Characterization of Stem Cells from Human Exfoliated Deciduous Anterior Teeth with Varying Levels of Root Resorption

Rachaita Chhabra* / Shama Rao** / B Mohana Kumar*** / A.Veena Shetty**** / Amitha M. Hegde***** / Meghna Bhandary******

Background: *Deciduous teeth undergo the physiologic process of resorption, during which the remnant pulp undergoes activation. However, the quality of stem cells obtained at various stages of root resorption has not been documented.* ***Objective****: To isolate and characterize stem cells from deciduous teeth with varying levels of root resorption.* ***Study design:*** *Healthy primary anterior teeth were extracted according to the treatment needs of the patient. The teeth were categorized into SHED(1/3)- teeth with 0 to 1/3rd root resorption, SHED(2/3)- teeth with 1/3rd to 2/3rd root resorption, and SHED(COMP)- teeth with more than 2/3rd root resorption. SHED were characterized based on their morphology, viability, proliferation rate, population doubling time, expression of cell surface markers, and in vitro differentiation potential into osteocytes and adipocytes.* ***Results:*** *SHED from all three groups demonstrated largely similar morphological and cellular characteristics. However, SHED(2/3) showed relatively better characteristics in terms of growth kinetics and phenotypic marker expression. Also, the differentiation ability for osteogenic and adipogenic cell lineages was slightly higher in SHED(1/3) and SHED(2/3) compared with SHED(COMP).* ***Conclusion:*** *Based on the cellular, phenotypic and biological characteristics, it is suggested that SHED (2/3) could be a useful source for tissue regeneration, and warrants further investigations.*

Keywords: *Stem cells from human exfoliated deciduous teeth, SHED, Root resorption, Cellular properties, Differentiation, In vitro*

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INTRODUCTION

The deciduous teeth are usually discarded as biological waste. Stem cells from human exfoliated deciduous teeth (SHED) have been recognized as a promising source for applications in tissue engineering and regenerative medicine.[1] The pulp of deciduous teeth is a unique source of stem cells as first demonstrated by Muira et al in 2003.[2]It has been suggested that the pulp of a naturally exfoliated deciduous tooth may be similar to an umbilical cord, providing a rich and distinctive source of stem cells showing a multipotent nature.[3]

Earlier studies have demonstrated the mesenchymal stem cell (MSC)-like properties of SHED with similar morphological features, cell surface marker expression and multilineage differentiation ability.[2,4,5,6] In comparative investigations, SHED have been shown to have equal or greater potency characteristics to that of bone marrow-derived MSCs (BMSCs).[7,8] They also possessed characteristics of more immature multipotent cells, such as higher proliferation rate, shorter population doublings and greater osteoinductive capacity compared to dental pulp stem cells (DPSCs).[9,10,11,12] Considering the ease of accessibility with ideal potency properties, SHEDs have been evaluated on their potential applications in tissue engineering and cell-based therapies.[13]

The primary distinguishing factor of deciduous teeth as compared to other sources of adult stem cells is the occurrence of

root resorption. It has been suggested that the dental pulp of deciduous teeth may play a role in the physiologic resorption of the tooth. The pulp of deciduous teeth showed higher expression of cytokines, such as receptor activator of nuclear factor κB ligand (RANKL) and colony-stimulating factor-1 (CSF-1), and a downregulation of osteoprotegerin (OPG), demonstrating its active state.[1,14] Bernadi *et al* have reported that the pulp obtained from deciduous teeth without root resorption does not form viable cell lines compared to those from teeth with resorbed roots.[15] However, studies on the influence of specific teeth, tissue source and conditions on the characteristics of SHED are very limited.[16] Further, to the best of our knowledge, comparison on the effect of different levels of root resorption on the establishment and potency properties of SHED has not been reported to date. For effective clinical applications, it is presumed that understanding the ideal and source time of obtaining the SHED based on the remnant root is crucial.

Keeping in view that all SHED are not the same, the present *in vitro* study was aimed to investigate their cellular and biological properties by isolating these cells from the teeth with varying levels of root resorption. Thus, identifying an ideal tissue source from exfoliated deciduous teeth could be useful for therapeutic considerations through tooth-banking and cell-based clinical applications

MATERIALS AND METHOD

Ethical approval

All procedures performed were in accordance with the Declaration of Helsinki on medical protocol and ethics, and were approved by the Institutional Ethics Committee of A. B. Shetty Memorial Institute of Dental Sciences (ABSMIDS) and the Institutional Committee for Stem Cell Research (IC-SCR), Nitte (Deemed to be University). Written informed consent was also obtained from every participant/parent/guardian. Children requiring the extraction of one or more primary anterior teeth were considered for the study. The vitality of the pulp was assessed at the time of extraction. Teeth with necrosed pulp or those "hanging by a thread" were not included. Carious teeth and those with a history of trauma and children with systemic disease or on medications were excluded in the study.

