration of 1% are the results inconclusive. The expression of negative surface markers is maintained at 1% O2 [57–59], but some studies showed a reduced expression of positive markers. Compared to normoxia, CD44 and CD105 reduction on the MSC surface from 90% to 75% and from 99.4% to 94.9%, respectively, was noted [57]. Upregulation of other stem cell markers as Oct4, REX-2, or NANOG was presented [38, 60].

Of no less importance is maintaining the appropriate morphology of MSCs growing in confluence. While increased cellular density and number of passages significantly change MSC’s morphology under normoxia and cause cell retraction at high density, hypoxic conditions retain the MSC’s spindle shape, and cells can divide even at high density, permitting multilayer formation [38]. Similarly, MSCs treated with a hypoxia-mimicking agent DFO did not alter their morphology. However, some intracellular vacuole-like structures may occur within the cells [61].

To summarize, the expression of stem cell surface markers is generally preserved under hypoxia but depends on the oxygen concentration, exposure time, tissue, and donor of MSCs. Up to date, there are no data on the influence of hypoxia-mimicking agents on MSC surface markers expressions.

3.2. Viability, Proliferation, and Clonogenicity. A high proliferation rate is critical for the successful implementation of stem cell-based therapy. The oxygen concentration and the incubation time may influence the overall hypoxia effect on stem cells, especially their viability, proliferation, and clonogenicity.

3.2.1. Hypoxia. As shown in Table 2, the proliferation, viability, and clonogenicity of the stem cells derived from various tissues were studied under at least 30 conditions different in terms of oxygen concentration and incubation time under reduced oxygen concentration.

In 19 conditions, an increase in proliferation or clonogenicity of MSCs was observed in the oxygen concentration ranged from 1% to 5%. Out of these 30 conditions considered, a decrease in cell viability was recorded in eight. This discrepancy is not related to oxygen concentrations since proliferation inhibition was observed at both 1% and 5% oxygen concentrations. It also does not depend on the time of cell growth under hypoxia because inhibition of proliferation was observed both after 2-day exposure to reduced oxygen concentration and after 21-day exposure at similar oxygen concentrations. These divergent effects can also be seen on one type of stem cell, e.g., BM-MSCs. Likewise, the impact of the test method on the results obtained cannot be attributed, e.g., Trypan Blue staining and counting cells under the microscope were used at the elaboration of conditions resulting in discrepant observations. It is, therefore, possible that more subtle molecular phenomena occurring in stem cells while growing under hypoxic conditions should be investigated, such as transcriptome or metabolome of hypoxia-treated cells.

The higher proliferation of MSCs could be attributed to the transition from aerobic to anaerobic respiration through oxidative phosphorylation and glycolysis, respectively [67]. The increase in glucose consumption and lactate generation in UC-MSCs in hypoxic culture may exemplify the metabolic changes described above and require enhanced glucose transport into the cells. Increased MSC proliferation under hypoxia enhances glucose uptake as the critical carbon source for the biosynthesis of essential nutrients. The involvement of metabolic pathways as glycolysis (lactate dehydrogenase A, LDHA), oxidative phosphorylation (3-phosphoinositide-dependent protein kinase 1, PDK-1), cellular glucose transport (cellular glucose transporter-1, GLUT-1), pentose phosphate pathway (glucose-6-phosphate dehydrogenase, G6PD), and the significant targets of hypoxia-inducible factor 1 alpha (HIF-1α) transcriptional factors was demonstrated in the diminished oxygen concentration [63, 68, 69].

HIF-1α is a crucial transcriptional factor, which regulates the adaptive response to hypoxia. Many proteins react directly with HIF-1α enhancing or reducing their activities. HIF-1α stabilization improves MSC’s proliferation rate and may augment their therapeutic potential [70].

The reduction of cellular senescence and inhibition of the telomere shortening was also observed in MSCs under hypoxic conditions [62, 63, 71, 72]. The mechanism of apoptosis suppression under hypoxia might relate to the cellular tumor antigen (p53) pathway inhibition [73]. The decreased O2 tension could also lead to the lower level of reactive oxygen species (ROS); the primary factor was attributed to increased cellular damage [74].

1) MSC’s Gene Expression. Cells’ adaptation to hypoxia requires changes in molecular pathways. Hypoxia regulates the transcription of hundreds of genes, which play a role in oxygen-dependent functions like angiogenesis, glycolysis, metabolism, proliferation, and apoptosis [75]. Most of these changes are HIF-1α-dependent and transcriptionally regulated. HIF-1α is also subject to epigenetic mechanisms such as histone modification, DNA methylation, and noncoding RNA-associated gene silencing [76]. Thus, epigenetic modifications are additional mechanisms regulating gene expression in hypoxia and enhancing or inhibiting their activity. However, the contribution of microRNA (miRNA) functioning during hypoxia and DNA methylation is not yet fully understood [77]. Furthermore, molecules of short noncoding RNAs and miRNAs, which regulate gene expression, are controlled by hypoxia in stem cell niches [78]. Some miRNA regulate vascular endothelial growth factor (VEGF), which stimulates angiogenesis and tightly controls hypoxia-induced cellular alteration [77, 79].

Beyond epigenetic mechanisms, hypoxia upregulates over 135 genes governing several physiological pathways, e.g., glycolysis, metabolism, proliferation/survival,
transduction, and signaling transduction in BM/umbilical cord blood- (UCB-) MSCs in the oxygen range from 1.3% to 10% [75, 82]. Short-term hypoxia downregulates pro-apoptotic genes such as BCL-2-associated X (BAX), B-cell lymphoma 2 (BCL-2), and caspase 3 (CASP-3) (Table 2), thus preventing cells from cellular damage after transplantation [57].

Hypoxic conditions (1-5% O2) increased expression of HIF-1α, Notch ligand, and JAGGED was observed, suggesting a link between hypoxia and Notch signaling pathway. Moreover, the augmented proliferation of hWJ-MSCs under hypoxia confirms the Notch-related proliferation [64].

(2) Reoxygenation of MSCs in Culture. As mentioned in Table 2, there is another way to grow cells with limited oxygen availability. It includes 15 min of preconditioning at 2.5% O2, 30 min of reoxygenation in ambient conditions, and the final conditioning at 2.5% O2 for 72 h. Such conditions were used for hUB-MSC culture, significantly improving the cell proliferation and migration in vitro.

The reoxygenation process following short hypoxia priming enhanced the prosurvival genes’ expression together with numerous angiogenic and trophic factors, such as the basic fibroblast growth factor (bFGF) and VEGF in MSCs [18, 60, 84, 86]. Moreover, other positive effects include the reduced release of lactate dehydrogenase, lower activity of apoptosis-related caspases, and diminished cell sensitivity to ischemia resulting from the reoxygenation of the MSC culture [57, 87].

