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Effect of Hypoxia on Stemness and Differentiation of Dental Pulp Derived Stem Cells

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Abstract: *Stem Cells are characterized by their properties of self renewal and multipotency. These stem cells are localized inside our body at specialized niches, which are known to have low oxygen tension. Dental pulp tissue found in the middle of the tooth is one such niche which experiences hypoxic conditions due to its anatomical location. However under in-vitro conditions, stem cells are cultured under normoxia condition (21% O_2 concentration). It has been known since decades that low oxygen tension in tissue cultures has positive effect on the cell growth. In this study, we tested this hypothesis on in vitro isolated, expanded, Dental Pulp Stem Cells (DPSCs) grown under low oxygen. The comparative evaluation was done between DPSCs grown in normoxia (21% O_2) and Hypoxia (3% O_2) on the basis of proliferation, aging, maintenance of stemness and differentiation potential. Our study indicates that DPSCs when grown in hypoxia exhibited better renewal capacity, lesser population time and hence decreased differentiation. Thus, this study emphasizes that hypoxic culture conditions is better in vitro culture condition for expansion of DPSCs in large scale and maintenance of their stemness for therapeutic purpose.*

Keywords: *Adult Stem Cells, Dental Pulp Stem cells, Differentiation, Hypoxia, Proliferation*

I. Introduction

Human body contains several microenvironment referred as Stem-cell (SC) niche inhabited by significant number of stem cells. Dental pulp is one such location situated in the middle of the tooth and has been shown to harbor diverse populations of multipotent stem/progenitor cells [1, 2]. DPSCs were first isolated in the year 2000, which mainly resides in the perivascular microenviroment of dental pulp. DPSCs have similar characteristics to bone marrow-derived mesenchymal stem cells (BM-MSCs) [3]. These DPSCs have edge over other adult stem cells because of their non-invasive procurement and ease of access [3]. Recently, it has been found that *in vivo* stem cells reside in niches with low oxygen tension [4, 5]. Dental pulp tissue is one such niche experiencing hypoxic conditions due to its anatomical location [6]. Researchers have reported that hypoxia leads to increased proliferation in human bone marrow cells [7], neural stem cells [8], fibroblasts [9] and others including dental pulp cells from various sources [10-14]. Although there is consensus on effect of hypoxia on proliferation [10-14], there is diverse view on effect of hypoxia on differentiation potential. Hypoxia has been reported to enhance as well as suppress the differentiation of MSCs into osteocytes and chondrocytes [15-17].

In this study, we have investigated the effect of hypoxia (3% O_2) on proliferation and differentiation potential of stem cells. DPSCs were obtained from impacted third molar and were grown under hypoxia and normoxia (21% O_2) as explant culture. These were examined on the basis of morphology, growth kinetic analysis, senescence assay, surface marker profile and tri-lineage differentiation potential.

II. Materials and Methods

2.1 Sample Collection

This study was approved by Institutional Committee for Stem Cell Research and Therapy (IC-SCRT). Ten impacted third molars were obtained from 10 individuals (age ranged between 16-18years) who required it to be removed for orthodontic treatment at Department of Orthodontics and Dentofacial Deformities, Centre for Dental Education and Research (CDER), All India Institute of Medical Sciences (AIIMS). Written informed consent was obtained either from the individuals or their legal representative. Following standard tooth extraction procedure, sample was transported to the Stem Cell Facility, AIIMS in cold transport media containing 1× Dulbecco's Modified Eagle's Medium- Low Glucose (DMEM-LG) (Life Technologies, USA), 200µg/ml of penicillin and 200U/ml of streptomycin (Life Technologies, USA) and 1X antimycotic (Life Technologies, USA) at 4ºC. The tooth samples were processed immediately for the establishment of primary culture.

2.2 Processing, isolation and establishment of DPSCs culture

Any extraneous soft connective tissue was removed from the outer surface of the tooth using needle or sterile scalpel blade. It was then cracked opened with 7" heavy duty bone cutter and pulp was carefully removed using sterile fine forceps and needle. The pulp was rinsed twice with 1X PBS containing antibiotics for 20 minutes. It was then cut into small pieces (<1mm) and explant culture were seeded into two 35mm Petri dishes (Becton Dickinson, USA) with DMEM-LG containing 10% Fetal Bovine Serum (FBS) (HyClone, USA), penicillin (100U/ml) and streptomycin (100µg/ml). One of the 35mm-petri dish was incubated at 37°C in humidified atmosphere containing 21% O_2 and 5% CO_2 and the second dish was incubated at 37°C in humidified atmosphere containing 3% O_2 and 5% CO_2. After 3 days, the media was changed and the subsequent media changes were done at every third day taking care not to dislodge the explants. After establishment of DPSC primary cultures of 70%-80% confluent, the cells were trypsinized using 0.25% trypsin-EDTA (TE) (Life-Technologies, USA) followed by incubation for 2-3 minutes at 37°C. Cells were split in 1:3 ratio and incubated at 37˚C, 5% CO_2 and respective O_2 concentration with media change on every third day.

