ORIGINAL RESEARCH

Antibacterial and Immunomodulatory Properties of Stem Cells from Human Exfoliated Deciduous Teeth: An *In Vitro* Study

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Abstract

Stem cells from human exfoliated deciduous teeth (SHED) provide an important autologous source for stem cell-based regenerative therapies with their easy acquisition and multipotency. However, the understanding of their antibacterial and immunomodulatory properties is limited. This *in vitro* research aims to determine whether SHED inhibits the growth of *Streptococcus mutans (S. mutans)* and *Enterococcus faecalis (E. faecalis)*, as well as whether or not it has immunomodulatory effects by measuring interleukins (ILs)-2 and -6 levels. SHEDs were derived from the pulp of deciduous teeth that had undergone up to two-thirds of their roots' resorption. Isolated SHEDs were characterized on their morphological features, viability, assessment of surface markers, and *in vitro* induction into osteocytes and adipocytes. SHED was tested for its antibacterial efficacy against *S. mutans* and *E. faecalis* using a colony-forming units (CFU) assay. Lastly, we checked the cytokine levels by enzyme-linked immune sorbent assay (ELISA) for assessing the immunomodulatory properties of SHED. The results showed that the established SHED had fibroblastic morphology with higher viability. The ability to differentiate into osteocytes and adipocytes, as well as the expression of stem cell-specific markers, demonstrated their potential and flexibility under *in vitro* settings. SHED demonstrated antibacterial characteristics by significantly ($p < 0.05$) lowering *S. mutans* CFU, whereas *E. faecalis* CFU was either unaffected by or just slightly affected by the cells. SHED also helped keep inflammatory indicators, including IL-2 and IL-6, at stable levels when compared to the control. The results indicate that SHED may aid in preventing or reducing an infection due to its antibacterial activity and may provide immunomodulatory activities by controlling the production of cytokines.

Keywords: Antibacterial activity, Immunoregulation, *In vitro,* Stem cells of human exfoliated deciduous teeth.

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Introduction

Recent studies[1] have shown that oral tissues, which dentists have easy access to, have a high concentration of stem cells. Stem cells from human exfoliated deciduous teeth (SHED) may differentiate into osteogenic/odontogenic, adipogenic, and neurogenic lineage cells similar to that of mesenchymal stem cells (MSCs).[2–4] Deciduous teeth are very often discarded as biological waste, and their pulp tissue serves as a unique source of SHED with less ethical concern.[2] Considering their unique abilities in terms of potency and plasticity, SHEDs are important for developing regenerative therapies.[4]

Stem cell-based therapy is being regarded a potential choice of treatment in various oral diseases for extraoral and maxillofacial defects, tissue repair, and regeneration.[4,5] As multipotent stem cells, SHED has been extensively characterized in terms of their biological properties for prospective therapeutic applications.[1,6] However, studies on deciphering their antibacterial and immunomodulatory properties are less.[7–9]

Dental caries is a disease caused by a specific type of bacteria, *Streptococcus mutans (S. mutans)*, which produces acid that destroys the tooth's enamel and dentin and acts as primary initiators.[10,11] Further, *Enterococcus faecalis (E. faecalis)* and its concomitants are known to be the main cause of pulpitis and a key pathogen of the root canal system.[12–14] *E. faecalis* was involved in opportunistic infections in the oral cavity, and studies showed it as a primary pathogen associated with endodontic treatment.[14] As bacteria and their secretions reach the pulp, the foremost pulpal cells to confront them are the odontoblasts as they possess a specialized innate response against pathogens.[15] Further, these cells offer a barrier function to the basal tissue by coordinating many inflammatory responses, including the production of cytokines involving various interleukins (ILs).[15–17]

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It has been reported that MSCs secrete antimicrobial peptides, thus exerting activities against gram-negative and gram-positive bacteria.[17–21] This property of MSCs is further supported by possessing significant immunomodulatory and nonimmunogenic properties, evidenced by experimental and clinical findings in the treatment of immune diseases.[17,21] However, a few studies explored the antibacterial and immunomodulatory properties of SHED. Therefore, this study was designed to evaluate the antimicrobial effect of SHED on *S. mutans* and *E. faecalis,* along

with its role in the secretion of selected cytokines, IL-2 and IL-6, during the inflammatory environment generated due to infection.

Materials and Methods

Collection of Sample and Establishment of SHED

The pulp tissues were collected from the deciduous teeth ($n = 3$) based on the root resorption level. After being collected in Minimum Essential Medium Eagle-α modification (MEMα) (Gibco, Invitrogen Corporation, Life Technologies, United States of America) supplemented with penicillin–streptomycin (1%) and 2.5 µg/mL of amphotericin-B (all from Gibco), the samples were brought to laboratory within 3 hours of extirpation. In this study, all procedures were performed according to the Declaration of Helsinki on Medical Protocol and Ethics and approved by the Institutional Ethics Committee and the Institutional Committee for Stem Cell Research, NITTE (Deemed to be University). Written informed consent was obtained from each participant/parent/guardian.

