Low Oxygen Tension Potentiates Proliferation and Stemness but Not Multilineage Differentiation of Caprine Male Germline Stem Cells

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Abstract

The milieu of testicular germline stem cells (mGSCs) is characterized as low oxygen (O_2) environment, whereas, there *in-vitro* expansion is typically performed under normoxia (20-21% O_2). Here, we evaluated and compared the culture and multilineage differentiation characteristics of enriched (through differential plating and percoll density centrifugation) caprine mGSCs (cmGSCs) under hypoxic (5% O_2) and normoxic (21% O_2) culture conditions. For this, in addition to growth characteristics and population-doubling time (PDT); viability, proliferation, senescence, and expression of key-markers of adhesion (β -integrin and E-Cadherin) and stemness (OCT-4, THY-1 and UCHL-1) were evaluated and compared under normoxia and hypoxia. Moreover, the extent of multilineage differentiation (neurogenic, adipogenic, and chondrogenic differentiation) was assessed. The survival, viability and proliferation were significantly promoted and PDT was reduced ($p < 0.05$), thus yielding a higher number of viable cells with larger colonies under hypoxia. Furthermore, expression of stemness and adhesion markers was distinctly increased under lowered O_2 condition. Conversely, the presence of differentiated regions and expression of differentiation specific key genes [C/EBP α (adipogenic), nestin and β -tubulin (neurogenic), and COL2A1 (chondrogenic)] were significantly ($p < 0.05$) reduced under hypoxic conditions. These data demonstrate that culturing cmGSCs under hypoxia augments the growth characteristics, and stemness but not the multilineage differentiation potential of cmGSCs as compared with normoxia. These data are important for the development of robust methodologies for *ex-vivo* expansion and lineage-committed differentiation of cmGSCs for clinical applications.

Introduction

The spermatogonial stem cells (SSCs), a group of most primitive spermatogonia in testis, are male germline stem cells (mGCs) with self-renewal and differentiation ability, and thus considered as the foundation of spermatogenesis through their pluripotent characteristics [16]. These cells are present on the basement membrane of seminiferous tubules (ST) and are almost completely enclosed by the Sertoli cells [33].

Existing reports have demonstrated that both intrinsic and extrinsic variables regulate growth and functional characteristics of mGCs [26]. Among these, oxygen (O_2) tension is an important variable, which closely associated to the maintenance of stemness and proliferation of mGCs [25]. However, the effect of lower O_2 tension on mGC proliferation, while maintaining stemness characteristics, under *in-vitro* culture system has rarely been reported [18]; hence, the appropriate mechanism of expansion and differentiation of these cells has remained an enigma.

The testis is considered as a naturally O_2 -deprived organ [13] as the ST are poorly vascularized and operate normally at lower O_2 levels i.e. approximately 20% of the testicular artery blood O_2 pressure (12 to 15 mm Hg) [8]. Therefore, *in vivo* niche of mGCs is characterized by low physiological O_2 tension [8,34]. In view of the fact that low O_2 level induce various responses in cultured cells [29], the derivation of cell

populations with stemness characteristics and their differentiation should therefore be affected by the hypoxic culture [29], although it is unknown whether caprine mGCs (cmGSCs) can do so. Moreover, culturing cmGSCs under low O₂ tension can mimic their natural intra-testicular niche and allow investigations of self-renewal and other biological characteristics of these cells [11,31]. However, the effect of hypoxia on culture characteristics of cmGSCs and maintenance of stemness through expression of pluripotency and adhesion markers, and extent of differentiation have yet to be investigated.

Therefore, in this study, we hypothesized that culturing cmGSCs under hypoxic conditions would have an impact on their culture and differentiation characteristics. For this, we compared the variation in functional properties of enriched cmGSCs such as the cell proliferation rate, viability, growth characteristic, senescence, immunophenotypic properties, stemness, multilineage differentiation (adipogenic, chondrogenic, and neurogenic); and expression of stemness, adhesion and differentiation specific key genes when under culture conditions with low (5%) and normal (21%) O₂ tension.

Material And Methods

Collection of tissue and cell harvest

Testes from four healthy prepubertal bucks (aged ~ 3 months) were collected and transported to the laboratory in a normal saline solution containing streptomycin (500 µg/mL; Sigma-Aldrich, Cat#S9137) and penicillin (400 IU/mL; Sigma-Aldrich, Cat#P3032), within 30 min of the sacrifice of the animals. All the experimental procedures were carried out in accordance with good veterinary practices and approved by the Animal Ethics Committee of the Institute.