Collection of pulpal tissue

The teeth were extracted under local anesthesia (2% lignocaine with 1:80,000 adrenaline), as atraumatically as possible (Fig. 1A). After extraction, the pulp was evaluated to be healthy by noting the red color and the presence of bleeding. The roots of the teeth were examined clinically to evaluate the zones of root resorption. The classification system was based on the maximal root wall present. A total of 15 samples were collected in this study. The teeth were categorized as follows: SHED (1/3)- teeth with 0 to 1/3rd root resorption (n=5), SHED (2/3)- teeth with 1/3rd to 2/3rd root resorption (n=5) and SHED (COMP)- teeth with more than 2/3rd root resorption (n=5) (Fig. 1B). The extracted tooth was washed with Dulbecco's phosphate-buffered saline (DPBS) (Gibco, Life Technologies, Thermo Fisher Scientific, USA), and the pulp was extripated. The pulp was obtained depending on the level of root resorption and collected in DPBS (Gibco) mixed with antibiotics and antimycotic solutions, such as penicillin, streptomycin (Pen-Strep, 1% solution, Gibco) and amphotericin-B (Gibco) (Fig. 1C & D).

Isolation of SHED and culture

The pulp was minced with a scalpel, and then digested in a mixture of 0.1% collagenase I (Gibco) for 45 min at 37°C. Following centrifugation at 1200 rpm for 5 min, the cell pellet was obtained along with partially digested pulp tissue remnants. The supernatant was aspirated and the cells were passed through a 70 µM filter (BD Falcon, USA) and cell suspension seeded onto a cell culture plate. The explants were placed on plastic tissue culture dishes containing Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 2.5 µg/ml of amphotericin B, 100 U/ml of penicillin and 100 µg/ml of streptomycin (Gibco) and cultured at 37°C in a humidified atmosphere of 5% CO2 in air. Once the pulp tissue explants firmly adhered to the surface of the plastic culture dish, fresh growth medium was supplemented. The medium was changed twice a week. When the culture reached 90% confluence, approximately after 15 days in incubation, a subpassage using 0.25% trypsin ethylenediaminetatraacetic acid (EDTA) (Gibco) was performed to dissociate and detach the cells from the culture plate. Cells were cultured at 37°C with 5% CO2 until 80 to 90% confluence for further passaging. All the analyses of SHED were carried out within the fifth passage.

Cell morphology

Cell morphology of SHED was assessed at all initial passages using a phase contrast microscope (Olympus, Japan). Cells were gently washed with DPBS to remove cells in suspension before photomicrography.

Cell Viability

The cell viability was assessed in triplicate for each cell line using 0.4% trypan blue (Gibco). The stained cells were counted in a hemocytometer with an inverted phase-contrast microscope (Olympus).

Cell proliferation and population doubling time (PDT)

All three types of SHED were analyzed for cell proliferation and PDT. Culture expanded cells were seeded at 1×10^3/well in a 12-well culture plate (BD Falcon, USA). The cells from each well were harvested on day 3, 6, 9 and 12 and counted with a hemocytometer under a phase-contrast microscope (Olympus, Japan). Three sets of cultures at defined passage were performed and the mean of the counts was calculated. The PDT was determined using the formula PDT = t (log 2)/ (log Nt – log No), where t represents culture time, No and Nt represent the cell number before and after seeding, respectively.

Flow cytometry analysis

SHED were analyzed for the expression of mesenchymal stem cell (MSC) markers (CD73 and Stro-1) and the absence of CD34 and CD45 (hematopoietic stem cell markers) using flow cytometry (FACSCalibur, Becton Dickinson, USA). SHED at 80% confluence were fixed with 3.7% paraformaldehyde (Sigma-Aldrich, USA) for 30 min. The cells then incubated with unconjugated CD73 (Biolegend, USA, 1:100), Stro-1 (E-bioscience, USA, 1:100), CD34 (Biolegend, 1:100) and CD45 (E-bioscience, 1:100) for 2 hrs at 37°C. Following washes with cell staining buffer (Biolegend), fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (E bioscience,

Fig. 1. A. Extraction of primary anterior tooth under local anesthesia (2% lignocaine and 1:80,000 adrenaline). B. Tooth samples collected with varying levels of root resorption (from top left: 0 to 1/3rd root resorption; 1/3rd to 2/3rd root resorption; and bottom row, 2/3rd to complete root resorption). C & D. Pulp extirpated from tooth samples for the isolation of stem cells from human exfoliated deciduous teeth (SHED).

