

Local Administration of Stem Cells from Human Exfoliated Primary Teeth Attenuate Experimental Periodontitis in Mice

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Objective: To evaluate the therapeutic effect of local injection of stem cells from human exfoliated primary teeth (SHED) on periodontitis in mice.

Methods: Fifteen female mice were randomly divided into three groups: normal control group, periodontitis group and SHED treatment group. A periodontitis model was established by ligating a 0.2 mm orthodontic ligation wire to the maxillary first molar. The SHED group was injected with SHED at 3 weeks post-ligation. All mice were sacrificed and their maxillae were dissected five weeks post-ligation. Clinical assessments, micro-computed tomography (micro-CT) scanning, and histologic examination were used to evaluate the outcome of tissue regeneration.

Results: Micro-CT analysis showed that SHED administration significantly increased periodontal regeneration and decreased the distance between the cemento-enamel junction and the alveolar bone crest. In addition, histopathological photomicrographs showed new regenerated bone, the number of TNF- α -positive, IFN- γ -positive and CD4⁺ cells decreased, and osteoclasts-positive decreased in the periodontal defect area in the SHED group compared with the periodontitis group.

Conclusion: SHED administration suppresses the expression of inflammatory factors, inhibits the production of osteoclasts, and promotes the regeneration of periodontal tissues.

Key words: periodontitis, stem cells from primary teeth, inflammation
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Periodontitis is a chronic inflammatory disease caused by bacteria. The clinical manifestation is the loss

of periodontal support tissue, including the periodontal ligament, alveolar bone and gingival tissue. It is the main pathogenic cause of adult tooth loss¹. In addition, periodontitis is closely related to cardiovascular diseases, diabetes and other systemic diseases². Therefore, there is an urgent need to find new and effective treatment strategies for periodontitis.

The goal of periodontitis treatment is to stop the progression of the disease, regenerate the damaged tissue and restore its original structure and function. At present, traditional treatment methods, whether surgical or non-surgical, are not effective in regenerating damaged periodontal tissues. Stem cell therapy is a promising therapeutic approach for the treatment of periodontitis³. A previous study showed periodontal regeneration in a rat periodontitis model after local injection of bone marrow mesenchymal stem cells (BMSCs) in suspension, and regeneration in periodontal defected areas in miniature pigs, after using periodontal ligament stem cells (PDLSCs)⁴. However, the limited sources of

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BMSCs and PDLSCs have greatly narrowed their therapeutic applications. Stem cells from human exfoliated primary teeth (SHED), which can be easily acquired from exfoliated primary teeth, have shown greater proliferation ability *in vitro* compared with BMSCs and PDLSCs, and are capable of generating robust amounts of bone *in vivo*⁵. Furthermore, SHED have the immunomodulatory ability to inhibit T lymphocyte proliferation and to release inflammatory cytokines⁶. Therefore, SHED may be a potential cell source for periodontal regeneration. Previous studies have shown that the inflammatory factors TNF- α and INF- γ were involved in the formation of osteoclasts⁷⁻⁹. However, whether TNF- α and INF- γ are involved in the periodontitis development is not known.

In this study, SHED was administered to treat periodontitis in mice, and it was able to alter the local inflammation, inhibit the osteoclasts generation and promote periodontal tissue regeneration. These results suggest that the use of noninvasive local injection of SHED may represent a new strategy for periodontitis treatment in the clinical practice.

Materials and methods

Animals

Healthy 6 to 8-week-old female Sprague-Dawley rats were purchased from Vital River Laboratory (Beijing Vital River Laboratory Animal Technology, China). Rats were housed under a 12 hours light/dark cycle, controlled temperature ($22 \pm 1^\circ\text{C}$) and had free access to food and water. All the experimental protocols for rat treatment were approved by the Institutional Animal Care and Use Committee, at Peking University (LA2013-92).

Cell culture

SHED were obtained from Oral Stem Cell Bank (Beijing, China). SHED were cultured in α -minimum essential medium (α -MEM) supplemented with 15% fetal bovine serum (FBS), 0.292 mg/ml glutamine, 100 U/ml penicillin and 100 U/ml streptomycin, and then incubated at 37°C in a humidified atmosphere containing 5% CO_2 . Cells from passage 3-5 were used. SHED were tested for multilineage differentiation capacity by growing them in a differentiation medium containing either osteogenesis or adipogenesis supplements, and stained with alizarin red S or oil red O (Cyagen Biosciences, Guangzhou, China) after 14 days of culture. Images were captured with a light microscope (BX60, Olympus, Japan). A cell

counting kit-8 (CCK8) assay (Dojindo Laboratories, Kumamoto, Japan) was used to assess SHED proliferation according to the manufacturer's instructions.

Induction and treatment of experimental periodontitis

Mice were randomly divided into three groups with five mice in each group: control, periodontitis and SHED group. The experimental process is depicted in Fig 1a. For all procedures, the mice were anaesthetised with 1% pentobarbital sodium (0.4 ml/100 g body weight). On day 0, the orthodontic ligature wire was passed through interdental space between first molar and second molar in the periodontitis and SHED groups. Subsequently, after lacerating the gingiva by a dental probe, the orthodontic ligature wire was ligated firmly to the dental cervix of the maxillary right first molars for 3 weeks (Fig 1b). On day 21, the ligature wire was removed from mice in the periodontitis and SHED groups, and the mice from the SHED group were injected with SHED at three sites (the mesial side of the molar, the distal side of the molar, and the middle of the molar); each site of the defect was injected with 1×10^6 SHED mixed in 0.1 ml of phosphate-buffered saline (PBS), whereas rats in the periodontitis group were injected with 0.1 ml of PBS at each site (Fig 1c). The needle was inserted from the mucosa to the bone surface. All mice were killed 5 weeks post-ligation.

