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ORIGINAL ARTICLE



# Osteogenic and odontogenic differentiation potential of dental pulp stem cells isolated from inflamed dental pulp tissues (I-DPSCs) by two different methods

Vellore Kannan Gopinath[a], S. Soumya[b] and Manju Nidagodu Jayakumar[b]

[a]Department of Preventive and Restorative Dentistry, College of Dental Medicine, University of Sharjah, Sharjah, UAE; [b]The Sharjah Institute for Medical Research, University of Sharjah, Sharjah, UAE

**ABSTRACT**

**Objective:** The objective of the present study is to isolate stem cells from inflamed dental pulp tissues (I-DPSCs) and study the characteristic such as surface markers, osteo/odontogenic differentiation potential between the outgrowth (OG) and enzymatic digestion (COL) methods.

**Materials and methods:** I-DPSCs harvested by both methods were analysed for Mesenchymal Stem Cell marker expression by flow cytometry. The metabolic activity of the isolated cells was assessed by MTT assay. The Alkaline Phosphatase (ALP) and Alizarin red staining was done to analyse the osteogenic potential of isolated cells. The osteo/odontogenic differentiation was done by checking the expression of Dentine Matrix Protein 1 (DMP1), Dentine Sialophosphoprotein (DSPP), ALP and Bone Gamma-Carboxyglutamate Protein (BGLAP) by Real time PCR.

**Results:** The isolated cells were positive for MSC markers such as CD-90, CD-105 and CD-73 and negative for CD-14, CD-45 and STRO-1. MTT assay indicated that the I-DPSCs from OG method showed higher metabolic activity than cells from COL. However, the osteo/odontogenic differentiation was in favour of cells isolated by COL method.

**Conclusion:** Although the cell metabolic rate was more in OG, the osteo/odontogenic differentiation was higher in COL, suggesting that the isolation method and culture conditions do affect the differentiation capacity of isolated cells.

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Dental pulp stem cells; dentine matrix protein 1; alkaline phosphatase; dentine sialophosphoprotein; flow cytometry

## Introduction

Dental pulp is located in the epicentre of the tooth enclosed by dentine. The cellular component of the dental pulp includes fibroblasts, odontoblasts precursors, endothelial cells, neurons, Dental pulp stem cells (DPSCs) and immune cells [1–3]. DPSCs are basically ectodermal cells which differentiate into mesenchymal stromal cells (MSCs) in the pulp [4–6]. These MSCs are multipotent in nature with high proliferative, regenerative and multilineage differentiation capability [7–9]. However, the dental pulp is encapsulated by dentine which protects the MSCs from the stimulus which can facilitate the differentiation of these cells, thereby helping in maintaining their stemness in the pulp tissues [10–12]. These undifferentiated mesenchymal stromal cells are considered as DPSCs.

The tissue source for *in vitro* isolation of DPSC is usually from intact permanent third molars or impacted third molars. The availability of these pulp tissues is certainly a limitation for DPSC culture for pulp-dentine regenerative work [13,14]. Therefore, researchers have investigated the feasibility of utilizing pulp tissues harvested from patients with irreversible pulpitis to be the tissue source for dental stem cell culture. Bacterial infection and oxidative stress induced morphological changes in the dentine-pulp complex leads to pulpitis, which may be acute or chronic in form. Once the pulpitis is diagnosed to be irreversible, the only solution is root canal treatment. Studies have shown that MSC derived from inflamed dental pulp retained the ability to proliferate, regenerate and have the same potential to differentiate as compared to stem cells isolated from healthy pulp, which proves the fact that inflamed pulp tissues still have progenitor/stem cells [15,16]. However, the inflamed pulp tissue is accumulated with free radicals and that high reactive oxygen species (ROS) levels cause cellular damage, but it is said that a low basal level of ROS is necessary for maintaining cellular proliferation and differentiation. Perhaps, at baseline, MSCs are known to express low levels of ROS that is advantageous to enhance osteogenesis [17,18].

The two most commonly used methods for DPSC isolation include the explant method and enzyme digestion method. In the explant method of isolation, the outgrowth of cells from pulp tissue to the culture plates takes place [19,20]. The cell sprouts get attached to the plastic surface and form colonies. In the enzyme digestion method, sterile inflamed dental pulp tissues are treated with Collagenase/Dispase and later cultured [21,22]. It is reported that the phenotypic characteristics of the isolated cells do change with the different culture conditions employed [23]. Previous studies on permanent and deciduous teeth have demonstrated that the

CONTACT Dr. Vellore Kannan gopinathvk@yahoo.com Department of Preventive and Restorative Dentistry, College of Dental Medicine, University of Sharjah, PO Box: 27272, Sharjah, UAE

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cells isolated through enzymatic method showed differences in surface marker expression and proliferation rates [24,25]. However, to the extent of our understanding, no such studies are reported which compares the osteogenic and odontogenic differentiation potential of Inflamed Dental Pulp Stem Cells (I-DPSCs) isolated by outgrowth and enzyme digestion method. Although DPSCs have a wide application in tissue regeneration research, there is a need to explore into alternative methods for osteogenic induction like the use of platelet rich plasma (PRP) which has shown favourable osteoinductive potential when used along with bone grafts [26]. In addition, evidence support the use of scaffolds such as decellularized bone extracellular matrix cultured with DPSCs favour osteogenic differentiation [27]. Owing to the immense therapeutic potential of DPSCs, it is necessary to identify the best isolation method, which provides good quality stem cells that in turn can be utilized for regenerative medicine application. Hence, the present study is an attempt to explore the feasibility of using outgrowth and enzyme-mediated digestion protocol in isolating DPSCs from inflamed pulp tissue. The study also focuses on the differentiation potential of the thus isolated stem cells mainly into osteogenic and odontogenic lineage.