1:100) was used as a secondary antibody and stained for 1 hr at room temperature. The isotype mouse immunoglobulin G controls conjugated with FITC (E-bioscience) were used to determine the positivity of the samples. Isotype-matched negative controls were run simultaneously and at least 10,000 events were acquired and analyzed with Cell Quest software (Becton Dickinson).

Osteogenic differentiation

Initially, all the three types of SHED were seeded at a density of 1×10^4cells/cm^{2}and cultured in maintenance medium consisting of DMEM with 10% FBS. When the cells reached 70% confluence, they were cultured in osteogenic differentiation medium consisting of DMEM, 10% FBS, 0.1 µM dexamethasone (Sigma-Aldrich), 10 mM sodium β-glycerophosphate (Sigma

Aldrich) and 100 µM ascorbic acid (Sigma-Aldrich) for 21 days. Control cultures were maintained with basal medium. Both media were changed twice a week. Alizarin red S (Sigma-Aldrich) staining was carried out to confirm the formation of calcified mineralized nodules. For staining, the medium was removed and the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich). The plates were rinsed three times with DPBS to remove non-adherent cells and stained with 40 mM Alizarin Red S (pH 4.2) for 10 to 30 min at room temperature. Cells were observed and images were captured using an inverted phase-contrast microscope (Olympus).

Adipogenic differentiation

A total of 1×10^4cells/cm^{2}of SHED were cultured in maintenance medium consisting of DMEM with 10% FBS. When the cells reached 70% confluence, the medium was replaced with adipogenic differentiation medium. Adipogenic induction media consisted of DMEM, 10% FBS, 10 µM insulin (Sigma-Aldrich), 200 µM indomethacin (Sigma-Aldrich), 500 µM isobutyl-methylxanthine (Sigma-Aldrich), and 1 µM dexamethasone (Sigma-Aldrich). The medium was replaced twice a week for 21 days. Untreated cells were maintained in DMEM with 10% FBS. For staining with Oil red O (Sigma-Aldrich), the medium was removed and the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich). The plates were rinsed three times with DPBS to remove non-adherent cells and stained with Oil red O solution. Cells were stained for 10 min at room temperature and excess dye was subsequently removed with 70% ethanol. Cells were then observed and images were captured using an inverted phase-contrast microscope (Olympus).

Statistical analysis

All data were expressed as the mean ± SD from at least three independent experiments. Analysis of variance (ANOVA) was performed by GraphPad Prism software (GraphPad, CA, USA) with Tukey's or Dunnet's post-hoc tests. $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Isolation and morphological features of SHED

Isolation of SHED from the three different groups of root resorption was successful in three samples (n=5 each) with a success rate of 60%. As depicted in Fig. 2A, C & E, SHED started emerging out from tissue explants during the second or third day of placement. Initially, SHED liberated from tissue explants had spindle-shaped cells without long processes. These cells showed high expansion activity and formed a monolayer on the culture plate. All types of SHED showed typical fibroblast-like morphology by day 15 in primary culture (Fig. 2B, D & F). After passaging, sub-cultured cells gradually became flattened and exhibited a more homogenous population of cells up to the fifth passage without any noticeable differences in morphological features between three types of SHED.

Viability of SHED

Viability assay of SHED was performed by the trypan-blue exclusion method from passage 1 to passage 4 using a hemocytometer and the results are presented in Fig. 3A. All cell cultures showed >90% viability at each passage, except in SHED (2/3) at the first passage, which showed values slightly lesser than 90% viability. However, from passages 2 to 4, SHED (2/3) displayed nearly 100% viability. In SHED (1/3), viability at passages 1 and 3 was around 92%. However, greater viability was observed at passage 2 (>95%) and passage 4 (96%) with no significant ($P>0.05$) differences. In SHED (COMP), viability at passages 1 and 2 was high with values of 99% and 100%. But the values were

reduced to 98% at passages 3 and 4, and did not show significant (P>0.05) differences. Further, there were no significant (P>0.05) differences in viability among three different types of SHED.

Proliferation and PDT of SHED

The cell proliferation assay was performed by counting the cells at days 0, 3, 6, 9 and 12 using a hemocytometer. All groups showed a slow growth phase from day 0 to day 3, after which the exponential cell growth was observed (Fig. 3B). Larger increase in cell number was noticed between days 6 and 12, and the difference in cell proliferation among the three types of SHED was significant (P<0.05). Among the three, SHED (1/3) showed the least number of cells on day 12. SHED (2/3) and SHED (COMP) had the maximum increase from day 6 to day 12 and showed significant (P<0.05) differences in values at these time points. For PDT, the values recorded were 57.60 hrs, 68.78 hrs and 58.23 hrs for SHED (1/3), SHED (2/3) and SHED (COMP), respectively. There was no significant (P>0.05) difference in PDT between SHED (1/3) and SHED (COMP). However, significant (P<0.05) differences were found in these values when compared to SHED (2/3), which showed a slightly higher PDT.

