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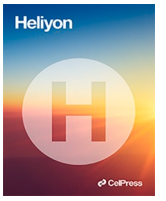

Research article

Effect of hypoxia on proliferation and differentiation of induced pluripotent stem cell-derived mesenchymal stem cells

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ABSTRACT

Although mesenchymal stem cells (MSCs) are extensively applied in the regenerative field, the majority of MSCs die after a few weeks of transplantation. Therefore, hypoxia pre-conditioning is a crucial step in increasing the MSCs' tolerance to physiological conditions. Meanwhile, induced pluripotent stem cell-derived MSCs (iMSCs) were proposed as a possible alternative to MSCs, and recently, the interest is growing in applying iMSCs in the regenerative field. This study examined the effect of hypoxia pre-conditioning on the proliferation, viability, and differentiation of iMSCs. Both iMSCs and MSCs were subjected to two rounds of severe short-term hypoxia (1 % O_2 for 24h). After that, iMSCs and MSCs were characterized by testing their surface markers' expression, proliferation, viability, oxidative stress, and differentiation potential. Our findings revealed that hypoxia did not have a consistent effect among all the analyzed lines: the severe short-term hypoxia (1 % O_2) reduced iMSCs proliferation, cell viability, and MMP while showing a benign effect on surface markers expression, colony formation, ROS accumulation, and osteogenic and adipogenic differentiation. Though hypoxia adversely affected iMSCs' proliferation, this does not necessarily mean that hypoxia is harmful to iMSCs; on the contrary, our results suggest that short-term hypoxia might have a beneficial long-term effect on the proliferation of iMSCs. Thus, the effect of hypoxia on proliferation, viability, and differentiation should also be tested after a long recovery period from iMSCs. Our next step will be to test the effect of hypoxia for a longer period besides uncovering the changes in the expression profile of hypoxic iMSCs.

1. Introduction

Mesenchymal stem cells (MSCs) represent a versatile cell population distinguished by their robust proliferation and capacity for differentiation [1]. These cells possess the remarkable ability to mature into various mesenchymal cell lineages, including adipocytes, osteocytes, and chondrocytes [2–4]. Recent investigations have revealed their potential to transcend lineage commitment, differentiating into cell types derived from ectodermal and endodermal origins [5,6]. Leveraging these properties, MSCs have emerged as promising candidates for cell-based therapies. However, this does not come without limitations, as some practical applications of cell-based therapies, such as stroke treatment, are time-sensitive. Collection procedures for MSCs, whether derived from bone marrow

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or adipose tissue, entail invasive measures. To circumvent these limitations, scientists are directing their focus towards induced pluripotent stem cells (iPSCs), which offer inherent advantages such as unlimited self-renewal and pluripotency [7,8]. iPSCs harbor the potential to differentiate into virtually any cell type, including mesenchymal stem cells (iMSCs), presenting a continuum of benefits such as an infinite supply, reduced risk of immune rejection, and personalised generation tailored to individual patients [9–11].

iMSCs align with the standard criteria the International Society of Cellular Therapy (ISCT) outlined for defining MSCs. They exhibit hallmark characteristics like adherence to plastic surfaces and expression of specific CD markers (CD73, CD90, CD105, and CD44), alongside the capacity for multilineage differentiation [9,12–14]. Moreover, recent endeavours have showcased the therapeutic potential of iMSCs in various applications, from ischemic stroke treatment to cancer cell inhibition and wound healing promotion [15,16].

Hypoxia, characterized by oxygen depletion within a microenvironment, plays a pivotal role in tissue development and stem cell behavior. Studies underscore the significance of hypoxia-inducible factors (HIFs) in orchestrating tissue biogenesis under low oxygen conditions [17,18]. Preconditioning MSCs with hypoxia modulates cellular proliferation, differentiation, angiogenic capacity, and homing [15,16,19], yet its impact on iMSCs remains largely unexplored. This knowledge gap may stem from ongoing investigations into the potential of iMSCs or disparities between iMSCs and conventional MSCs.

In our study, we aimed to elucidate the effects of hypoxia on iMSC proliferation and differentiation. We anticipated hypoxia would enhance iMSC proliferation and viability, but its influence on differentiation outcomes remained uncertain. Utilizing a protocol established by Karam et al., we derived iMSCs from iPSCs and subjected them to severe hypoxia conditions [20]. Our investigation encompassed an analysis of proliferation, differentiation, clonogenicity, and viability under hypoxic stress. By understanding how hypoxia impacts iMSC behavior, we aim to advance the utilization of iMSCs in regenerative medicine, thereby enhancing their resilience in vivo environments.

2. Materials and methods

2.1. Ethical approval

This study was approved by the Institutional Review Board (IRB) No: IRB/7/2019 at the Cell Therapy Center (CTC)/University of Jordan.

2.2. Generation of iMSCs

Four iPSC lines previously produced from dermal fibroblasts were used in this study [21]. iPSCs were cultured on Matrigel (Corning)-coated plates and maintained in mTeSR (Stem Cell Technologies). Monolayer iPSC cultures were detached using 0.5 M EDTA (Gibco, USA), and cellular clumps were seeded on ultra-low attachment plates and maintained in MSC differentiation medium to produce EBs. The MSC differentiation media consisted of Minimum Essential Medium Eagle-Alpha Modification (Alpha MEM, Gibco) supplemented with 15 % fetal bovine serum (FBS, Hyclone), 1 % 100X Glutamax (Gibco, USA), and 1 % 100X antibiotic-antimycotic mixture (Gibco, USA). On days 2 and 4 of differentiation, the medium was replaced with fresh medium supplemented with 10 μM RA and 0.1 μM RA, respectively (Sigma-Aldrich). Then, RA was removed on day 6. On day 7, the EBs were moved into matrigel-coated plates and maintained in MSCs differentiation media, which was exchanged every two days. Then, on day 12, the media was supplemented with 2.5 ng/ml basic fibroblast growth factor (bFGF) and exchanged every two days. The iMSCs were passaged once reaching 80–90 % confluency. Then, iMSCs were cryopreserved in FBS containing 10 % DMSO and stored in liquid nitrogen (LN).

2.3. Cell culture and hypoxia

The adipose tissue-derived MSC (ADMSCs) lines of four age-matched donors were previously produced at the CTC and used in this study. iMSCs and ADMSCs were expanded in cell culture media (CCM) aMEM medium supplemented with 10 % FBS, 1 % of 100X Glutamax and 1 % of 100X antibiotic-antimycotic mixture. Cells from both groups were incubated in standard culture conditions at 37 °C and 5 % CO_2 and passaged once they reached 70–80 % with 1X TrypLE (Gibco, USA) for 4–6 min at 37 °C to dissociate the adherent cells.

The iMSCs and ADMSCs cultured on T75 cm^2 were exposed to two cycles of hypoxia for 24 h each, using 1 % O_2 anaerobic sachets 2.5 L (AnaeroGen, Oxoid, UK) in zip lock bags. After each cycle of hypoxia, the CCM was exchanged with fresh media, and cells were left for 24 h before further passaging. Normoxic samples for all cell lines were run parallel to serve as normal controls.