### Table 1: Effect of hypoxia on MSC surface markers.

| Treatment conditions | Types of stem cells | The effect compared to normoxia (method of analysis) | Ref. |
|----------------------|---------------------|----------------------------------------------------|------|
| O2 concentration    | Time/passage        |                                                    |      |
| 1%                   | 2 d                 | hBM-MSC; no changes positive (CD44 and TF) and negative markers (CD11b, CD19, CD34, CD45, and HLA-DR); decreased level of CD73, CD90, and CD105 (flow cytometry) | [57] |
| 1%                   | 10 d                | hBM-MSC; no changes positive (CD73, CD90, and TF) and negative markers (CD45, CD34, CD11b, CD19, and HLA-DR); decreased level of CD105 and CD44 (flow cytometry) | [57] |
| 1%                   | 14 d                | hBM-MSC; no changes positive (CD73, CD90, CD105, CD106, CD 146, and MHC class I) and negative markers (CD34, CD34, and HLA-DR) (flow cytometry) | [58] |
| 1%                   | 21 d                | hAD-MSC; no changes positive (CD90 and CD105); increased level of negative markers (CD34, CD54, and CD 166) (flow cytometry) | [62] |
| 2%                   | 2 d                 | hBM-MSC; no changes positive (CD73, CD44, CD90, and CD105) and negative markers (CD11b, CD19, CD45, CD34, and HLA-DR) (flow cytometry) | [57] |
| 2%                   | 10 passages         | hBM-MSC; no changes positive (CD73, CD105, CD90, CD44, CD10, CD29, and CD13) and negative markers (CD14, CD34, CD33, CD45, and HLA-DR) (flow cytometry); increased level of DNMT3B, CRABP2, IL6ST, IFITM1, GRB7, IMP2, LIN28, and KIT (RT-PCR) | [64] |
| 2.5%*                | >72 h*              | hUCB-MSC; no changes positive (CD44, CD73, CD90, and CD105) and negative markers (CD14, CD34, CD45, CD271, and HLA-DR) (flow cytometry); upregulation of Oct4, NANOG (RT-PCR) | [60] |
| 2.5%*                | >72 h*              | hUCB-MSC; no changes positive (CD73, CD44, CD105, and CD90) and negative markers (CD14, CD45, and CD106) (flow cytometry) | [65] |
| 5%                   | 2 d                 | hBM-MSC; no changes positive (CD44, CD90, and CD73) and negative markers (CD11b, CD19, CD34, CD45, and HLA-DR); reduced level of CD105 (flow cytometry) | [57] |
| 5%                   | 14 d                | hBM-MSC; no changes positive (CD73, CD90, CD44, CD105, and STRO-1) and negative markers (CD34); reduced level of CD146 and CD45 (flow cytometry) | [65] |
| 5%                   | Primary cells and passage 2 | hBM-MSC; no changes positive (CD29, CD73, CD90, CD44, CD105, and STRO-1) and negative markers (CD45 and CD34) (flow cytometry) | [66] |

*Hypoxic preconditioning in 2.5% O2 for 15 minutes, then reoxygenation at 21% O2 for 30 minutes, and again hypoxia preconditioning at 2.5% O2 for 3 days; h: human; d: day/days; UCB: umbilical cord blood.
Table 2: Effect of hypoxia on MSC viability, proliferation, and clonogenicity.

| Treatment conditions | Time/ passage | Types of stem cells | The effect compared to normoxia (methods of analysis) | Ref. |
|----------------------|---------------|---------------------|------------------------------------------------------|------|
| O2 concentration     |               |                     |                                                      |      |
| **1%**               | 2 d           | hBM-MSC             | Proliferation (DNA Quant-iT PicoGreen assay), clonogenicity (Giemsa staining), and metabolic activity (Vybrant assay) increased; *HIF-1α* downregulated (qRT-PCR), the proapoptotic genes: BAX, BCL-2, and CASP-3 downregulated (qRT-PCR) | [57] |
| 1%                   | 2 d           | rBM-MSC             | The proliferation decreased (Trypan Blue staining, cell count) | [80] |
| 1%                   | 7 d           | hBM-MSC             | Proliferation significantly reduced (MTS proliferation assay) | [58] |
| 1%                   | 7 d           | hBM-MSC             | *HIF-1α* upregulated three-folds (qRT-PCR) | [58] |
| 1%                   | 9 d           | hAD-MSC             | Proliferation increased 1.7-folds (Trypan Blue staining, cell count) | [62] |
| 1%                   | 10 d          | hBM-MSC             | Proliferation (DNA Quant-iT PicoGreen assay) decreased, and metabolic activity increased (Vybrant assay), *HIF-1α* downregulated (qRT-PCR) | [57] |
| 1%                   | 14 d          | mBM-MSC             | Viability (MTT viability assay) and proliferation (BrdU cell proliferation assay) increased, the main metabolic regulators like *Hk2* upregulated (sqRT-PCR); shift to anaerobic glycolysis, the *Slc16a3* (MCT-4) gene upregulated under prolonged hypoxia (qRT-PCT), the MCT-4 level increased under prolonged hypoxia (WB) | [68] |
| 1%                   | 14 d          | rBM-MSC             | Clonogenicity increased (crystal violet staining) | [80] |
| 1%                   | 21 d          | hAD-MSC             | Cell aging reduced, telomeres longer 1.5-folds (qPCR) | [62] |
| 1%                   | 21 d          | hBM-MSC             | A slowdown of cell cycle progression, accumulation in G1 phase under prolonged hypoxia (flow cytometry) | [58] |
| 1-3%                 | 16 h          | hBM-MSC             | Viability and proliferation (flow cytometry) maintained, Akt signaling pathway activated (WB) | [81] |
| 1.5%                 | 1 d           | hBM-MSC hUCB-MSC    | Proliferation increased (Trypan Blue staining, cell count) and the cell cycle faster progression (flow cytometry), *HIF-1α* increased (WB) | [82] |
| 1.5%                 | 3 d           | hUC-MSC             | Proliferation decreased (Trypan Blue staining, cell count), *LDHA*, *GLUT-1*, and *PDK-1* upregulated (RT-PCR), glutamate production decreased (HPLC), glucose consumption significantly increased (YSI 2700 analyzer) | [69] |
| 2%                   | 2 d           | hBM-MSC             | Proliferation (DNA Quant-iT PicoGreen assay), clonogenicity (Giemsa staining), and viability (flow cytometry) increased | [57] |
| 2%                   | 2 d           | hWJ-MSC             | Expression of the genes *HIF1-α*, *HIF-2α*, *Notch2*, and *JAGGED1* increased (RT-PCR) | [64] |
| 2%                   | 7 d           | hBM-MSC             | A high growth rate maintained even after confluency–multilayer formation (cell count, growth curve), *HIF-2α* upregulated (RT-PCR) | [38] |
| 2%                   | 7 d           | hBM-MSC             | Clonogenicity increased (crystal violet staining) | [63] |
| 2%                   | 12 d          | hBM-MSC             | Higher proliferation rate (Trypan Blue staining, cell count), the number of actively dividing cells significantly increased (PKH26 Red Fluorescent Cell Linker kit), the cellular division started earlier in the cell cycle (PKH26 staining, flow cytometry) | [63] |
| 2%                   | 20 d          | hBM-MSC             | Clonogenicity (colony count from microscopic images) and doubling time (cell count and growth curve) maintained, cellular senescence reduced (*β*-galactosidase staining, histochemistry) | [71] |
| 2%                   | Passages 2-7  | hBM-MSC             | Higher cell number in each passage from 2 to 7 (Trypan Blue staining, cell count) | [38] |
| 2%                   | 10 passages   | hWJ-MSC             | Faster growth rates and higher total cell number yielded (cell area count, image analysis), normal karyotype maintained (Giemsa staining) | [64] |
| 2%                   | 64 d          | hBM-MSC             | Homogenous morphology of rapidly self-renewing cells maintained up to 52 d (microscopy analysis) | [83] |
| 2.5%                 | 3 d           | hUC-MSC             | Proliferation increased (cell counting under a microscope), *HIF-1α* increased (WB), *PDK-1, GLUT-1*, and *LDHA* upregulated (RT-PCR), glutamate production diminished (HPLC), glucose consumption significantly increased (YSI 2700 SELECT analyzer) | [69] |
| 2.5%                 | >3 d*         | hUCB-MSC            | Cell viability (at 24 h and 2 d) increased (Trypan Blue staining, cell count, and MTT); proliferation (at 3 d) increased (Trypan Blue staining, cell count), CFU-F number *in vitro* significantly enhanced (Giemsa staining) | [60] |
| 2.5%                 | >3 d*         | hUCB-MSCs           | | [84] |
| Treatment conditions | Time/ passage | Types of stem cells | The effect compared to normoxia (methods of analysis) | Ref. |
|----------------------|--------------|---------------------|-----------------------------------------------------|-----|
| **O₂ concentration** | **Time/ passage** | **Types of stem cells** | **The effect compared to normoxia (methods of analysis)** | **Ref.** |
| 3% | ~100 d Passage 1 | hBM-MSC | Cell metabolic activity (MTT), CFU-F number (Giemsa staining), and proliferation (at 2 and 3 d) (Trypan Blue staining, cell count) increased, doubling time reduced (at 2 and 3 d) (Trypan Blue staining, cell count), cell death inhibited (at 2 and 3 d) (microscope analysis) | [33] |
| 3% | Over 25 passages | hBM-MSC | Proliferative lifespan with additional 10 PD improved (flow cytometry), transcription of hypoxia-related the genes encoding VHL, HIF-1, PH-4, HYOU1, HIF1AN, HIG, and HIG unaltered (qPCR) | [33] |
| 3% | 2 d | hBM-MSC | Cell growth improved (Trypan Blue staining, cell count), population doublings increased (Trypan Blue staining, cell count), oxidative stress reactions (DHE, flow cytometry) and nuclear alterations such as damage of DNA, telomere shortening, and chromosomal abnormalities (DAPI, Q-FISH, Breast Aneusomy Multicolor Probe kit) limited, glycolysis increased (OCR/ECAR, F96 Flux analyzer) | [32] |
| 5% | 3 d | hUC-MSC | Proliferation rate lowered (DNA Quant-iT PicoGreen assay), clonogenicity (Giemsa staining), and metabolic activity elevated (Trypan Blue staining, cell count) | [57] |
| 5% | 4 d | rBM-MSC | Proliferation increased (Trypan Blue staining, cell count), LDHA, PDK-1, and GLUT-1 upregulation (RT-PCR) | [69] |
| 5% | 14 d | hBM-MSC | Clonogenicity decreased at primary cells and the passage 1 but increased at the passages 2 and 3 (crystal violet staining) | [66] |
| 5% | 20 d | hBM-MSC | Colony formation significantly reduced (colony count from microscopic images), doubling time maintained (Trypan Blue staining, cell count), cellular senescence reduced (β-galactosidase staining, histochemistry, blue stained cell count) | [71] |
| 5% | Passage 1-10 | hBM-MSC | The number of population doublings increased (Trypan Blue staining, cell count), cellular senescence reduced (β-galactosidase staining, histochemistry, blue stained cell count) | [72] |