Flow cytometry analysis

SHED were subjected for the expression of mesenchymal stem cell (MSC) markers (CD73, and Stro-1) and absence of CD34 and CD45 (hematopoietic stem cell markers) using flow cytometry. The results of percentage expression are depicted in Fig. 3D and the representative images are presented in Fig. 4. All the types of SHED were positive for CD73, with different intensity of expression levels. CD73 was highly expressed in SHED (2/3) with 76.25%. However, the expression of CD73 was slightly lesser in SHED (1/3) and SHED (COMP), 55.57% and 69.81%, respectively. The expression of Stro-1 was low in all types of SHED and the values ranged from 6.95% to 9.4%. Further, SHED progeny were negative (<1.0%) for CD34 and CD45.

Osteogenic differentiation

The potential of SHED for *in vitro* osteogenic differentiation was assessed and the cytochemical staining images are presented in Fig. 5. Alizarin red S staining was performed on day 21 after osteoinduction. All three types of SHED in control medium maintained fibroblast-like morphology. The deposition and staining of mineralized nodules was observed in all SHED (1/3), SHED (2/3) and SHED (COMP) examined. However, marginally higher intensities of staining were observed for SHED (1/3) and SHED (2/3) when compared with SHED (COMP).

Adipogenic differentiation

The adipogenic potential of SHED was assessed and the cytochemical staining images are presented in Fig. 6. The cells in basal medium retained a fibroblast-like morphology after 21 days of culture. Oil red O staining clearly showed the accumulation of neutral lipid globules or clusters in SHED from all the three groups. However, slightly stronger staining of oil droplets was noticed for SHED (1/3) and SHED (2/3) compared with SHED (COMP).

Fig. 2: Primary culture establishment and growth features of SHED. A, C and E. Cells emerging out from tissue explants during the second or third day after their placement (arrows). B, D and F. Plastic adherent cells showed proliferation activity after attaining typical fibroblast-like morphology by day 15 of culture.) (Images: ×10).