After 3 to 4 washings with Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich, Cat#D5773) and removal of connective tissue, testes were processed for collection and enrichment of cmGSCs. Isolation of cmGSCs was performed by two-step enzymatic digestion method, as described earlier [15], with minor modifications. Briefly, for the first digestion, minced tissue was suspended in DMEM (5 fold v/w) containing 1 mg/mL collagenase IV from *Clostridium histolyticum* (Sigma-Aldrich, Cat#C5138), 1 mg/mL hyaluronidase type II from sheep testes (Sigma-Aldrich, Cat#H2126), 5 µg/mL DNase type I (Sigma-Aldrich, Cat#DN25), 1 mg/mL trypsin (Sigma-Aldrich, Cat#T7409) and antibiotics (1% w/v), and incubated at 37°C in an orbital shaker for 45 min. The supernatant was discarded after washing at 1000 × rpm for 5 min with DMEM media containing antibiotics for the removal of interstitial cells. Subsequently, the second digestion was then given for 30 min, as described for the first digestion. After second digestion, twice washing of cells was done with DMEM with 10 % (v/v) fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA, Cat#10082-147) to stop the enzymatic digestion. The supernatant after centrifugation was filtered through nylon mesh filters (80 µm and 60 µm pore-size; Merck Millipore, Cat#NY6002500) to enrich the cmGC population.

Enrichment and expansion of cmGSCs

The enrichment of putative cmGSCs was performed by differential plating and Percoll density gradient centrifugation methods as described previously [15]. The enriched cmGSCs thus obtained were grown under either normoxic [5 % CO₂, 95 % air (20% O₂), 37 °C; Control] or hypoxic (5 % O₂, 5 % CO₂, and remaining N₂, 37 °C; Treatment) culture conditions. For all assays, the cultured cells of both the groups were used and all experiments were performed in triplicates.

Survival rate and growth kinetics

The cells were evaluated for a period of 7 days to analyze the survival rate and growth characteristics in culture system with low and normal O₂ tension. The number of total and live cells were counted during different days in culture (day 0, 3, 5 and 7) using an automatic cell counter (Countess™ II FL, Invitrogen Inc.), and survival rate was calculated according to the formula i.e. Survival rate (%) = (Live cell count / Total cell count) × 100

For the initial 3 days and then for subsequent days, the number of cell divisions was measured with the formula i.e. number of cell divisions = Log₂ (N/N₀), where N₀ and N are the initial and final number of cells after either 3 or 7 days of culture, respectively [17]. For this, each observation the cells were counted by trypan blue method after trypsinization with 0.25% Trypsin-EDTA (Sigma Aldrich, Cat#T4049), using an automatic cell counter (Countess™ II FL, Invitrogen Inc.).

To examine Population Doubling Time (PDT), cells were seeded at a density of 2 × 10³ per cm² and PDT calculated for two different time intervals of the culture of cmGSCs using an algorithm available online (<http://doubling-time.com>) [30]

$$\text{Population Doubling Time} = \frac{\text{Duration} \times \log(2)}{\log(\text{final cell count}) - \log(\text{initial cell count})}$$

Morphometry and alkaline phosphatase (AP) staining

After 3rd day of culture, cell growth and the cell colonies in each 24-well plates in normoxia or hypoxia were observed by inverted microscopy and the areas under the colonies were measured using ZEISS Zen 3.1 software (Carl Zeiss Microscopy, GmbH Carl-Zeiss-Promenade 10 07745 Jena, Germany). The mean of five largest colonies of three wells of the culture plates were used for comparison in each independent experiments that were performed in triplicate. Alkaline phosphatase (AP) staining was performed according to the manufacturer's instructions (Sigma-Aldrich, Cat#86R) and protocol described earlier [14].

Cellular and metabolic viability assays

Colony-forming Assay

To examine cellular viability of cultured cells through assessment of colony forming units under normoxic and hypoxic conditions, the cell colonies were washed with PBS and fixed with 4% paraformaldehyde solution for 15 min at RT before staining with 1% aqueous solution crystal violet (Sigma-Aldrich, Cat#V5265) for 30 min at RT. After through washing with distilled water, colonies were observed under an optical microscope and then stain was solubilized with 1% SDS solution for 10 min at room temperature. The absorbance was measured as 590 nm using microtiter plate reader (Sun Rise, Tecan, Männedorf, Switzerland). Each test was conducted in triplicate.

