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Original article

Study of optimal conditions for growth and osteogenic differentiation of dental pulp stem cells based on glucose and serum content

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ABSTRACT

Dental pulp stem cells (DPSCs) have shown promising characteristics in terms of their proliferation and osteogenic differentiation potential, which could be of greater benefit in regenerative dentistry. However, obstacles remain in the in vitro cultivation of DPSCs, which significantly affect their growth and differentiating ability. Therefore in this study, we demonstrated the growth and osteogenic differentiation of DPSCs in the presence of media containing different combinations of serum and glucose to get an optimized combination of both. DPSCs were cultured in media containing combinations of low glucose (LG), low serum (LS), high glucose (HG), and high serum (HS). The proliferation and osteogenic differentiation were assessed in DPSCs cultured with these different combinations of culture conditions. High glucose high serum condition significantly inhibited the proliferation of DPSCs and also affected their clonogenic potential, as evidenced by colony-forming units. Irrespective of the serum content, high glucose in the media also decreased the osteogenic potential of DPSCs confirmed by functional staining, and downregulation of osteogenesis-related genes. High glucose content in the culture media affects the growth and differentiation potential of the DPSCs. Hence, the culture conditions for the DPSCs should be reconsidered to utilize their maximum potential.

1. Introduction

In the last decade, mesenchymal stem cells have shown promising results for the management of degenerative diseases, via positive clinical trial outputs (Gronthos et al., 2000; Hollands et al., 2018; Kolind et al., 2014; Ledesma-Martínez et al., 2016). Dental stem cells, especially DPSCs, are not far behind in clinical translation and are being studied extensively worldwide. DPSCs are unique because of their neural crest origin and while showing the typical characteristics of stem cells like self-renewal and multilineage differentiation with the ability to differentiate into odontoblasts, adipocytes, osteoblasts, chondrocytes, myocytes, cells from liver, and neural cells (Ducret et al., 2015; Gronthos et al., 2002). DPSCs are emerging as an ideal source of mesenchymal stem cells, because of their abundant availability, less morbidity during sample extraction, and high stem cell content in comparison to bone marrow-derived mesenchymal stem cells (BMMSCs) in recent times (Gronthos et al., 2002; Kolind et al., 2014; Ledesma-Martínez et al., 2016; Shi et al., 2005).

DPSCs are a stem cell population showing the high proliferative potential capacity for self-renewal, multi-lineage differentiation, and no allogenicity and tumor formation upon transplantation. This makes them an ideal candidate for cellular and regenerative therapy (Alkhalil et al., 2015; Bakkar et al., 2017; Ledesma-Martínez et al., 2016; Patil et al., 2018; Xiao and Nasu, 2014).

Abbreviations: DPSCs, dental pulp stem cells; HGLS, high glucose low serum; HGHS, high glucose high serum; LGLS, low glucose low serum; LGHS, low glucose high serum.

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DPSCs could be extremely useful in the new field of regenerative dentistry from cell-based therapies to tissue engineering based on their greater propagation rate, enhanced clonogenicity, and elevated osteoinductive capacity in vivo compared to MSCs of other origins (D'Alimonte et al., 2017; Ducret et al., 2015; Ferro et al., 2012). Moreover, there is a very less degree of ethical or legal concerns about the therapeutic and medicinal use of these cells; therefore, the interest of the researchers and clinicians is continuously increasing in the DPSC research (Ferro et al., 2012; Kolind et al., 2014; Viña-Almunia et al., 2017).

The main hurdle in the therapeutic applications of DPSCs is their efficacy (Bakopoulou et al., 2017; Bonnamain, 2013; Kanafi et al., 2013; Patil et al., 2018; Suchánek et al., 2013). Considering the need to develop the defined culture conditions, we speculate that an ideal culture condition would modulate the functional activity of DPSCs in terms of differentiation potential and the degree of differentiation, both qualitatively & quantitatively. Optimal changes in the supplementation in maintaining DPSCs are likely to augment their efficacy to treat a particular disorder.