2.3 Culture Conditions

Hypoxic culture conditions were maintained by culturing of DPSCs (hereafter referred as DPSC-H) in 3% O_2, 5% CO_2, and 92% N_2 in a 95% humidified atmosphere. Control cells were kept under normoxic conditions (hereafter referred as DPSC-N) (21% O_2). [18]

2.4 Characterization of DPSCs

2.4.1 Morphological Analysis

Both DPSC-H and DPSC-N were regularly viewed under Phase Contrast Inverted Microscope (Nikon 80i, Japan) for any morphological changes. Any signs of contamination were also monitored.

2.4.2 Immunophenotyping

Immunophenotype of cultured DPSCs (N=10) was determined by flow cytometry. DPSC-H and DPSC-N at passage 3 were harvested using 1X TrypLE Express (Life Technologies, USA). Single cell suspension of 1×10^6 cells/ml was prepared and from this 100 µL of cell suspension (1×10^5 cells) were incubated with anti-human antibodies CD73-Phycoerythrin (PE) , anti-CD90- Phycoerythrin conjugated with Cyanine dye derivative (PECy5) , anti-HLA Class I-Allophycocyanin (APC), anti-HLA Class II- Fluorescein Isothiocyanate (FITC) (Becton Dickinson, USA), anti-CD29- FITC and anti-CD105- APC (eBioscience, USA) at room temperature in dark for 1 hour. Unstained cells were included as controls. The cells were acquired in LSR II flow cytometer (Becton Dickinson, USA) with at least 5000 events for each sample and analyzed with FACs DIVA software (version 6.1.2). [19]

2.4.3 Measurement of Metabolic Activity by MTT Assay

Proliferation rate of DPSCs (N=5) was measured by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. DPSC-N and DPSC-H passage three cells were seeded in triplicates onto 96 well flat bottom plate at a concentration of $5x10^3$ cells per well. On days 1, 3, 6, 9, 12, 15, 22 and 28, media was replaced with 180µl fresh media and 20µl MTT reagent (5mg/ml in PBS) was added and further incubated for 4 hours at 37˚C in respective incubating conditions. The supernatant media was removed carefully and 150µl DMSO (Sigma, USA) was added to each well to dissolve formazan crystals and incubated at 37˚C for 30 min in dark. The optical density (OD) was measured at 570nm and 660nm by spectrophotometer (BioTek, USA).

2.4.4 Proliferation Assay

DPSCs for each sample (N=5) were seeded at a density of $25x10^3$ cells per 35mm petri dish (Becton Dickinson, USA) and grown under normoxia and hypoxia condition as mentioned earlier. After 3 days, DPSC-H and DPSC-N were harvested, enumerated and assessed for viability using Trypan Blue dye exclusion assay. The PDT was obtained by the formula [20]:

PDT=T−To Log2 (LogN−LogNo)
Where, T: Time of harvesting
To: Time of seeding
N: Number of cells harvested
No: Number of cells seeded

2.4.5 Senescence Assay

In order to evaluate senescence, β-galactosidase staining was performed using commercially available kit (Cell Signalling Technology, USA) according to manufacturer's protocol. Briefly, DPSC-N and DPSC-H of 3rd passage (N=5 each) were seeded in duplicates and incubated in respective O_2 concentration until they become confluent (75-80%). Cells were washed with 1XPBS and fixed with 2% formaldehyde/0.2% glutaraldehyde solution for 15 minutes. Followed by two washes with PBS β-galactosidase chromogenic substrate solution containing 1mg/ml 5-bromo-4-chloro-3-indolyl-β-galactosidase (X-Gal), 40mM citric acid (pH 6.0), 5mM potassium ferrocyanide, 5mM potassium ferricyanide, 150mM NaCl and 2mM $MgCl_2$ was added. The petri dishes were then incubated at 37°C overnight. Percentage of cells expressing β-galactosidase was measured in three random fields under inverted phase contrast microscope (Nikon 80i, Japan) [20].

2.4.6 Tri-lineage Differentiation of DPSCs

DPSC-H and DPSC-N of passage 3 (N=5 each) were used for tri-lineage differentiation. Undifferentiated cells were used as experimental controls.

2.4.6.1 Osteogenic Differentiation

DPSC-H and DPSC-N were induced with DMEM-LG (Life Technologies, USA) supplemented with 10% FBS (Life Technologies, USA), 50 μM ascorbic acid-2- phosphate, 0.1μM dexamethasone and 10mM ß-glycerophosphate (Sigma-Aldrich, USA) for 28 days. Osteogenic differentiation was confirmed by Alizarin Red S staining (Himedia, India).