2.4. Flow cytometry

To characterize hypoxic and normoxic iMSCs and ADMSCs, cells were assessed for the absence of three pluripotency markers: Nanog, Tra-1-60 and Tra 1–80, and the presence of hMSCs surface markers; CD90, CD105, CD73 and CD44. Three iPSC lines were used as a positive control for staining the pluripotency markers. Before running the experiment, samples were washed twice with 1X PBS and resuspended in 400 μl staining buffer of 2 % BSA in 1X PBS. Then, each sample was aliquoted in four test tubes containing 100 μl of the cell suspension and 5 μl of the proper antibodies (Supplementary Table 1). The cells were incubated in the dark for 40 min with shaking and then washed twice with 1X PBS. Finally, pellets were resuspended in 200 μl PBS, processed on BD FACS Canto II, and analyzed by BD FACSDiva software.

To assess hMSCs markers, the Stem Flow hMSC Analysis kit (BD Biosciences, USA) consisted of a cocktail of fluorescently conjugated antibodies against MSC-positive markers: CD90, CD105, CD73, and CD44. Their isotype controls were freshly prepared, and the harvested iMSCs and ADMSCs at early passage (passage <8) were resuspended in 800 μl staining buffer. Each sample was aliquoted in four test tubes containing 200 μl of the cell suspension, centrifuged and resuspended with 100 μl of the appropriate antibody cocktail and incubated in the dark for 30 min. After that, cells were washed twice with PBS and resuspended in 200 μl 1X PBS, and samples were assessed using BD FACS Canto II and analyzed by BD FACSDiva software.

2.5. Cell proliferation assays

2.5.1. MTT, real-time Glo MT, and CFU

The difference in metabolic activity between iMSCs and ADMSCs cultured under normoxic and hypoxic conditions was evaluated using MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, ATCC, USA) colourimetric assay. After the second hypoxic exposure, iMSCs and ADMSCs were seeded in three 96-well plates with a seeding density of 7×10^3 cells/well in 100 μl CCM to measure the absorbance after 24, 48, and 72 h. On the day of measurement, 10 μL of MTT reagent were added to each well, and cells were incubated for 3 h at 37 °C. Then, 100 μl solubilization stop solution was added into each well and incubated at 37 °C for 30 min. The absorbance was then recorded at 570 nm on Biotek Cytation 5 and analyzed using Bioteck Gen 5 data analysis software (BioTek, USA). Absorbance was measured after 24, 48, and 72 h.

RealTime-Glo MT assay was used to monitor cell proliferation via RealTime-Glo™ cell viability bioluminescent assay (Promega, USA). iMSCs and ADMSCs on passage five were plated in white surface tissue culture-treated 96-well plates (Costar, Corning, USA) at a seeding density of $5x10^3$ cells/well. Then, the reagent solution was prepared by adding 10 μl of NanoLuc Enzyme and 10 μl of MT Cell Viability Substrate to 5 ml μl of CCM to make 2X concentration. The reagent solution was distributed equally by adding 50 μl in each well and 50 μl of the growth medium to reach a 1X final concentration. Cells were then incubated at 37 °C for 1 h. The luminescence was monitored and recorded at the desired time points (4, 20, 22, 24, 26, 36, 44, 46, 48, 70, 72, 74, 92, 96 and 98 h) using Biotek Cytation 5 and analyzed by Bioteck gen 5 data analysis software (BioTek, USA). The Luminometer wavelength detection range was between 350 and 650 nm.

After the second hypoxic exposure, both iMSCs and ADMSCs were harvested and seeded in duplicates with different seeding densities of 100, 200 and 300 cells/well and incubated for nine days under standard culture conditions and medium exchange every three days. Then, cells were washed with 1X PBS, fixed with methanol for 15 min, and stained with 5 % crystal violet for 5 min. Then, the stained cells were washed twice with distilled water, and the plates were left to air-dry. Colonies of ≥50 cells were counted manually under the microscope. The average number of colonies from duplicate wells was divided by the seeding density to calculate the percentage of cells that formed the colonies.

2.6. Apoptosis

According to the manufacturer's instructions, cell apoptosis was detected using FACS® Annexin V-FITC Apoptosis Detection Kit (R&D systems, USA). Briefly, samples of hypoxic and normoxic iMSCs and ADMSCs were harvested and washed with 1X PBS. Cells were resuspended in 100 μl of Annexin V reagent and incubated in the dark for 15 min. Then, samples were directly assessed using BD FACS Canto II and analyzed using BD FACSDiva software.

2.7. ROS level measurement

According to the manufacturer's instructions, the reactive oxygen species level was detected using the Total Reactive Oxygen Species (ROS) Assay Kit 520 nm (Invitrogen, USA). For each sample, 7×10^3 cells/well were seeded in a black surface tissue culture treated 96-well plates (Costar, Corning, USA) and incubated for 48 h at 37 °C and 5 % CO_2. Then, 1X ROS stain was prepared by adding 10 μl of 500X stock reagent to 5 ml prewarmed serum-free media. The CCM was aspirated, 50 μl of the 1X ROS stain solution was added, and the plates were incubated at 37 °C for 60 min. For control wells, 200 μM tert-butyl Hydrogen Peroxide (TBHP) was prepared in serum-free media and added after the first 30 min. After that, the fluorescence was detected at 488 nm excitation and 520 nm emission on Biotek Cytation 5 and analyzed using Bioteck Gen 5 data analysis software (BioTek, USA).

2.8. Mitochondrial membrane potential (MMP)

Mitochondrial membrane potential was tested using MitoProb™ JC-1 Assay Kit (Invitrogen, USA). For each sample, 7×10^3 cells/well were seeded in black tissue culture-treated 96-well plates (Costar, Corning, USA) and incubated for 48 h. Then, 100 μl of freshly prepared 2 μM JC-1 (5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) in serum-free media were added to all wells and incubated for 60 min at 37 °C and 5 % CO_2. Then 50 μM CCCP (carbonyl cyanide 3-chlorophenylhydrazone) was added to control wells in the last 30 min. Then, cells were washed with PBS, and the fluorescent intensities were measured using Biotek Cytation 5 (BioTek, USA) at excitation 485 nm, emission 528 nm for green J-Monomers, and 535 nm excitation, 590 nm emission to detect Red J-aggregates and images were taken on Bioteck gen 5 data analysis software (BioTek, USA). Finally, the ratio of red/green was measured by dividing the area of red fluorescent regions by the area of green fluorescent regions. Calculations were done using Bioteck Gen5 data analysis software (BioTek, USA).

2.9. iMSCs and ADMSCs differentiation

After the second exposure to hypoxia, the hypoxic and normoxic iMSCs and ADMSCs samples were tested for their ability to differentiate into osteogenic and adipogenic lineages.