In this study, we focus on methodological aspects of stem cell culture isolated from human dental pulp tissue by providing different culture conditions for glucose and serum content to give a piece of useful information about the right combination of serum and glucose, which maximizes the potential of DPSCs.

2. Materials and methods

2.1. Culture and expansion of human DPSCs

Human DPSCs from healthy premolar tooth at passage 2 were obtained from Regenerative Medicine Laboratory, Dr. D. Y. Patil Dental College & Hospital, Pimpri, Pune – 411018, India. The DPSCs were further expanded in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and antibiotic–antimycotic solution at 37 °C and 5% CO_2. The culture medium was replenished twice weekly, and the cell growth, health, and morphology were monitored regularly with an inverted phase-contrast microscope. At 70–80% confluence cells were detached using 0.25% Trypsin-EDTA solution (Invitrogen, Carlsbad, CA, USA) and then continuously passaged in the 1:2 ratio for further experiments. Cells from passage 3 to 6 were used in the experimentation.

2.2. Surface markers characterization using flow cytometry

For flow cytometry analysis, confluent DPSCs were harvested with trypsinization and washed with PBS twice. Cells were then incubated for 30 min at 4 °C withAnti-human-CD73-APC, Anti-human-CD90-APC, Anti-human-CD105-APC, and Anti-human-HLA-DR-APC antibodies (all monoclonal) (Miltenyi Biotec, Auburn, CA, USA). Antibody-stained cells were washed twice with PBS, and 20,000 cells per sample were acquired on Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA). Isotype control was used for the detection and to differentiate between positive and negative signals.

2.3. Osteogenic differentiation

The cell density of 2500 cells/cm^2 was used in a 24-well plate (Nunc, Rochester, NY, USA) with a complete growth medium. After 24 h, the complete growth medium was replaced with osteogenic induction medium, which is DMEM with 1% antibiotic–antimycotic, 0.1 μM of dexamethasone, 50 μM of ascorbate-2-phosphate, and 10 mM of β-glycerophosphate (Sigma-Aldrich Corp., St. Louis, MO, USA), the medium was prepared with different serum and glucose conditions [17, 18] that are low glucose-low serum (1 gm/L, 1%), low glucose high serum (1 gm/L, 10%), high glucose low serum (4.5 gm/L, 1%), and high glucose high serum (4.5 gm/L, 10%). The medium was replaced with a fresh induction medium with the same composition twice a week. To analyze the differentiation after 21 days towards osteogenic lineage, the cells were fixed with 4% paraformaldehyde, and 2% alizarin red S (pH 4.1–4.3) staining was performed for 20 min. The quantitation of alizarin red S stained osteoblasts was done by dissolving stained cells in 4% NaOH [17], and the dissolved stain was read spectrophotometrically at 450 nm.

2.4. Adipogenic differentiation

For adipogenic differentiation, the DPSCs were seeded in a 24-well plate (2500/cm^2) (Nunc, Rochester, NY, USA) complete growth medium Adipogenic media (DMEM supplemented with 10% FBS, 1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin, and 0.5 mM isobutyl-methylxanthine (Sigma-Aldrich Corp., St. Louis, MO, USA)) was introduced to the cells twice a week for three weeks. Differentiated adipocytes were fixed with 4% paraformaldehyde and confirmed by performing the 0.3% oil red O for oil droplets for 1 h.

2.5. Chondrogenic differentiation

The cell density of 2500 cells/cm^2 was used in a 24-well plate (Nunc, Rochester, NY, USA) with a complete growth medium. After 24 h, the complete growth medium was replaced with a chondrogenic induction medium, which is DMEM with 1X-ITS, 1 mM of sodium pyruvate, 100 nM of dexamethasone, 50 μg/ml of ascorbate-2-phosphate, 40 μg/ml of L-proline, and 10 ng/ml of TGF-β3 (Sigma-Aldrich Corp., St. Louis, MO, USA). Cultures were incubated for 28 days at 37 °C in a 5% CO_2 incubator; the medium was replaced with a fresh medium every 2–3 days. For analysis for differentiation towards chondrogenic lineage, alcian blue staining was performed on fixed cells after 28 days. Cells were fixed with 4% paraformaldehyde and stained with 0.1% alcian blue for 30 min.