2.4.6.2 Adipogenic Differentiation

For adipogenic differentiation, DPSC-H and DPSC-N were cultured in DMEM- LG with 10% FBS supplemented with 100μM Indomethacin, 1μmol/L dexamethasone, 500μM 3-isobutyl-1-methylxanthine (IBMX), 1μg/ml Insulin (Sigma-Aldrich, USA) for 21 days. Media was changed every third day for 28 days. The differentiation was confirmed by Oil Red O staining (Himedia, India) [19].

2.4.6.3 Chondrogenic Differentiation

Commercially available kit (Life Technologies, USA) for chondrogenic differentiation was used. Briefly, micromass culture was generated by seeding 10μl droplets (consisting about $1x10^5$ cells) of DPSC-H and DPSC-N cell solution in 35mm petri dish and incubated for 2 hours at 37ºC with 5% CO_2 in respective oxygen concentration followed by addition of chondrogenic differentiation media for 21 days. The differentiation was confirmed by Alcian Blue staining (Sigma-Aldrich, USA).

2.4.7 Reverse Transcriptase-PCR (RT-PCR)

DPSCs-H and DPSCs-N (osteo and chondro induced cells, N=5 each) were lysed using TRIzol (Sigma-Aldrich, USA) and RNA was isolated using Phenol-Chloroform method. cDNA was prepared using 1μg of RNA by Reverse transcriptase (RT) enzyme (Promega, USA). RNA was reverse transcribed by mixing 1μg of RNA with 0.5μg of oligo-dT followed by 5 minutes incubation at 70°C. Final volume was made 20μl by further addition of RT-Buffer (1X), 200U M-MLV RT, 2mM dNTPs and 40U RNasin plus RNase inhibitor (all Promega, USA). Reaction mixture was incubated at 37°C for 1 hour. For confirmation of osteogenic and chondrogenic differentiation, expression profile of osterix and collagen- II was checked by semi-quantitative RT-PCR respectively and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene (BioRad, USA).

Table 1 Primer Sequences and Reaction Conditions for PCR Sequence

S. No	Gene	Sequence	Annealing (ºC)	Size (bp)
1	GAPDH	Forward:5'-GAG TCA ACG GAT TTG GTC GT-3' Reverse: 5'-GAC AAG CTT CCC GTT CTC AG-3'	56	200
2	Osterix	Forward: 5'-GGC ACA AAG AAG CCG TAC TC-3' Reverse: 5'-CAC TGG GCA GAC AGT CAG AA-3'	57	284
3	Collagen-II	Forward: 5'-ACC AAA GGG ACA GAA AG-3' Reverse: 5'-CAG CTT CAC CAT CAT CAC C-3'	56	400

2.4.8 Statistical Analysis

All values were stated as mean ±standard deviation (SD). Student's t test was applied for MTT assay and Population doubling time. Two-sample Wilcoxon rank-sum (Mann-Whitney) test was used for Flow cytometry- and senescence assay- Fisher's exact test. The degree of significance was set at $p<0.05$.

III. Results

3.1 Establishment of DPSC Primary Cultures

Initial migration of cells from explants was observed within 6-7 days of culture among DPSC-H where as in DPSC-N migration was observed in 10-12 days (Fig 1). DPSC-H cells established monolayer of 70-80% confluence in 25-30days while DPSC-N took 6-7days more to establish monolayer with similar confluence. Cells were passaged once they reached 70-80% confluency.

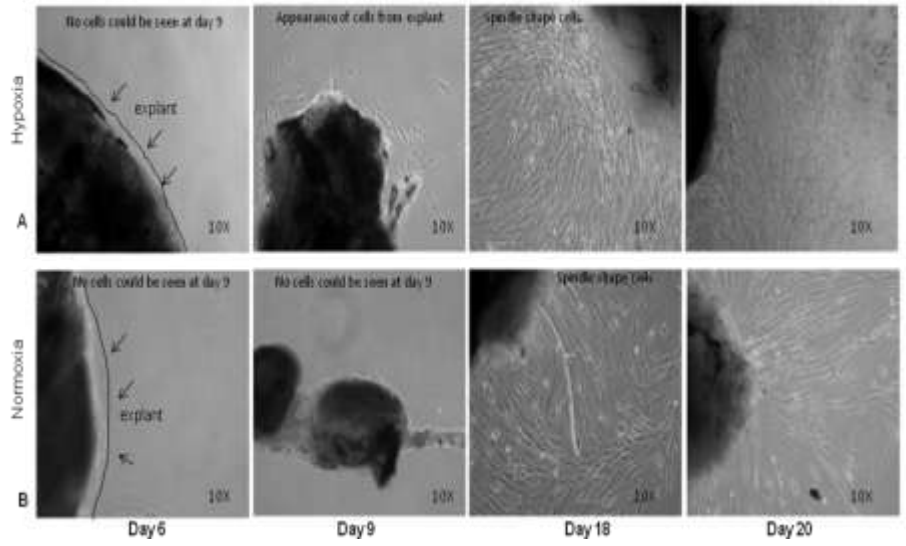

Figure 1 Explant culture of DPSCs from Dental Pulp

DPSCs (10X magnification) migrating out of explants while grown in (A) Hypoxia and (B) Normoxia from day 6 to day 20.