Stem cells from human exfoliated deciduous teeth (SHED) was established by treating pulp tissues with 0.1% collagenase I (Gibco) at 37°C for 45 minutes. Tissue remnants were partially digested, then cultured in MEM*α* (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 gm/mL penicillin, 100 gm/mL streptomycin, and 2.5 gm/mL amphotericin B, (Gibco) at 37°C in 5% CO_2. Primary cells (P0) were cultivated for 15 days to 70–80% confluence and then sub-passaged using 0.25% trypsin-ethylenediaminetetraacetic acid (Gibco). A series of subcultures were performed, and the assays were conducted using the cells up to passage 5.

Morphology and Viability Analyses

The morphological features of SHED were observed by an inverted phase-contrast microscope (Olympus, Japan) at the start of each passage. Cells were gently rinsed in Dulbecco's phosphate-buffered saline (DPBS, Gibco) to remove free-floating cells prior to photomicrography. Viability was assessed using 0.4% trypan blue (Gibco) in triplicates by counting in a Neubauer hemocytometer.

Flow Cytometry for Surface Marker Analysis

Stem cells from human exfoliated deciduous teeth (SHED) was analyzed for the expression of MSC-specific markers [cluster of differentiation (CD)29, CD44, and CD90] and the lack of marker expression specific to hematopoietic stem cells (CD34 and CD45). Using a flow cytometer (BD FACSCalibur, Becton Dickinson, United States of America), 3×10^5 cells were incubated with the primary antibodies (1:100 dilution) of CD29 (Biolegend, United States of America), CD44 (Biolegend), CD90 (E-bioscience, United States of America), CD34 (Biolegend), and CD45 (E-bioscience, United States of America) at 37°C for 2 hours. Later, cells were washed with DPBS and stained with a fluorescein isothiocyanate-conjugated secondary antibody for an hour at room temperature (Biolegend, 1:100). An isotype-matched negative control served as a reference. At least 10,000 events were acquired and processed with the Cell Quest program (Becton Dickinson).

Osteogenic and Adipogenic Differentiation

For osteogenesis, SHED was seeded at a density of 1×10^4 cells/well on 12-well culture plates with 10% FBS in MEMα. At 70% confluence, the cells were cultured in osteogenic induction media consisting of MEMα, 10% FBS, 0.1 µM dexamethasone (Sigma-Aldrich, USA), 10 µM sodium-glycerophosphate (Sigma-Aldrich), and 100 µM ascorbic acid (Sigma-Aldrich) for 21 days. The cells in control were maintained in a basal medium. The media was changed twice a week. After induction, cells were stained with a 40 µM Alizarin Red S (Sigma-Aldrich, pH 4.2) in 4% paraformaldehyde solution for 30 minutes (Sigma-Aldrich). Images were captured using a phase-contrast microscope (Olympus).

For adipogenesis, SHED was seeded at a density of 1×10^4 cells per well onto 12-well culture plates in MEMα with 10% FBS. After 70% confluence, the cells were cultured in an adipogenic induction medium that included MEMα, 10% FBS, 10 µM of insulin, 200 µM of indomethacin, 500 µM of isobutyl-methylxanthine, and 1 µM dexamethasone (All from Sigma-Aldrich). The media was changed twice a week for 3 weeks. The cells in control were maintained in MEMα supplemented with 10% FBS. Oil red O staining (Sigma-Aldrich) was performed by fixing cells in 4% paraformaldehyde. After staining with 0.0125% Oil red O, the plates were observed under an inverted phase-contrast microscope (Olympus).

Assay for Antibacterial Activity of SHED

Preparation of Bacterial culture and Serial Dilution Assay

Brain heart infusion (BHI) broth and agar and Mutans-Sanguis Agar (MSA) were used for culturing *E. faecalis* (ATCC 29212) and *S. mutans* (MTCC 497), respectively. Around 2–3 colonies of *E. faecalis* and *S. mutans* were emulsified into BHI broth. Sterile petri plates containing sterilized media were prepared, inoculated, and incubated at 35 ± 2°C overnight. The optical density value of the suspension was recorded at 600 nm. Dilutions were prepared as per the standard and known optical density values. 30 µL was inoculated into each well respective of the organism, and after 6 hours of incubation, 100 µL from each well was used to prepare 10-fold dilutions for $1:10^1$, $1:10^2$, $1:10^3$, and $1:10^4$. Later, 20 µL from the above dilutions was used to perform the spread plate method on BHI agar and MSA. Plates were incubated overnight at 37°C in a CO_2 incubator, and colony counts were recorded. Colony-forming units (CFU) per milliliter were determined by using an appropriate dilution factor.