Metabolic viability and cellular senescence assay

A tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric kit (Merck Millipore, Cat#CT02) was used for the determination of metabolic viability of cmGSCs in different culture conditions. Four thousand cells per well were seeded into a 96-well flat-bottom microplate with 0.1 mL culture media. After 24 h of culture at 37 °C in CO₂ incubator, the cells were treated with 10 µL of an MTT solution per well and then incubated at 37 °C for an additional 4 h. After the addition of 0.1 mL of isopropanol with 0.04 N HCl to each well and mixed thoroughly, absorbance was measured at 570 nm using a microtiter plate reader, and data were analyzed with Magellan 4.0 Software (Männedorf, Switzerland).

A senescence-associated β -galactosidase (SA- β -gal) activity was evaluated to assess the senescence in cmGSCs grown under different culture conditions following the manufacturer's protocol (Cellular Senescence Assay kit; Merck Millipore, Cat#KAA002).

BrdU incorporation assay

The proliferation of cmGSCs was determined using immunocytochemical staining based on 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich, Cat#B5002) incorporation by DNA synthesis. For this, cmGSCs were seeded in a 24-well culture plate in triplicates and incubated under either normoxic or hypoxic culture conditions. After 3 days of incubation, culture media was replaced with the media containing 30 µg/mL BrdU. The cells were incubated at 37 °C for 2 h in a CO₂ incubator before they were washed and fixed with cold 70% ethanol. Then, the cells were treated with 1.5 M HCl for 30 min at RT. After twice washing with 1× PBS, blocking, a process of permeabilization (2% BSA in 1× PBS and 0.3% Triton X 100) was done for 60 min at RT. Further, the cells were incubated with a detection antibody (anti-BrdU-Alexa 488 antibody; Merck Millipore, Cat#FCMAB101A4; 1:200) and DAPI (Sigma-Aldrich; Cat#D8417, 300 ng/mL in blocking buffer) for staining of cells and nuclei, respectively. To estimate proliferation rate, cells were counted on three wells and four images/well. The ratio of BrdU and DAPI positive (proliferative), and BrdU negative and DAPI positive (non-proliferative) cells was calculated out of the total number of cells.

Immunocytochemistry

cmGSCs were seeded into 24-well plates and cultured in normoxic or hypoxic conditions to obtain about 80% cell confluence. After 7 days of culture, the cells were fixed with 4% paraformaldehyde for 15-20 min.

and permeabilized them by incubating with Triton X-100. Thereafter, blocking was performed with 4% bovine serum albumin for 60 min at RT. The fixed and permeabilized cmGSCs were incubated overnight at 4 °C with affinity-purified rabbit Ab specific for pluripotent markers [rabbit anti-OCT 4 (Sigma-Aldrich, Cat#AB3209; 1:200 dilution), rabbit anti-THY-1 (Invitrogen, Cat#PA5-11917; 1:500 dilution) and rabbit anti-PGP9.5 or UCHL-1 (Invitrogen, Cat#PA1-10024; 1:500 dilution)]. After rinsing in PBS (three-times, 5 min each), cells were exposed to Donkey anti-rabbit IgG (Alexa Fluor 488, Invitrogen, Cat#R37118; 1:1000 dilution) as a secondary antibody for 60 min in the dark at RT. Additionally, cell nuclei were counterstained with DAPI with antifade mountant (ProLongTM Gold Antifade Mountant with DAPI, Invitrogen, Cat#P36931). All the steps for negative control were processed in a similar to the samples except for the use of primary Ab and the imaging of the labelled cells were performed by fluorescence microscope (Zeiss Axiovert A1, Germany).

Neurogenic, adipogenic and chondrogenic differentiation of cmGSCs

The cmGSCs from passage (P) three were selected for differentiation that were grown in 24 well plates. Once the cultured cells reached the 60-70% confluency in basal medium, the cells were grown in the specific induction media that permit the differentiation of cmGSCs into different lineage such as neuron-like cells, chondrocytes, or adipocytes. The differentiating cells were observed and images were obtained with an inverted microscope (Nikon ECLIPSE TE2000-U).