3.2 DPSCs Morphology

DPSC-N and DPSC-H both exhibited fibroblast like spindle shaped morphology and expanded in whirlpool like manner bone marrow derived mesenchymal stem cells (BM-MSCs) (Fig 2). During the long term cultivation no visual signs of culture degeneration or spontaneous differentiation could be observed in both the groups.

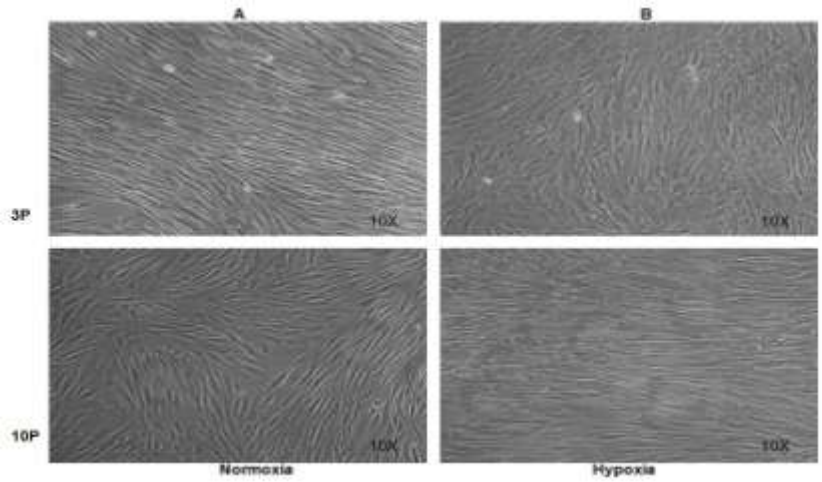

Figure 2 Morphological characteristics of DPSCs

Fibroblast like morphology of DPSCs at early (passage 3) and late (passage 10) passage when grown in (A) Normoxia (B) Hypoxia under phase contrast microscope (10X).

3.3 Surface Marker Profiling

Both DPSC-H and DPSC-N groups were found to be positive for CD29, CD73, CD90, CD105, HLA-I and negative for HLA II and CD34/45. However, no significant difference was observed (p=0.796) (Fig 3).

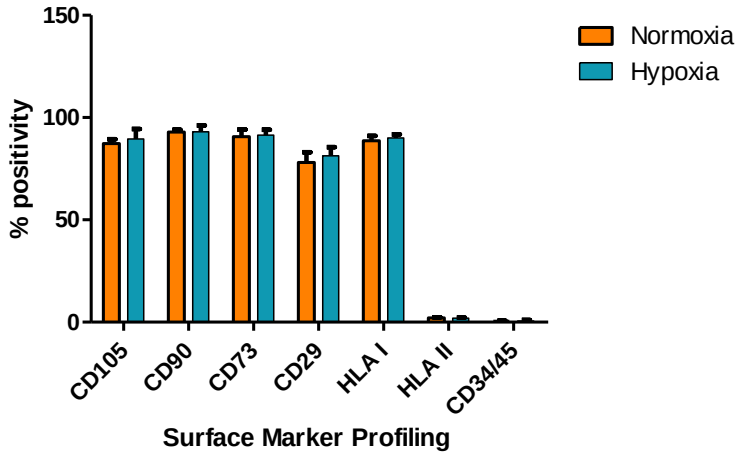

Figure 3 Surface Marker Profiles of DPSCs

3.4 Growth Kinetics

To detect the effect of hypoxic condition on DPSCs proliferation MTT assay was performed over a span of 28 days. On all assay days cell proliferation in hypoxic cultures was greater than that of normoxic group. However, statistically significant difference in proliferation rate was observed only on assay 1, 6, 12 and 22 days (Fig 4A).

Furthermore, PDT for hypoxic group was 26.75 ±2.76 hours while the normoxic group had significantly longer doubling time of 38.59 ± 4.25 hours (Fig 4B).

