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Low oxygen tension affects proliferation and senescence of caprine bone marrow mesenchymal stem cells in *in vitro* culture condition

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ABSTRACT

The culture system of bone marrow mesenchymal stem cells (bmMSCs) in the normoxic environment does not imitate the hypoxic milieu of typical biological conditions, thus hypoxic culture conditions may improve survival, and growth attributes of bmMSCs during *in vitro* culture. Therefore, the present study was conducted at ICAR-CIRG, Makhdoom during year 2020 with the objective to investigate the changes in biological characteristics of cultured caprine bmMSCs (cbmMSCs) including the cellular senescence, survival, rate of proliferation, immuno-phenotypic characteristics, and gene expression pattern in a normal and hypoxic microenvironment condition. For this, cbmMSCs isolated from bone marrow collected from iliac crest were enriched and grown under either hypoxic (5% O_2) or normoxic (20% O_2) conditions. Thereafter, the outcome of hypoxic (5% O_2) culturing of cbmMSCs on growth characteristics, proliferation, senescence, and expression profile of important stemness-associated (*OCT-4*) and oxidative stress [glutathione peroxidase (*GPx1*) and copper-zinc superoxide dismutase (*CuZnSOD*)] marker genes was evaluated. cbmMSCs cultivated in hypoxic conditions showed higher proliferation and decreased population doubling time and senescence-associated β-GAL expression; however, the immune-phenotypic characteristics of the cells remain unchanged. Furthermore, the culture of cbmMSCs in hypoxia increased the expression of OCT-4, *GPx1*, and *CuZnSOD*, compared with the cells grown under normoxia. In conclusion, the culture condition with low O_2 level improved the growth characteristics and proliferation of cbmMSCs. These outcomes would provide information to formulate strategies for the collection and efficient *in vitro* expansion of bmMSCs from goats and other farm animals before their downstream applications.

Keywords: Bone marrow mesenchymal stem cell, Goats, Growth characteristics, Hypoxia

The normoxic condition in *in vitro* culture systems does not imitate a typical physiological niche of bone marrow mesenchymal stem cell (bmMSCs) since the physiologic O_2 pressure in bone marrow [4–7%; (Spencer *et al.* 2014)], is much lower than the atmospheric or *in vitro* O_2 pressure (150 mm Hg; 20% O_2). Therefore, the typical niche of MSCs in these tissues with restricted O_2 availability may activate several stress pathways (Peck *et al.* 2021) and can impair stem cell actions through a variety of mechanisms (Mas-Bargues *et al.* 2019). Thus, culturing bmMSCs under low O_2 levels can imitate their normal microenvironment and allows studies of proliferation, senescence, and related biological activities of these cells (Samal *et al.* 2021).

The low O_2 concentration in the physiological niche is important to maintain stem cell characteristics such as self-renewal, undifferentiated state, and pluripotency (Mas-Bargues *et al.* 2019, Singh *et al.* 2021). Moreover, few recent investigations demonstrated that the growth features of human MSCs such as proliferation, differentiation, and survival rate are modulated by low O_2 levels in the culture system (Kim *et al.* 2016, Kwon *et al.* 2017, Adolfsson *et al.* 2020). Copper-zinc superoxide dismutase (*CuZnSOD*) and Glutathione peroxidase (*GPx*) are two protagonist oxidoreductase enzymes and are reported as the important oxidative stress markers (Ye *et al.* 2020). These enzymes are up-regulated when the body undergoes stressful situations and tend to reduce the condition by converting toxic free radicals like superoxide radicals to molecular O_2 and hydrogen peroxide in the *CuZnSOD* case and free hydrogen peroxide to water in the case of *GPx* (Yoo *et al.* 2016). Nonetheless, the information on the effects of hypoxic conditions on *in vitro* growth, proliferation, senescence, and expression of antioxidants in cbmMSCs is unavailable.

Thus, the present study aimed to investigate the difference in biological characteristics of cultured cbmMSCs comprising the cellular senescence, survival, rate of proliferation, immuno-phenotypic characteristics, and gene expression pattern in a normal or hypoxic microenvironment.