## Materials and methods

### *Isolation of dental pulp stem cells from inflamed dental tissues (I-DPSCs)*

The dental pulp tissue samples were collected from young patients (20–30 years) with signs and symptoms of irreversible pulpitis in decayed permanent molar, which were indicated for endodontic treatment. The samples were collected at the urgent care clinic at UDHS (University Dental Hospital Sharjah) after obtaining informed consent from patients, in accordance with the World Medical Association Declaration of Helsinki. The present research was approved by Research and Ethics Committee, University of Sharjah (Ref No. REC-19-03-04-01). Pulp tissue samples from six young patients diagnosed with irreversible pulpitis were collected in DMEM media containing antibiotics and were transferred to the laboratory for I-DPSC isolation. The sample was split into two pieces; one for outgrowth method and one for enzymatic digestion using Collagenase and Dispase.

In the cell outgrowth method the inflamed pulp tissues were washed several times in phosphate buffered saline supplemented with penicillin-streptomycin. The washed tissues were transferred to a 35 mm petridish and were minced into 1–2 mm fragments. The fragments were then transferred to T-25 flask with appropriate amount of DMEM (Dulbecco's Modified Eagles Medium; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20% of FBS (Fetal Bovine Serum; Sigma-Aldrich), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 25 μg/mL of amphotericin B; and incubated at 37 °C with 5% $CO_2$. Medium was changed every three days without disturbing the pulp tissues. After 1 week, cell sprouting could be observed around the pulp tissues. The cells after confluency were subcultured using 0.25% Trypsin-EDTA (Sigma-Aldrich). The optical microscopic images of the isolated I-DPSCs were acquired using IX53 Inverted Microscope (Olympus, Shinjuku, Tokyo, Japan).

For the enzymatic method of I-DPSC isolation, a cocktail of Collagenase and Dispase were employed. The minced tissues of 1–2 mm fragments were treated with 0.3% Collagenase/Dispase (Sigma-Aldrich) for 1 hour at 37 °C. The enzyme was inactivated by adding equal amount of DMEM complete medium and the cell aggregates were removed by passing through a 70 μm filter. Single cell suspension of I-DPSCs was centrifuged at 1500 rpm for 5 min to get the cell pellet. The cell pellet was resuspended in DMEM medium and were plated in T-25 flask and kept at 37 °C incubator with 5% $CO_2$. The media was changed at an interval of every three days. Once the cells reached 70–80% confluency, it was subcultured for the next passage using 0.25% Trypsin-EDTA. The optical microscopic images of the isolated I-DPSCs were acquired using IX53 Inverted Microscope (Olympus).

### *Phenotypic characterization of isolated I-DPSCs by flow cytometry*

The isolated I-DPSCs by the two methods; outgrowth and enzymatic were analysed for the CD marker expression by flow cytometry. The cells ($1 \times 10^6$) from the third passage were harvested and fixed using 4% paraformaldehyde (PFA) for 15–20 min at room temperature. The cell pellet was further incubated with Anti-CD73 antibody [AD2] (Phycoerythrin), Anti-CD105 antibody [MEM-226], prediluted (Allophycocyanin), Anti-CD90/Thy1 antibody [5E10] (PE/Cy5), Anti-STRO1 antibody [STRO-1] (Phycoerythrin), Anti-CD45 antibody [B-A11] (FITC) and Anti-CD14 antibody [MEM-15] (FITC) at room temperature for 15–20 min in dark. All the antibodies for the flow cytometry were purchased from Abcam, Cambridge, United Kingdom. After the incubation, the cell suspension were washed thoroughly with PBS and analysed for the surface markers using Accuri C6 Flow Cytometer equipped with 488 nm argon lasers (Becton Dickinson, Franklin Lakes, NJ, USA). Commercially available DPSCs (CLS cell line services GmbH, Eppelheim, Germany) were used as the positive control and the experiments for the positive control were carried out in the BD FACSAria III (Becton Dickinson). A minimum of 10,000 events were acquired for each of the samples and the data was analysed by FlowJo software version 10, Becton Dickinson, Ashland, OR, USA.

### *Cell metabolic activity by MTT assay*

The metabolic activity of the isolated I-DPSCs by outgrowth and enzymatic method, was analysed by MTT assay. $1 \times 10^4$ cells were seeded in a 96 well plate and were incubated for 1, 3 and 7 days. After the incubation period, the medium was replaced with 50 μl of MTT (1 mg/ml in serum free medium, Sigma-Aldrich) and the plates were incubated for 4 h at 37 °C incubator with 5% $CO_2$. The formazan crystal formed was dissolved by adding 100 μl of isopropanol and the plates were swayed for 10 min to mix the solution. The absorbance were measured at 570 nm in the microplate

spectrophotometer (Thermo Fisher Scientific™ Multiskan™ GO Microplate Spectrophotometer, Vantaa, Finland).

### Osteogenic differentiation efficiency of isolated I-DPSCs

The osteogenic differentiation ability of the isolated I-DPSCs were analysed by measuring the ALP (alkaline phosphatase) activity using the colorimetric ALP kit from Abcam and the calcium mineral quantification by Alizarin Red Assay (Sigma-Aldrich).