Neurogenic differentiation of cmGSCs and confirmation

After P3, cmGSCs were seeded onto 24 well culture plates containing induction medium [90% DMEM, 10% FBS, 5.5×10^{-5} M β -mercaptoethanol, 1×10^{-7} M retinoic acid, and 5×10^{-4} M 3-isobutyl-1-methylxanthine]. After 15 days of induced differentiation, cmGSCs were stained with toluidine blue and cell morphology was assessed by microscopy. The neurogenic differentiation was confirmed by expression analysis of specific genes (nestin and β -tubulin) by RT-PCR [32].

Adipogenic differentiation of cmGSCs and confirmation

For adipogenic differentiation, cmGSCs were cultured in two laboratory-made media formulations [32]. The induction media were referred as medium A (90% DMEM, 10% FBS, 5.5×10^{-5} M β -ME, 1 μ M Decaderol, 0.1 mg/L insulin and 5 μ M IBMX) and medium B (90% DMEM, 10% FBS, 5.5×10^{-5} M β -ME, and 0.1 mg/L insulin). Induction medium A was added for three days followed by induction medium B for a day. The procedure of using media A and B were repeated for three times. Then the cells were maintained in medium B until day 15 of experiment. During entire experiment, culture plates were kept at normoxic or hypoxic condition in the incubators.

After 15 days, differentiating cmGSCs were assessed by cell morphology and staining of lipid droplets with Oil Red O. To quantify differentiation into adipocytes, the cells were fixed with 4 % paraformaldehyde for 30 min at RT and stained with 0.5% Oil Red O solution in isopropanol (Sigma-Aldrich, Cat#O1391). The adipogenic differentiation specific genes PPAR γ and C/EBP α were detected by RT-PCR [32].

Chondrogenic differentiation and confirmation

The cmGSCs were harvested with trypsinization and centrifuged into a pellet. The cells were seeded with commercially available stempro chondrogenic supplement (Gibco, Cat#A10064-01) mix with stempro basal differentiation medium (Gibco, Cat#A10069-01). The medium was changed every third day of culture until d 15. Chondrogenic differentiation was evaluated first by microscopic observations. After differentiation steps were complete, gently wash the cells with DPBS (Sigma-Aldrich, Cat#D8537), and colonies were fixed in 4% paraformaldehyde for 30 min at RT. Then, after twice washing with DPBS, the cells were stained with 1% Alcian blue (Sigma-Aldrich, Cat#A3157) solution prepared in 0.1 N HCl for 30 min. After three times rinsing with 0.1 N HCl, distilled water was added to neutralize the acidity, and visualized under a light microscope. Moreover, the expression profile of COL2A1 gene was evaluated to confirm chondrogenic differentiation [7].

cDNA synthesis and quantitative real time-PCR (qRT-PCR)

The total RNA was isolated by using total RNA extraction reagent (RNAiso Plus) as per the manufacturer's protocol (DSS Takara, Japan, Cat#9108). After quantification of RNA using Quantus™ Fluorometer (Promega, USA) and synthesis of respective cDNA using Primescript™ 1st strand cDNA synthesis kit (DSS Takara, Japan, Cat#6110A) using thermocycler (C1000™ Thermal Cycler, Biorad), qRT-PCR reactions were performed using SYBR green chemistry with respective primers (Supplementary Table 1) in a StepOnePlus® Real time PCR system (Applied Biosystem, USA). No template control has been kept as a reaction negative control along with the GAPDH gene as an endogenous control in all the cmGSCs samples. $\Delta\Delta CT$ method as described by [22] was used in determining the relative expression (fold change) of target genes.

Statistical analysis

Statistical analysis was done on the final relative fold change (RQ) i.e. $2^{-\Delta\Delta CT}$. The n-fold expressions of different groups of samples were compared with the normal samples. For two-way comparisons, e.g., normoxia vs. hypoxia, an unpaired t-test was performed (version 20.0 of the SPSS software package). For multiple comparisons, data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Data are presented as mean $\pm$ SEM and differences of $p < 0.05$ were considered significant.

Results

Morphological characteristics of cmGSCs and alkaline phosphates (AP) staining

In both the culture conditions, cmGSCs began to adhere after 4-6 h of seeding. Gradually the cells grown and formed paired and cluster colonies in 3-4 days in culture. Cells had grown to 80-85% confluency after 7-8 days and 9-10 days under hypoxic and normoxic conditions, respectively [Supplementary Fig.1 (a)]. The proliferation activity reduced with the advancement of days in culture in both normoxia and hypoxia.