2.6. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The metabolic activity of DPSCs was measured using the MTT assay. The cells were seeded into 96-well plates and incubated with appropriate media for 48 h. Following this, MTT solution (Sigma-Aldrich Corp., St. Louis, MO, USA) at a concentration of 0.5 mg/ml was mixed in each well after mixing plates were incubated for 4 h at 37 °C. Subsequently, the medium was removed, and 100 μL dimethyl sulfoxide (DMSO) (Sigma-Aldrich Corp., St. Louis, MO, USA) was added to each well. The absorbance was measured at 570 nm using a Multiskan Spectrum spectrophotometer (Thermo Scientific, San Jose, CA, USA).

2.7. Carboxyfluorescein succinimidyl ester (CFSE) cell proliferation assay

Cell proliferation was assessed flow cytometrically by using the CellTrace CFSE Cell Proliferation Kit (Invitrogen, Waltham, MA, USA). Briefly, 1 μL of CellTrace stock solution in DMSO was added to the cell suspension in phosphate-buffered saline (PBS) (Sigma, St. Louis, MO, USA) for a final working solution. The cells were incubated for 20 min at room temperature or 37 °C, protected from light. The reaction was stopped by adding a large volume of culture medium (6 times the initial volume) to the cells and incu-

bated for 5 min. The cells were pelleted by centrifugation and resuspended in the fresh pre-warmed complete culture medium. The cells were then incubated for at least 10 min before analysis to allow the CellTrace reagent to undergo acetate hydrolysis and subsequently seeded into the culture dishes with different media compositions. After incubation for 48 h, the cells were acquired on the flow cytometer.

2.8. Colony forming unit assay

To assess the clonogenic ability of cells, a colony-forming unit assay was performed. 5×10^2 cells per well seeded in a 6-well plate (Nunc, Rochester, NY, USA) in culture media (DMEM with 10% FBS). After 24 h of incubation, the cells were cultured for seven days with different media compositions. After seven days, cells were washed with PBS and stained with 0.3% crystal violet (Sigma-Aldrich Corp., St. Louis, MO, USA).

2.9. Real-time quantitative PCR for analysis of gene expression

Cells were trypsinized (up to 5×10^6 cells) and washed twice with PBS. The total RNA was extracted using the GeneJET RNA purification kit (Thermo Scientific, Vilnius, Lithuania). RNA (2 μg) was reverse transcribed using a cDNA synthesis kit (High Capacity, Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's guidelines. Total 100 ng cDNA was used for the total reaction volume of 20 μg for each gene. Quantitative analysis of genes of interest was carried out using the SYBR Green PCR master mix (Applied Biosystems, Austin, TX, USA) on a Real-Time PCR system (QS5, Applied Biosystems, Foster City, CA, USA). Expressions of target genes related to stemness, pluripotency, and differentiation were normalized to ß-actin as a reference gene using the ΔΔCt method. The list of genes and primers (IDT, Coralville, IA, USA) is given in Table 1.

2.10. Statistical analysis

All the experiments were repeated three times and the samples were run in triplicates (n = 5). All the numeric values in the obtained data were presented as mean ± standard deviation. All the groups in the experiments were evaluated by unpaired *t*-test (two-tailed). For all analyses, p-value < 0.05 is considered significant in all the data analyses (*p < 0.05 and **p < 0.01).

Table 1
List of primers.