### Alkaline phosphatase activity

The I-DPSCs isolated by outgrowth and enzymatic method at passage 3 were harvested and seeded on to 6 well plates at a density of 50,000 cells per well in DMEM normal media (NM). Upon confluency, the media was changed to osteogenic media (OM), which contains 10% DMEM containing 10 nM dexamethasone (Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich) and 50 μg/ml ascorbic acid (L-ascorbic acid 2-phosphate, Sigma-Aldrich). The cells were cultured for a period of 21 days in both osteogenic and normal medium. The media was changed at regular intervals i.e. once in every 3 days. The cells in NM and OM at day 1 and after 21 days were compared for the ALP activity. After the desired incubation periods, the cells were trypsinized and washed in PBS and the cell lysate was prepared by treating the cells with cell lysis buffer. The cell pellet was vortexed for every 20 s for every 10 min up to 4–5 times. The samples were further sonicated for 20 s 3 times in a probe sonicator and were centrifuged at 12,000 *g* at 4 °C for 15 min. The supernatant was collected for the analysis of ALP activity and was measured following the manufacturer's instructions. Briefly, to the cell lysate, 50 μl of 5 mM pNPP (*p*-nitrophenyl phosphate) substrate was added and the plates were incubated at 25 °C for 60 min in dark. The ALP enzyme activity is determined by its ability to convert pNPP substrate to Coloured p-Nitrophenol (pNP). The optical density was measured after stopping the reaction by adding 20 μl of stop reaction in a microplate reader (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer) at 405 nm. The ALP enzyme activity was calculated from the concurrently plotted pNP standard calibration curve.

### Calcium mineral deposition – quantification

For analysing the mineralization ability of the isolated I-DPSCs, cells (passage 3–6) at a density of 50,000/well were seeded onto 6 well plates and maintained in DMEM normal medium. Upon confluency, the media was changed to osteogenic media (OM). The cells were cultured for a period of 21 days in both osteogenic and normal medium. Media change was given once in three days. After 1 and 21 days, the plates were taken and were fixed using 4% PFA for 20 min at room temperature. Later the plates were washed with distilled water and stained with 40 mM Alizarin Red S solution (pH 4.1) for 20–30 min at room temperature with gentle shaking. Afterwards, the dye was removed and the cells were washed well with distilled water and the stained mineral deposits were captured using IX53 Inverted Microscope (Olympus).

Calcium mineral quantification was done by Alizarin Red S based on a protocol previously reported [28]. Briefly, 800 μl of 10% (v/v) acetic acid was added to all the wells and were incubated for 30 min at room temperature with gentle shaking for extracting the dye. The cell layer was scraped off using a cell scraper and the extracted dye in acetic acid was transferred to a 1.5 ml Eppendorf tube. The samples were vortexed for 30 s and were heated at 85 °C for 10 min and further cooled down by transferring the samples to ice for 5 min. The samples were then centrifuged at 12,000 rpm for 15 min and the supernatant was removed to a new 1.5 ml vial. The neutralization of acid was done by adding 200 μL of ammonium hydroxide (10% (v/v)) to the supernatant collected. 100–150 μL of the supernatant was then transferred to a 96 well plate to measure the optical density at 405 nm in a microplate spectrophotometer (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer).

### Osteo/odontogenic differentiation of I-DPSCs by real time PCR analysis

For analysing the osteo/odontogenic differentiation potential of the isolated cells, I-DPSCs were seeded onto 6 well plates at a density of 100,000 cells/ml of culture media for a specified time interval of 1 and 21 days. The cells once confluent is supplied with media (OM) supplemented with osteo/odontogenic factors. At definite time intervals, total RNA was extracted from cells using RNA isolation with the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instruction. The quality of isolated RNA was analysed using an agarose gel electrophoresis with the identification of specific bands of 28 S rRNA and 18 S rRNA and the quantity was measured in a nanodrop spectrophotometer (ND-1000, Wilmington, DE, USA) system. The total RNA thus isolated was transcribed to cDNA using $RT^2$ HT First Strand Kit (Qiagen), in a PCR system. The real time PCR analysis was carried out using GoTaq® qPCR (Promega, Madison, WI, USA) in triplicates in a Rotor-Gene Q5 PCR machine. Briefly, the reaction volume (20 μl) included 10 μl PCR master mix, 1 μl each of forward and reverse primers (200 nM) and 2 μl of cDNA. The PCR cycling conditions included an initial hot Start Polymerase activation at 95 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 15 s and an annealing at 60 °C for 1 min. A dissociation curve analysis was also done using the default setting temperatures of the instrument. GAPDH was used as the internal control. The expression levels of target genes were calculated by normalizing the Ct values with that of the reference gene and the fold change was expressed using the formula $2^{-\Delta\Delta Ct}$. The genes and the primer sequences used for the study is represented in Table 1.

### Statistical analysis

All results were obtained from six different patients and each experiment was done in triplicate and the statistical

significance was analysed by One Way Anova and Tukey's Post-hoc test. Data are expressed as mean ± SD and $p$-values $\leq .01$ were considered as statistically significant.

## Results

### I-DPSC isolation and culture

During the isolation process of I-DPSCs, spindle shaped cells attaching to the plastic of T-25 flask could be seen as early as day 4 in the COL method. Whereas, the first appearance of cell sprout from the pulp tissues could be seen only on day 7 for the OG method . The results are in favour of COL method which resulted in early isolation of cells. The cells isolated by both methods demonstrated the typical spindle shaped morphology as that of the mesenchymal stem cells (Figure 1).