The results of morphometry of cmGSCs grown under either normoxic or hypoxic culture conditions are presented in Supplementary Table 2.

The AP staining of cmGSCs was performed to identify the pluripotent characteristics of cmGSCs cultured under normoxia or hypoxia. The cells grown in different culture conditions were positive for AP staining, though the intensity of staining varied among the groups. The significantly ($p < 0.05$) higher intensity was observed in hypoxia compared to normoxia on day 7 and 10 of culture (Supplementary Fig. 1b).

Survival rate and growth characteristics

The comparison of total cell count and count of live cells after different days in culture under normoxic or hypoxic conditions is presented in Fig. 2. The results suggest that the number of total and live cells were similar in normoxia or hypoxia up to day 3 in culture. Thereafter, on day 5 and 7 significantly ($p < 0.05$) higher survival, and number of total and live cells were recorded in the hypoxia group compared to the normoxia (Supplementary Fig. 2).

The PDT of cmGSCs expanded under hypoxic condition was 1.8 fold (on day 5) and 1.3 fold (on day 7) lower than cmGSCs cultured under normoxic conditions (Fig. 1a). The overall PDT (from day 0 to d 7) was 1.4 fold lower under hypoxic culture conditions (2.86 days) compared to the normoxic condition (3.99 days). Similarly, the number of cell division were higher in hypoxic conditions (2.46) compared to the normoxia (1.77). The shorter PDT and more cell cycles indicate a higher growth rate of cmGSCs under hypoxic condition compared to the normoxic conditions (Fig. 1a). Thus, low O_2 tension in *in vitro* culture system prevents the increase in PDT and decrease in the number of cell division of cmGSCs over time.

Cellular viability, MTT, SA- β -GAL senescence and BrdU proliferation assays

The colony-forming unit assay by crystal violet staining was performed to assess the cellular viability and proliferative potential of cmGSCs grown under normal or lowered O_2 level. A representative image and result of absorbance are presented in Fig 1 c and d. A significantly ($p < 0.05$) higher number of colonies and optical density in hypoxia compared with normoxia, indicate favorable effect of hypoxic conditions on proliferation of cultured cmGSCs.

cmGSCs obtained from three different donors were cultured for 7 days under hypoxia (5% O_2) or normoxia (20% O_2), and the effect of O_2 level on viability and proliferation were studied during different days (day 3 and d 7) in culture. For viability assay, MTT assay was performed on day 3 and day 7. On day 7, significantly higher optical density (0.88 ± 0.05 vs 1.061 ± 0.10 ; $p < 0.05$) indicates a higher number of viable cells under hypoxic conditions compared to the normoxic conditions [Fig. 2(a)]. The senescent phenotypes of cultured cmGSCs were identified by the expression of SA- β -GAL. For this, the SA- β -GAL staining was performed at day 3 and 7 of cmGSCs cultured under normoxic or hypoxic conditions (Fig. 4 a-c). The number of senescent cells was similar on day 3, whereas significantly ($p < 0.01$) higher number of SA- β -GAL-positive cmGSCs were observed under normoxic conditions than in hypoxia. The proportion of SA- β -gal-positive cells were $52.33 \pm 2.33\%$ and $28.00 \pm 1.15\%$ of total cmGSCs

counted under normoxia or hypoxia, respectively (Fig. 2b). Representative images of BrdU proliferation assay of cmGSCs cultured in normoxic or hypoxic conditions at day 3 and 7 of culture are presented in Fig. 3. Over time, higher expression of BrdU in the cmGSCs cultured in hypoxic conditions demonstrate that the proliferation was distinctly higher for cmGSCs expanded under hypoxic condition than for those under normoxic conditions (Fig. 1a). Overall, hypoxic culture condition improves viability and increased proliferation whereas decrease senescence in cmGSCs compared to the normoxic culture conditions.