2.9.1. Osteogenic differentiation

Cells were harvested and seeded in 6-well tissue culture plates at 200 × 10^3 cells/well seeding density. Cells were maintained in CCM until reaching at least 50 % confluency. Then media was replaced with a complete osteogenic differentiation medium consisting of Minimum Essential Medium Eagle-Alpha Modification (Alpha MEM, Gibco) supplemented with 15 % fetal bovine serum (FBS, Hyclone), 1 % 100X Glutamax (Gibco, USA), 1 % 100X antibiotic-antimycotic mixture (Gibco, USA), 10 mM dexamethasone, 50 μg/ml ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate (Carbosynth, USA) for 21–28 days; until calcium deposition was observed. Control cells were maintained in CCM, and media was exchanged thrice weekly. Differentiated samples were pelleted for further analysis using qRT-PCR or Alizarin Red stain. The red-stained calcium crystals were examined and imaged using the EVOS XL Core Imaging System (Thermo Fisher, USA).

2.9.2. Adipogenic differentiation

iMSCs and ADMSCs were harvested and seeded in 6-well tissue culture plates at a seeding density of 200 × 10^3 cells/well. Cells were maintained in adipogenic differentiation media consisting of Minimum Essential Medium Eagle-Alpha Modification (Alpha MEM, Gibco) supplemented with 15 % fetal bovine serum (FBS, Hyclone), 1 % 100X Glutamax (Gibco, USA), 1 % 100X antibiotic-antimycotic mixture (Gibco, USA), 10 mM dexamethasone, 500 μM 3-isobutyl-1-methylxanthine (IBMX), 0.2 mM indomethacin, and 10 μg/ml insulin for 14–21 days; until fat vacuoles were observed under the microscope. Control cells were maintained in CCM. Media was exchanged every 2–3 days, and when differentiation signs were observed under the microscope, samples were either stained with Oil Red-O stain or harvested for further analysis using qPCR. The red-stained fat vacuoles were examined and imaged using the Thermo Fisher EVOS XL Core Imaging System.

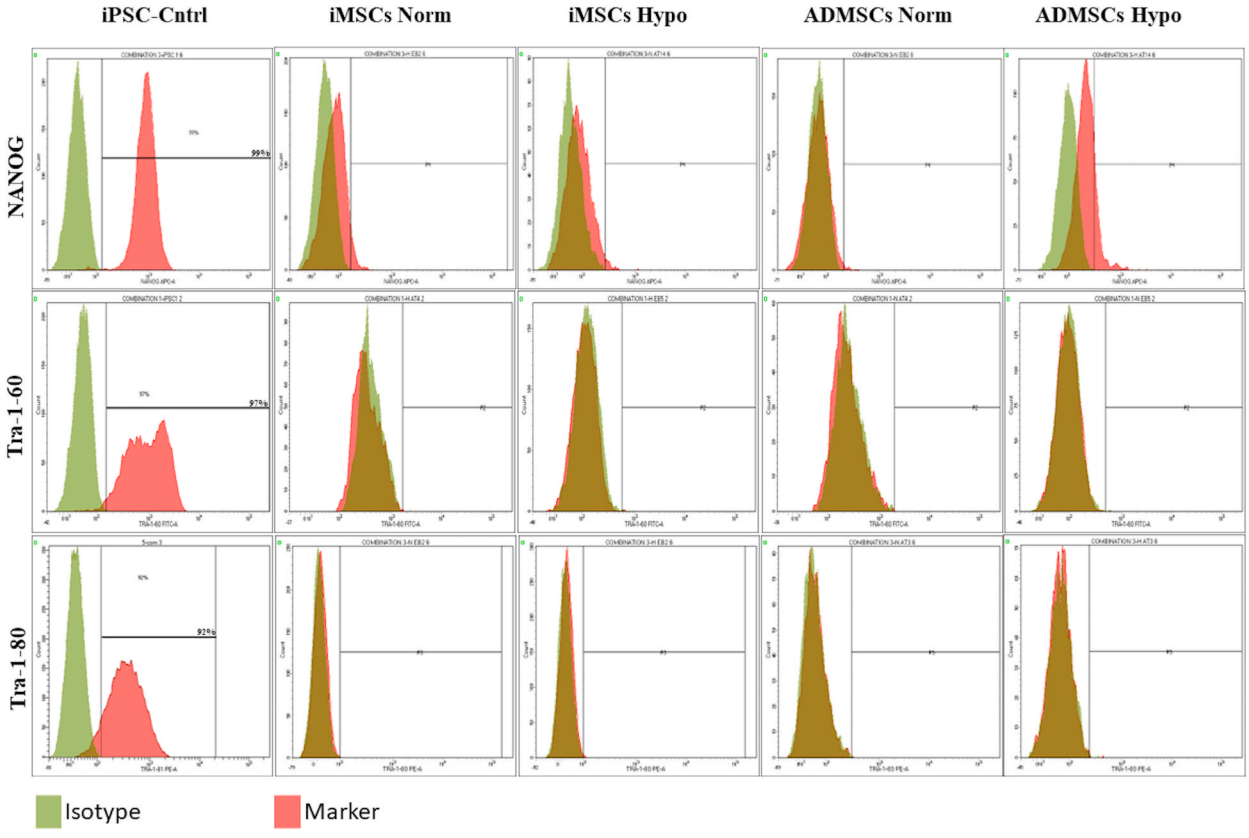

Fig. 1. Flow cytometry analysis of the expression of pluripotency surface markers for the hypoxic and normoxic iMSCs and ADMSCs. Histogram of Nanog, Tra-1-60 and Tra-1-80 flow cytometric analysis in normoxic and hypoxic iMSCs and ADMSC samples compared to control iPSCs.

2.10. Quantitative real-time PCR (qRT-PCR)

RNA extraction was performed using RNeasy® Mini Kit (Qiagen, Germany) following the manufacturer's instructions. RNA concentration was measured on a NanoDrop 8000 Spectrophotometer (ThermoFisher, USA). cDNA was synthesized using 1 μg RNA in 20 μl total volume using PrimeScript™ RT Master Mix (Takara, Japan) according to the following program: 37 °C for 15 min, 80 °C for 5 s, and then at 4 °C until further storage at −20 °C.