Effect of SHED on S. mutans and E. faecalis

For assessing the effect of SHED *on S. mutans* and *E. faecalis,* eight groups were established. Namely, group I: media without antibiotics (penicillin–streptomycin), group II: media + SHED, group III: media + SHED + *S. mutans,* group IV: media + SHED + *E. faecalis,* group V: media + antibiotics + *S. mutans,* group VI: media + antibiotics + *E. Faecalis,* group VII: media without antibiotics + *S. mutans,* and group VIII: media without antibiotics + *E. faecalis*. Briefly, SHED in 24-well plates (2×10^5 cells per well) in MEMα with 5% FBS were infected with 300 CFUs of *S. mutans* and *E. faecalis* and incubated at 37°C for 6 hours. Then, aliquots of culture medium were collected from each well of *S. mutans* and *E. faecalis*, serially diluted with sterile DPBS, and plated on MSA and Müller-Hinton agar, respectively. Counting of colonies was performed following overnight incubation at 37°C.[17,19]

Effect of SHED on the Levels of IL-2 and IL-6

Enzyme-linked immune sorbent assay (ELISA) was performed for the estimation of human IL-2 and IL-6 levels by following the kit protocol (Biolegend ELISA MAX kit, Biolegend). Briefly, first, a 96-well plate was coated with a particular monoclonal antibody. Cytokines were allowed to attach to the immobilized capture antibody, and standards were introduced independently of samples. To complete the antibody-antigen-antibody sandwich, biotinylated anti-human

detection antibodies (IL-2 and IL-6) were added. After adding avidin-horseradish peroxidase, a blue color developed that correlated with the concentration of a certain cytokine in the sample. Finally, a stop solution was added, and color changes were recorded with absorbance at 450 nm by a microplate reader (Thermo Fisher Scientific, United States of America).

Statistical Analysis

GraphPad Prism was used for one-way or two-way analysis of variance, and the data has been presented as the mean ± standard deviation from at least triplicate experiments (GraphPad, California, Unted States of America). Tukey's test was employed for *post hoc* comparisons. The significant level was tested at $p < 0.05$.

Results

Isolation of SHED, their Morphology, and Viability

Pulp tissues were collected and processed for the successful establishment of SHED (Fig. 1A). Minced tissues as explants were placed in cell culture plates. By the 8th day of primary culture, small spindle-shaped cells started emerging from the tissue explant cultured in MEMα supplemented with 10% FBS (Fig. 1B) and displayed fibroblast-like morphological features by the 15th day of primary culture (Fig. 1C). Further, SHED exhibited a proper adherence and fibroblastic morphology after being sub-cultured from primary culture (P0) to passage 1 (Fig. 1D). SHED showed more than 98% viability at passage 1 (P1), P2 and P3 (Fig. 1E). However, in P1, the viability of SHED was significantly ($p < 0.05$) higher than P2 and P3.

Expression of Cell Surface Markers in SHED

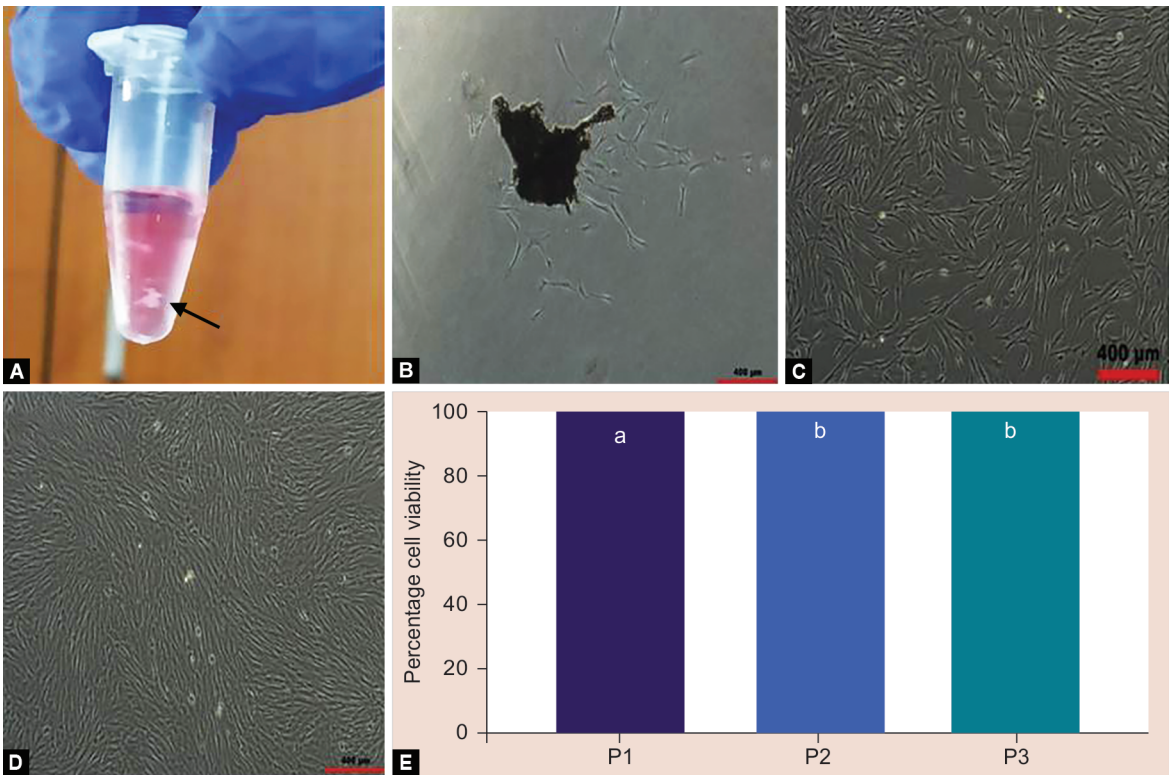
The flow cytometry data showed that SHED expressed CD29, CD44, and CD90 (MSCs) at higher levels (>80%) and CD34 and CD45 (hematopoietic stem cell markers) at low levels (<5%) (Fig. 2). Data acquired from 10,000 cells is presented.