Immunophenotypic characterization

The immunophenotype of cmGSCs expanded under normoxia or hypoxia for 5-8 days were characterized by staining for GC markers (octamer-binding transcription factor-4, OCT-4; T-cell antigen, THY-1 and ubiquitin carboxyl-terminal esterase L-1, UCHL-1) and the representative images of immunofluorescence staining of cultured cmGSCs are presented as Fig. 6. As confirmed by immunocytochemical analysis, the expression of the pluripotency specific cell surface (THY-1) and intracellular (OCT-4 and UCHL-1) markers was higher in cmGSCs cultured under hypoxia compared to the cells in normoxia (Fig. 4), implying that hypoxia improves the expression of pluripotent stem cell characteristic of cmGC over time in culture.

Neurogenic differentiation

Neurogenic differentiation of cmGSCs was confirmed by toluidine blue staining (Fig. 5a). After feeding cmGSCs with neurogenic-inducing media, blue color neuron-like cells were seen with toluidine blue staining. The neuron-like structures were relatively larger and more confluent in cmGSCs culture under normoxia compared to hypoxic conditions (Fig. 5a). The expression of nestin, β -tubulin, and doublecortin mRNA was detected (Supplementary Fig. 3), indicating differentiation of cmGSCs into neuronal cells.

Adipogenic differentiation

Adipogenic differentiation of cmGSCs was confirmed by Oil Red-O staining. After culturing of cmGSCs with adipogenic-inducing media for 15 days, oil droplets were observed in the cytoplasm (Fig. 5b). The more number of cells with larger oil droplets were appeared by bright field microscopy and specific staining of differentiated cmGSCs under normoxia compared to the hypoxic condition. The results indicated that cmGSCs had differentiated into fat cells following adipogenic induction in both the culture conditions, though the higher number were present in the normoxia compared to the hypoxia group.

Chondrogenic differentiation

After 15 days of induction in culture with differentiation media, chondrogenic differentiation of cmGSCs was confirmed by Alcian blue staining (Fig. 5c). The cmGSCs cultured with induction media under normoxia or hypoxia showed the formation of the distinct lacuna structure of cartilage (Fig. 5c). Comparing the effect of culture conditions on chondrogenic differentiation of cmGSCs by bright field and specific staining method, cartilage lacunae and representative morphological structures were more distinctly found under hypoxic culturing compared to the normoxia. The results indicated that cmGSCs

had differentiated into chondrocytes following chondrogenic induction with normoxia as a more favorable condition for such differentiation.

Real-Time PCR for quantification of pluripotency and adhesion-related genes

The expression of pluripotency (OCT-4, THY-1 and UCHL-1) and adhesion (β -integrin and E-cadherin) related functional genes were investigated using cmGSCs cultured under either normoxic or hypoxic condition for 1 week. Their expressions in different culture systems were studied by comparing their expression levels relative to the house-keeping gene (GAPDH). The qRT-PCR based gene expression analysis showed that the expression of pluripotency and adhesion genes were up-regulated (OCT-4 = 2.22 ± 0.28 fold; THY-1 = 1.63 ± 0.18 fold; UCHL-1 = 1.95 ± 0.05 fold; β -Integrin = 2.09 ± 0.31 fold and E-Cadherin = 1.25 ± 0.10 fold) in cmGSCs cultured under hypoxia compared to normoxia (Supplementary Fig. 3a).

The qRT-PCR results represent that the cultured cmGSCs showed the amplification of differentiation markers such as adipogenesis (PPAR γ and C/EBP α), neurogenesis (nestin, β -tubulin, and doublecortin) and chondrogenesis (COL2A1). The expressions level of these genes in different culture systems were studied by comparing their levels relative to the house-keeping gene. Under hypoxic conditions, the expression of differentiation markers was significantly ($p < 0.05$) down regulated in hypoxic conditions compared to the normoxia. The expression level of C/EBP α , nestin, and COL2A1 were downregulated by 3.76 ± 2.68 , 4.50 ± 1.67 , and 6.34 ± 1.17 folds compared to normoxia, respectively. The results indicate lower extent of multilineage differentiation of cmGSCs under hypoxic conditions compared to the normoxia.

Discussion

The level of O₂ is an important variable of stem cell milieu and plays an important role in the regulation of biological characteristics of stem cells. Therefore, O₂ tension in the culture system is believed to play a vital role in cell proliferation, development, and homeostasis [25]. Usually, *in-vitro* expansion of stem cells is performed under ambient O₂ level (20-21% O₂); which is nearly 4–10-folds higher than the O₂ level in natural milieu of mGSc [8]. Thus, the testis has been considered as a naturally O₂-deprived organ [13] and mGC population preferentially resides in a low O₂ (avascular) microenvironment along the basement membrane of the testis. Therefore, as an initial step to better understand the responsiveness and functional characteristic of cmGSCs to the O₂ tension, in this study we investigated the effect of low O₂ concentration on expansion and multiple differentiation of cmGSCs through cell viability, proliferation rate, senescence, immunophenotype and gene expression of specific markers, and compared with the normoxic condition.