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MATERIALS AND METHODS

Enrichment and culture of cbmMSCs: The isolation and enrichment of cbmMSCs were performed following the minor modification in the protocol described earlier (Jena *et al.* 2020). Briefly, before the bone marrow aspiration from four healthy male goats of 1–2 years of age during sub-tropical weather conditions (March – May, 2020), the entire pelvic region was shaved and sterilized by using ethyl alcohol (70%). After sedation with Xylazine (0.05 mg/kg BW, IM), Lignocaine hydrochloride (5 mL) was infused near the site of the incision. Further, a stab incision of about 1 cm was made on the iliac crest for insertion of a 16 G sterile bone marrow aspiration needle. After appropriate localization of a needle, bone marrow was collected into a syringe with an anticoagulant (EDTA). After collection, the antiseptic lotion was applied to the puncture area and a schedule of antibiotics was followed.

For isolation and enrichment of cbmMSCs using a density gradient cell separation medium, the BM samples were loaded slowly through the wall onto the Histopaque®-1077 (Sigma Aldrich, Catalog#10771) in sterilized tubes and centrifuged (830 × *g*, 30 min, 25°C). The buffy coat was collected without disturbing the lower layers and washed twice with PBS (5200 × *g* for 10 min, 25°C) and the final suspension of cells was made in complete DMEM/F12 media.

The enriched cbmMSCs were then cultivated in either normoxia [5% CO_2, 9% air (20% O_2), 37 °C] or hypoxia (5% O_2, 5% CO_2, and remaining N_2, 37 °C). The assays were performed in triplicates using cultured cells of both groups.

Growth kinetics: The growth kinetic study was conducted for seven days to analyse the growth potential of bmMSCs in a culture system with low O_2 tension. After trypsinization of the cultured cells using 0.25% Trypsin-EDTA, counting of cells was performed by a cell counter (Countess™ II FL, Invitrogen). Thereafter, the following formulas were used for estimation of the number of cell divisions and population doubling time of cultured cbmMSCs, as described earlier by Jena *et al.* (2020).

Number of cell divisions = $Log_2 (N/N_0)$ (Kim *et al.* 2016)
Population Doubling Time = (Log_2 × Duration) / [Log (N) – Log (N_0)] (VR, 2006),
N_0, Number of cells seeded (at d 0 or d 4) and N, Cell count after either 3 days or 1 week of culture.

Cell senescence assay: The senescent cbmMSCs in different culture conditions were detected through the identification of SA-β-gal activity, as per the manufacturer's instructions (Merck Millipore, Catalog#KAA002, Darmstadt, Germany). Briefly, cbmMSCs were grown onto the 24-well culture plates until 70–80% confluence, washed with DPBS, fixed (using ice-cold 70% ethanol), and treated overnight with SA-β-gal solution at 37ºC. Subsequently, after washing with PBS, the stained cells were examined under a bright-field microscope. The blue-stained cells represent the senescent cells whereas normal and healthy cells remain unstained. The number of SA-β–gal positive cells among the total number of cells was used to determine the portion of senescent cells in each group.

Proliferation assay: The proliferation rate of cbmMSCs in each culture condition was estimated by 5-Bromo-2-deoxyuridine (BrdU) cell proliferation assay. For this, cbmMSCs were seeded onto a 24-well cell culture plate to cultivate for 3 days under either normal or low O_2 tension. Thereafter, the culture media was removed and media comprising BrdU (30 µg/mL, Sigma-Aldrich, Catalog#B5002) was added. The cells were then incubated for 2 h at 37°C and further fixed with ice-cold 70% ethanol. Next, the denaturation of DNA and cell permeabilization were performed at RT by incubation of fixed cells with 1.5 M HCl (for 30 min) and permeabilization buffer (0.3% Triton X 100; for 1 h), respectively. Thereafter, anti-BrdU antibody (Merck-Millipore, Catalog#FCMAB101A4, Germany; 1:250 dilution in blocking buffer) and 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Catalog#D8417, 250 ng/mL in PBS) were applied for cellular and nuclear staining, respectively. To determine the rate of proliferation, the proportion of proliferative (DAPI and BrdU positive) and non-proliferative (DAPI positive and BrdU negative) cells among the total number of cells were assessed in both the groups.