*Hypoxic preconditioning in 2.5% O₂ for 15 minutes, then reoxygenation at 21% O₂ for 30 minutes, and again hypoxia preconditioning at 2.5% O₂ for 3 days; d: day/days; h: human; m: mouse; r: rat; PD: population doublings; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BrdU: 5-bromo tetrazolium inner salt-20-deoxyuridine; MTS: tetrazolium inner salt; WB: Western Blotting.
Table 3: Effect of pharmaceutically and chemically derived hypoxia on MSC viability, proliferation, and clonogenicity.

| Treatment conditions | Time | Stem cell type | The effect compared to normoxia (methods of analysis) | Ref. |
|----------------------|------|----------------|--------------------------------------------------------|------|
| DFO/0.1 μM           | 53 d | hBM-MSC        | Proliferation increased (Incucyte HD Imaging system)   | [61] |
| DFO/10 μM            | 2 d  | hBM-MSC        | The genes related to glycolysis (HK2, PDK-1, BNIP3, LDHA), viability, and survival upregulated (microarray analysis) | [61] |
| DFO/10 μM            | 53 d | hBM-MSC        | Proliferation inhibited at concentrations of 10 μM and higher (Incucyte HD Imaging system) | [61] |
| DFO/50 μM            | 12 h | hBM-MSC        | Proliferation as effective as for 2 d in 3 μM DFO (Incucyte HD Imaging system), HIF-1α upregulated (microarray analysis) | [99] |
| DFO/50 μM            | 1-3 d| rBM-MSC        | Viability increased (MTT)                               | [100]|
| DFO/50-500 μM        | 1 d  | hAD-MSC        | Viability unchanged (CellTiter 96 Aqueous kit)         | [102]|
| DFO/100 μM           | 12 h | rBM-MSC        | HIF-1α increased (qRT-PCR)                             | [100]|
| DFO/100 μM           | 1 d  | rBM-MSC        | HIF-1α increased (WB)                                  | [100]|
| DFO/100 μM           | 1-3 d| rBM-MSC        | Viability increased (MTT)                               | [100]|
| DFO/100 μM           | 2 d  | hWJ-MSC        | HIF-1α increased (WB)                                  | [115]|
| DFO/120 μM           | 2 d  | hUC-MSC        | Viability increased (MTT)                               | [100]|
| CoCl₂/50-300 μM      | 1 d  | hAD-MSC        | Cell viability was DPO concentration-dependent, cell viability decreased above 120 μM DFO (MTT) | [97] |
|                     |      | hDP-MSC        | Viability increased (MTT)                               | [102]|
| CoCl₂/100 μM         | 1-2 d| hUC-MSC        | Viability increased (MTT)                               | [53] |
|                     |      | hAD-MSC        |                                                                 |      |
|                     |      | hDP-MSC        |                                                                 |      |
| CoCl₂/100 μM         | 2 d  | hUC-MSC        | HIF-1α increased in DP- and UC-MSC and maintained in AD-MSC (WB) | [53] |
|                     |      | hAD-MSC        |                                                                 |      |
| CoCl₂/100 μM         | 2 d  | hUC-MSC        | Viability decreased above 100 μM CoCl₂ (MTT)           | [97] |
| CoCl₂/100 μM         | 6 d  | hBM-MSC        | The higher proliferation of hBM-MSC in coculture (crystal violet staining), reduced viability of hBM-MSC | [52] |
| Treatment conditions | An agent/concentration | Time | Stem cell type | The effect compared to normoxia (methods of analysis) |
|----------------------|------------------------|------|----------------|-----------------------------------------------------|
| CoCl₂/0.5 mM         | 1 d MSC, HUVEC         |      | rAD-MSC       | Reduced viability (MTT)                              |
| DMOG/100 μM + SD     | 1 d rBM-MSC            |      |                | Proliferation maintained (Trypan Blue staining, cell count), PI3K/Akt signaling activated (WB), HIF-1α increased (WB) |
| DMOG/0.5 mM + SD     | 1 d rBM-MSC            |      |                | Proliferation maintained (Trypan Blue staining, cell count), PI3K/Akt signaling activated (WB), HIF-1α increased (WB) |
| DMOG/0.5 mM + 1%O₂   | 2 d rBM-MSC            |      |                | HIF-1α increased (WB)                                |
| DMOG/0.5 mM          | 6 d hBM-MSC, HUVEC     |      |                | The higher proliferation of hBM-MSC in coculture (crystal violet staining), increased viability of hBM-MSC |
| DMOG/1 mM            | 1 d rBM-MSC            |      |                | Viability increased in vitro (Hoechst 33342 staining), HIF-1α increased (WB), glucose transporter 1 increased (WB), the pAKT level increased (WB), increase survival of MSC after transplantation into ischemic heart (a rat model) (TUNEL assay), time-dependent protective effect against cell death in vitro (Trypan Blue staining, cell count) |
| DMOG/1 mM + SD       | 1 d rBM-MSC            |      |                | Proliferation maintained (Trypan Blue staining, cell count), PI3K/Akt signaling activated (WB), HIF-1α increased (WB) |
| DMOG/5 mM + SD       | 1 d rBM-MSC            |      |                | Proliferation decreased (Trypan Blue staining, cell count) |
| ISO/2%               | 4 h hBM-MSC            |      |                | Cell metabolic activity increased after 4 h, significantly reduced after 6 h at ISO concentrations above 2% (MTT), HIF-1α increased (WB), the PI3K/Akt signaling activated (WB), the percentage of apoptotic cells significantly reduced after treatment with 1-2% ISO for 6 h (flow cytometry) |
| DNP/0.25 mM*         | 20 min* rBM-MSC CM     |      |                | The viability significantly increased (PKH26, flow cytometry) |

*20 minutes of treatment with 0.25 mM and then reoxidation either 2 or 24 hours in 21% O₂; d: day/days; h: human; m: mouse; r: rat; SD: serum deprivation; CM: cardiomyocytes; HUVEC: human umbilical vein endothelial cells.
(3) Spheroids. Using a spheroid with short-term hypoxia in 1% O₂ poses an advantage over transplantation of individual cells. Spheroids better mimic cellular behavior in native tissue, improving viability, angiogenesis, and immunomodulatory properties [88]. Moreover, interactions of MSCs with endogenous ECM within spheroids increase proliferation and maintain osteogenic differentiation potential influencing bone tissue repair. The synergy of MSC priming with hypoxia and MSC spheroid transplantation is believed to be a good cellular therapy due to increased survival, angiogenic potential, and bone formation. Moreover, spheroids enhance interaction with ECM and promote osteogenesis. Thus, MSC priming under hypoxia and spheroids grafting can be effective in regenerative medicine [89].