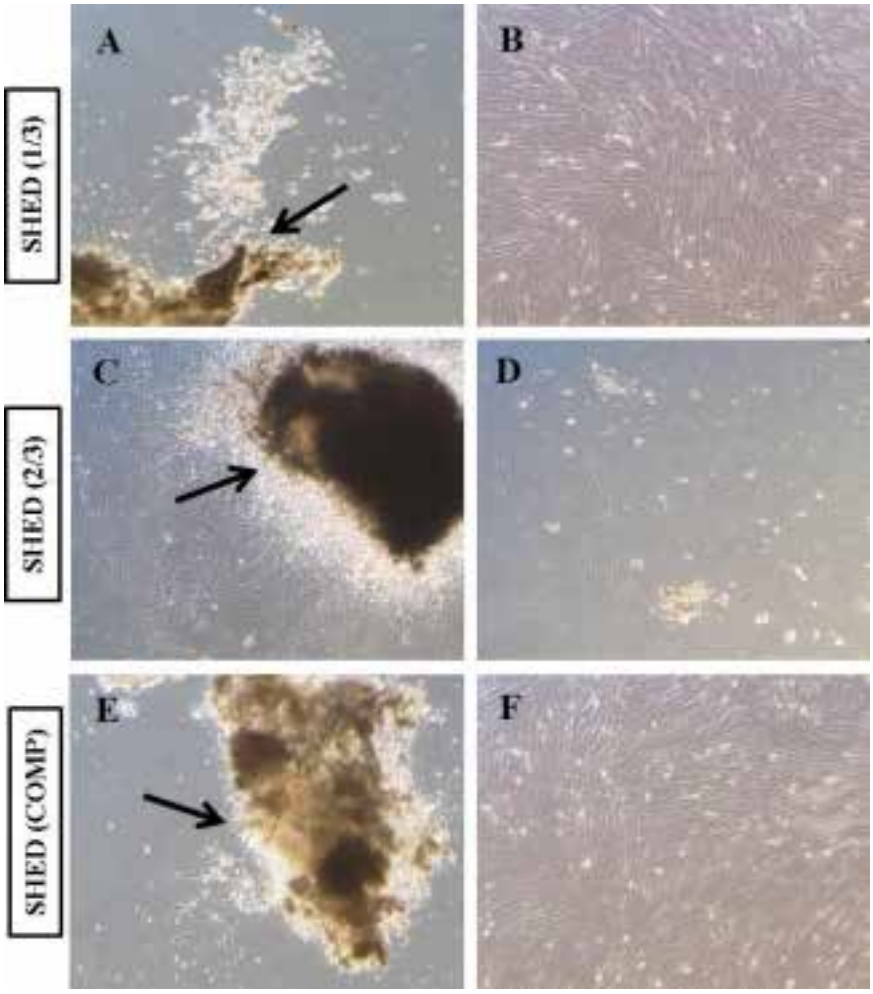


Fig. 3. Cellular properties and cell surface markers expression in SHED. A. Percentage cell viability from passage 1 (P1) to P4. B. Cell proliferation assay at days 0, 3, 6, 9 and 12 of culture. C. Population doubling time (PDT) in hours. D. Percentage expression of cell surface markers. Values are represented as mean ± standard deviation (SD) of triplicates. * and a, b, c, indicate significant (P<0.05) difference between three types of SHED.

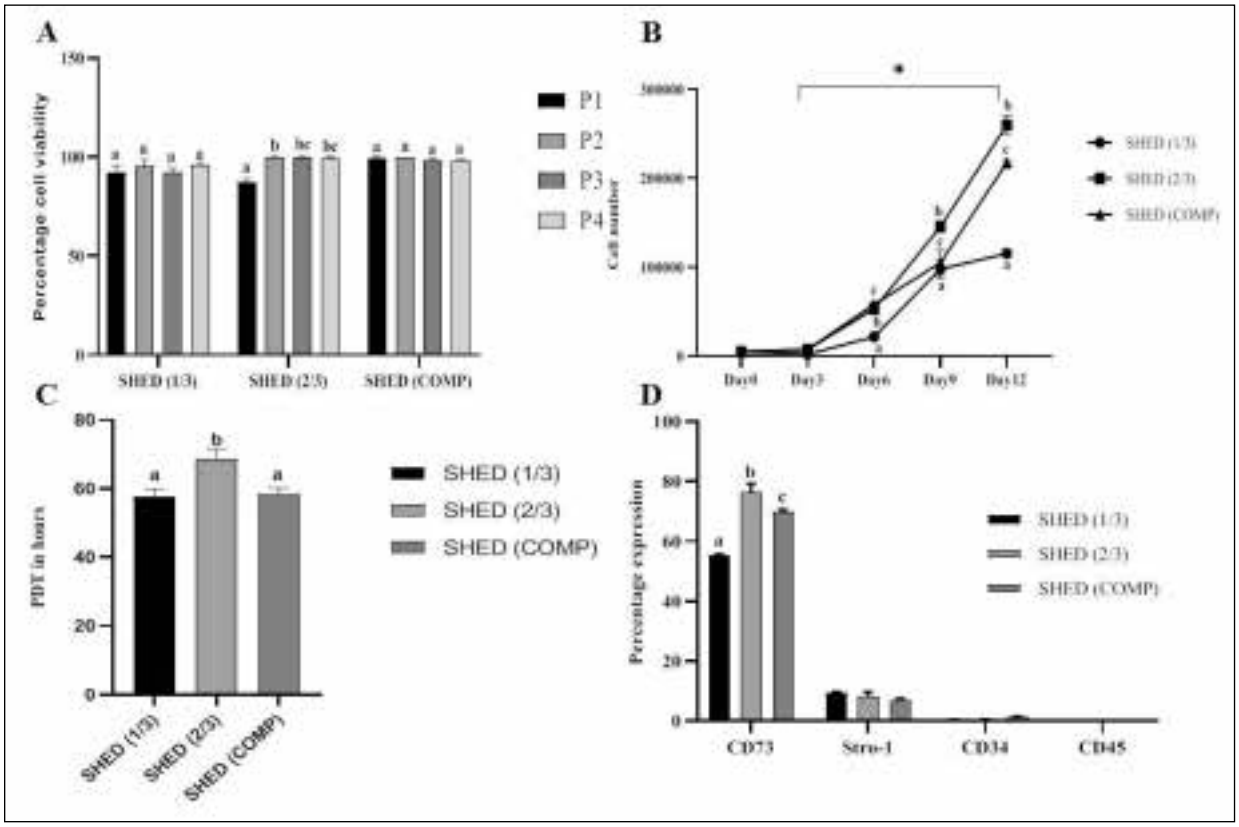


Fig. 4. Flow cytometry analysis of cell surface marker expression in SHED. Cells were stained with antibodies as indicated. In merged images, dark histograms indicate signal of isotype control and pink histograms show the positive reactivity with a specific antibody. Representative images indicating the marker expression profile are shown. A total of 10000 cells were analyzed.