Osteogenic and Adipogenic Potential of SHED

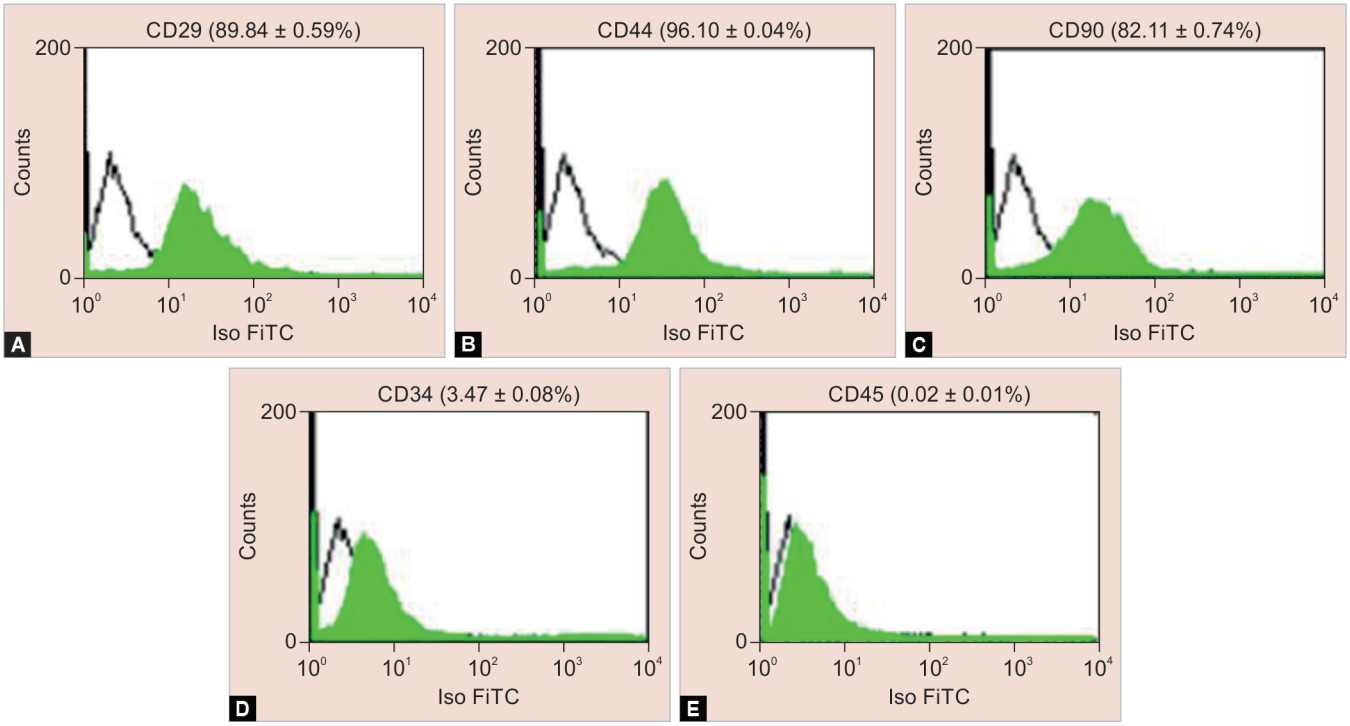
Stem cells from human exfoliated deciduous teeth (SHED) cultured in basal medium for 21 days retained their fibroblastic morphology without showing any differentiation ability (Figs 3A and C). Following induction in osteogenic-specific media, SHED displayed their strong ability to form osteocytes as indicated by their positivity to Alizarin red (arrows) (Fig. 3B). Upon adipogenic media induction, SHED demonstrated their intensity to form neutral lipids as confirmed by Oil Red O (arrows) (Fig. 3D).