3.2.2. Pharmacological and Chemical Hypoxia-Mimetic Agents. Among commercially available pharmacological/chemical hypoxia-mimetic agents, the following are discussed below: DFO, DMOG, DNP, CoCl₂, and ISO.

DFO is a chelating agent used to remove an excess of iron or aluminum from the body [90]. DFO stabilizes HIF-1α under normoxia; thus, it is a suitable hypoxia-mimetic agent [91]. DMOG is a prolyl hydroxylase inhibitor. DMOG regulates HIF-1α and phosphorylation under hypoxia. DMOG acts via inhibition of factor inhibiting HIF-1α (FIH-1) and the prolyl hydroxylases via competitive inhibition of 2-oxoglutarate (2-OG). It indicates that DMOG can be an effective drug for diabetes due to HIF-1α regulation [92, 93]. DNP increases oxygen consumption due to the enhancement of oxidative metabolism [94]. CoCl₂ artificially induces hypoxia and can block the degradation of HIF-1α protein, thus inducing its accumulation [52, 95-97]. ISO is a volatile anesthetic agent. Because of its cytoprotective capacities, it is a good candidate to be a hypoxia-mimetic agent that activates HIF-1α [98].

(1) Cytotoxicity. Table 3 presents the results of the MSC viability upon pharmacologically- or chemically induced hypoxia.

Most studies have been carried out with DFO. It was used in a concentration range of 0.1-500 μM. DFO did not impair the viability of MSCs until 120 μM [97, 99, 100]. The standard preconditioning protocol of MSC treatment with DFO (48h at a concentration of 3 μM) can be substituted with treatment for 12 hours at a concentration of 50 μM [99]. Fujisawa et al. showed significant cytotoxicity of DFO at a concentration of 10 μM towards BM-MSCs but only after long-term treatment of 53 days [61].

The viability of BM-, UC-, AD-, and DP-MSCs was preserved when CoCl₂ was used for 24-48 hours at a concentration of 100 μM [101]. CoCl₂ at a concentration of 500 μM significantly decreased MSC viability [102]. DMOG is non-cytotoxic until it reaches a concentration of 5 mM [103]. DMOG also increased the proliferation of cocultured cell BM-MSC and human umbilical vein endothelial cells (HUVEC) [52]. ISO increased hBM-MSC metabolism at a concentration of 2% and incubation time of 4 h [98]. DNP at a concentration of 0.25 mM did not injure rBM-MSCs in the coculture with cardiomyocytes, but the treatment period was very short (20 min). Otherwise, this compound could be highly toxic. The cells were slightly shrunk but regained normal morphology after their reoxidation for 2-24 hours. Thus, these results imply that the differences in culture protocols and compound concentrations may be crucial for successfully implementing hypoxia and hypoxia-mimetic agents in regenerative medicine.

(2) Metabolome. The metabolic changes occur in the cells upon adaptation to hypoxia. Metabolome analysis revealed that both hypoxia treatment and DFO administration influence cellular metabolism.

MSCs exhibited metabolic changes in Krebs tricarboxylic acid (TCA) cycle, amino acids, creatine, uric acid, and purine and pyrimidine metabolism upon both types of treatment. DFO-derived hypoxia affected TCA cycle-related metabolism by increasing aconitate, alpha-ketoglutarate (α-KG), and citrate concentrations and decreasing malate and fumarate via reductive carboxylation in reverse Krebs cycle. These effects were more visible for DFO-induced than natural hypoxia (increase only in the α-KG level) [61]. α-KG provides energy for the cellular oxidation of nutrients. The increased α-KG level is required during enhanced cell proliferation. As a precursor of glutamate and glutamine, α-KG acts as an antioxidant agent and directly reacts with hydrogen peroxide. DFO stronger upregulated α-KG in comparison to hypoxia, providing better protection against ROS [104].

The low level of malate and fumarate during hypoxia had a positive effect on cells. In contrast, high levels of these compounds were harmful and led to cancer development (by mediating chronic proliferative signals) [105, 106].

The impairment of purine and pyrimidine metabolism is also detrimental to cells, and elevated uric acid levels generated from the purines’ metabolism may be responsible for human diseases such as vascular inflammation, atherosclerosis, articular, and gout degenerative disorders [107]. Since phosphoribosyl pyrophosphate (PRPP) is an enzyme involved in synthesizing purine and pyrimidine nucleotides, its level raised under DFO-derived hypoxia [61, 108].

Additionally, 1% hypoxia upsurges the level of the 1-methyl adenosine, a stress marker, compared to DFO-primed MSCs [61, 109]. Further detailed investigations on this topic are required [61, 110, 111].

To summarize, DFO-induced hypoxia affects minor MSC metabolic changes compared to hypoxia. Up to now, detailed metabolome studies have been done only for DFO. Metabolome studies of other hypoxia-mimetic agents are needed to understand the mechanism of their actions and possible short- and long-term side effects.

(3) MSC’s Gene Expression. All hypoxia-mimetic agents discussed here increase the expression of HIF-1α, the central controller of adaptive cellular response to hypoxia, and enhance glycolysis similarly to hypoxia [4, 53, 61, 98, 99]. DFO upregulates the genes related to glycolysis: hexokinase 2 (HK2), PDK-1, BCL-2 interacting protein 3 (BNIP3), and
Table 4: Effect of hypoxia pre/treatment on MSC differentiation.