Quantitative RT-PCR was performed using TB Green® Premix EX Taq™ II (Tli RNase H Plus) kit (Takara, Japan) in Bio-Rad CFX96 cycler (Bio-Rad, USA). Specific primers amplify HIF-1 α, LPL, PPAR-γ, Adipsin, OCN 2, and RUNX 2. GAPDH was used as a housekeeping gene, and cDNA samples were diluted to 10 ng/μl. Samples were run in triplicates in a 96-well qRT-PCR plate. The cycling conditions were as follows: 95 °C for 30 s, then 40 cycles of 95 °C for 5 s and 60 °C for 1 min, at different annealing temperatures based on the primer set. (All primer sequences and optimal temperatures are described in Supplementary Table 2)

2.11. Statistical analysis

All data were analyzed on GraphPad Prism version 9.1.0 (GraphPad Software, LaJolla, CA, USA). An unpaired student's *t*-test was used for a two-group comparison for the CFU assay. For the rest of the assays, one-way and two-way analysis of variance (ANOVA)

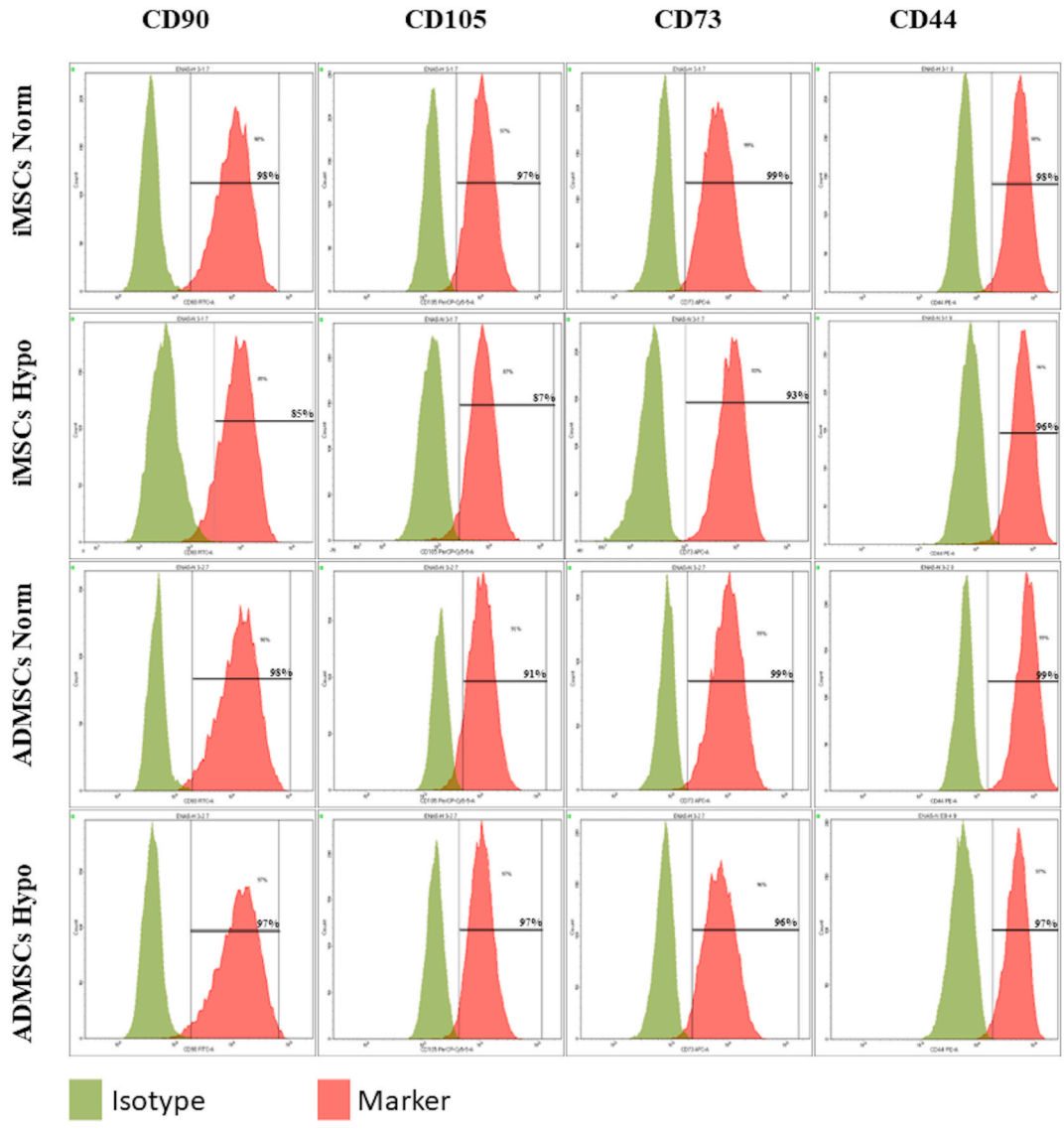

Fig. 2. Flow cytometry analysis of the expression of human MSC surface markers for the hypoxic and normoxic iMSCs and ADMSCs. Histograms of flow cytometric analysis of human MSC surface markers for normoxic and hypoxic iMSCs and the ADMSCs.

were used, followed by the Bonferroni test when indicated. The *p*-value ≤0.05 was considered significant (*≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001).

3. Results

3.1. Characterization of iMSCs and ADMSCs surface markers by flow cytometry

The pluripotency surface markers (Nanog, Tra-1-60, and Tra-1-80) were absent in all iMSC samples, suggesting the successful differentiation of the iPSCs towards iMSCs (Fig. 1). All iMSC samples have successfully expressed -hMSC markers (CD 90, CD105, CD73, and CD44) similarly to the ADMSCs (the control). Hypoxia did not affect the expression of hMSC markers in both iMSC and ADMSC samples (Fig. 2).