Immunocytochemistry: cbmMSCs were cultivated onto the 24-well plates under normal or low O_2 tension for one week. After one week, cells were treated with paraformaldehyde (4%; for 15 min) and then for 30 min with Triton X-100 at RT for fixation and permeabilization, respectively. Thereafter, cells were treated with blocking solution (4% BSA solution) for 60 min at RT to block unoccupied spaces. Then, the cells were co-incubated overnight with rabbit anti-OCT 4 primary antibody at 4°C (Sigma-Aldrich, Catalog#AB3209; 1:250). After three washings with PBS (5 min each), Alexa Fluor 488 (donkey anti-rabbit IgG, Thermo Fisher Scientific, Catalog#R37118; 1:1000) was added and incubated for 60 min in dark at RT. Then after five washings with PBS, the DAPI solution (same as done in proliferation assay) was added to stain nuclei of the cells. For the negative control, all steps were followed except the use of the primary antibody. Finally, the bright field and fluorescence microscopy were performed for cell imaging (Zeiss Axiovert A1, Germany).

Isolation of RNA from cbmMSCs: The total RNA was isolated by TRIzol™ LS reagent method (Invitrogen™, ThermoFisher Scientific, USA, Catalog#10296028) method following the manufacturer's protocol. Briefly, the cbmMSCs were treated with 1 mL TRIzol™ LS reagent and mixed thoroughly by vortexing followed by incubation for 5 min. Subsequently, after adding 0.2 mL chloroform and incubating for 5 min at RT, the mixture was centrifuged (15 min at 12,000 × *g* at 4°C). The resultant solution comprising RNA was shifted to a fresh tube. In the ensuing stage, 0.5 mL of ice-cold isopropanol was added and incubated at RT for 20 min. followed by centrifuging at 12,000 × *g* at 4°C for 10 min. Then, the supernatant was removed and 1 mL of 75% ethanol (75% Ethanol V/V in DEPC water) and mixed thoroughly to dissolve the pellet followed by centrifugation twice at 7500 × *g* for 5 min at 4°C. Then, after removing the supernatant, RNA pellet was air-dried for about 10 min and the pellet was dissolved in 25 µL of DEPC water before assessment of the quantity and quality of RNA at A260 nm/280 nm using Bio-photometer® plus (Eppendorf, USA).

cDNA synthesis and qRT-PCR: The cDNA was synthesized using a cDNA synthesis kit according to the manufacturer's protocol (TaKaRa, Japan, Catalog#6110A). To make 1st reaction mix, 1.0 µg RNA sample, 1.0 µL Random hexamer Primer, and 1.0 µL of 10 mM dNTP Mix were mixed and incubated at 65°C for 5 min and then kept on ice. Thereafter, to prepare the 2nd reaction mix, 4.0 µL 5× Primescript buffer, 4.5 µL RNase free water, 0.5 µL RNase inhibitor, and 1.0 µL primescript RTase were added. Thereafter, 1st reaction mix was mixed with 2nd reaction mix and kept sequentially at 30°C (10 min), 42°C (1 h), and finally at 75°C (15 min) for cDNA synthesis in a thermocycler.

To investigate transcript expression of *CuZnSOD* and *GPx* genes and their fold changes, the quantitative reverse transcription real-time PCR (qRT-PCR) reactions were executed using SYBR green in a CFX96™ Real-time PCR machine (Bio-Rad, USA). The details of respective primers are presented in Table 1 and reaction controls such as no reverse transcription controls and no template control were kept along with GAPDH as a housekeeping gene.

Gene expression data and statistical analysis: ΔΔCT method was followed to define the relative expression (fold change) of target transcripts (Livak and Schmittgen 2001). Initially, the ΔCT (cycle threshold) values for each treatment were figured out by deducting CT values of GAPDH from the CT values of the transcripts of interest (*CuZnSOD* and *GPx*). The average ΔCT for samples from the control group was used as a calibrator (ΔCT calibrator). The n-fold expression of different groups was compared using the students' t-test as implemented in version 20.0 of the SPSS.