| O₂ concentration | Treatment conditions | Time/passage | Type of stem cells | The effect compared to normoxia (methods of analysis) | Ref. |
|------------------|----------------------|--------------|-------------------|-----------------------------------------------------|-----|
| 1%               | Pretreatment for 2 d  | rBM-MSC      | Osteogenesis increased (ALP activity, 7 d; Alizarin Red S, 21 d) | [80] |
| 1%               | Pretreatment for 3 d  | hBM-MSC      | Osteogenic potential of MSC maintained, for high-density spheroid osteogenic potential enhanced; increase in the ALP activity related to the spheroid cell density (ALP staining after 14 d), osteocalcin level maintained (ELISA) | [89] |
| 1%               | Pretreatment for 14 d | hBM-MSC      | Adipogenic differentiation impaired (Oil Red staining, 14-20 d), osteogenic potential reduced (Alizarin Red staining, 21 d, calcium precipitates detected) | [58] |
| 1%               | Pretreatment for 21 d | hAD-MSC      | Chondrogenic potential increased (Alcian Blue staining, 21 d); high expression of the SOX9 and COL2A1 genes (RT-PCR), osteogenic potential slightly reduced (Von Kossa staining, 21 d, manual counting of calcified areas); lower expression of ALP and unchanged OPN (RT-PCR), adipogenic potential slightly reduced (Oil Red staining, 21 d); lower expression of the ADPN and LPL genes (RT-PCR) | [62] |
| 1%               | Treatment for 9 d    | Coclure of hBM-MSC and HUVEC | Osteogenic potential maintained (Alizarin Red stain, 9 d), expression of the osteogenic RUNX2 and ALP genes retained and upregulation COLIA1 (qRT-PCR) | [52] |
| 1.5%             | Pretreatment for 1 d | BM-MSC       | Osteogenic potential (Von Kossa staining, 14 d) and expression of the RUNX2 gene retained (RT-PCR), adipogenic differentiation potential retained (Oil Red staining, 21 Coll type II d) and its corresponding marker gene PPAR-α (RT-PCR), chondrogenesis (Coll type II detection, 21 d), and expression of the SOX9 gene increased (RT-PCR) | [82] |
| 2%               | Pretreatment for 7 d | hAD-MSC      | Osteogenesis enhanced (Von Kossa staining, 22 Coll type II d), adipogenesis enhanced (Oil Red staining, 21 Coll type II d) | [114] |
| 2%               | Pretreatment for 8 d | mAD-MSC      | Early chondrogenesis increased (Alcian Blue staining, sGAGs assay kit, 6 d), osteogenesis after 7 d maintained (ALP activity, colorimetric assay), after 21 d—decreased (Alizarin Red staining) | [116] |
| 2%               | Pretreatment at passage 2 and/or treatment for 21 d | hBM-MSC | Osteogenic potential reduced (Alizarin Red staining, 21 d), osteogenic potential reduced due to hypoxia pretreatment in cells grown in normoxia and hypoxia conditions | [71] |
| 2%               | Treatment for 14 d  | hBM-MSC      | Osteogenic potential maintained (Von Kossa staining and ALP activity, 14 d), adipogenic potential maintained (Oil Red staining, 14 d) | [63] |
| 2%               | Treatment for 14 d  | hBM-MSC      | Osteogenic potential preserved (Von Kossa staining, 14 d), the above capacities preserved up to the seventh passage | [38] |
| 2%               | Treatment for 14-17 d|hWJ-MSC       | Chondrogenic potential maintained (Alcian Blue staining, 14-17 d) | [64] |
| 2%               | Treatment for 18 days|hWJ-MSC       | Adipogenic potential increased (Oil Red staining, 18 d) | [63] |
| 2%               | Treatment for 21 days|hWJ-MSC       | Adipogenic potential preserved (Nile Red staining, 21 d) up to the seventh passage | [38] |
| 2%               | Treatment for 21 days|hWJ-MSC       | Osteogenic potential increased (Von Kossa staining, 21 d) | [64] |
| 2.5%*            | Pretreatment for more than 3 days* | hUC-MSC  | Osteogenic (Alizarin Red staining, 21 d), adipogenic (Oil Red staining, 21 d), and chondrogenic (Alcian Blue staining, 21 d) potential preserved | [84] |
| 3%               | Pretreatment for 7-10 d and/or treatment for 21 d | hBM-MSC | Both hypoxia pretreatment and hypoxic treatment during differentiation preserve osteogenic (Alizarin Red staining, 21 d) and adipogenic (Oil Red staining, 21 d) potential for primary cells only, diminished adipogenesis and inhibited osteogenesis with increase in the passage number from 1 to 4, the genes ALPL, IBSP, FABP4, and LPL downregulated (qRT-PCR) with increase in the passage number from 1 to 4, osteogenic differentiation (passage 1) stimulated upon transition from 3 to 20% O₂ | [33] |
| O₂ concentration | Treatment conditions | Type of stem cells | The effect compared to normoxia (methods of analysis) | Ref. |
|------------------|----------------------|-------------------|------------------------------------------------------|-----|
| 1-3%             | Pretreatment for 16 h | hBM-MSC           | Osteogenic (Alizarin Red staining), adipogenic (Oil Red staining), and chondrogenic (Alcian Blue staining) differential potential maintained | [81] |
| 1-5%             | Treatment for 21 d    | hBM-MSC           | Osteogenic differentiation reduced (Alizarin Red assay; 7, 14, and 21 d), ALP and OPN expressed at low levels below 5% O₂ (WB) | [125] |
| 5%               | Pretreatment at passages 2-4 and/or treatment for 21 d | hWJ-MSC           | Osteogenesis (Von Kossa staining, 21 d) and cartilage differentiation (Masson’s trichrome staining, 21 d) maintained at the same level, hypoxic/normoxic pretreatment and treatment did not affect MSC differentiation potential | [126] |
| 5%               | Pretreatment up to passage 2 and/or treatment for 28 d | hBM-MSC           | Osteogenic potential (Alizarin Red staining, 28 d), and the expression of the ALPL and RUNX2 genes increased (RT-PCR), adipogenic potential (Oil Red staining, 28 d) increased; the expression level of the LPL and PPARγ genes maintained (RT-PCR), both hypoxia pretreatment and hypoxic treatment increased differentiation potential | [66] |
| 5%               | Treatment for 14 d    | hBM-MSC           | Osteogenic potential (ALP activity, 14 d) and the BGLAP, RUNX2, and COL2 gene expression increased (RT-PCR) | [65] |
| 5%               | Treatment for 21 d    | hBM-MSC           | Adipogenesis reduced (Oil Red staining, 21 d) | [65] |
| 5%               | Treatment for 28 d    | hBM-MSC           | Chondrogenesis reduced (Alcian Blue staining, 28 d) | [65] |
| 8%               | Treatment for 8 d     | mBM-MSC           | Adipogenesis increased after 8 d (Sudan Black staining) | [123] |
| 8%               | Treatment for 14 d    | mBM-MSC           | Oct4 inhibited (qRT-PCR) | [123] |

*Hypoxic preconditioning in 2.5% O₂ for 15 minutes, then reoxygenation at 21% O₂ for 30 minutes, and again hypoxia preconditioning at 2.5% O₂ for 3 days; d: day/days; h: human; m: mouse; r: rat.*
**Table 5: Effect of pharmaceutically and chemically derived hypoxia pretreatment on MSC differentiation.**

| Treatment conditions | Stem cell type | The effect compared to normoxia (methods of analysis) |
|----------------------|----------------|------------------------------------------------------|
| DFO/3 μM Treatment for 4 d | hBM-MSC | Osteogenic (Alizarin Red staining, 14 d) and adipogenic (Oil Red staining, 14 d) potential decreased [61] |
| DFO/15 μM Treatment for 7 d | hBM-MSC | ALP increased (WB, 7 d) [120] |
| DFO/15 μM Treatment for 21 d | hBM-MSC | Osteogenic potential increased (Alizarin Red staining, 21 d), ALP, RUNX2, and OC upregulated (qRT-PCR), osteogenesis through β-catenin signaling increased (WB) [120] |
| DFO/50 μM Treatment for 21 d | hBM-MSC | Chondrogenesis (Alcian Blue, 21 d) and SOX9 expression (RT-PCR, 7 d) slightly increased [124] |
| DFO/120 μM Treatment for 8 d | mBM-MSC | Adipogenic potential preserved (Sudan Black staining, 8 d), Oct4 expression maintained (qRT-PCR) [123] |
| DFO/120 μM Treatment for 21 d | hUC-MSC | Osteogenic (Von Kossa staining, 21 d), and adipogenic (Oil Red staining, 21 d) potential preserved [97] |
| CoCl₂/100 μM Pretreatment for 1-2 d | mC3H/10T1/2-MSC | Chondrogenesis (Alcian Blue, 28 d) in AD- and UC-MSC increased, in DP-MSC—not detected, cell-source dependent changes of the expression of the following genes: COL2A1, ACAN, SOX9, and VCAN (RT-PCR): DP-MSC—SOX9 (after 7 d up, and after 14, 21, and 28 d downregulated), VCAN (after 7, 14, 21, and 28 d upregulated), COL2A1 and ACAN not detected [101] |
| CoCl₂/100 μM Pretreatment for 2 d | hDP-MSC, hUC-MSC, hAD-MSC | Osteogenesis (Alizarin Red staining, 18 d) and expression of the Col I, Bglap, and Alp genes (RT-PCR, 10 d) enhanced, adipogenesis (Oil Red staining, 5 d) and expression of the Ap2, Ciecna, and Ppary genes (RT-PCR, 5 d) reduced, chondrogenesis (Alcian Blue staining, 14 d) and expression of SOX9, Col II, and ACAN genes (RT-PCR, 10 d) enhanced [93] |
| CoCl₂/100 μM Treatment for 8 d | mBM-MSC | Adipogenic potential preserved (Sudan Black staining, 8 d), Oct4 expression maintained (qRT-PCR) [123] |
| CoCl₂/100 μM Treatment for 9 d | Coculture hBM-MSC/HUVEC | Osteogenic potential retained (Alizarin Red, nine days), expression of RUNX2, ALP, and COLIA1 maintained (qRT-PCR) [52] |
| CoCl₂/100 μM Treatment for 21 d | hUC-MSC | Osteogenic (Von Kossa staining, 21 d), and adipogenic (Oil Red staining, 21 d) potential preserved [97] |
| CoCl₂/100 μM Treatment for 21 d | hBM-MSC | Chondrogenesis (Alcian Blue, 21 d) and SOX9 expression (RT-PCR 7d) maintained [124] |
| DMOG/200 μM Treatment for 21 d | hBM-MSC | Chondrogenesis (Alcian Blue, 21 d) and SOX9 and RUNX2 expression (RT-PCR, 7 d) increased [124] |
| DMOG/500 μM Pretreatment for 2 d | rBM-MSC | Osteogenesis (Alizarin Red S, 21 d) and ALP activity (7 d) increased [80] |
| DMOG/500 μM Pretreatment for 2 d | rBM-MSC | Osteogenesis (Alizarin Red S, 21 d) and ALP activity (7 d) increased [80] |
| DMOG/500 μM Treatment for 9 d | Coculture hBM-MSC/HUVEC | Osteogenic potential retained (Alizarin Red, 9 d), expression of RUNX2 maintained (qRT-PCR), expression of ALP and COLIA1 increased (qRT-PCR) [52] |