In the study, we demonstrated that cultured cmGSCs under hypoxia had significantly ($p < 0.05$) higher proliferation rates than cmGSCs cultured under normoxia. We observed a faster expansion and earlier attainment of confluency while culturing cmGSCs under lower O₂ conditions. The higher growth rate and

viability coupled with lower senescence of cmGSCs under hypoxic conditions resulted in shorter PDT of the cells. These findings are in agreement with earlier reports that demonstrated higher proliferation of other stem cells such as human BM-MSCs cultured under hypoxia [12,27]. The proliferative effect of cmGSCs under low O₂ tension suggests that the relative physiological environment may provide a suitable condition for the viability and proliferation of these cells. Overall, we found that the culturing cmGSCs under hypoxic condition augments their biological characteristics during *ex-vivo* expansion. These results could be a useful to develop methodologies for improving the output of cmGSCs-based cell therapies.

Senescence adversely affects cell proliferation as the senescent cells lose their pluripotent characteristics, and undergo cell cycle arrest during aging [3]. The delay in the development of senescence in cmGSCs under hypoxic conditions may be due to the maintenance of their higher rate of proliferation under hypoxia than normoxic culturing. This may be applicable to obtain larger population of cmGSCs with desired characteristics during long-term culture.

The higher survival and proliferation of cmGSCs under hypoxic condition may be because such an environment represents the physiological growing conditions these cells [8,34]. It is also suggested that mGSCs preferentially reside in hypoxic regions of the ST, which facilitates metabolic pathways that are integral for the ongoing regenerative capacity of the cells [23]. Though beneficial effects of hypoxic culture on proliferation and differentiation potentials of specific cell lines have been suggested by several groups [12,27,19], other studies showed contrasting results with negative or no effects of hypoxia on stem cell proliferation and differentiation [2,21]. These discrepancies in the results may be due to the variation in the O₂ level, the duration of trial, range of biological characteristics compared with type and number of stem cell population, and the variation in the sensitivity of system that was used to regulate the O₂ level. Moreover, the duration and degree of hypoxia used in previous studies vary greatly and may account, in part, for the inconsistent effects of hypoxia on the functional characteristics of cmGSCs [10,24].

In the present study, we demonstrated that cmGSCs cultured under hypoxia for 7 days had higher rates of cell proliferation and expression of stem cell markers (OCT-4, THY-1, UCHL-1) and adherence specific marker genes (β -integrin and E-cadherin) than cells cultured under normoxia. Higher expression of adhesion molecules (both β -integrin and E-cadherin) and subsequently higher adhesion rate of cultured cells onto the surface may be important for faster expansion of the cells under hypoxic conditions [5]. Overall, the O₂ has been shown to act as a communication signal that can affect characteristics of stem cell such as self-renewal, stemness, and differentiation properties [1,28].

In this study, we also observed that the exposure of cmGSCs to a hypoxic environment reduced their differentiation into the neurogenic, adipogenic, and chondrogenic lineages. The absence of significant difference in doublecortin (marker of immature neurons) gene among the groups suggests a similar extent of cmGSCs differentiation up to the state of immature neurons in both the culture conditions. A similar effect of hypoxic condition was reported earlier for chondrogenic or osteogenic differentiation of

adipose tissue-derived stem cells [24]. Further studies into the possible effects of long- and transient/short-term hypoxic culturing with different O₂ levels on lineage-committed differentiation of cmGSCs are required.

In the present study, we have shown that the hypoxic condition (5% O₂) had a reassuring effect on viability, proliferation and stemness, and delayed the senescence of cmGSCs. Our results indicate that under hypoxic conditions, cmGSCs overexpress key makers of stemness and adhesion, thereby positively influencing their self-renewal and proliferation. Nonetheless, differentiation of cmGSCs into different cell types is inhibited by hypoxic culturing. Thus, faster expansion of cultured cmGSCs coupled with the differential expression pattern of stemness, adhesion, and differentiation-specific key markers in cmGSCs provides correlative evidence that low O₂ microenvironment (5% O₂) provides a better culture condition for the self-renewal and proliferation of cmGSCs without adversely affecting their stemness properties. Overall, low O₂ tension is supportive for maintaining proliferation and stemness but not for multilineage differentiation (plasticity) of this stem cell population. These observations add to the understanding of cmGSCs responses to defined culture conditions, which is the most critical issue for their maintenance and clinical applications.