Antibacterial Activity of SHED

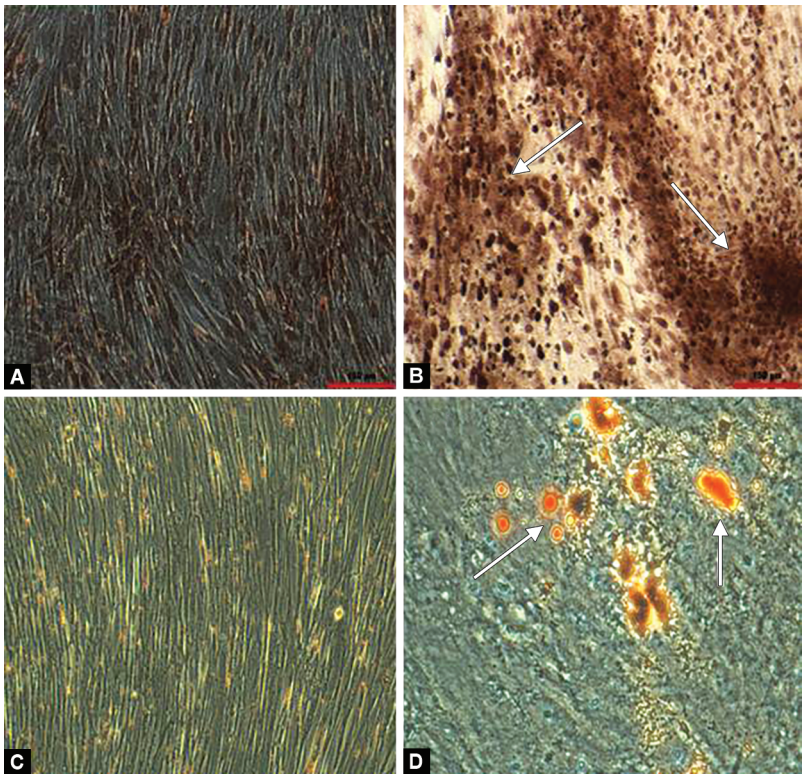
To evaluate the antibacterial properties of SHED against *S. mutans* and *E. faecalis*, eight experimental groups were formed, and the results are presented in Figures 4A to E. In the no dilution group as a positive control, the bacterial CFU values were 22.5 and 30 for S. mutans and E. faecalis, respectively (Fig. 4A). Group III (Medium + SHED+ *S. mutans*) had a considerably (*p,* 0.05) lower number of *S. mutans* and was essentially identical to *S. mutans* treated with antibiotics groups. In the group of *E. faecalis*, no reduction in CFU values in group IV was observed (media + SHED + *E. faecalis*). However, *E. faecalis* treated with antibiotics displayed a significant decrease in CFU. Thus, SHED had very limited or no antimicrobial



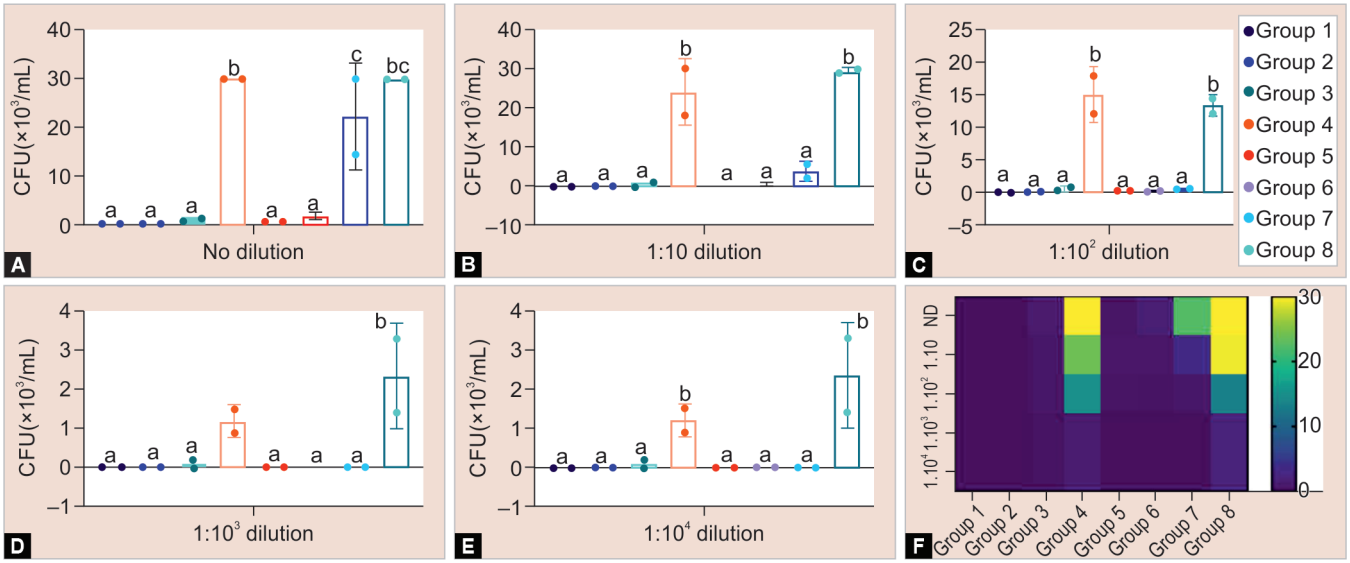
Figs 1A to E: Sample collection, isolation, culture, and viability of stem cells from exfoliated deciduous teeth (SHED); (A) Tissues collected in MEMα (arrow); (B) Minced tissue explants placed in cell culture plates; (C) Plastic adherent cells showing proper fibroblast-like morphology by the 15th day of primary culture; (D) SHED retaining the proper adherence and fibroblastic morphology after being subcultured from primary culture (P0) to passage 1; (E) SHED showed more than 98% viability at passage 1 (P1), P2, and P3. Superscripts a and b indicate statistical differences tested at $p < 0.05$