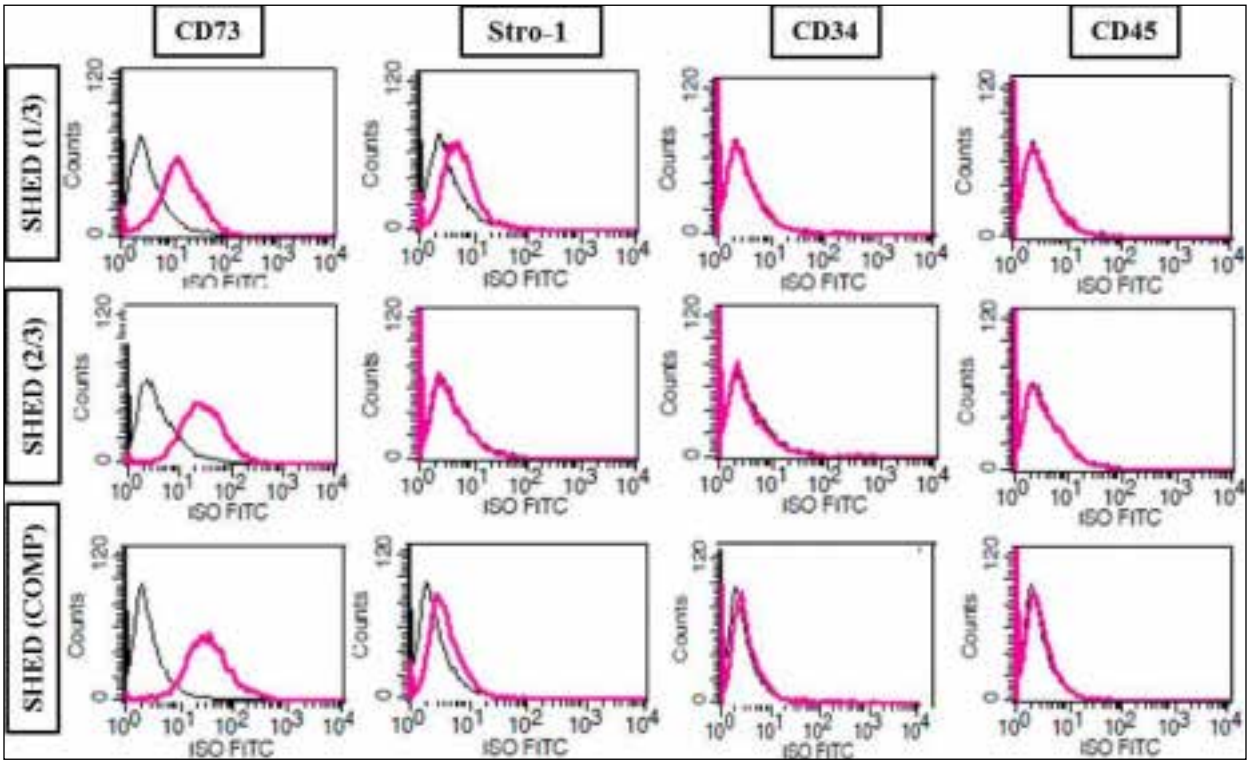


Fig. 5. Osteogenic differentiation of SHED. Cells cultured in basal medium showed fibroblast-like morphology. Mineralization of calcium nodules was exhibited by differentiated osteocytes from SHED as assessed by Alizarin red-S staining (arrows). (Images: ×20).