RESULTS AND DISCUSSION

cbmMSCs were cultivated for one week in hypoxic or normoxic culture conditions, and the effect of O_2 level on proliferation and senescence was evaluated. The PDT of cbmMSCs grown in hypoxic conditions was 1.5 fold (0–3 days) and 1.6 fold (4–7 days) lower than cbmMSCs cultured in normoxic conditions (Fig. 1a). Similarly, cbmMSCs incubated in hypoxic conditions demonstrate a significantly ($p<0.01$) higher number of cell divisions than under normoxic conditions (Fig. 1b). The number of cell divisions in cbMSCs during days (0–3) and days (4–7) under normoxia and hypoxia were 1.4 ± 0.48 vs. 4.05 ± 0.65, and 0.95 ± 0.35 vs. 2.53 ± 0.45, respectively (Fig. 1b). Thus, lower O_2 levels facilitate a drop in PDT by the increase in cell divisions of cbmMSCs.

The O_2 tension of the niche is a vital factor for the normal physiological properties of stem cells. The O_2 has been demonstrated to activate a component that can regulate biological features of stem cells including survival, growth, differentiation, and stemness properties (Mohyeldin *et al.* 2010, Abdollahi *et al.* 2011). In most cases, the *in vitro* cultivation of stem cells is made under a microenvironment with a normal O_2 level (20–21% O_2). However, the cbmMSCs in *in vivo* conditions are never exposed to such a higher O_2 tension, i.e. about 4–10 fold higher compared with the physiological level of O_2 in the normal niches (Antoniou *et al.* 2004).

In the present investigation, the outcome of low O_2 tension was studied on cbmMSCs through alteration in proliferation rate, cellular senescence, immunophenotypic characteristic, and expression of stress-related genes compared with the normoxic conditions. The results revealed that cbmMSCs grown under low O_2 conditions had an improved rate of proliferation than the cells grown in normoxic conditions. Similar results were reported earlier if human bmMSCs are cultured under lower O_2 conditions (Hung *et al.* 2012, Mohd Ali *et al.* 2016). The molecular mechanism associated with the relative expression of oxidative stress-related marker genes, such as *GPx* and *CuZnSOD*, may affect the survival, growth, and proliferation of stem cells.

The senescent cbmMSCs in normoxic or hypoxic culture conditions were recognized by the blue colouration of the cell colonies (Fig. 1 c-d). The SA-β-GAL staining was done on day 7 of cbmMSCs culture. A lower count of SA-β-GAL-positive bmMSCs was detected when cells were grown in hypoxia compared with the normoxia. The percentage of cells showing positive SA-β-gal reaction was 54.0±2.08% and 24.67±2.40% of total P3 bmMSCs cultured in normoxic or hypoxic conditions, respectively (Fig. 1e).

Table 1. List of primers used for the characterization of cbmMSCs

Gene of interest	Oligo sequences* (5′→3′)	References
Glutathione peroxidase 1(*GPx1*)	F: ACATTGAAACCCTGCTGTCC R: TCATGAGGAGCTGTGGTCTG	Wang *et al.* 2021
Copper zinc superoxide dismutase (*CuZnSOD*)	F: TGCAGGCCCTCACTTTAATC R: CTGCCCAAGTCATCTGGTTT	Wang *et al.* 2021
GAPDH (House Keeping)	F: GGTGATGCTGGTGCTGAGTA R: TCATAAGTCCCTCCACGATG	Chaussepied *et al.* 2010

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase. *Forward (F) and reverse (R) primers used to detect mRNA expression of the indicated gene of interest.