Figs 2A to E: Expression of cell surface markers in stem cells from exfoliated deciduous teeth (SHED) by flow cytometry analysis; the cell surface markers are represented as filled-green histograms, and isotype control is represented as dark-lined histograms; expression levels are presented as mean ± standard deviation (SD)



Figs 3A to D: Osteogenic and adipogenic differentiation potential of stem cells from exfoliated deciduous teeth (SHED); (A and C) SHED cultured in basal medium for 21 days; (B) Positivity of osteocytes to Alizarin red staining (arrows); (D) The presence of lipid vacuoles by Oil Red O staining (arrows); magnification: 4× and 10×



Figs 4A to F: Antibacterial activity of stem cells from exfoliated deciduous teeth (SHED); group I: media without antibiotics (Pen–Strep), group II: media + SHED, group III: media + SHED + *S. mutans,* group IV: media + SHED + *E. faecalis,* group V: media + antibiotics + *S. mutans,* group VI: media + antibiotics + *E. Faecalis,* group VII: media without antibiotics + *S. mutans,* and group VIII: Media without antibiotics + *E. faecalis;* (A) No dilution; (B) 1:10 dilution; (C) $1:10^2$ dilution; (D) $1:10^3$ dilution; (E) $1:10^4$ dilution, superscripts a, b, and c indicate statistically significant differences at $p < 0.05$; (F) Antibacterial activity through graphical presentation of heat map of various groups

effect on *E. faecalis*. Similar statistical observations were recorded in different dilutions as well (Figs 4B to E) (1:10 to $1:10^4$ dilution) with a gradual reduction in bacterial load in every dilution.

The results of antibacterial activity through the graphical presentation of heat maps of various groups are depicted in Figure 4F. Group I served as blank, which consisted only of cell culture media without antibiotics, which showed zero colonies (purple color indicated zero colonies, as it moves above in the scale bar, color ranges from purple to yellow, indicating an increased number of bacterial colonies). Group II (media + SHED) also did not show any colony formation, indicating that culture was free from any of microbial contamination. Group III (media + SHED + *S. mutans*) showed a low bacterial load in the no-dilution group. However, as the dilution increased, the formation of *S. mutans* colony reduced and reached zero from 10^3 dilution onward. However, the *S. mutans* load in the positive control group, that is, group VII (media + *S. mutans*), was itself low, and colony formation significantly reduced as the dilution increased. Group IV (media + SHED + *E. faecalis*) showed almost equal (no significant variation) CFU ability to the positive control group (group VIII). This was considerably reduced on dilution, but no significant ($p > 0.05$) difference was observed between groups IV and VIII. This showed that SHED had very minimal or no antibacterial effect on *E. faecalis*. However, antibiotics-treated groups showed a significant reduction in CFU for both bacterial species.