### Flow cytometric evaluation of MSC specific surface markers

Cell characterization by flow cytometry indicated positive expression for CD-90, CD-105 and CD-73; whereas negative for STRO-1, CD-14 and CD-45 for the cells isolated by OG and COL method. The commercial DPSCs showed all ~100% positive for MSC specific markers CD-90, CD-105 and CD-73 and ~45% expression for STRO-1 and did not express haematopoietic stem cell markers. No significant difference was observed between COL and OG methods for the expression of CD-90, CD-105 and CD-73. (Figures 2 and 3).

Table 1. The primer sequences used in the qPCR study.

| Gene | Primer sequence (5′-3′) | Gene bank accession numbers |
|---|---|---|
| DMP-1 | AGGAAGTCTCGCATCTCAGAG<br>TGGAGTTGCTGTTTTCTGTAGAG | NM_004407.3 |
| DSPP | ATATTGAGGGCTGGAATGGGGA<br>TTTGTGGCTCCAGCATTGTCA | NM_014208.3 |
| ALP | ACGTGGCTAAGAATGTCATC<br>CTGGTAGGCGATGTCCTTA | NM_000478.5 |
| BGLAP | CATGAGAGCCCTCACA<br>AGAGCGACACCCTAGAC | NM_199173.5 |
| GAPDH | GAAGGTGAAGGTCGGAGTC<br>GAGATGGTGATGGGATTT | NM_002046.3 |

### MTT assay

Assessing the metabolic activity of cells from passage 3 revealed significantly higher cellular metabolic activity in OG method compared to COL method at day 3 ($p < .0001$). However, by day 7, the metabolic rate declined in both OG and COL (Figure 4).

### Osteogenic differentiation of I-DPSCs

ALP activity and quantification of calcium deposition indicated osteogenic differentiation potential was higher in COLOM and OGOM compared to COLNM and OGNM at day 21. Comparing between the methods, COLOM was significantly higher than OGOM ($p < .0001$) (Figures 5 and 6). The results are favourable towards I-DPSCs cultured by the COL method. Stained calcium mineral deposits at day 21 also indicated higher percentage of stained mineral in COLOM when compared to OGOM (Figure 7).

### Real time PCR analysis for osteo/odontogenic gene expression

To analyse the differentiation potential of the isolated cells into osteo/odontogenic lineages, real time PCR analysis was carried out. The results showed not much expression for the osteo and odontogenic related genes from OG and COL cells at day 1. By day 21, the induced cells showed upregulated expression of osteo and odontogenic related gene expression in both the COL and OG. The odontogenic related genes mainly DMP1 and DSPP expression was significantly upregulated in induced cells from COL method compared to OG method. However, no statistical significance was observed for the expression of ALP and BGLAP (Figure 8).

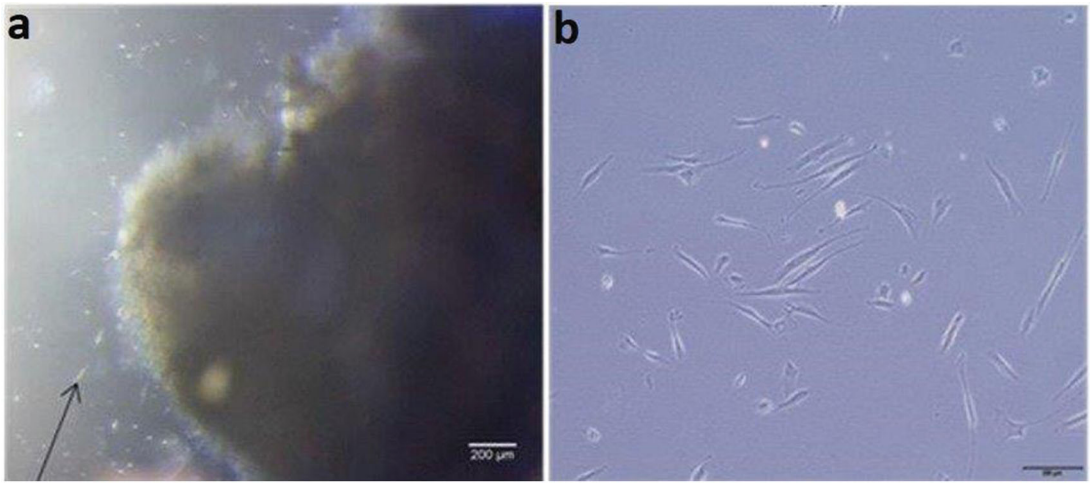


Figure 1. Phase contrast images of the isolated I-DPSCs. (a) Microscopic image of the I-DPSCs isolated by outgrowth method on day 7. (b) I-DPSCs isolated by enzymatic digestion on day 7. The cells showed the typical spindle shaped morphology as that of the Mesenchymal stem cells. The scale bar shows 200 µm.