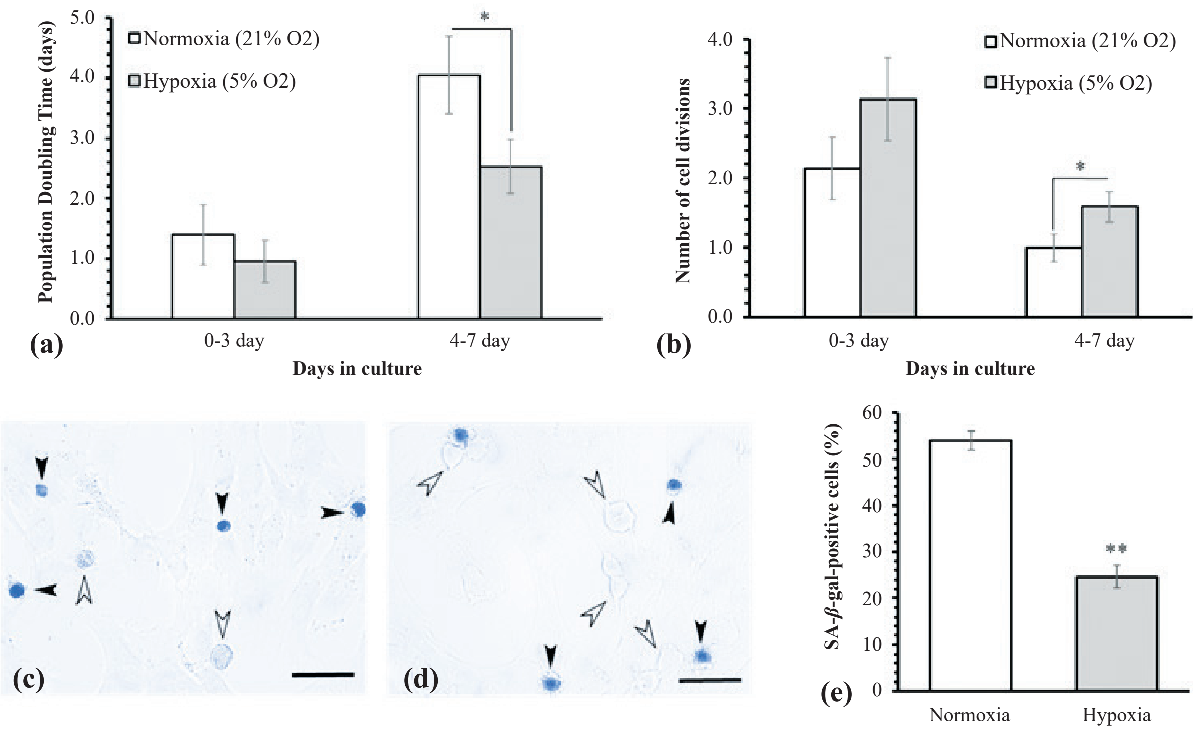

Fig. 1. (a) Time-dependent effect of O_2 tension on PDT, and (b) cell divisions of caprine bone marrow-derived mesenchymal stem cells (cbmMSCs) cultured in normoxic or hypoxic conditions during different days of culture. (c and d) Representative images of senescence-associated (SA)-β-GAL staining at d7 of cbmMSCs grown in the conditions of normal (c) or low (d) O_2 tension. The senescent cells are identified by blue colour staining (filled arrowhead) and growing cells remain unstained (empty arrowhead); (e) Quantification of Fig. c and d to present data as mean ± SEM and statistical differences are presented significant as *, $p<0.05$ and **, $p<0.01$, scale bar, 50 µm.

The higher proliferation rates of cultured cbmMSCs are associated with a higher cell division rate under hypoxic conditions. The cbmMSCs in hypoxia had lower expression of SA β-GAL than the normoxic cultured cells. Similarly, Tsai *et al.* (2012) described higher OCT-4 expression and inhibition of senescence of human MSCs cultured under low O_2 tension.

The proliferation and immune-phenotype of cultured cbmMSCs were characterized by BrdU and OCT-4 immunostaining, respectively, and the representative images are presented in Fig. 2a and 2b. The higher BrdU expression in cbmMSCs demonstrates a higher proliferation of cbmMSCs expanded under low O_2 tension compared with the normoxia (Fig. 2a). Similarly, the expression of undifferentiated pluripotent cell surface marker (OCT-4) was relatively higher in cbmMSCs grown under hypoxic conditions compared with the cells at normal O_2 level (Fig. 2b). The differential growth kinetics of cbmMSCs grown under normoxic or hypoxic culture conditions are presented in Table 2.