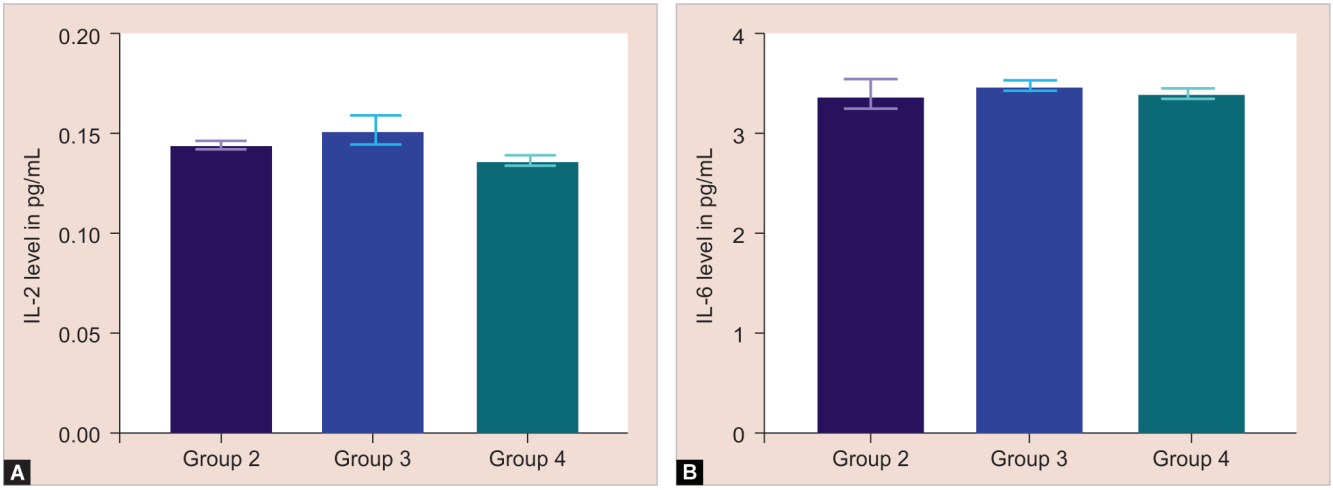
Immunomodulatory Properties of SHED

Levels of IL-2 and IL-6 after stimulation with *S. mutans* and *E. faecalis* were analyzed by ELISA, and the results are depicted in Figures 5A and B. Significant differences ($p > 0.05$) were not found in the levels of IL-2 (range: 0.13–0.15 pg/mL) and IL-6 (range: 3.3–3.5 pg/mL) among the groups II, III, and IV (Figs 5A and B).

Discussion

The present study evaluated the potency characteristics of SHED along with their antibacterial and immunomodulatory properties *in vitro*. The permanent teeth contain DPSCs, while deciduous teeth are a rich source of SHED.[4] The existence of physiologic root resorption in the deciduous teeth is the primary distinction between the formation of essential and permanent teeth[22] that leads to morphological changes in the pulp, vascular and cellular changes, such as the absence of odontoblasts and increase in inflammatory and elastic cells.[6,22] However, cells from deciduous teeth without obvious root resorption were unable to multiply *in vitro* and further, cells from teeth that had undergone root resorption still managed to proliferate and differentiate well.[5,22] In this study, SHEDs were successfully isolated from pulp tissues collected from the teeth with resorption up to two-thirds level, similar to our report published previously.[5] The cells showed plastic-adherent properties with fibroblastic features possessing >90% viability at every subpassage examined.[1,5,22,23] These observations confirmed that the isolation and *in vitro* expansion methods resulted in the generation of a sufficient number of viable SHEDs for prospective clinical use.

Flow cytometry was used to examine cell surface marker expression in SHED. As per the findings, SHEDs were positively stained for CD29, CD44, and CD90 (>80%) but negatively stained for CD34 and CD45 (<5%). CD29, also known as integrin β1, is expressed on a wide variety of cell types, from mesenchymal to epithelial, and plays a role in the interaction of cells with extracellular matrix proteins such as collagen, laminin, and fibronectin.[24,25] Cell adhesion, organ development, tissue healing, and homeostasis have all been linked to CD29 functional importance.[25] Inducing odontoblastic differentiation in DPSCs has been linked to the expression of CD44, a nonkinase transmembrane proteoglycan.[26,27] CD90 (Thy1) also helps monocytes and leukocytes migrate and adhere to endothelial cells and fibroblasts, demonstrating its importance in cell-to-cell interactions.[1,4,28] Higher expression of these markers in SHED supports the preservation of stem cell properties, such as proliferation, cell migration, and adhesion, which are required for the development of cell-based therapies.[28,29] Moreover, consistent with earlier results, SHED revealed very low



Figs 5A and B: Levels of inflammatory cytokines after stimulation with *S. mutans* and *E. faecalis;* (A) There was no significant difference ($p < 0.05$) observed in IL-2 levels between groups II, III, and IV; values are expressed as mean ± SD; each condition was performed in triplicates; (B) There was no significant difference ($p < 0.05$) observed in IL-6 levels among the groups II, III, and IV; values are expressed as mean ± SD; each condition was performed in triplicates; group II: media + SHED; group III, media + SHED + *S. mutans;* group IV: media + SHED + *E. faecalis*

levels of markers of hematopoietic stem cell and higher levels of MSCs-specific markers.[3–5,28,29]

The differentiation ability of SHED is crucial for assessing the potential to regenerate specific lineage tissues.[3] In this study, SHEDs were cultivated in osteogenic induction media and stained with Alizarin red to indicate that they could differentiate into osteocytes. Further, the cells were expanded in adipogenic media, and staining with Oil Red O showed the presence of adipocytes. The SHEDs have been found to be capable of osteogenic and adipogenic differentiation *in vitro*.[1,2,5,19,22] However, a few studies have shown a lesser propensity of SHED towards adipogenesis.[1,7] The highest osteogenic lineage potential of SHED makes them a preferred candidate for tissue engineering applications.