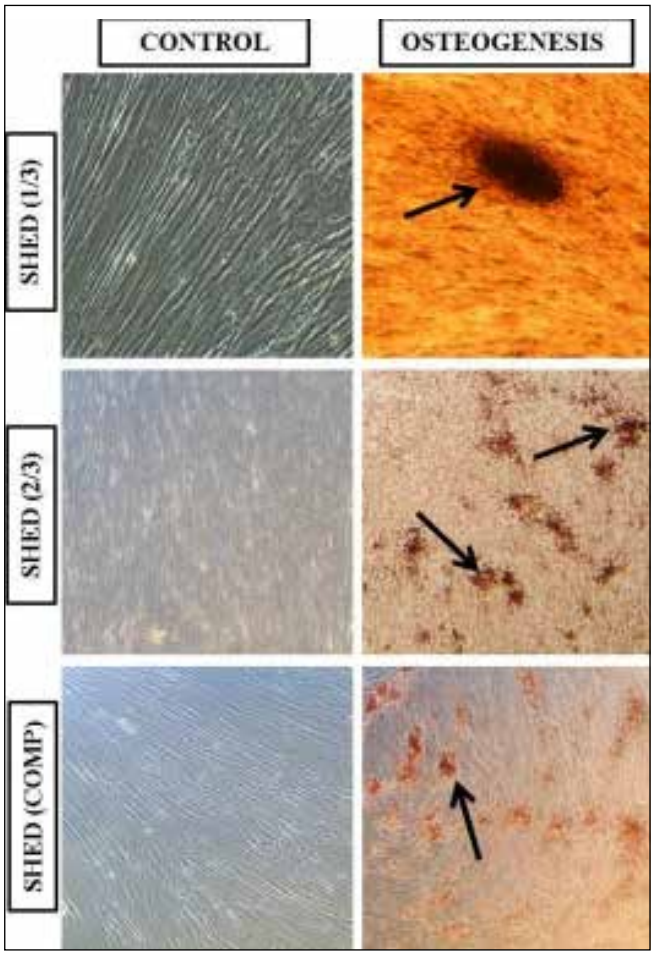
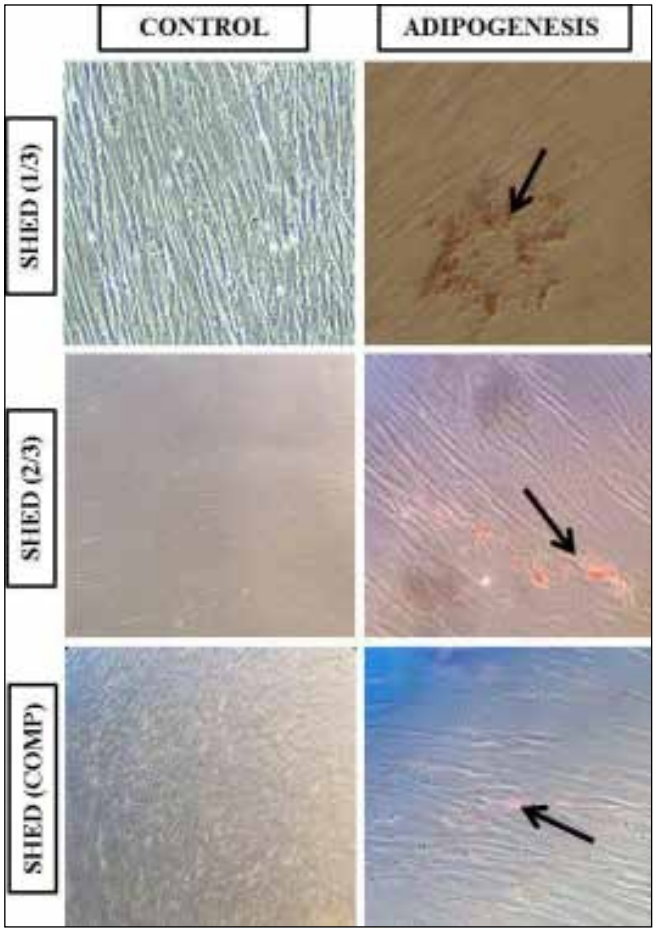


Fig. 6. Adipogenic differentiation of SHED. Cells cultured in basal medium exhibited with fibroblast-like morphology. Adipogenic induction was observed by the accumulation of neutral lipid clusters after 21 days of culture by oil red O staining (arrows). Many droplets of lipids were found in a single cell of SHED. (Images: ×20).



DISCUSSION

SHED play a crucial role in the emerging fields of regenerative medicine and tissue engineering.[13] SHED are a unique population as they are readily accessible and a naturally disposable postnatal human tissue source.[17] The major difference in the pulp of primary and permanent teeth is the occurrence of physiologic root resorption of the deciduous teeth.[14] It has been reported that deciduous teeth without any visible root resorption were unable to proliferate *in vitro*, whereas, those in an advanced state of root resorption showed good proliferation and differentiation potential.[15] The present study was conducted to decipher the quality of stem cells derived from deciduous anterior teeth with varying levels of root resorption for potential therapeutic applications. Three types of SHED were isolated and assessed on their morphology, viability, proliferation rate, PDT, expression of cell surface markers and differentiation potential into osteocytes and adipocytes.

The establishment of primary cultures of SHED was achieved with a success rate of 60% from all the three groups. Particularly, in teeth with more than 2/3rd root resorption, the shorter root length resulted in an extremely small quantity of pulpal tissue available for primary culture. This is in accordance with earlier study by Shekar and Ranganathan,[9]suggesting that deciduous teeth have a significantly lesser amount of dental pulp compared to third molars used for the isolation of DPSCs. However, an improved success rate of 81.8% was demonstrated recently with a suggestion on the requirement of a still higher success rate for clinical applications.[16] Further, the authors opined that successful isolation of SHED seems to be dependent on the patient's age, length of the remaining root, mechanical stress and other factors.