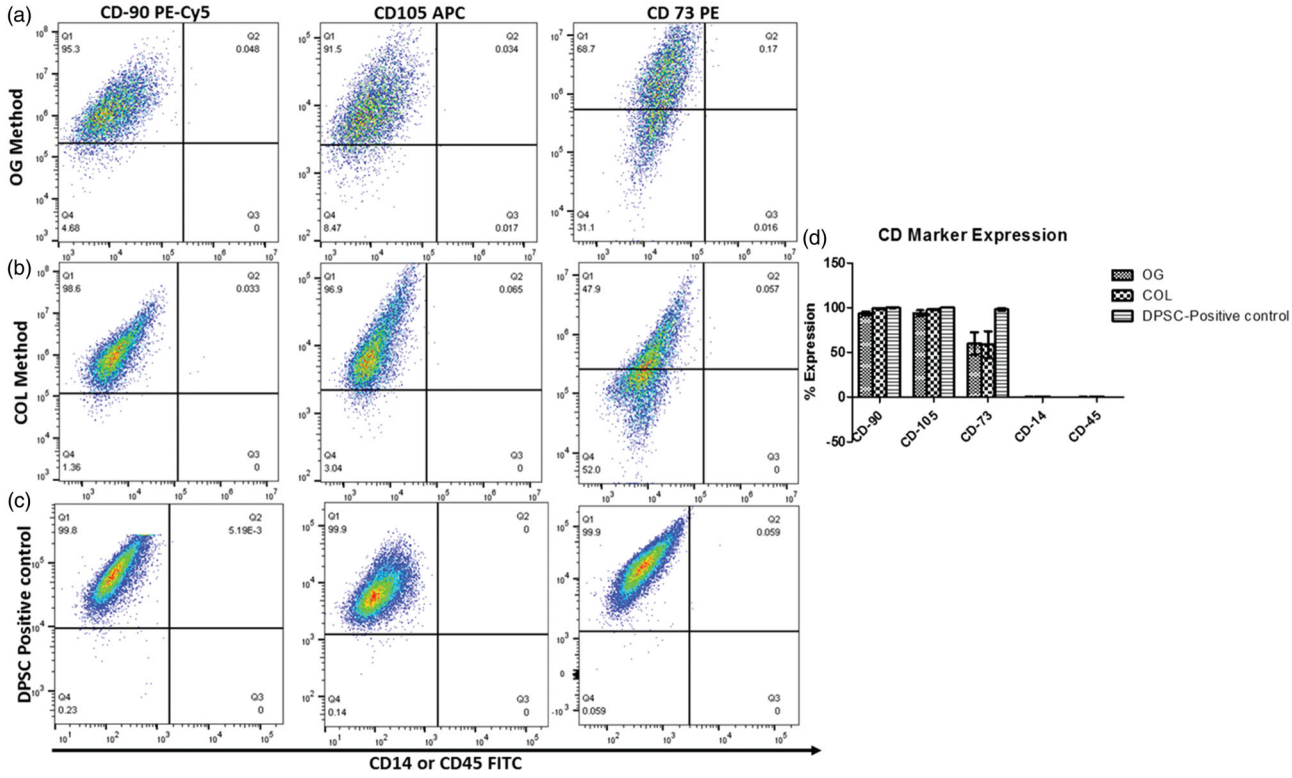


**Figure 2.** Representative flow cytometric characterization data of the isolated I-DPSCs. (a) shows the stained I-DPSCs isolated by outgrowth method showing positive expression for CD-90, CD-105 and CD-73; whereas negative for CD-14 and CD-45, (b) represents the same as that of the upper panel for the I-DPSCs isolated by enzymatic digestion. The cells were positive for CD-90, CD-105 and CD-73 and negative for CD-14 and CD-45, (c) represents the flow cytometric analysis of the commercially available DPSCs showing all positive for CD markers CD-90, 73 and 105 with negative expression for haematopoietic markers CD-14 and CD-45. (d) Represents the quantitative expression of CD markers for the outgrowth, enzymatic and DPSC positive control. Cells from the passage 3 and 4 were used in all the experiments. Data represented as dot plots using the FlowJO software and the gates were set for each samples according to the isotype control.

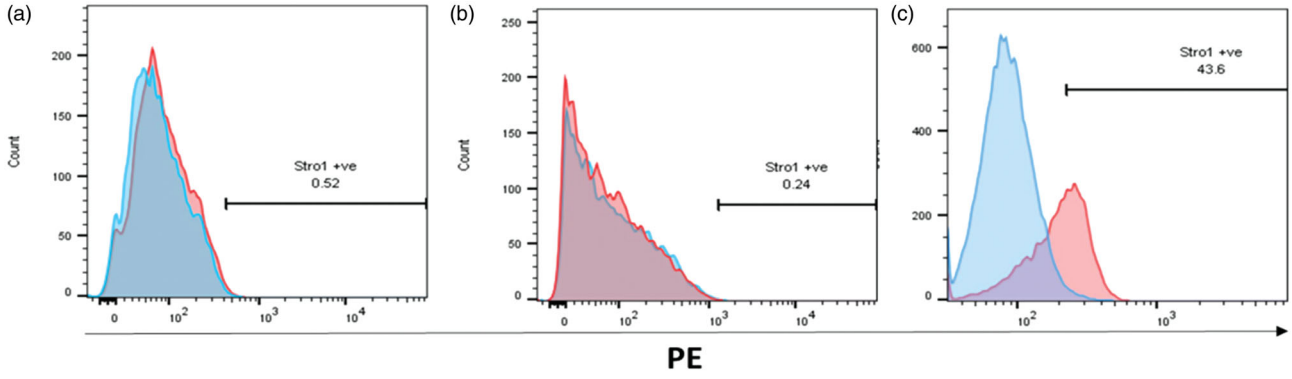


**Figure 3.** Flow cytometric characterization of STRO-1 marker expression compared between (a) OG, (b) COL and (c) DPSC positive control. OG and COL cells demonstrated no expression for STRO-1compared to DPSC positive control (43.6%). Data represented as histogram using FlowJo software with blue colour representing the cells without the antibody and red the positive population.