Gene		Primer sequence
Human RUNX2	F	5′-GTG CCT AGG CGC ATT TCA-3′
	R	5′-GCT CTT CTT ACT GAG AGT GGA AGG-3′
Human Osteopontin	F	5′-CCA TCT CAG AAG CAG AAT CTC CTT-3′
	R	5′-GGT CAT GGC TTT CAT TGG AAT T-3′
Human Osteocalcin	F	5′-GGC GCT ACC TGT ATC AAT GG-3′
	R	5′-TCA GCC AAC TCG TCA CAG TC-3′
Human Osterix	F	5′-TGC TTG AGG AGG AAG TTC AC-3′
	R	5′-AGG TCA CTG CCC ACA GAG TA-3′
Human NANOG	F	5′-TTT GTG GGC CTG AAG AAA ACT-3′
	R	5′-AGG GCT GTC CTG AAT AAG CAG-3′
Human OCT4	F	5′-GTG GAG GAA GCT GAC AAC AA-3′
	R	5′-ATT CTC CAG GTT GCC TCT CA-3′
Human SOX2	F	5′-CCA GCA GAC TTC ACA TGT CC-3′
	R	5′-ACA TGT GTG AGA GGG GCA GT-3′
Human KLF4	F	5′-CCC AAT TAC CCA TCC TTC CT-3′
	R	5′-CAG GTG TGC CTT GAG ATG G-3′
Human Beta Actin	F	5′-AGA GCT ACG AGC TGC CTG AC-3′
	R	5′-AGC ACT GTG TTG GCG TAC AG-3′

3. Results

3.1. DPSCs have MSC-like morphological characteristics

The morphological characteristics like shape, confluency, and size of DPSCs were observed at regular intervals with phase-contrast microscopy. DPSCs showed typical mesenchymal stem cells like-elongated spindle-shaped morphology (Fig. 1A).

3.2. DPSCs show surface expression for MSC-specific surface markers

DPSCs showed expression of cell surface markers (>90%) CD73, CD90, and CD105, which are well known mesenchymal stem cell markers (Fig. 1B); however, the expression of HLA-DR (MHC class-II cell surface receptor) was found to be negative in all DPSCs (Fig. 1B). The results of flow cytometry-based analysis clearly showed that the cells used for further differentiation were having all the characteristic cell surface markers described for mesenchymal stem cells.

3.3. DPSCs possess expression for stemness-related transcription factors

The expression of pluripotency genes such as OCT4, SOX2, NANOG, and c-Myc is one of the characteristics of stem cells and is essential for self-renewal and stemness maintenance. Quantitative real-time PCR data confirmed the expression of the pluripotency genes by DPSCs (Fig. 1C).

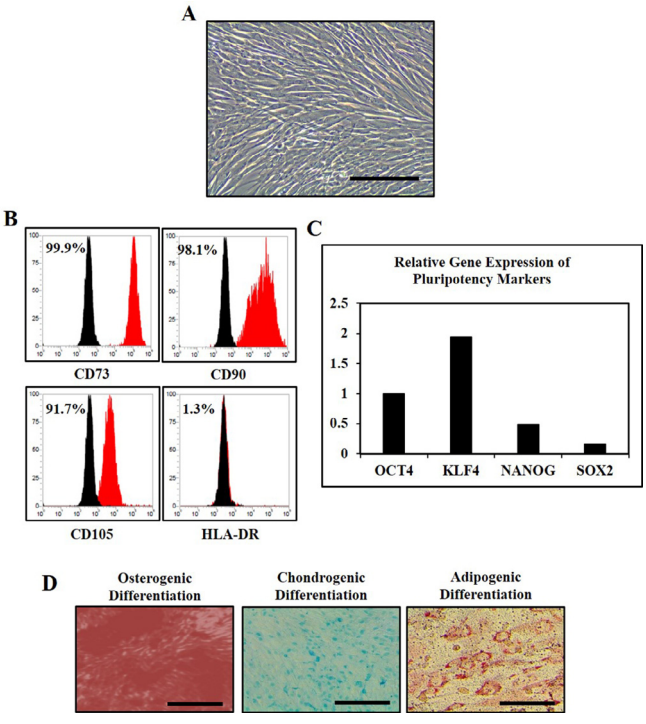

Fig 1. Culture and characterization of DPSCs. (A) DPSCs at Passage 3. Scale bar = 100 μm. (B) Flow Cytometry analysis of DPSCs for MSC-specific cell surface markers Positive markers: CD73, CD90, CD105, Negative marker: HLA-DR. Dark areas are isotype control and red areas are stained cells with specific antibodies. (C) Gene expression analysis of pluripotency-related markers OCT4, KLF4, NANOG, and SOX2 normalized to beta-actin. (D) Tri-lineage differentiation of DPSCs into osteoblasts, chondrocytes, and adipocytes was carried out using specific induction media and functional staining was done using specific dyes (n = 5). Scale bars = 100 μm.