Declarations

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Conflicts of Interest The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Availability of data and material The datasets pertain to the current study are available from the corresponding author on reasonable request.

Code availability Not applicable

Author contributions **Conceptualization:** Shiva Pratap Singh, Suresh Dinkar Kharche; **Methodology:** Shiva Pratap Singh, Manisha Pathak; **Formal analysis and investigation:** Shiva Pratap Singh, Ravi Ranjan, Yogesh Kumar Soni; **Writing - original draft preparation:** Suresh Dinkar Kharche; Manoj Kumar Singh, Ramasamy Pourouchottamane; **Writing - review and editing:** Shiva Pratap Singh, Manmohan Singh Chauhan; **Funding acquisition:** Shiva Pratap Singh; **Supervision:** Manmohan Singh Chauhan

Ethical approval All the experimental procedures were carried out were approved by the Animal Ethics Committee of the Institute

Consent to participate Not applicable

Consent for publication All the authors agree and approve the manuscript.

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Figures

Figure 1

(a) Differential response of O₂ levels on population doubling time (days), and (b) number of cell divisions in caprine male germline stem cells (cmGSCs) cultured in normoxic or hypoxic conditions during different days in culture. (c) Crystal violet staining of cells grown under normoxia and hypoxia was performed in triplicate after 3 passages. Scale bar, 200 μ m. (d) The optical density of normoxia and hypoxia group was compared after solubilization of stain. * indicates a significant difference ($p < 0.05$) among the groups

Figure 2

(a) MTT assay for metabolic viability. Optical density of the caprine male germline stem cells (cmGSCs) growing under normoxic or hypoxic culture conditions after day 3 and 7 of cell seeding. Results are presented as mean $\pm$ SEM (**: $p < 0.01$). b-d Cellular senescence occurring during culture of cmGSCs. (b) Quantification of Figure (c-d), data are presented as mean $\pm$ SEM of three independent experiments and statistical differences were calculated significant as **: $p < 0.01$. Representative images of senescence-associated (SA)- β -GAL staining at day 5 of cmGSC culture in normoxic (c) or hypoxic (d) conditions. Senescent cells are stained with dark blue color (indicated by a solid arrowhead) whereas; growing cells remain unstained (indicated by an unfilled arrowhead). Scale bar, 50 μ m

Figure 3

Representative images of BrdU proliferation assay of caprine male germline stem cells (cmGSCs) cultured in normoxic or hypoxic conditions at day 3 and 7 in culture. For negative (-ve) control, all the steps were followed except use of the primary antibody (rabbit anti-BrdU antibody). Scale bar, 50 μ m

Figure 4

Representative images of immunofluorescence staining of cultured caprine male germline stem cells (cmGSCs) stained for stem cell markers (octamer-binding transcription factor-4, OCT-4; T-cell antigen, THY-1 and ubiquitin carboxyl-terminal esterase L-1, UCHL-1). Cells were grown on the 24 well culture plates under normoxic or hypoxic condition. After 5-8 days of culture at P3, cells were stained for markers with specific primary antibodies and Alexa flour 488 secondary antibody followed by counter-stained with DAPI. Merged images are after double-immunofluorescence staining with anti-primary antibody and DAPI. Cells positive for specific marker appear green and DAPI positive cells stained blue. In negative (-iv) control, primary antibody was excluded. Scale bar, 50 μ m

Figure 5

Representative images of morphology of differentiation caprine male germline stem cells (cmGSCs) under normoxic or hypoxic culture conditions. Appearance under the microscope (bright field) and specific staining properties were used to identify the cells. Cells were grown on the 24 well culture plates under normoxic or hypoxic condition. After 15 days of culture in corresponding differentiation media at P3, the cells were stained for specific staining (a: Neuronal differentiation: Toluidine blue; b: Adipogenic differentiation: Oil Red O, and c: Chondrogenic differentiation: Alcian blue). Scale bar, 50-100 μm

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