## Discussion

The present study compared the efficacy of cell outgrowth (OG) [29–31] and enzyme digestion method (COL) [32,33] to isolate dental pulp stem cells from inflamed dental tissues (I-DPSCs), with an intention to determine if the isolation method influences the cell proliferation and differentiation potential. While isolating the stem cells from inflamed dental pulp tissues it was observed that in the COL method the cells were isolated earlier, probably due to the digestion of the pulp tissues with enzymes and creating a single cell suspension for culture. Whereas, in the OG method it takes time for the cells to sprout out of the pulp tissues in the culture. Findings similar to the present study showed clusters of dental pulp stem cells as early as day three in the enzyme digestion method whereas the cell sprouts from the pulp tissues could be seen only on day five in the cell outgrowth method [34,35].

As suggested by the International Society for Cellular Therapy (ISCT), if the isolated cells to be confirmed as MSC they should satisfy the minimum requirements such as plastic adherence, expression of positive surface antigens and its multipotent differentiation ability under suitable culture conditions [36]. Herein; both the isolated cells displayed the spindle shaped fibroblastic morphology satisfying the first

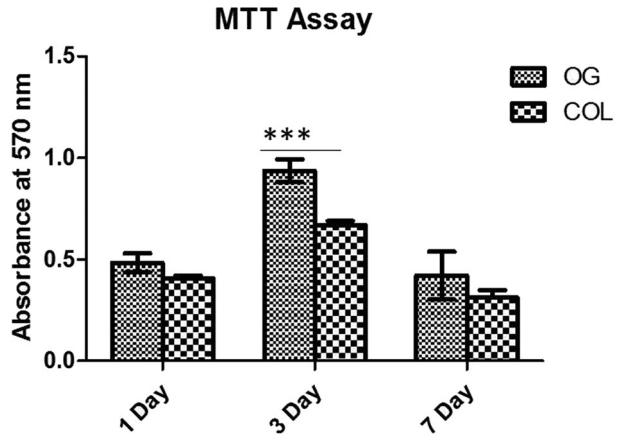


**Figure 4.** MTT assay showing the metabolic activity of the I-DPSCs at 1, 3 and 7 days. At day 3 the IDPSCs from outgrowth method showed more cell proliferation than I-DPSCs isolated by enzymatic method. By day 7, the cell proliferation rate declined in both OG and COL. Comparison between the test groups was performed by unpaired t-test and the results are represented as mean ± standard deviation from six separate experiments done in triplicates. ***represents $p$-value $< .0001$.

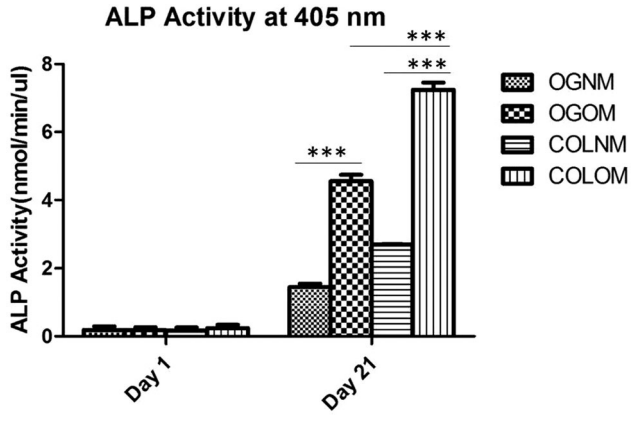


**Figure 5.** Alkaline phosphatase activity of I-DPSCs isolated by outgrowth and enzymatic method over a period of 21 days. Osteogenically induced I-DPSCs from enzymatic treatment cultured in differentiation media showed significant increase in enzyme activity than cells from outgrowth method after 21 days of induction. NM and OM represents cells cultured in normal media and osteogenic media. Comparison between the test groups was performed by one-way Anova and the results are represented as mean ± standard deviation from six separate experiments done in triplicates. ***represents $p$-value $< .0001$.

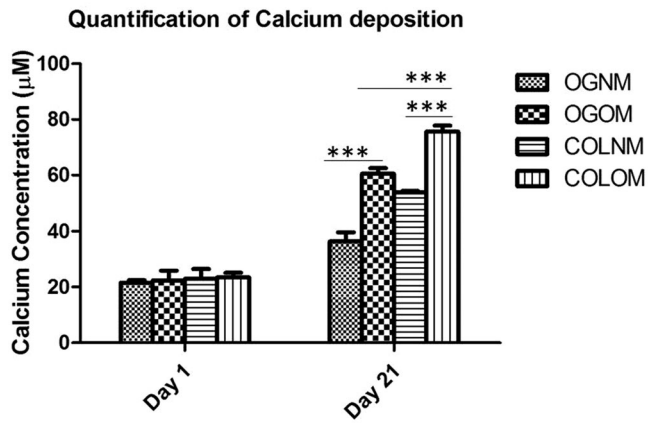


**Figure 6.** Represents the calcium quantification by Alizarin Red S extraction assay. I-DPSCs from enzymatic method showed enhanced calcium deposition compared to I-DPSCs isolated by outgrowth method. NM and OM represents cells cultured in normal media and osteogenic media. Cells from passage 3–5 were utilized for the study. Comparison between the test groups was performed by one-way Anova and the results are represented as mean ± standard deviation from six separate experiments done in triplicates. ***represents $p$-value $< .0001$.