The study presents the supporting effect of low O_2 levels on proliferation, senescence, and expression of stem cell marker (OCT-4) and antioxidant enzymes (*GPx* and *CuZnSOD*) in cbmMSCs. The similar beneficial properties of hypoxia on the rate of proliferation of human bmMSCs have been shown earlier (Hung *et al.* 2012, Mohd Ali *et al.* 2016, Kwon *et al.* 2017). These effects of hypoxia provide a basis to consider that the condition with low O_2 levels may be the original niche or preferable microenvironment for the *in vitro* culture of bmMSCs (Mohyeldin *et al.* 2010). These results suggest that *ex vivo* expansion of bmMSCs under low O_2 conditions could be useful to overcome the issues of bmMSC culture under normoxia such as slower growth with a higher senescence rate. However, in contrast to this, some other studies demonstrated contrasting results with adverse or absence of any significant effects of hypoxic culture conditions on MSCs (Malladi *et al.* 2006, Holzwarth *et al.* 2010, Roemeling-van Rhijn *et al.* 2013). These discrepancies may account, in part, due to the disparity in the O_2 level, the length of the trial (the degree and duration of hypoxia), and biological characteristics compared (Malladi *et al.* 2006, Holzwarth *et al.* 2010).

The expression pattern of functional genes related to oxidative stress-related (*GPx* and *CuZnSOD*) in cbmMSCs was investigated after 1 week of culturing. The qRT-PCR based gene expression analyses displayed upregulation in the expression of *GPx* and *CuZnSOD* in hypoxia compared with the normoxic condition by 1.95 and 10.77-folds, respectively.

A transcription factor OCT-4 (POU5F1) is crucial for the survival and self-renewal of MSCs (Tsai *et al.* 2012), and *GPx* and *CuZnSOD* are two important oxidative stress markers of stem cells in the culture system (Yoo *et al.* 2016, Vono *et al.* 2018). In the present study, it was confirmed that cbmMSCs grown in culture conditions with low O_2 tension for one week had improved cell proliferation