Mesenchymal stem cells (MSCs) have been shown to exhibit antibacterial action against both gram-negative and gram-positive bacteria in recent investigations.[17,19] Further, data suggests that MSCs secrete antimicrobial peptides and proteins, which play a role in eliciting powerful antimicrobial responses through many indirect and direct routes.[20] Keeping this in view, we next examined the antibacterial properties of SHED against *S. mutans* and *E. faecalis*. *S. mutans* has been considered one of the critical microorganisms implicated in the occurrence of dental caries.[10] Further, *E. faecalis* is the major microbial factor in causing endodontic infections and is found as a monoculture in retreated root canals.[12,14] To rule out the influence of factors, such as contamination, SHEDs were cultured with or without supplementation of antibiotics in the media. A total of eight groups were formulated for antibacterial assays. The results indicated that at no dilution and the dilutions of 1:10, $1:10^2$, $1:10^3$, and $1:10^4$, group III (medium + SHED + *S. mutans*) had a bacterial count virtually as low as the antibiotic-treated *S. mutans* groups. In the presence of SHED, a dramatic reduction in the *S. mutans* population was readily visible, and these cells exhibited significant antimicrobial activity. In contrast, *E. faecalis* treated with antibiotics exhibited a noticeable reduction in CFU, but there was no decrease in CFU values in group IV (media + SHED + *E. faecalis*). Thus, SHED did not exert any considerable antimicrobial effect on *E. faecalis*. Collectively, our results showed that SHED could attenuate microbial growth, particularly that of *S. mutans,* and this effect can be due to the secretion of antimicrobial factors or peptides.

Previously, two vital properties of MSCs have been shown to be associated with their antimicrobial potential.[19] They are the presence of toll-like receptors and formyl peptide receptors and the production of LL-37, a cathelicidin family of antimicrobial protein.[17,19,21] Apart from these, other antimicrobial peptides secreted by MSCs, such as defensins, cystatin C, elafin, and lipocalin 2, have also been implicated in mediating antibacterial activities.[17,18,21] However, the role of these antimicrobial peptides in SHED is not yet clearly understood and needs to be analyzed extensively on their regulatory functions.

Lastly, the immunomodulatory properties of SHED were analyzed by determining the expression levels of IL-2 and IL-6. The data indicated that the expressions of IL-2 and IL-6 were unaltered after the stimulation with *S. mutans* and *E. faecalis,* and the values were statistically insignificant ($p > 0.05$). These observations suggest the possible immunosuppression of IL-2 and IL-6 levels by SHED in the presence of the stress factors *S. mutans* and *E. faecalis*. In view of bacterial infection, the effectiveness of responsiveness is determined by the inflammatory milieu and the reactivity of immune cells.[21] IL-2 is known to have a wide range of impacts on the immune system apart from encouraging the growth and differentiation of CD4 and CD8 T cells into effector T cells and memory T cells.[7,9] Moreover, infections and tissue damage act as stress inducers that trigger an immediate and temporary production of IL-6.[28] While under pressure, such as early childhood caries and endodontic infections, the cytokines play a critical role as pro-inflammatory and anti-inflammatory factors, and the levels of these immunological markers were elevated.[7,17] In agreement with these findings, SHED significantly suppressed the proliferation of T-lymphocytes, altered the levels of cytokines involved in pro- and anti-inflammatory responses, and enhanced the production of regulatory T cells,[9] and suggested that these cells may be considered therapeutic agents for treating allergic diseases. With these observations, it is opined that SHED plays a role in suppressing or maintaining the levels of pro-inflammatory markers and might be protective in dental caries and endodontic infections.

In conclusion, our results showed that the established SHED had MSC-like properties with their cellular potency and plasticity under *in vitro* conditions. Further, SHED displayed antibacterial effects

by drastically decreasing *S. mutans* CFU but had little to no impact on *E. faecalis*. Also, SHED contributed to reducing or keeping the inflammatory markers IL-2 and IL-6 at constant levels when compared to the control. Based on these results, it seems that SHEDs may help restrict the spread of infection by inhibiting bacterial growth, and they may also have immunomodulatory effects by controlling the production of cytokines. Nevertheless, in order to fully understand their expanded potential in therapeutic applications, further functional investigations of SHED are necessary.

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