requirement of plastic adherence. However, further cellular and molecular level characterization is necessary to qualify the cells isolated to be as of the mesenchymal lineage. There are different methods available in literature for cellular and molecular level characterization. This includes immunofluorescence, flow cytometry and immunoblotting. In this particular study, flow cytometry was employed to study the phenotypic characterization of cells isolated [37]. For the positive marker expression studies by flow cytometry, a panel of three positive cluster of differentiation markers such as CD-90, 73 and 105 were selected along with negative haematopoietic markers CD-14 and CD-45. The surface characterization of cells isolated by these methods showed >95% expression for CD-90 and CD-105 in both the cell groups with CD-73 showed only ~60% positivity. In contrary, the perivascular marker STRO-1 was negative in both the isolated cell groups compared to DPSC positive control. Our results were in accordance with one of the studies wherein, the isolated DPSCs from healthy permanent teeth were negative for STRO-1 [38]. Though STRO-1 is employed nowadays to identify the Mesenchymal stem cell population in tissues, studies suggests that it cannot be considered as a single marker for stem cells as it shows varying degrees of expression in tissues [39]. In one of the studies by Alongi et al. [13], the STRO-1 expression analysed for IDPSCS between different patients showed remarkable differences in expression with one patient tissue showing almost 21% and the other one with ~2%. Interindividual variation among donors could be one of the reasons for varying expression of STRO-1. The absence of haematopoietic cell markers such as CD-14 and CD-45 proved the cells are having the mesenchymal origin than the haematopoietic origin [40].

Metabolic activity indicating cell proliferation was in favour of OG method on day 3 of culture but was not very different between the methods on day 7. This indicates that I-DPSC isolated by the OG method proliferate faster and the reason for the decline in the metabolic activity on day 7 could be attributed to the over proliferation of the cells in the culture plate. This might have resulted in a lack of space for the cells to grow resulting in the reduction in the metabolic activity at day 7. Higher proliferation rate of I-DPSC observed in the OG methods has not been previously reported. However, dental pulp stem cells isolated from human third molars showed higher proliferation rate in digestion method when compared to outgrowth method [41]. Although, a direct comparison between the studies is not possible as the dental pulp stem cells have been harvested from different sources.

The isolated cells should also be characterized to measure their multilineage potential such as osteoblast, adipocytes, neuronal cells, chondrocytes etc. Herein, we have checked the bilineage potential of these cells into osteoblasts and odontoblasts. ALP is an early marker of osteoblastic

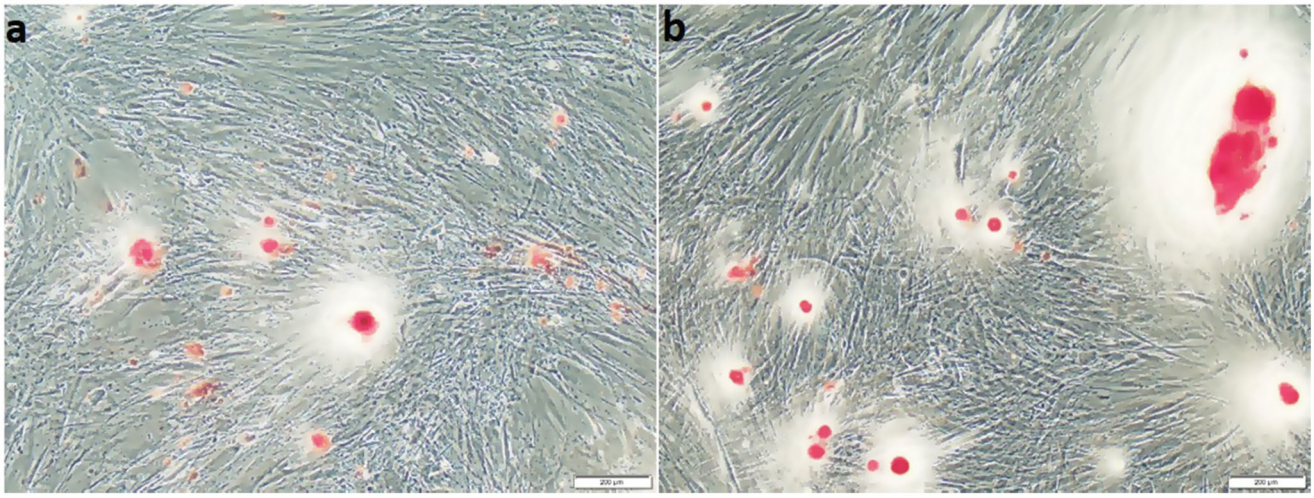


**Figure 7.** Alizarin Red S stained IDPSC in OGOM (a) and COLOM (b) at day 21. As evident from the figure, more calcium deposits were seen on cells cultured in COLOM compared to I-DPSCs in OGOM.