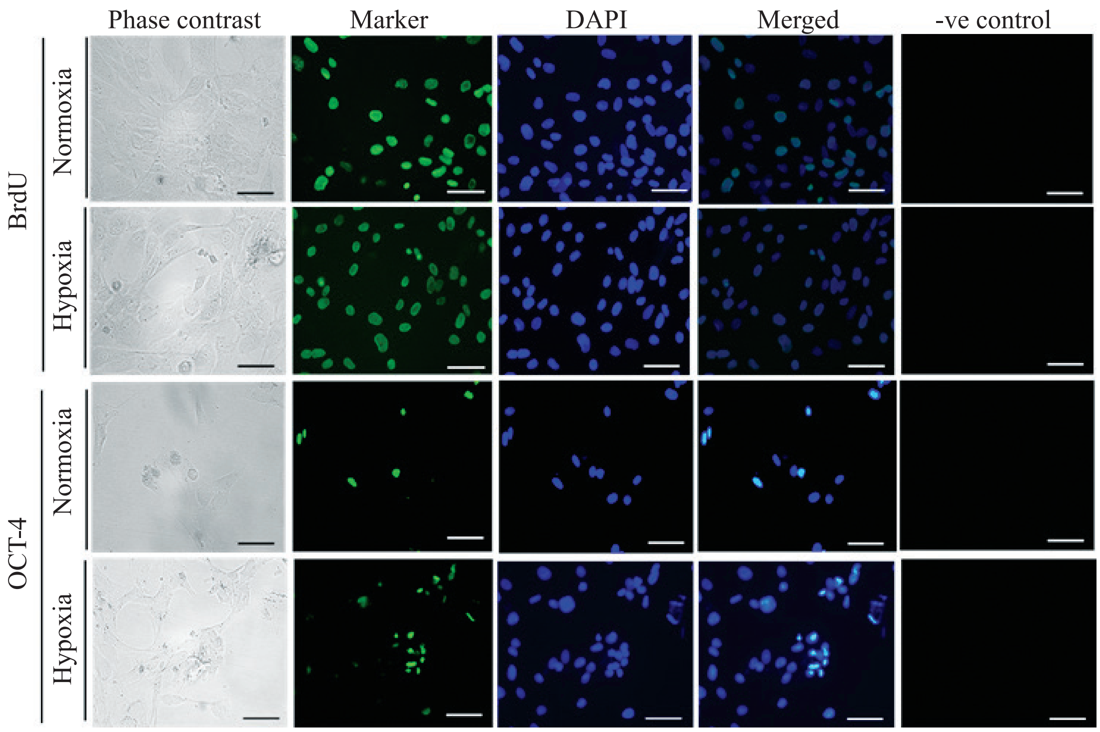

Fig. 2. Representative images of BrdU incorporation assay and immunophenotypic characterization (OCT-4 staining) of cbmMSCs cultivated under normoxia (a) or hypoxia (b) for one week. For the negative (-ve) control groups, the primary antibodies (anti-BrdU or anti-OCT-4 antibodies) were omitted. Scale bar: 50 µm.

rate, expression of OCT-4, and upregulation of *GPx* and *CuZnSOD* transcript expression. The higher expression of OCT-4 suggests that the hypoxic microenvironment enhances stemness characteristics in cbmMSCs. Similarly, Hung *et al.* (2012) reported that bmMSCs expanded under 1% O_2 improved the expression of stemness-related genes such as OCT-4 and NANOG.

Based on the response to the general free radical invasion, the first line of defense antioxidants including *SOD* and *GPx* are important in the general defense mechanism of the body (Ighodaro 2018). In the present experiment, it was observed that both *GPx* and *CuZnSOD* were up-regulated. However, the relative expression of *CuZnSOD* was way higher than that of the *GPx* counterpart signifying the fact that *CuZnSOD* activity was highly up-regulated in hypoxia-induced cells. *CuZnSOD* is identified as a factor with the therapeutic potential of adipose-derived MSCs by maintaining antioxidant enzyme levels in the culture system (Yoo *et al.* 2016) and reported that it enhances the amelioration of ischemic stroke in neural stem cells (Sakata *et al.* 2012). The oxidative stress is an important factor for senescence and aging in MSCs (Vono *et al.* 2018), therefore, the overexpression of *GPx* and *CuZnSOD* in the hypoxic cultured cbmMSCs may contribute to lower senescence and indicate the greater ability of bmMSCs cultured under low O_2 to ameliorate harmful effects of free radicals compared to normoxic cultured bmMSCs.

In conclusion, the hypoxic culture condition with 5% O_2 tension had a supportive effect on cell proliferation, delayed senescence, and maintenance of stemness characteristics of cbmMSCs. Thus, the culture of cbmMSCs under hypoxic conditions may be a useful strategy for faster multiplication and preserving their biological characteristics. The over-expression of two important antioxidative enzymes, i.e. *GPx* and *CuZnSOD* under hypoxic conditions indicate that such micro-environments support to combat the deleterious effects of free radicals, thereby positively affecting survivability and proliferation of cbmMSCs. Overall, a culture condition with a low O_2 level is suitable to maintain the higher proliferation rate and improved viability of cbmMSCs and their downstream clinical applications.

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Table 2. Growth kinetics of cbmMSCs under normoxic or hypoxic culture conditions

Treatment group	No. of cells ($\times 10^5$ cells/mL)							
	d 0*	d 1	d 2	d 3	d 4	d 5	d 6	d 7
Normoxia (20% O_2)	0.59±0.05	1.34±0.38	2.37±0.61	2.62±0.59	3.49 0.60^a	4.05 0.31^a	3.76 0.47^a	2.92 0.30^a
Hypoxia (5% O_2)	0.42±0.06	1.07±0.33	2.60±0.64	3.71±0.68	4.95 0.63^b	6.35 0.70^b	9.01 0.28^b	8.81 0.42^b

* d 0 was the day of seeding. Means with different superscripts (a, b) within a column differ significantly (p<0.05).

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