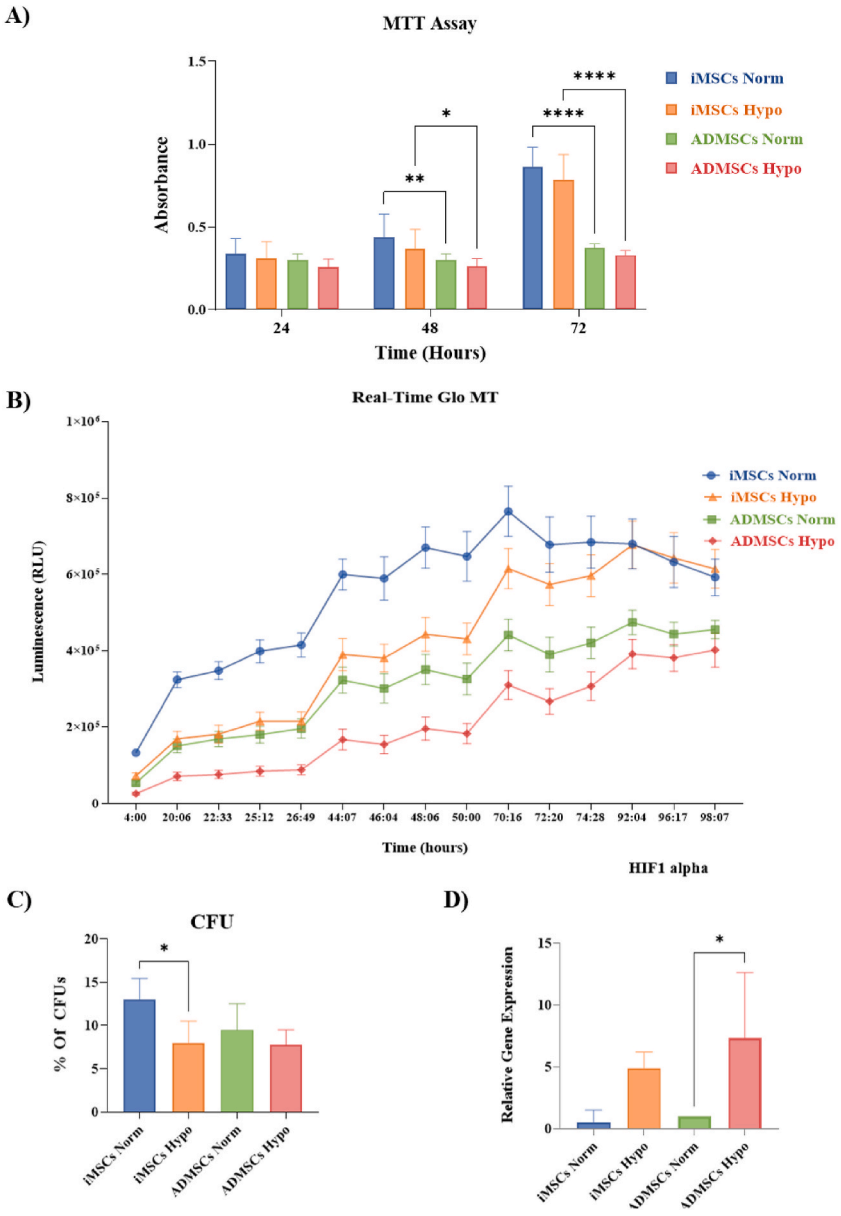

Fig. 3. The effect of hypoxia on cell viability and proliferation. a) Absorbance measurements in normoxic and hypoxic samples of both iMSCs and ADMSCs after 24, 48, and 72 h of cell seeding. **b)** Real-time detection of cell proliferation during 98 h of cell seeding. **c)** The effect of hypoxia on the percentage of clonogenic cells among iMSCs and ADMSCs. **d)** The difference in HIF-1α gene expression between iMSCs and ADMSCs after hypoxia exposure (**p*-value ≤0.05, ** *p*-value ≤0.01, ****p*-value ≤0.0001).

3.2. Cell viability and proliferation

We used the MTT assay to detect the changes in the proliferation rate of all iMSC and ADMSC samples. At 24 h, no significant differences were detected among all samples of iMSCs and ADMSCs. However, at 48 h, we noticed a significant difference between normoxic iMSCs and ADMSCs (p-value $\leq$0.01) and between hypoxic iMSCs and ADMSCs (p-value $\leq$0.05). This difference continued to increase at 72 h until reaching a p-value of $\leq$0.0001 between normoxic iMSC and ADMSC samples and hypoxic iMSC and ADMSC samples. In other words, at 48 h and 72 h, iMSCs samples (normoxic and hypoxic) showed a higher proliferation than ADMSCs (hypoxic and normoxic). At all time points, hypoxia did not affect the proliferation rate of all iMSCs and ADMSCs groups (Fig. 3A).

Although MTT showed that hypoxia did not affect proliferation, real-Time-Glo MT showed a significant reduction in viable cells on multiple levels: the first was between normoxic and hypoxic iMSCs, the second was between normoxic and hypoxic ADMSCs, the third

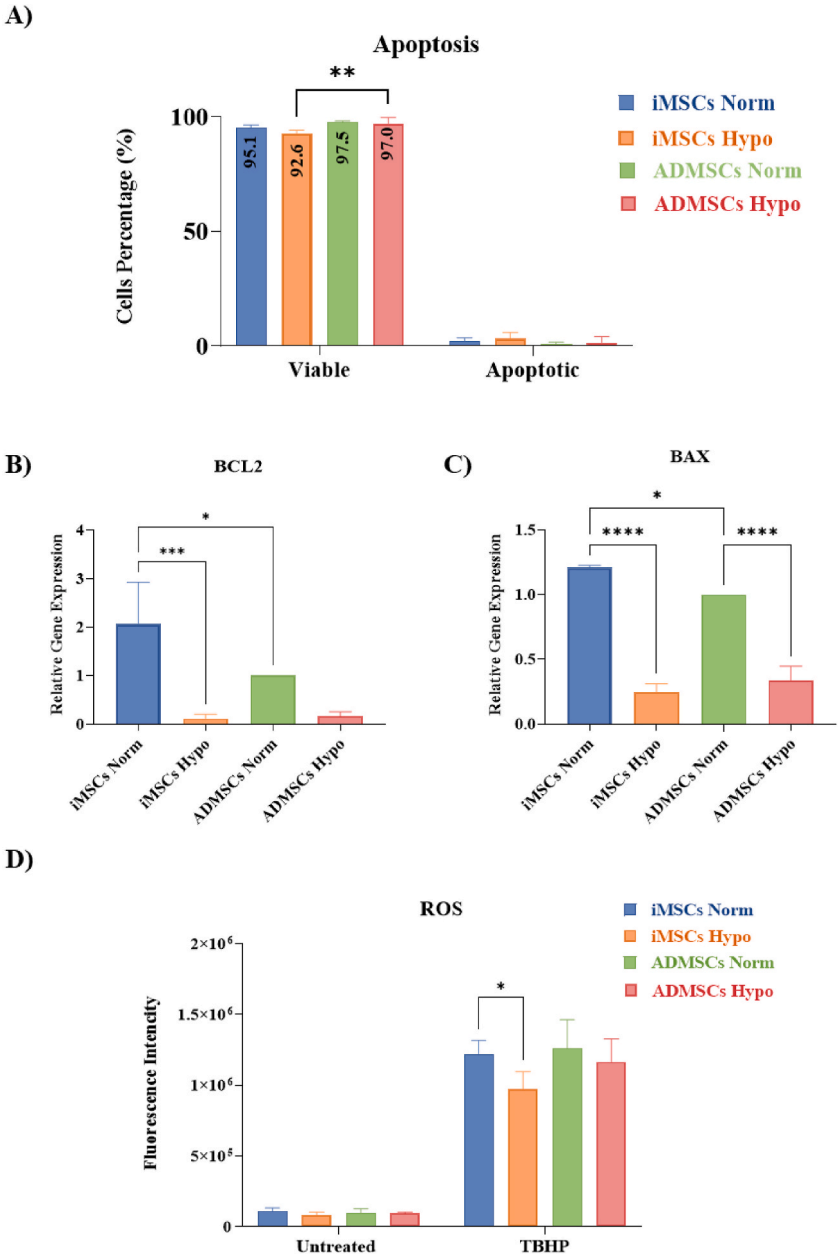

Fig. 4. The effect of hypoxia on apoptosis and ROS production. a) The graph illustrates the percentage of viable and apoptotic cells in hypoxic and normoxic samples of both iMSCs and ADMSCs. **b-c)** The difference in the anti-apoptotic BCL2 gene and pro-apoptotic BAX gene expression in iMSCs and ADMSCs after hypoxic exposure. **d)** Fluorescent intensities of total ROS production in normoxic and hypoxic samples from both iMSCs and ADMSCs. TBHP was used as a positive control to induce ROS production. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$).

was between normoxic iMSCs and ADMSCs, and the fourth was between hypoxic iMSCs and ADMSCs (Fig. 3B). This significant reduction started at 4 h and lasted to 50 h, with a *p*-value ranging between 0.0001 and 0.01. However, the significant difference between hypoxic iMSCs and ADMSCs persisted for a longer time (p-value ≤0.025 at 98 h), while for normoxic iMSCs and ADMSCs, the difference persisted for a slightly shorter time (*p*-value ≤0.01 at 74 h) (Fig. 3B).