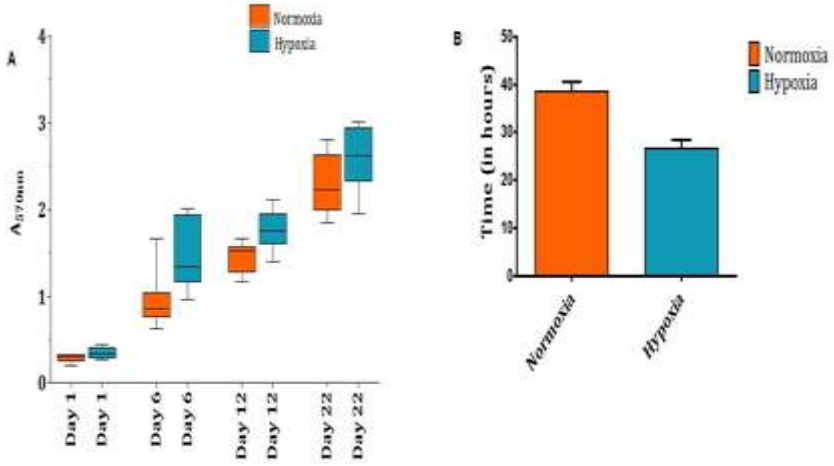

Figure 4 Proliferation patter and Population Doubling Time of DPSCs

(A) MTT assay depicting proliferation rate of DPSCs and (B) Comparison of Population Doubling Time under Normoxia and Hypoxia.

3.5 Senescence Assay

It was observed that at 10^{th} passage DPSCs-H and DPSCs-N exhibited senescence in 0.5% and 1% of cells, respectively (Fig 5).

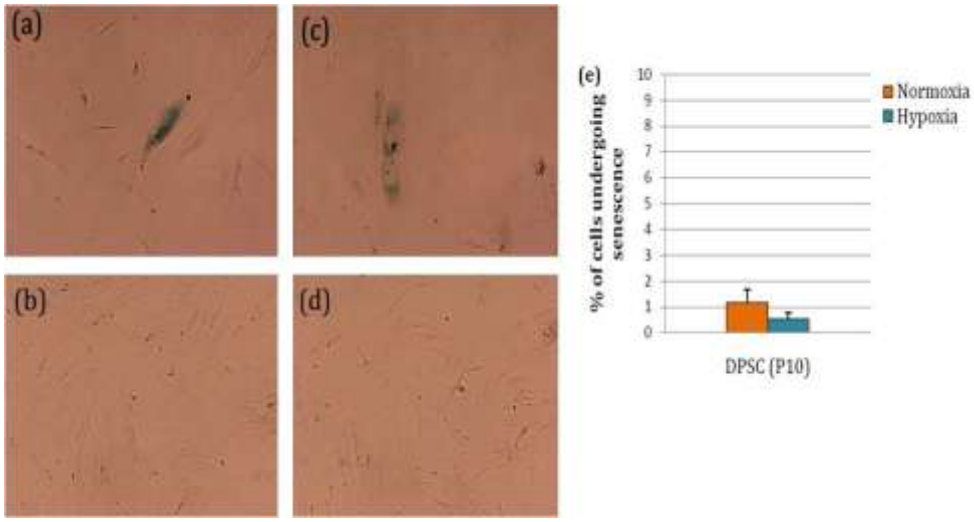

Figure 5 Senescence Rate in DPSCs

Microscopic observation (10X) of DPSCs grown under Hypoxia (a, b) and Normoxia (c, d). Graphical representation of % of cells found positive for senescence in two groups (e).

3.6 Tri-lineage Differentiation Potentials

DPSCs cultured in hypoxic and normoxic conditions were able to differentiate towards osteogenic lineage. Controls grown under normoxic and hypoxic condition during the experimental period did not show any spontaneous induction; rather the cells grew in confluency and resulted in confluency. Osteoinductive cultures of DPSC-N and DPSC-H stimulated for four weeks, exhibited osteogenic nodule formation (Fig. 6 & 7) and chondrogenic lineages (Fig. 8 & 9) after four weeks of culture in appropriate differentiation medium. Uninduced DPSC-H and DPSC-N did not show any visual signs of spontaneous differentiation during the experimental period of four weeks. This was confirmed by their Alizarin Red S and Alcian Blue staining. On an average, osteogenic nodules took about 15-16 days to develop in normoxia condition while in hypoxia it took them about 16-18 days. Similar trend was observed for chondrogenic differentiation where chondrogenic nodules in normoxic condition took 3-4 days to develop while in hypoxic condition they took 6-7 days. These differentiations were further successfully confirmed using RT- PCR detection of osterix (osteogenic marker) (Fig.7) and collagen-II (chondrogenic marker) (Fig. 9). However, we were not able to achieve adipogenic differentiate in any of our DPSC-N and DPSC-H cells (Fig.10).