*d: day/days; h: human; m: mouse; r: rat.*

LDHA [61]. DFO upregulates NUPR and pI6 expression, improving cell survival [99]. It also induces an increase in the level of HIF-1α by 50-110% while DMOG elevates HIF-1α level by 2-3 times, which is less than CoCl₂ stimulating HIF-1α by 2-5 times compared to normoxia. ISO demonstrated the highest impact on the HIF-1α expression (a 150-400% increase). Moreover, DMOG via increasing of HIF-1α expression and activation of the phosphoinositide 3-kinases/protein kinase (PI3K/Akt) signaling pathways regulates cell survival and apoptosis [103]. DMOG lowers...
3.3. Diﬀerentiation. This subchapter presents the effects of hypoxia and pharmaceutical/chemical hypoxia-mimetic factors on MSC diﬀerentiation. The ability to the multidirectional diﬀerentiation is a crucial hallmark of MSC. Furthermore, the diﬀerentiation potential and proliferation rate of MSC depend on the type of cells source.

3.3.1. Hypoxia. As described above, stem cells adapt metabolically to hypoxia in vitro [113]. The question is whether they diﬀerentiate equally eﬃciently in hypoxia compared to normoxia. The cells can be grown under hypoxia before induction of the diﬀerentiation process by the appropriate media (a pretreatment), or lower oxygen tension may be maintained in cultures during diﬀerentiation (a treatment). In Table 4, we summarize the available data on the inﬂuence of hypoxia on the fate of MSCs cultured in the growth or diﬀerentiation media.

Hypoxia pretreatment and treatment can maintain or reduce MSC’s osteogenic potential. These eﬀects were observed at the oxygen concentration ranging from 1 to 5% for BM-, AD-, UCB-, UC-, and WJ-MSCs. It may be related to the low expression of the ALP and ALPL genes coding for alkaline phosphatases and the IBSP gene coding for an integrin-binding sialoprotein in AD- and BM-MSCs. However, Boyette et al. noted increased BGLAP, RUNX2, and COL2 in hBM-MSC [65].

Hypoxia pretreatment and diﬀerentiation in low oxygen conditions (1-5% O2) preserve BM-, AD-, UCB-, UC-, and WJ-MSC capability for adipogenic diﬀerentiation [84, 114]. In BM-, AD-, UCB-, UC-, and WJ-MSCs, the expression of the following adipogenic marker genes, lipoprotein lipase (LPL), PPARα, peroxisome proliferator-activated receptors (PPARγ), and fatty acid-binding protein 4 (FABP4), was preserved or even increased.

Nevertheless, inconclusive observations concern the ability to diﬀerentiate into cartilage. Chondrogenic potential might be elevated under hypoxia pretreatment [62, 81, 82, 84, 116] and maintained or reduced during hypoxic

### Table 6: Effect of hypoxia on MSC engraftment, migration, and secretion profile.

| Treatment conditions | Time/passage | Stem cell type | The effect compared to normoxia (methods of analysis) | Ref. |
|----------------------|--------------|----------------|-------------------------------------------------------|------|
| 1% O2                | 1 d          | hBM-MSC        | CX3CR1 and CXCR4 upregulated (qRT-PCR)                | [172]|
| 1% O2                | 2 d          | hBM-MSC        | VEGF secretion in spheroids increased (ELISA) on a rat model, collagen deposition (Masson’s trichrome stain) enhanced, vascularization and bone formation promoted (high-resolution radiographs), and healing after transplantation of primed MSC spheroids improved compared to transplantation of individual cells | [89]|
| 1% O2                | 2 d          | hBM-MSC        | VEGF and NANOG upregulated (qRT-PCR)                  | [57]|
| 1% O2                | 2 d          | hBM-MSC        | VEGF upregulated (RT-PCR), VEGF increased (WB, ELISA) | [80]|
| 1% O2                | >2 d*        | mBM-MSC        | Cxcr4 downregulated (qRT-PCR)                         | [143]|
| 1% O2                | 10 d         | hBM-MSC        | VEGF and NANOG upregulated (qRT-PCR)                  | [57]|
| 1% O2                | 14 d         | mBM-MSC        | On a myocardium infarction (MI) mouse model, cardiomyocyte survival reduced due to MCT-4 (WB) increase, and ﬁbrosis in cardiac tissue initiated | [68]|
| 1-3% O2              | 16 h         | hBM-MSC        | Migration potential increased (scratch test)          | [81]|
| 2% O2                | Up to 7      | hBM-MSC        | ECM secretion enhanced (ﬁbronectin and collagen type II ﬂuorescent staining, CLSM), expression of connexin-43 increased (ﬂuorescent staining, CLSM) | [38]|
| 2.5% O2              | >3 d*        | hUCB-MSC       | Migration potential increased                          | [60]|
| 5% O2                | >8 h***      | mBM-MSC        | CXCR4, MMP 9, and 14 increased (WB), after MI treatment on the rat model the left ventricular (LV) ﬁbrosis reduced, improved LV function | [152]|
| 5% O2                | 4 d          | hBM-MSC        | VEGF increased (ELISA)                               | [65]|
| 5% O2                | 10 d         | hBM-MSC        | MMP7-16 and TIMP1-3 upregulated (qRT-PCR)             | [65]|

*Hypoxic pretreatment 4-48 hours at 1% O2 and then reoxygenation 8 hours at 21% O2; **hypoxic preconditioning in 2.5% O2 for 15 minutes, then reoxygenation at 21% O2 for 30 minutes, and again hypoxia preconditioning at 2.5% O2 for 3 days; ***hypoxic pretreatment 8 hours at 5% O2 and then 30 minutes of reoxygenation at 21% O2; h: human; m: mouse; r: rat.
**Table 7: Effect of pharmaceutically and chemically derived hypoxia on MSC engraftment, migration, and secretion profile.**

| Treatment conditions | An agent/ concentration | Time | Stem cell type | The effect compared to normoxia (methods of analysis) | Ref |
|----------------------|-------------------------|------|----------------|-----------------------------------------------------|-----|
| DFO/10 μM            | 2 days                  | hBM-MSC | VEGF upregulated (RT-PCR) | [61] |
| DFO/50-300 μM        | 1 day                   | hAD-MSC | VEGF increased, the higher DFO concentration induced the higher VEGF expression (qPCR) | [102] |
| DFO/60-600 μM        | 20 h                    | hBM-MSC | CX3CR1 and CXCR4 upregulated (RT-PCR), CX3CR1 and CXCR4 increased (WB) | [172] |
| DFO/100 μM           | 1-3 days                | rBM-MSC | Cxcr4 upregulated (RT-PCR), homing capacities in a NIHL rat model enhanced via PI3K/AKT signal transduction pathway (WB) | [100] |
| DFO/100 μM           | 2 days                  | hWJ-MSC | VEGF upregulated (qRT-PCR), mobilization and homing capacities increased | [115] |
| DFO/150 μM           | 1 day                   | hAD-MSC | VEGF increased (ELISA) | [102] |
| CoCl2/50-300 μM      | 1 day                   | hAD-MSC | VEGF increased, the higher CoCl2 concentration the higher VEGF expression in the range of 50-150 μM, at 300 μM slightly dropped compared to VEGF expression at 150 μM (qPCR) | [102] |
| DMOG/500 μM          | 1 day                   | hBM-MSC | VEGF increased (WB), angiogenesis increased (tube formation test in the Matrigel), engraftment ability improved, cardiac function improved (left ventricular ejection fraction evaluation), rat model of MI | [92] |
| DMOG/500 μM +1%O2    | 2 days                  | rBM-MSC | VEGF upregulated (RT-PCR), VEGF increased (WB, ELISA), angiogenic capability increased in vitro and in vivo (tube formation test, Matrigel, rat bone defect model) | [80] |
| DNP/0.25 mM          | 20 min                  | rBM-MSC | The cardiomyogenic genes (Anp, Gata-4, Nkx2.5, Vegf, and Con43) upregulated (RT-PCR); improvement in cardiac function and significant reduction in scar formation in the rat model of MI | [159] |
| DNP*/0.25 mM         | 20 min*                 | rBM-MSC | Igf, Hgf, Vegf, II-7, and II-7r upregulated (RT-PCR) | [94] |
| ISO/2%               | 4 h                     | hBM-MSC | CXCR4 increased (WB), cell migration increased (hematoxylin and eosin staining, cell count) on a rat stroke model, engraftment and recovery improved | [98] |

* 20 minutes of treatment with 0.25 mM and then reoxygenation either 2 hours or 1 day in 21% O2, h: human; r: rat.