The colony-forming ability of iMSC samples was impaired after short-term exposure to hypoxia (p-value ≤0.05) (Fig. 3C). Although the percentage of CFUs in iMSCs was higher than in ADMSCs, the difference remained insignificant (p-value >0.05). Quantitative RT-PCR results showed a significant increase in HIF-1α expression of hypoxic ADMSC samples (*p*-value ≤0.0001), while the increase in HIF-1α expression of hypoxic iMSCs remained insignificant. (Fig. 3D). Though hypoxic ADMSCs showed higher HIF-1α expression than hypoxic iMSCs, the difference was negligible.

3.3. Annexin V flow cytometry detection of apoptosis

Although hypoxia caused a slight reduction in the viability of normoxic ADMSCs and iMSCs, this reduction remains insignificant (*p*-value <0.05)) (Fig. 4A). The viability of hypoxic ADMSC was notably higher than hypoxic iMSC (*p*-value ≤0.01).

qRT-PCR analysis of the anti-apoptotic gene (BCL2) and pro-apoptotic gene (BAX) showed the following results: First, the expression levels of both BCL2 and BAX in normoxic iMSCs were higher than in normoxic ADMSCs (*p*-value ≤0.05). Second, the hypoxic exposure led to a sharp drop in BCL2 expression levels among iMSCs (*p*-value <0.001). Third, the expression of BAX dropped in both iMSCs and ADMSCs after exposure to hypoxia (*p*-value ≤0.0001) (Fig. 4B–C).

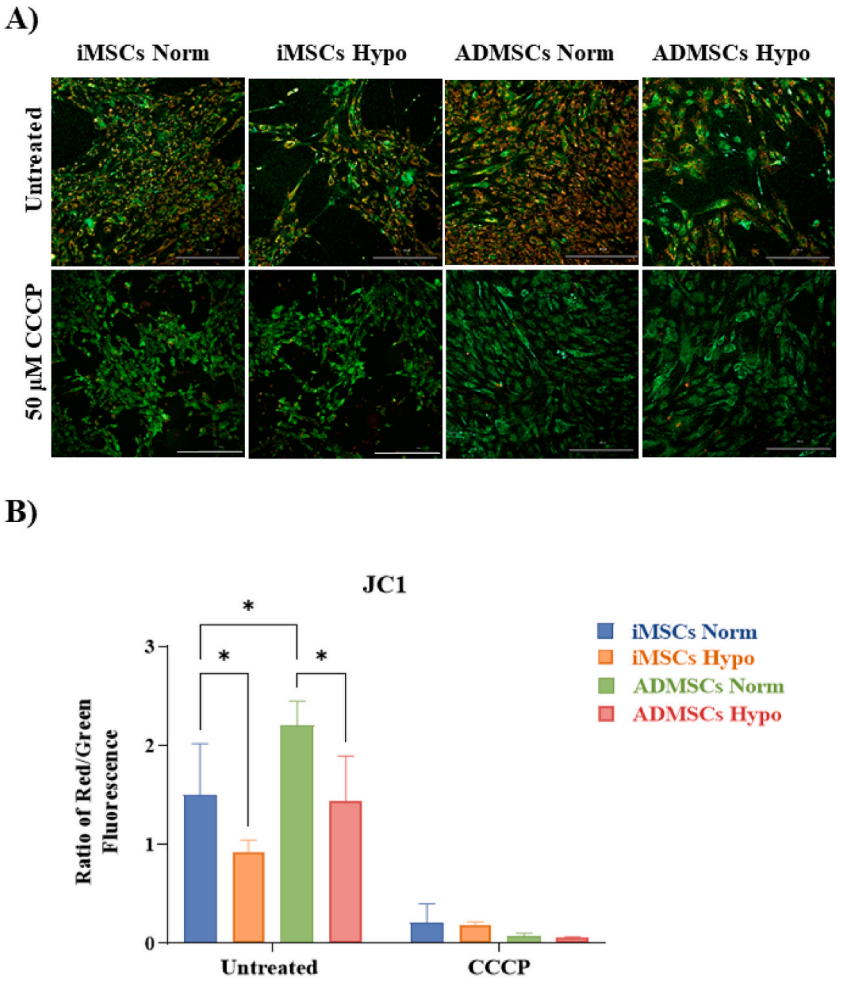

Fig. 5. The effect of hypoxia on mitochondrial membrane potential (MMP). a) Fluorescent images represent the red aggregates (indicating healthy mitochondria) and the green monomers (indicating damaged mitochondria) in all untreated samples compared to control CCCP-treated samples. **b)** The red/green fluorescence ratio in all untreated and CCCP-treated samples. The scale bar was 500 µm, *p ≤ 0.05 *p ≤ 0.05.

3.4. Effect of hypoxia on the induction of reactive oxygen species (ROS)

The fluorescent intensity of total ROS levels was similar in all iMSC and ADMSC groups (hypoxic and normoxic). Hypoxia did not affect total ROS production in iMSCs and ADMSCs (Fig. 4D). However, the hypoxic effect was evident in hypoxic iMSCs treated with TBHP (p-value $\leq$0.05).

3.5. Effect of hypoxia on the mitochondrial membrane potential (MMP)

According to the fluorescent images, normoxic ADMSCs showed abundant red fluorescent aggregates compared to normoxic iMSCs, meaning that ADMSCs had healthier mitochondria compared to iMSCs (Fig. 5A) as revealed by red/green fluorescence ratio, the p-value was less than 0.05 between normoxic ADMSCs and iMSCs (Fig. 5B). Hypoxia reduced the red/green ratio in both iMSCs and ADMSCs (p-value $\leq$0.05).

3.6. Osteogenic and adipogenic differentiation of iMSCs and ADMSCs

Both hypoxic and normoxic iMSC samples could differentiate into osteocytes. Yet, the calcium-deposits areas of the differentiated iMSCs appeared lighter and fewer under the microscope than those of the differentiated ADMSCs. The short-term hypoxia-treated iMSCs and ADMSCs appeared under the microscope with slightly lighter and smaller red-stained areas compared to the normoxic samples of both groups. (Fig. 6A).