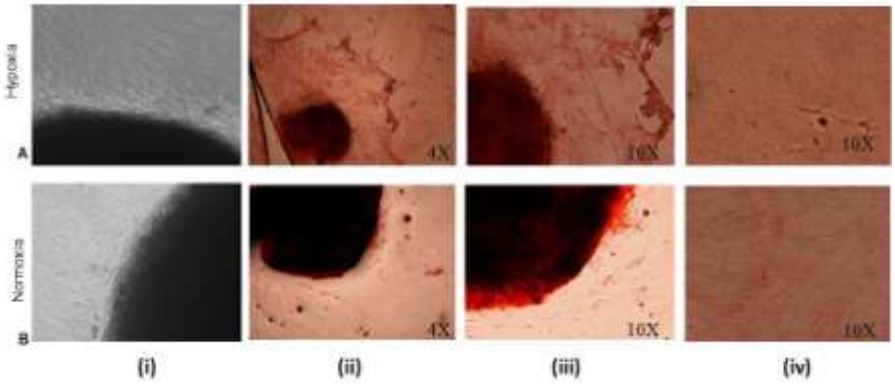

Figure 6 Alizarin Red S Staining for confirmation of Osteogenic differentiation

Representative images showing Osteoinductive cultures of DPSCs under Hypoxia (A) and Normoxia(B) indicated the deposition of mineralized matrix within the osteogenic nodules as confirmed with Alizarin Red S staining. Bright field images of induced cultures at 10x (i), Alizarin Red S staining at 4x and 10x (ii, iii) uninduced cells at 10x (iv).

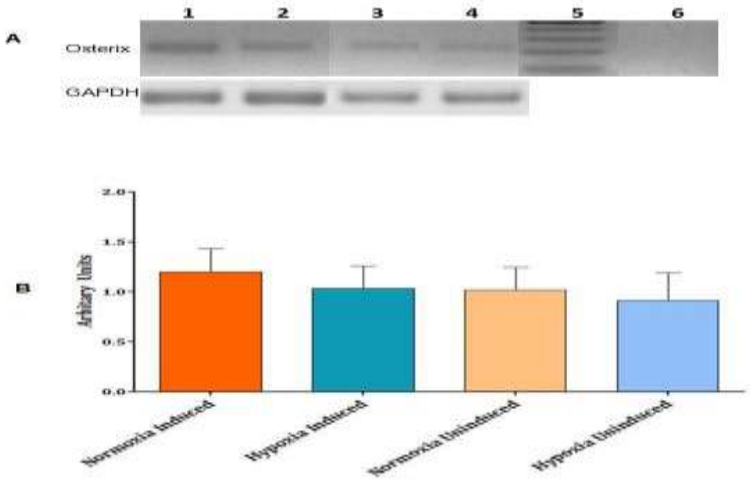

Figure 7 mRNA expression of Osterix for confirmation of Osteogenic differentiation

(A) Agarose gel electrophoresis image (Lane 1: Induced DPSC-N, Lane 2: Induced DPSC-H, Lane 3: uninduced DPSC-N, Lane 4: uninduced DPSC-H, lane 5: Ladder, Lane 6: negative control. Normalization of each band obtained was done with respect to its GAPDH. (B) Densitometry pattern of osterix in induced cultures under Hypoxia and Normoxia.

(B)

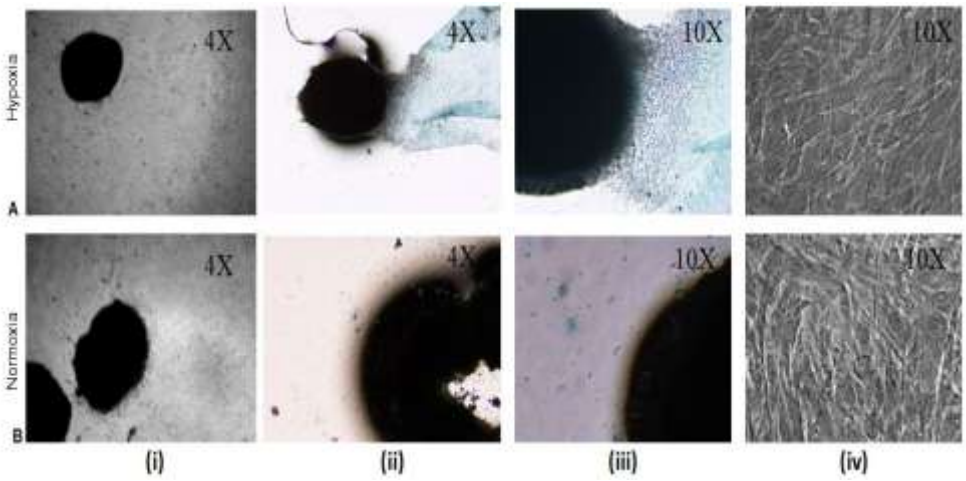

Figure 8 Alcian Staining for confirmation of Chondrogenic differentiation

Representative images showing Chondrogenic cultures of DPSCs under Hypoxia (A) and Normoxia(B) Bright field images of induced cultures at 4x(i), Alcian Blue staining staining at 4x and 10x (ii, iii), uninduced cells at 10x (iv).