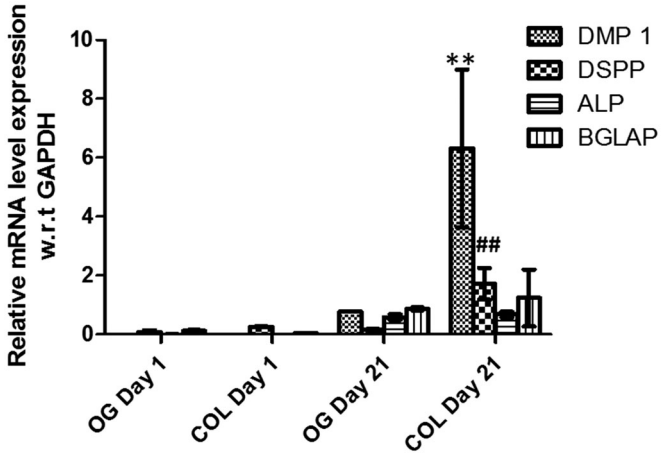


**Figure 8.** Real time PCR analysis showing the DMP1, DSPP, ALP and BGLAP expression in OG and COL at day 1 and day 21. Normalized expression with GAPDH showed increased DMP1 and DSPP expression on I-DPSCs from enzymatic treatment cultured in differentiation media than cells from outgrowth method after 21 days of induction. Data are shown mean ± SD from six separate experiments done in triplicates. ** and ## indicates $p < .01$.

differentiation. Differentiating osteoblasts are known to synthesize ALP enzyme which in turn helps in the deposition of inorganic calcium phosphate [42–44]. Here, the differentiation ability of isolated I-DPSCs was analysed by checking its ALP activity and mineral deposition for a period of 21 days. Osteogenic differentiation as evaluated by calcium mineral deposition and ALP activity were in favour of I-DPSCs isolated by COL methods. This indicated that cells isolated by the COL method have an enhanced capacity to form mineral structure, which is in favour of using these stem cells for tissue repair and restorative medicine applications. Similar findings were reported on human dental pulp stem cells obtained from primary and permanent teeth isolated by enzyme digestion method to have favourable mineral deposition potential while compared to cells isolated by outgrowth method [28,38].

The osteo/odontoblastic differentiation analysed semi quantitatively by qPCR demonstrated an upregulated expression of all the genes at day 21 compared to day 1. Herein, we have checked the gene expression of four potential genes, which are mainly involved in osteogenic and odontogenic differentiation of DPSCs. As discussed earlier, ALP is a phosphatase enzyme, which helps in the deposition of calcium phosphate in bone and dentine. A higher ALP activity could be found during the differentiation phase of bone and dentine. During the odontoblastic differentiation, a higher mineralized dentine matrix is deposited and the main non-collagenous proteins present are DMP1 and DSPP. We have used DMP1 and DSPP in our experiment as this protein is existent in odontoblasts, dentinal tubules and its presence are known during dentine matrix mineralization [45–48]. The upregulated mRNA level expression of DMP1 and DSPP is indicative of odontogenic differentiation and was found to be higher in cells cultured in COL osteogenic media compared to OG at day 21. In a study similar to ours, compared the expression of Dentine Sialophosphoprotein (DSPP) in dental pulp stem cells isolated by outgrowth and enzyme digestion methods, no significant difference was observed in the DSPP expression by the DPSC differentiated odontoblasts between the methods [38]. However, in our study we found that the expression of DMP1, which is considered to regulate the DSPP gene transcription was higher in IDPSC isolated by COL method, compared to the OG method. The expression of other genes such as ALP and BGLAP were enhanced by day 21 in both the cell groups but no significant difference were observed between the groups. These finding were contradictory to the ALP activity and Alizarin Red S staining analysed biochemically wherein, the cells isolated by COL method showed better activity and mineral deposition compared to OG method. Despite the fact that the isolated cells were negative for STRO-1, the stem cell specific marker, the differentiation ability of the cells were not compromised in our case. The cells isolated by both the methods were able to differentiate into osteoblastic and odontogenic lineages, with the COL method showed better differentiation ability than OG method. Our study also

favoured the concept that the inflamed dental pulp tissue, which is discarded as a waste in the endodontic procedure, contains putative stem cell population, which retains similar stem cell characteristics as that of the cells isolated from healthy tissues. Comparable to the present work it is interesting to note that human periapical cyst-mesenchymal stem cells (hPcy-MSCs) derived from excised periapical cyst, showed characteristics similar to DPSC [49]. However, it should be noted that hPcy-MSCs cells favoured osteogenic lineages whereas I-DPSCs isolated in the present study favoured odontogenic differentiation [50].

Stemness is the property of stem cells to show self-renewal and differentiation ability as seen in I-DPSCs isolated in our study by both methods. These cells harvested from inflamed dental pulp tissues can be considered as a potential source of mesenchymal stem cells with a varied application in tissue and dental pulp regeneration. It should also be noted that the isolation method and the different culture conditions employed do affect the differentiation capacity of the isolated cells.

## Conclusion

In the present study, although the I-DPSCs isolated by enzymatic method showed higher osteo/odontogenic differentiation potential, these cells were preferably directed towards odontogenic lineage. Hence, it is important to select the proper isolation method for employing these cells for targeted tissue regeneration studies. In future, a detailed investigation on the dentine tubule formation by these I-DPSCs should be carried out to determine whether the cells from inflamed pulp could be used as a potential source for pulp tissue repair and regeneration applications.

## Limitations

Multilineage differentiation potential of I-DPSCs isolated by outgrowth and enzyme-mediated digestion method need to be assessed to determine the true potential of these MSCs. Whereas the present study investigated only on the osteogenic and odontogenic lineage which can be considered as a limitation of this work.

## Ethical approval

The present research was approved by Research and Ethics Committee, University of Sharjah (Ref No. REC-19-03-04-01). All procedure performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

## Informed consent

Informed consent was obtained from all patients for the collection of the samples used in this study.

## Disclosure statement

The authors declare no conflict of interest.

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## ORCID

Vellore Kannan Gopinath http://orcid.org/0000-0002-8456-641X
S. Soumya http://orcid.org/0000-0002-1947-253X

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