However, qRT-PCR analysis showed that iMSC and ADMSC samples (hypoxic and normoxic) exhibited no significant difference in the mRNA levels of the osteogenic-related genes (*RUNX2* and *OCN2*). Also, hypoxia did not significantly alter the mRNA levels of both genes (Fig. 6B–C). iMSCs and ADMSCs maintained in CCM were used as controls for the qRT-PCR test.

As for adipogenic differentiation, iMSC samples had limited fat-stained areas compared to ADMSC samples, indicating the weak adipogenic differentiation ability of iMSCs (Fig. 6D). The qRT-PCR analysis showed a significant variation in the mRNA levels of LPL, adipsin, and PPAR -γ between the iMSC and ADMSC samples, with p-values ranging between 0.0001 and 0.05 (Fig. 6E–G). Short-term hypoxia treatment did not alter the expression of the genes mentioned above in both iMSC and ADMSC samples.

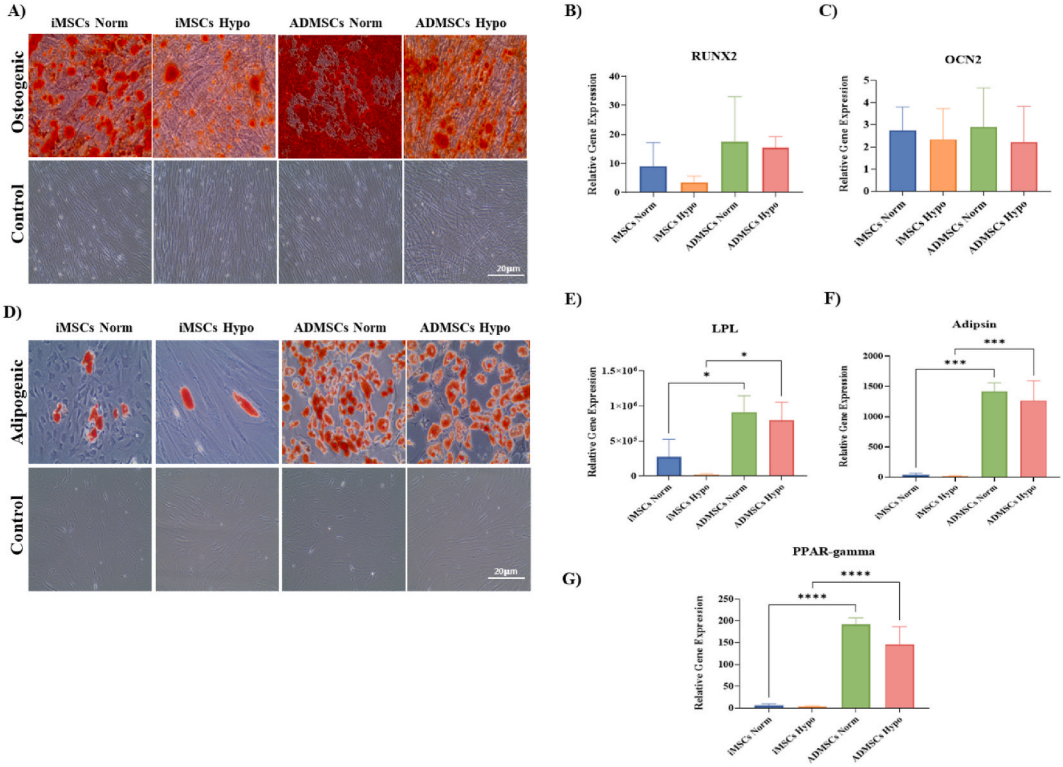

Fig. 6. The effect of hypoxia on osteogenic and adipogenic differentiations of iMSCs and ADMSCs. a) Alizarin Red staining of calcium deposits in the differentiated osteogenic cells. **b-c)** The differences in expression of RUNX2 and OCN2 osteogenic-related genes among the normoxic and hypoxic samples of iMSC and ADMSC groups (p-value >0.05). **d)** Oil Red O staining of fat vacuoles in the adipocyte differentiated cells. **e-g)** The difference in the expression of adipogenic-related genes (LPL, Adipsin, and PPAR-γ) among the iMSC and ADMSC normoxic and hypoxic samples. *p-value $\leq$0.05; ***p-value $\leq$0.001.

4. Discussion

Clinical research has shown that 99 % of transplanted MSCs do not survive physiological conditions in mice after one week of transplantation [22,23]; hypoxia pre-conditioning appealed, which is the answer to this question. Previous studies have shown accumulating evidence of the role of hypoxia in improving stemness, proliferation and differentiation of MSCs [24–26]. Nowadays, iMSCs have gained special interest in regenerative medicine due to their higher proliferation and longer expansion capacity than primary MSCs [27]. However, we believe that iMSCs should also be pre-exposed to hypoxia to increase their tolerance to the physiological conditions in transplanted tissues. In light of that, this study is the first to elucidate the effect of hypoxia on the proliferation, viability, and differentiation of iMSCs.

Several previous studies reported the ability of hypoxia to improve the stemness of ADMSCs by increasing the expression of stem cell markers, including Nanog [28,29]. However, we did not observe any significant difference in the expression of pluripotency markers or the hMSC surface markers of both iMSCs and ADMSCs. Both normoxic and hypoxic iMSCs retained their ability to express hMSC markers in a pattern similar to ADMSCs. Furthermore, the proliferation potential of both iMSC groups (hypoxic and normoxic) exceeded the proliferation potential of both ADMSC groups. This finding is consistent with previous studies in which iMSCs showed a higher proliferation than bone marrow-derived MSCs and equine adipose-derived stem cells (E-ASCs) [30–32]. Our results showed that hypoxia reduced ADMSCs proliferation. At first; we thought this depletion was due to severe hypoxic conditions (1 % O_2) that were used compared to physiological conditions of ADMSCs, which ranged between 3.2 % and 11.3 % [33]. However, a recent systematic review by Garcia et al. concluded that four studies showed that 1 % O_2 enhanced ADMSC proliferation [34–38]. Besides the doner variations, this inconsistency between the findings might be related to the method used for hypoxic treatment and the exposure time to hypoxia.

The real-time Glo MT showed a significant reduction in the proliferation potential of iMSCs following hypoxia. After 50 h, the growth of hypoxic iMSCs increased until it slightly exceeded the growth of normoxic iMSCs. This finding leads us to question whether short-term hypoxia causes a persistent effect on the proliferation of iMSCs that appears in the long run.

The clonogenic potential of iMSCs was higher than ADMSCs among the normoxic and hypoxic groups. Yet, contrary to our expectations, hypoxia significantly reduced the colony-forming ability of iMSCs while causing an insignificant reduction in the percentage of CFUs of ADMSCs. Previous studies demonstrated the efficiency of hypoxia in increasing the clonogenicity of MSCs; at 1 % O_2, ADMSCs had the highest percentage of CFU compared to 5 % and 10 % O_2 [37]. Tamama et al., for instance, reported an increase in MSCs clonogenicity at 1 % O_2 even during the absence of HIF-1α since HIF-1α usually improves the proliferation and clonogenic potentials of MSCs [39].