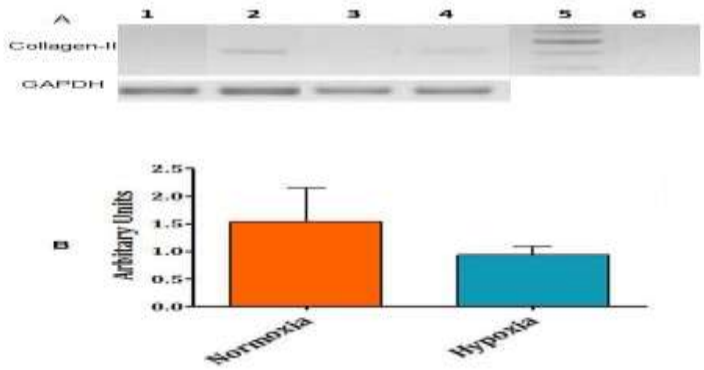

Figure 9 mRNA expression of Collagen-II for confirmation of Chondrogenic differentiation
(A) Agarose gel electrophoresis image (Lane 1: Uninduced DPSC-N, Lane 2: Induced DPSC-N, Lane 3: uninduced DPSC-H, Lane 4: Induced DPSC-H, lane 5: Ladder, Lane 6: negative control. Normalization of each band obtained was done with respect to its GAPDH. (B) Densitometry pattern of collagen-II in induced cultures under Hypoxia and Normoxia.

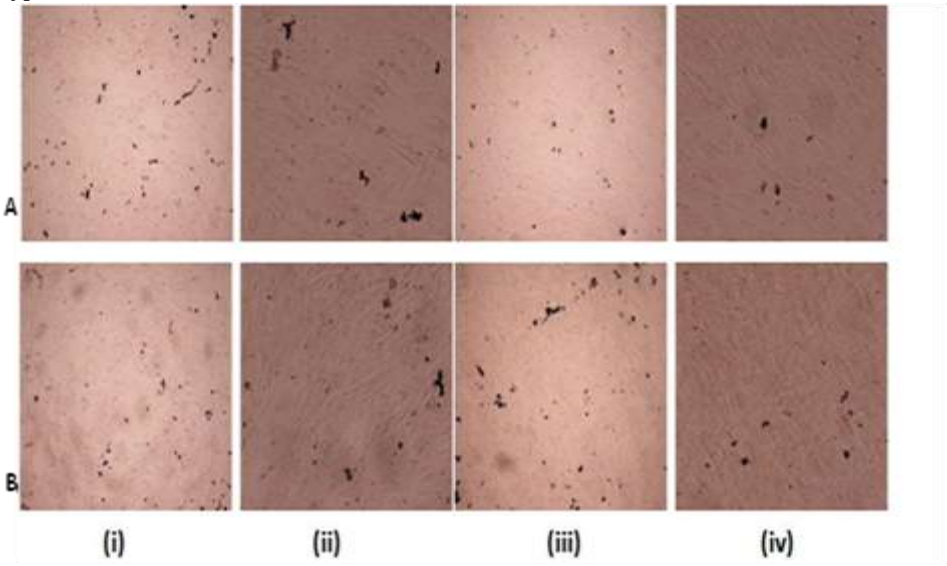

Figure 10 Oil Red O Staining for confirmation of Adipogenic differentiation

Representative images showing adipogenic cultures of DPSCs under Hypoxia (A) and Normoxia(B) Bright field images of induced cultures at 4x and 10x (i,ii), uninduced cells at 4x and 10x (iii, iv). None of the samples induced (A and B) showed Oil Red O stained lipid globules inside the cells.

IV. Discussion

During the last few decades stem cells have been derived from various tissue sources including dental pulp. DPSC are now being recognized as vital source in regenerative dentistry. Due to their easy availability and multipotency characteristics, DPSCs might have clinical implication beyond dentistry. Expansion of these cells while preserving their stem cell characteristics or stemness is of prime importance for their successful use in clinical practices. Among all factors, hypoxia has been shown to have positive effect on cell proliferation [21].
In the current study, we demonstrated the effect of hypoxia on DPSCs proliferation, stemness, differentiation and aging. We could isolate healthy, spindle shaped cells using explants method in both hypoxia and normoxia These were cultured and expanded successfully in DMEM-Low Glucose (LG) media with 10% FBS which is in sync with previous reports [22, 23]. However, Govindasamy *et al,* observed low cellular proliferation, higher senescence rate and abnormal morphology in DPSCs cultivated in DMEM-LG [24]. Also, Lizier *et al,* demonstrated MEM-α and DMEM/F12 as most appropriate media for DPSCs expansion [25]. Hence, we have shown that DMEM-LG is the simple media that can be used for successfully isolation and expansion of DPSCs both in normoxia and hypoxia.