Differentiation in WJ- and BM-MSC in 1-2% O2 [64, 65]. The expression of chondrogenic marker genes SRY-box transcription factor 9 (SOX9) and collagen type II alpha 1 chain (COL2A1) followed the above pattern in AD-, BM-, and UCB-MSCs.

Hypoxia pretreatment/treatment influences the MSC differentiation process with effectivity related to passage numbers. In primary cell lines and at low number of passages, MSCs maintain their differentiation potential compared to the cells passaged many times in the in vitro culture under hypoxia [31, 66, 116]. The down-regulation of the FABP4, LPL, ALPL, and IBSP genes accompanied this diminished capacity of MSCs.

Moreover, individual stem cells under hypoxia are characterized by the enhanced level of plasticity-dependent marker genes such as NANOG, REX-1, or Oct4 [117]. The increase in osteogenic potential of individual MSCs was observed compared to monolayer cell culture under normoxia [118]. Oct4 is an essential transcription factor for self-renewal, and it is present in MSCs at low levels on each passage (the higher passage number, the lower Oct4 level). Improved stemness due to higher expression of Oct4 can result in increased differentiation potential of hypoxia primed stem cells [89, 119].

### 3.3.2. Pharmacological and Chemical Hypoxia-Mimetic Agents

According to Table 5, the DFO-derived hypoxia treatment during differentiation preserves osteogenic potential and the level of its corresponding marker genes ALP and Runt-related transcription factor 2 (RUNX2).

DFO treatment maintains or reduces adipogenic potential while increasing chondrogenesis and the expression of SOX9. These effects were observed in BM- and UC-MSC after 14-21 days of treatment [61, 97, 120, 121].

CoCl2-derived hypoxia pretreatment increased osteogenesis and upregulated the Alp, Col1, and osteocalcin (Bglap) genes while treatment during differentiation maintained osteogenic potential and the expression of RUNX2, ALP, and COL11. These effects were observed on mC3H/10T1/2 MSCs and UC-MSCs for 1-9 days [52, 97, 101]. Murine C3H10T1/2 cells are embryogenic cells with features of mesenchymal stem cells and thus represent interesting research objects. They have the potential to be an attractive alternative source of primary BM-MSCs in studies of
osteogenic and chondrogenic differentiation for regenerative medicine [122]. CoCl₂-derived hypoxia pretreatment decreases adipogenesis and the marker genes Apetala 2 (aP2), CCAAT/enhancer-binding protein α (C/ebpα), and Pparγ in mC3H/10T1/2 MSC for 24-48 hours. On the opposite, CoCl₂-derived hypoxia treatment maintained adipogenicity in BM-MSCs for eight days [101, 123]. CoCl₂-derived hypoxia pretreatment and treatment increase chondrogenesis and the expression of the chondrogenic marker genes SOX9, Coll2a1, VCAN, and aggrecan (ACAN) in mC3H/10T1/2, BM-, UC-, AD-, and DP-MSCs for 2-21 days [53, 101, 124].

DMOG-derived hypoxia treatment of BM-MSCs maintained osteogenesis and RUNX2 expression and upregulated ALP and COL11A1 for nine days [52] as well as chondrogenesis and SOX9 marker for 21 days [124].

DMOG-derived hypoxia treatment of BM-MSCs maintained osteogenesis and RUNX2 expression and upregulated ALP and COL11A1 for nine days [52] as well as chondrogenesis and SOX9 marker for 21 days [124].

Unfortunately, up to date, no data on the influence of DNP or ISO on MSC differentiation are available in the literature. Concerning the published results, it may be assumed that the effect of pharmaceutical/chemical hypoxia-mimetic agents on MSC differentiation is similar to hypoxia. However, the period of this enhancement has not been studied yet. The currently available scientific data also do not allow concluding whether hypoxia-inducing chemical agents could efficiently reduce the time required for cell differentiation.

3.4. Engraftment, Migration, and Secretion Profile. Successful MSC engraftment is crucial in regenerative medicine. The high proliferation rate and prominent expression of chemokine receptors on MSCs are attributed to young cells providing migration and potential therapeutic increase after transplantation [45].

The latest data indicate that chemokines and their receptors are critical in migration, chemotaxis, and homing in vitro and in vivo [130]. There are different BM-MSC-
related chemokine receptors, such as CXC, but insufficient data are available on their function in cell therapy [131]. The rat brain ischemia model shows that chemokines C-C motif chemokine ligand 2 (CCL25) and C-X-C motif chemokine ligand 1 (CX3CL1) can also influence MSC chemotaxis [131]. Moreover, CC-type chemokines are involved in cellular implantation and remodeling following transplantation [130].

HIF-1α causes upregulation of chemokine receptors on MSCs [132]. Under hypoxic conditions, the stabilized HIF-1α is shifted into the nucleus to bind the HIF-1β forming heterodimer. Subsequently, the heterodimer attaches to hypoxia response elements (HREs) linked with CREB-binding protein/p300 protein (CBP/p300) [133, 134] and increases the expression of chemokine receptors C-X3-C motif chemokine receptor 1 (CX3CR1), C-X-C chemokine receptor type 7 (CXCR7), and C-X-C motif chemokine receptor 4 (CXCR4). Hypoxia can increase CXCR4 expression [135]. Hypoxia-induced upregulation of CXCR4 may result from HIF-1α stabilization [136]. Metabolic flexibility is one of the features represented by MSCs, helping them survive under ischemic stress and maintaining their multipotency [137]. HIF-1α is one of the master regulators controlling the cellular response to the tension caused by low oxygen levels [138].

HIF-1α is also involved in the CXCR4 expression induced by the activation of HREs in the Et1 promoter, a transcription factor of CXCR4. Changes in the oxygen level are an essential regulator of CXCR4 expression. Hypoxia stabilizes CXCR4 transcripts, contributing to an increase in the CXCR gene expression. It suggests that hypoxia-regulated RNA binding proteins could influence CXCR4 stabilizing its mRNA at the posttranscriptional level [139].

Angiogenesis is vital in tissue engineering because of tissue blood flow restoration and new blood vessel formations [140]. Proangiogenic factors (VEGF and matrix metalloproteinases (MMPs)) and antiangiogenic factors (endostatin and tissue inhibitor of metalloproteinases (TIMPs)) are involved in angiogenesis regulation [141]. Applications of proangiogenic proteins in stroke and myocardial infarction treatment have been reported [142].

3.4.1. Hypoxia. As shown in Table 6, hypoxia increases MSC migration via upregulation of chemokine receptors CXCR1 and CXCR4.

This effect was observed in the O2 concentration ranged from 1 to 5% when BM- and UC-MSC were grown for 8-48 hours [60, 81]. The CXCR4 gene expression decreased in C57BL/6 murine BM-MSCs exposed to acute hypoxia compared to normoxia. The reduction of the CXCR4 gene expression could result from the long-term culture of cells in normoxia followed by acute hypoxia shock. In the next step, MSC reoxygenation after hypoxia led to the CXCR4 gene expression decreasing. The reduction of the CXCR4 gene expression during the second stage of reoxygenation could have been caused by the compatibility of cells to new oxygen conditions—hypoxia following the suppressive effect of normoxia on the CXCR4 promoter [143].

Hypoxia also increases the angiogenic capacity of MSCs. This effect might be observed upon O2 concentration ranging from 1% to 5% after incubating BM-MSCs for 2-4 days [57, 80, 89]. The VEGF gene expression increased under hypoxic conditions [119, 144]. VEGF and Angiopoietin 1 (Ang-1) play a crucial function in angiogenesis, and their increase is essential for successful stem cell transplantation [102, 145]. Decrease of high mobility group box protein 1 (HMGB1) nuclear protein under hypoxia is believed to protect tissue from damage [80]. MSC’s spheroids promote vascularization and bone formation [89].