3.4. DPSCs differentiate into adipogenic, osteogenic, and chondrogenic lineages in vitro

The MSCs free from any abnormal activity in terms of proliferation, and the degree of differentiation has a unique value in the therapeutic applications. DPSCs induced with appropriate differentiation cocktails showed tri-lineage differentiation capacity into osteoblasts, chondrocytes, and adipocytes (Fig. 1D), thus confirming their functional equivalence.

3.5. The proliferation and clonogenic ability of DPSCs are inhibited by high glucose content

In our study, we observed that high glucose and high serum in culture media thwarted their clonogenic potential as there were no colony-forming units (Fig. 2C). Also, we found that the proliferation of DPSCs was slowed down by high glucose and high serum media demonstrated by both MTT and flow Cytometry-based CFSE assay (Fig. 2A & 2B).

3.6. Osteogenic differentiation of DPSCs is affected by high glucose content

Our experimental results reveal that all cultures with different combinations of glucose and serum could differentiate DPSCs into the cells of osteogenic lineage producing mineralized nodules following treatment with osteogenic induction medium when observed in phase-contrast microscopy. Alizarin Red S staining was performed to confirm the occurrence of osteogenic differentiation (Fig. 3A); further, the Alizarin Red S staining quantification also gave similar results (Fig. 3B).

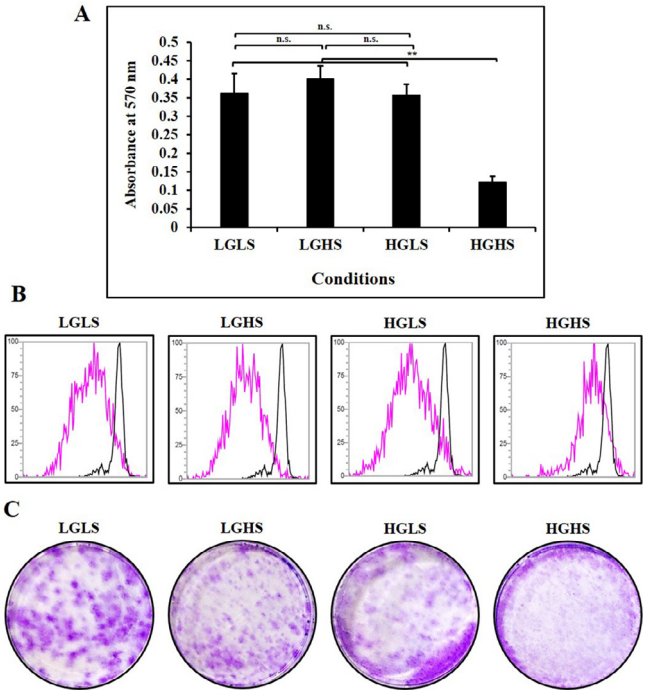

Fig 2. Proliferation and clonogenic potential of DPSCs cultured in different conditions. (A) The metabolic activity of DPSCs cultured in different conditions was assessed by MTT assay. (B) The proliferation of DPSCs was assessed by flow cytometry after staining the cells with CFSE labeling before incubation. The stained population (magenta coloured population) is shifting with more proliferation from day 0 control (black coloured population) towards the left. (C) CFU assay was performed to evaluate the clonogenic ability of DPSCs culture in different conditions (n = 5). LGLS: low glucose low serum, LGHS: low glucose high serum, HGLS: high glucose low serum, HGHS: high glucose high serum. n.s. not significant, *p < 0.05, **p < 0.001.