We employed explants culture method for DPSC isolation and got successful isolation in both normoxia and hypoxia. This explant culture method fulfils the criteria of an ideal source of mesenchymal stem cells for clinical application because they are harvested using procedures that have minimal invasiveness and morbidity, and high content of stem cells. Also, this is a simple, gentle, economic and enzyme free method. Time taken for initiation and establishment of culture is lesser in hypoxia as indicated by early emergence of cells spreading out of the explants, which indicates the advantage of hypoxia in scaling up of stem cells for future therapeutic purpose in lesser duration.

There was no morphological difference observed between DPSCs grown in normoxia and hypoxia and they exhibited fibroblast like spindle shaped morphology as reported for BM-MSCs [26]. Surface marker profiling which is hallmark of mesenchymal stem cells, demonstrated that DPSCs of both groups were positive for MSCs markers CD105, CD90, CD73 and CD29 and were negative for hematological markers CD 34/45 which is in sync with other previous studies [27, 28]. Moreover, DPSCs were also found to be positive for immunological marker HLA I and negative for HLA II. Expression of MSCs markers substantiates the fact that these cells have mesenchymal characteristics due to their ecto-mesenchymal origin. Quantitatively, no

significant difference was observed in the percentage of cells expressing these markers in the two groups under investigation [29,30].

Growth kinetics studies done by MTT showed that DPSCs-H exhibited higher proliferation than DPSC-N for up to 21 days with significant difference and significantly lower PDT as compared to DPSCs-N. These cells could be successfully propagated for prolonged period of time (5 months; 14 to 21 passages for hypoxic cultures and up to 14 passages for normoxic culture) in undifferentiated state without any visual signs of spontaneous differentiation and culture degeneration. This was further confirmed by the expression of surface markers, which were found to be above 85% up to 15^{th} passage in hypoxia (data not shown), which depicts that hypoxia helps maintain its stem cell characteristics. Also, senescence assay depicted that cells were healthy and about 1% of cells in normoxia and 0.5% cells in hypoxia were undergoing senescence though, the difference was not statistically significant which is in line with previous reports [20].

Apart from self renewal, we also checked effect of hypoxia on tri-lineage differentiation potential of DPSCs. In our study, DPSCs successfully differentiated into osteogenic and chondrogenic lineages. Osteogenic differentiation was confirmed by Alizarin red S staining and RT-PCR for Osterix gene. Densitometry analysis of PCR amplified cDNA of osterix from two groups revealed marginally lower expression of this osteogenic marker gene in induced hypoxic culture [7,31]. Similar observation has been recently reported in case of Adipose Stem Cells, where hypoxia was found to inhibit osteogenic differentiation [32,33]. Similarly, Chondrocytes differentiation was confirmed by Alcian blue staining and RT-PCR for Collagen-II. Densitometry analysis for collagen-II from induced samples of two groups revealed decreased expression of this chondrogenic marker in hypoxic culture, which is in sync with earlier report for decreased chondrogenesis of adipose stem cells in hypoxia [32]. Meanwhile contradictory findings have been reported showing enhanced chondrogenesis of MSCs in hypoxia [22,34]. For, adipogenic differentiation, we could not see any adipocytes upon induction in both the groups. These results are in agreement with previous studies where either researcher were not able to differentiate these cells into lipid laden adipocytes or observed very low differentiation using same inducers [3,20], and this difference in degree of differentiation might be their tissue specific behaviour. In 2010, a study was published suggesting that a more potent adipogenic inductive culture can induce DPSCs to form oil red O positive lipid containing adipocytes containing hydrocortisone instead of dexamethasone [35]. Similarly, densitometry analysis of PCR amplified cDNA of collagen-II from induced samples of two groups revealed decreased expression of this chondrogenic marker in hypoxic culture, which is in sync with earlier report for decreased chondrogenesis of adipose stem cells in hypoxia [32]. Meanwhile contradictory findings have been reported showing enhanced chondrogenesis of MSCs in hypoxia [22,34].

Role of hypoxia in proliferation and differentiation has been investigated since years. However, we were not able to study the underlying mechanism. Growing literature states the role of Hypoxia Induced Factor (HIF) interaction with notch signaling in maintenance of undifferentiated state [34]. The HIF-1α is induced after low oxygen exposure in certain cell types [36]. However, in present study we did not study its role in maintaining stemness of the stem cells.

V. Conclusions

We demonstrated that DPSCs can be successfully isolated from human third molars using explant method, which is cost effective and enzyme free. These can be cultivated successfully in DMEM-LG + 10% FBS. Hypoxia i.e. low oxygen tension enhances proliferation, maintains stemness and suppresses tri-lineage differentiation of these cells. Therefore, hypoxia culture may be used for scale up of stem cells required for further therapeutic purpose.

Acknowledgements

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