Plastic adherent cells were observed in all cultures, irrespective of the level of root resorption, as per the minimal criteria to define MSCs.[18] Cells in all established primary cultures showed typical fibroblast-like morphology, as observed in MSCs from other adult tissue sources.[7, 8, 10] However, the results are in contrast to Shekar and Ranganathan,[9] as they found a higher proportion of epitheloid cells in SHED culture. Also, Samiei *et al*,[11] have reported SHED cultures with spindle shaped morphology. In this study, we noticed a few spindle-shaped cells in the initial stages of culture, but they gradually disappeared during subsequent culture and formed a homogeneous population of cells.

Trypan blue staining enabled the assessment of viable or nonviable cells. We observed >90% viability in all cultures of

SHED at each passage from P1 to P4, except in SHED (2/3) at the first passage, which showed values slightly lesser than 90% viability. An earlier study reported average viability of 77.9% in the first passage.[15] However, average viability of 90.6% was recorded after long-term cultivation of SHED.[4]

In the present study, we evaluated the cellular proliferation and PDT. SHED (2/3) and SHED (COMP) showed significantly higher proliferative potential than SHED (1/3). The proliferation rate for SHED cultures has been reported to be significantly higher than DPSCs and BMSCs.[8,19,20] The PDT values in this study ranged from 57.6 hrs to 68.78 hrs, with slightly higher PDT recorded in SHED (2/3). Most of the previous investigations reported a slightly shorter PDT for SHED compared to DPSCs, with values varying from 16 hrs to 25 hrs.[5, 9,11] In contrast, Suchanek *et al* [4] found a PDT of 41.3 hrs for SHED when compared to 24.5 hrs for DPSCs. Collectively, the cellular growth parameters of SHED are comparable to earlier reports and their lower PDT facilitates in obtaining larger number of cells in a shorter time for potential *in vivo* applications.

In this study, flow cytometry analysis showed the expression of CD73 and Stro-1 markers in all types of SHED at varied levels. The positivity of these markers indicated the stemness and undifferentiated status of SHED. It is known that CD73 plays an active role in stromal interactions, migration activity and MSC mediated modulation of adaptive immunity.[21] The results of earlier studies support the high expression of CD73 in SHED cultures.[4,5,6,11,12] However, we observed a relatively low expression of Stro-1 in all three types of SHED. This observation is in agreement with earlier studies, where only 9% of the SHED expressed the Stro-1 protein.[2,22] Also, a few investigations have reported a negative result for Stro-1 expression,[23] in contrast to higher expression of Stro-1 in SHED.[10,19] Differences in the tissue sources and culture conditions could be the reasons for these variations in the expression of Stro-1 marker in SHED.[4]In addition, the negative to very low expression of CD34 and CD45 in this study confirmed that the SHED obtained are indeed of mesenchymal lineage as reported previously.[4,5,6,11,12]

The ability of differentiation into other types of cells is a significant feature of SHED.[1]In the present study, the osteogenic and adipogenic differentiation of SHED was evaluated using Alizarin red S and Oil red O staining, respectively. The results indicated that all the three types of SHED had the potential for differentiation into osteocytes and adipocytes. However, the intensities of staining demonstrated that the ability for osteogenic and adipogenic lineage cell formation was marginally higher in SHED (1/3) and SHED (2/3) than that of SHED (COMP). Earlier studies have showed the strong capability of SHED towards osteogenic differentiation by staining with Alizarin red[2,5,6,10,24] as well as by using von Kossa staining.[12] The adipogenic differentiation potential of SHED was also reported by various authors.[2,6,10,12,24] However, a few researchers have shown the weaker ability or inability to induce *in vitro* adipogenic differentiation of SHED attributing their pre-commitment towards the osteogenic lineage.[1,13,25]

The present study demonstrated the successful isolation of SHED from teeth of varying levels of root resorption with largely similar morphological and cellular characteristics. However, SHED from teeth with 1/3rd to 2/3rd root resorption showed relatively better characteristics, based on the growth kinetics as well as the phenotypic marker expression. In addition, the differentiation ability for osteogenic and adipogenic lineages was higher in SHED (1/3) and SHED (2/3) compared with SHED (COMP). Based on these observations, SHED (2/3) appeared to be useful for prospective applications in dentistry and hence, warrants further investigations.

Conflicts of interest

The authors declare that they have no conflict of interest.

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