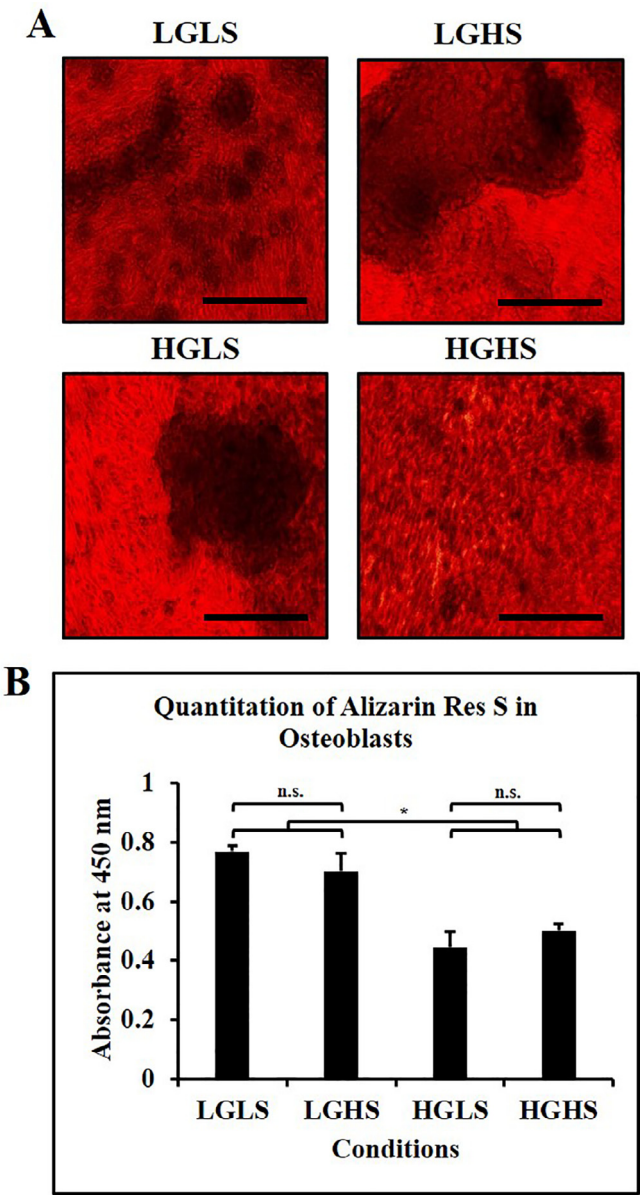

Fig 3. Differentiation of DPSCs into Osteoblasts with Different Culture Conditions and Quantitation of Mineralization in Differentiated DPSCs. (A) Differentiation of DPSCs was carried out using an osteogenic differentiation cocktail and the differentiation was assessed by functional staining with Alizarin Red S. Scale bars = 100 μm. (B) Quantitation of differentiated and stained DPSCs was done by dissolving stained cells in 4% acetic acid and the absorbance was measured colorimetrically at 450 nm (n = 5). n.s. not significant, *p < 0.05, **p < 0.001.

Real-time PCR was performed to compare the osteogenesis-related genes RUNX2, Osteopontin (OPN), Osterix (OSX), and Osteocalcin (OCN) in all the groups against DPSCs not induced with the osteogenic medium as a control group (Fig. 4). The data of this experiment is perfectly correlated with the data obtained in the differentiation experiment.

4. Discussion

An ideal source of MSCs for clinical application should be easily accessible and minimally invasive and should contain a good number of viable stem cells. Dental pulp satisfies all these criteria and