Cell migration, vascularization, and tissue remodeling in bone are MMP/TIMP dependent. The family of TIMP proteins controls MMP’s function. MMP-2, MMP-9, MMP-13, and TIMP-1 are crucial in bone formation and repair [65]. MMP-9 and MMP-13 are involved in the recruitment and activation of osteoclasts [146–148]. MMP-2 is essential for generating spatial osteolytic structures and mineralization [149]. A loss of its function can disrupt proliferation and osteoblastic differentiation, disturbing skeletal development [150], and mutation in the MMP-2 gene might cause bone diseases [151]. Hypoxic preconditioning showed upregulation of many MMP and TIMP genes in 5% O2 up to ten days in BM-MSCs [65, 152]. Long-term hypoxic cultivation upregulates MMP7-16 and TIMP1-3 but downregulates MMP2. There are few experiments on this topic, but it requires further investigation.

Heart damage is one of the common diseases of modern civilization [153]. Cardiomyocytes, endothelial cells, fibroblasts, and perivascular cells are crucial in heart homeostasis. Transplantations of two cell types, cardiomyocytes (CMs) and vascular cells, exhibited better therapeutic effects in infarcted hearts [154]. Moreover, the coculture of myocytes with endothelial cells enhances myocytes’ survival in vitro[155]. New, more efficient strategies are still needed. Mathieu et al. noted that hESC could reenter pluripotency under hypoxia conditions, and this dedifferentiation depends on HDAC activity [156]. The iPSC research seems to be very promising, as it does not raise ethical questions such as the hESC [157]. Practical methods for differentiating murine iPSC-derived cardiomyocytes, combining hypoxia and bioreactor controlling culture conditions, have already been described [158]. Extracellular vesicles (EVs) are attracting the attention of researchers because of their ability to mimic all the therapeutic effects induced by the MSCs (e.g., anti-inflammatory, proangiogenic, or antiﬁbrotic) [159]. Thus, MSC-derived EVs can modulate tissue response to a broad spectrum of injuries [160] and are considered a substitute for cell-based therapies. The clinical studies using exosomes in the treatment of cardiovascular disease are at an early stage [161–163]. For example, the exosomes derived from BM-MSCs [161, 163] or umbilical cord- (UC-) MSCs [164] showed the positive inﬂuenced cardiac function (preclinical model of MI) [165]. Hypoxia and DFO preconditioning of MSC for EV delivery is the developing strategy for regenerative medicine [166, 167].

3.4.2. Pharmacological and Chemical Hypoxia-Mimetic Agents. As shown in Table 7, pharmaceutical/chemical hypoxia-mimetic agents can improve the migration and angiogenic capabilities of MSCs.
An increase in migration was observed after BM- and WJ-MSC incubation with DFO and ISO for 4-72 hours [98, 100, 115]. The increased expression of VEGF was noted in WJ-, AD, and BM-MSCs after their treatment with DFO, DMOG, or DNP for 20 min and 48 hours [94, 115, 168]. Preconditioning of MSC with DMOG was applied in the harvesting of cells for application in the treatment of heart ischemia [4, 92], cartilage regeneration [124], and bone regeneration in an aged population [45, 80]. DNP has already been used as a hypoxia-mimetic agent on numerous cell types such as neonatal cardiomyocytes, neurons, H9C2, and embryonic cardiac cells [169–171]. Preconditioning of stem cells with DNP improved their adhesion, survival, homing capacities, and cardiomyogenic genes such as Gata-binding protein 4 (Gata-4), NKX2 homeobox 5 (Nkx2.5), Connexin 43 (Con43), atrial natriuretic peptide (Anp), and Vegf [168]. MSC priming with DNP was used in the myocardium regeneration process [94] and improved cardiac function [168]. Similarly, preconditioning of MSCs with ISO improved their migration and engraftment into the ischemic brain (the rat model of stroke) [98].

Hypoxia increases migration and vascularization of MSCs and protects them against apoptosis. It was revealed that pharmaceutical/chemical hypoxia-mimetic agents stronger enhance the expression of chemokine receptors and VEGF than hypoxia. The exact effect depends on the hypoxia-mimicking agent. Moreover, chemokine receptor studies were performed only for DFO and ISO. There is no data about the influence of other hypoxia-derived agents on chemokine expression. Moreover, there was no information about essential proteins and MMP/TIMP changes upon treatment of MSCs with hypoxia-mimetic agents.

4. Conclusions

Clinical applications of MSCs gave insufficient effects due to low survival, retention, or the insufficiency of cell differentiation. Hypoxia conditions mimic the natural tissue environment preserving embryonic development and the pluripotency of stem cells and enhancing angiogenesis. The knowledge on MSC priming is critical in evaluating safety procedures and potential use in clinics. Hypoxia preconditioning in vitro uses 2-5% oxygen concentration. It preserves MSC's differentiation potential, upregulates chemokine receptors, and delays cell senescence in a source-dependent manner. There are clear pieces of evidence that both hypoxia pretreatment and treatment are beneficial for MSC differentiation. Hypoxia priming has been proved as a practical approach for ischemic stroke and other disability treatment.

A growing group of pharmaceutical/chemical hypoxia-mimetic agents concur with hypoxia chambers and incubators, acting similarly according to the current knowledge (Figure 1). Pharmaceutical/chemical hypoxia-mimetic agents can also increase cell proliferation, preserve or enhance differentiation potential, increase migration potential, and induce neovascularization in a concentration- and stem cell source-dependent manner. According to the current knowledge, they act via upregulation of HIF-1α, leading to changes in the metabolism, e.g., increasing glycolysis. Pharmaceutical/chemical hypoxia-mimetic agents might find several applications in human medicine. DFO can be used in the general preconditioning of stem cells in regenerative medicine (due to contrary data on osteoblastic differentiation, its application in bone regeneration requires further investigation). CoCl2 is proposed for cartilage regeneration. DMOG has been applied in myocardial infarction, ischemic heart, brain, and bone regeneration in the aged population. Moreover, it is a better candidate for cartilage tissue regeneration compared to DFO and CoCl2. DNP is believed to promote cardiac regeneration, and ISO can be used in ischemic brain treatment.

However, current literature still shows certain contradictory data on the influence of hypoxia on MSC functions. This phenomenon stems from differences in the protocols used, culture conditions, media composition, hypoxia conditions and timing, and the heterogeneity of cell donors. At least on some hypoxia inducers, our knowledge of the mechanisms is not sufficiently comprehensive, affecting their potential use. Up to now, DFO is the most studied agent for MSC priming and seems to be a quite safe choice. Metabolome changes in DFO-derived hypoxia are less harmful to MSCs compared to hypoxia. Many new hypoxia-mimetic agents have not yet been fully characterized. One of these agents is DMOG, which is going to have great potential in MSC preconditioning.

DFO and hypoxia-mimetic agents in optimized treatment conditions can improve MSC lifespan and maintain or increase their differentiation potential, migration, and immunomodulatory properties for successful engraftment in a hypoxia inducer concentration-dependent manner. The optimal culture conditions and pharmaceutical/chemical agent concentration should be optimized for priming stem cells to translate the results from in vitro effectiveness to in vivo conditions.

To summarize, preconditioning using DFO and other pharmacological/chemical hypoxia-mimetic agents positively affects MSC viability and other properties. They have not been studied so wildly as hypoxia but are believed to find application as pretreatment for many diseases considering their low cost and ease of use.

Abbreviations

AD: Adipose-derived
α-KG: Alpha ketoglutarate
ALP: Alkaline phosphatase
ANP: Atrial natriuretic peptide
aP2: Adaptor protein 2
BAX: BCL-2-associated X
BCL-2: B-cell lymphoma 2
bFGF: Basic fibroblast growth factor
BGLAP: Bone gamma-carboxyglutamic acid-containing protein (osteocalcin)
BM: Bone marrow
CASP-3: Caspase 3
CD: Crohn’s disease
C/EBPα: CCAAT enhancer binding proteins
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