The HIF 1-α protein is the master regulator of cellular pathways during hypoxia. Hence, HIF-1α regulates the expression of more than a thousand genes related to metabolism, proliferation, differentiation, apoptosis, and more [40,41]. During normoxic conditions, the poorly expressed HIF-1α protein is directly degraded by prolyl hydroxylase (PHD) [42], but during hypoxia, the expression of HIF-1α is upregulated and stabilised. In a recent report, Martinez et al. shared an interesting finding where HIF-1α mRNA levels were considerably higher in short-term hypoxia compared to long-term hypoxia in cancer cells [42]. He explained that although HIF-1α was highly stable and active during long-term hypoxia, chronic oxygen depletion restricts the activation of HIF transcription genes, which ultimately leads to a decrease in HIF-1α mRNA levels [42]. Whether these findings in cancer cells apply to MSCs or not, the expression of HIF-1α was widely reported to be increased under both short-/long-term hypoxia in MSCs [35,36,43], and our study was no exception. However, the increase was insignificant in iMSCs.

Although iMSCs demonstrated better proliferation potential than ADMSCs, our data show that ADMSCs have slightly better, though not significant, viability than iMSCs. HIF-1α regulates apoptosis by interfering in different pathways, including the expression of the apoptotic genes (the anti-apoptotic BCL2 gene and the pro-apoptotic BAX gene). In this study, after hypoxic exposure, HIF-1α reduced the expression of BAX in iMSCs and ADMSCs, however, this reduction in BAX was not accompanied by an increase in viability of both iMSCs and ADMSCs; this suggests a complex interplay of other factors that affect cell viability: reduction in anti-apoptotic proteins like BCL2 and BCL-XL, accumulation of other pro-apoptotic proteins like Bak, activation of non-apoptotic cell death pathways (necrosis or autophagy), and induction of senescence [44–46]. In light of that, the iMSCs showed a significant reduction in the expression of BCL2, which might explain why iMSCs were more affected by hypoxia than ADMSCs.

When studying apoptosis, BCL2, BAX, ROS and mitochondria are all inseparable; they all have interconnected and indispensable roles in regulating apoptosis. BAX, for example, induces apoptosis by destabilising cytochrome C channels in mitochondria [25], while high ROS accumulation induces apoptosis [47]. The mitochondrial respiratory chain controls ROS production; therefore, any changes in oxygen tension will directly affect ROS production [47]. In his work, Zhang et al. explained how the increase in HIF-1α, under hypoxic conditions, reduces the mitochondrial number and eventually reduces ROS production [48]. Our results showed that hypoxia caused a slight, insignificant, reduction in ROS production in both iMSCs and ADMSCs.

We all know the vital role of mitochondria in cell metabolism; a high number of mitochondria and a stable intra/extracellular environment are crucial for mitochondria's proper structure and function. Mitochondria maintain their physiological functions by keeping a healthy MMP. Therefore, any alteration in the inrta/extracellular environment will directly alter mitochondrial functionality by reducing its MMP, which leads to cellular apoptosis [47]. In our study, the initial mitochondrial number of ADMSCs and MMP was higher than iMSCs, which explains the insignificantly higher viability of normoxic and hypoxic ADMSCs compared to iMSCs. Besides the significant increase in HIF-1α expression, this finding explains why iMSCs had less viable cells than ADMSCs: the initial mitochondrial number and MMP were lower than those of ADMSCs. The latter conclusion might be explained by the higher BAX starting concentrations in normoxic iMSCs compared to ADMSCs; the higher BAX concentration leads to lower mitochondrial membrane potential and stressed mitochondria [49]. Although the MMP of both iMSCs and ADMSCs was reduced after hypoxia, the MMP of

ADMSCs was still, though not significant, higher than the MMP of iMSCs. Thus, hypoxia affected iMSCs more than ADMSCs in terms of viability.

The differentiation process is hectic for MSCs; they consume high energy while differentiating into a specific cell lineage. Since mitochondria are the powerhouse of cells, a considerable shift in energy production accompanies this high energy demand. MSCs tolerate this shift by increasing the size and number of mitochondria [50], which affects MMP and ROS production, and eventually, the differentiation process. At low concentrations, ROS can improve osteogenic and adipogenic differentiation [51]. Since HIF-1α regulates both mitochondria and ROS production, it is no surprise for HIF-1α to regulate osteogenic and adipogenic differentiation. HIF-1α was associated with suppressing the expression of the osteogenesis master regulator—RUNX2 [52]. Hypoxia was reported to inhibit the osteogenic differentiation potential of MSCs by decreasing the expression of osteogenic genes such as RUNX2, OCN, and ALP [39, 52–54]. However, some studies reported enhancement in the osteogenic ability of MSCs after hypoxic exposure [55,56]. In our study, RUNX2 and OCN levels remained the same after short-term hypoxic exposure, and the osteogenic differentiation potential of iMSCs was similar to ADMSCs. Noteworthy, iMSCs were previously reported to have equal or less osteogenic differentiation potential compared to MSCs [27,57,58].

For adipogenic differentiation, iMSCs were reported to have poor differentiation potential compared to primary MSCs [9,58]. Although hypoxia has been reported to favour adipogenic differentiation over osteogenic differentiation by inducing mitochondrial acytel-co release [59], several studies highlighted the role of HIF-1α in suppressing PPARγ expression during hypoxia [60,61]. Other studies reported the positive effect of hypoxia on adipogenic differentiation [55,56], but like osteogenesis, our findings show that short-term hypoxia did not affect the differentiation potential of both iMSCs and ADMSCs.

To conclude, although hypoxia did not improve iMSC proliferation, viability, and differentiation in our study, hypoxic preconditioning is still essential for better utilization of iMSCs. Hypoxia seems to be a double-edged sword; it can either switch on the apoptotic pathways or sustain and improve the viability, proliferation, and differentiation of MSCs. In this study, we were unable to further test the effect of hypoxia on the expression profile of iMSCs, but we could test the impact of long-term hypoxia on iMSCs. In addition, our results suggest that short-term hypoxia might have a sustainable positive effect on the proliferation of iMSCs. The variation between our and other research findings was related to the hypoxic conditions used. Therefore, our next is to test the long-term effect of short-term hypoxia on iMSCs and the variation in the expression profiles of iMSCs under different hypoxic conditions; to find the best ones that might ultimately suit our iMSCs.

Ethical statement

The Institutional Review Board (IRB) (IRB No.7/2019) at the Cell Therapy Center (CTC)/University of Jordan approved this study.

Data availability

The authors declare that this study's data are not deposited in any publicly available repository but will be made available on request.

CRediT authorship contribution statement

Enas Alwohoush: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Mohammad A. Ismail:** Methodology. **Ban Al-Kurdi:** Methodology. **Raghda Barham:** Methodology. **Sabal Al Hadidi:** Methodology. **Abdalla Awidi:** Supervision, Conceptualization. **Nidaa A. Ababneh:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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