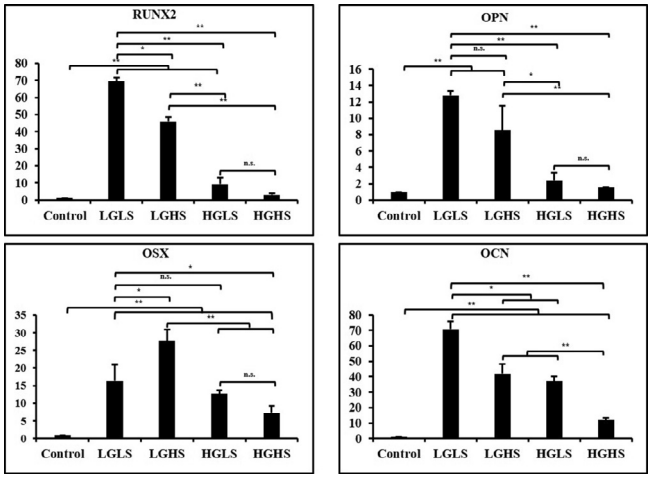

Fig 4. Comparative gene Expression Analysis of Differentiated DPSCs into Osteoblasts with Different Culture Conditions. Differentiation of DPSCs was carried out using an osteogenic differentiation cocktail and the total RNA was extracted from the differentiated osteoblasts. Real-time quantitative PCR was performed to compare the osteogenesis-related genes RUNX2, OPN, OSX, and OCN in all the groups against DPSCs not induced with the osteogenic medium as the control group. n.s. not significant, $^{*}p < 0.05$, $^{**}p < 0.001$.

is, therefore, the desired tissue to acquire cells of clinical significance (Bakopoulou et al., 2017; Kanafi et al., 2013; Ledesma-Martínez et al., 2016; Patil et al., 2018). Isolation and culture of the cells from the tissue should also be easy, and without any chemical or mechanical stress and during the culture process the health and mesenchymal morphology should be maintained. It was evident by some detailed findings that the ideal culture conditions can establish an in vitro microenvironment that augments the property of MSCs to maintain stemness and to carry identical features over time (Bakopoulou et al., 2017; Ducret et al., 2015; Kolind et al., 2014).

It is a well-known fact that stem cells optimally grow and differentiate in a specific milieu, this microenvironment affects the vital cellular processes of cellular homeostasis, and for stem cells from different origins, these requirements can be different. The proliferation data indicates that the rate of proliferation in DPSCs is dependent upon a particular combination of glucose and serum added in the medium that is high glucose – high serum (HGHS). Researchers can avoid this specific combination during the phase of experimentation, where only the proliferation of DPSCs is required. Another combination of low glucose – high serum (LGHS) is having a slight edge in terms of the rate of proliferations indicated in the data that can be used during the proliferation of DPSCs. The results are describing the bioenergetic (Glucose), and nutritional (Serum) needs of DPSCs for their optimal proliferation; however, further experiments should be done incorporating the gene expression analysis for cell cycle controlling genes in different combinations of glucose and serum to correlate the data at the molecular level.

Changing the physical and chemical culture conditions of MSCs showed enhanced differentiation potential, as reported before (Bakkar et al., 2017; Bonnamain, 2013; Kanafi et al., 2013; Suchánek et al., 2013; Viña-Almunia et al., 2017). Our data indicate that the combinations HGHS and HGLS both are having less differentiation in comparison to combinations LGLS and LGHS. Results are suggesting that the presence of higher glucose in the medium decreases the osteogenic differentiation potential, and the presence of low glucose amount in the medium is having optimal osteogenic differentiation while LGLS has a slight edge on other combinations. The combination of LGLS can be utilized in that phase of experimentation where DPSCs need to be differentiated. Higher expression of all four osteogenic differentiation markers in LGLS and LGHS than HGLS and HGHS proves that differentiation is higher in the first two combinations when compared with the latter two combinations while LGLS has a slight edge on LGHS. At gene expression level also the presence of high glucose in media decreases the osteogenic differentiation potential of DPSCs.

5. Conclusion

Our study highlights that the experimentation on DPSCs can be done in two phases where they can be rapidly expanded in the presence of low glucose and high serum levels, in the second phase when cells are treated with osteogenic induction medium for differentiation glucose and serum levels can be switched to low glucose and low serum for optimal osteogenic differentiation. The study deduces a perfect combination of glucose and serum for proliferation as well as for differentiation; however, further experiments are required to dissect the bioenergetics of proliferation and differentiation.

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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