Micro-CT examination

The maxillae of mice were dissected and a stereo microscope (SWZ1000, Nikon, Japan) was used to record the buccal surface of the maxillae. Then, maxillae were fixed in 10% buffered formalin for 48 hours and stored in 70% alcohol. Mice maxillae were scanned using micro computed tomography imaging (micro-CT) (Sky-scan1174, Bruker micro-CT, Belgium). The scanning parameters used were the following: rotation angle 360° , source voltage 50 kV, source current 800 μA , X-ray exposure time 4,750 ms and scaled image pixel size 18.11 μm . Three-dimensional (3D) images were constructed and analysed using the computer software CTvox (Bruker, Billerica, MA, USA). Two-dimensional (2D) images were obtained and analysed using computer software CTAn (Bruker, Billerica). The probing bone loss (PBL) was evaluated by measuring the distance between the buccal cement-enamel junction (CEJ) and the alveolar bone crest at five sites for each defect in the 3D image, in particular in the middle of: mesial root; distal root; middle root; mesial furcation; and the distal furcation (Fig 1d). Subsequently, the measurement of

the PBL distance was conducted using ImageJ software (1.37v, National Institutes of Health, USA). The final result corresponded to the average of the 5 sites of the PBL, with the greater value representing more periodontal bone loss. The bone regeneration rates (BRR) of the maxillary first molars were examined by 2D image to measure the intrabony defects in the three groups. All measurements of the distal aspects of the first molar were performed with ImageJ software (1.37v). The apex-cusp (AC) and apex-alveolar ridge (AR) distances were measured, and the BRR was calculated according to: $AR/AC \times 100\%$ (Fig 1e). A greater BRR value indicated less bone loss.

Histology analysis

After the maxillae were scanned by micro-CT, they were decalcified using 0.5 M disodium ethylenediaminetetraacetic acid (EDTA) for 8 weeks and subsequently embedded in paraffin. The paraffin blocks were serially cut in the mesial and distal direction of the first molar, 5- μ m-thick sections were dewaxed in xylene and rehydrated using ethanol baths, and stained with haematoxylin and eosin (HE) to observe structures under an inverted microscope system (Eclipse 80i, Nikon, Tokyo, Japan).

Immunohistochemical staining

The sections were enzyme-treated for antigen retrieval with Trypsin and 20 μ g/ml Proteinase K at 37°C for 30 min, and then blocked with 5% fetal calf serum at room temperature for 30 min. Sections were incubated at 4°C overnight with primary antibodies including: anti-CD4⁺ (diluted 1:100), anti-TNF- α (diluted 1:100) and anti-IFN- γ (diluted 1:100). Positive immunoreactivity was detected using a two-step DAB detection kit (Zhongshan Golden Bridge Biotechnology, Beijing, China) according to the manufacturer's instructions. Controls for each antibody consisted of incubating a secondary antibody in the absence of the primary antibody. Sections were counterstained with haematoxylin for 3 min and then rinsed under running water for 10 min. Photomicrographs were taken using an inverted microscope system (Eclipse 80i).

Tartrate-resistant acid phosphatase staining

Sections were deparaffinised to perform the tartrate-resistant acid phosphatase (TRAP) test using the acid phosphatase, leukocyte kit (387A, Sigma, USA) according to the manufacturer's protocol. Alveolar

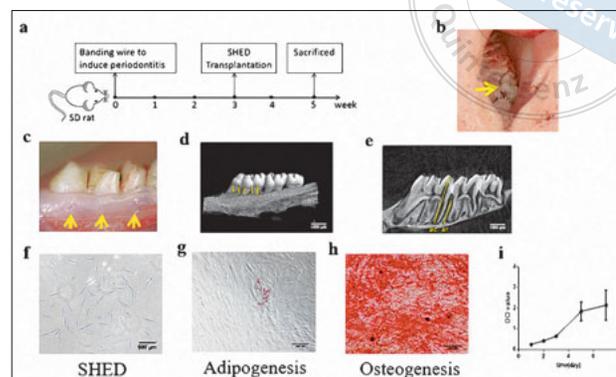


Fig 1 The schema of SHED treatment for periodontitis in rat models and the characterisation of SHED. **(a)** Experimental procedure for the treatment of periodontitis in rats by local injection of SHED. **(b)** Banding wire around the rats right maxillary first molars to induce periodontitis. **(c)** SHED local injection sites as the arrows indicate. **(d)** The probing bone loss (PBL) was evaluated by measuring the distance between the cement-enamel junction (CEJ) and the alveolar bone crest at five sites in each defect. **(e)** The apex-cusp (AC) and apex-alveolar ridge (AR) distances were measured to calculate the bone regeneration rates (BRR). **(f)** SHED exhibited a fibroblastic morphology with a bipolar spindle shape. **(g)** Positive staining for lipid droplets with oil red O. **(h)** Positive staining for mineral nodules with alizarin red S. **(i)** Cell counting kit-8 (CCK8) proliferation assay: SHED demonstrated robust cell proliferation.

bone between the first and second molar was observed. TRAP-positive multinucleated (> 3 nuclei) cells that attached to the alveolar bone surface were counted. Photomicrographs were taken using an inverted microscope system (Eclipse 80i).

Statistical analysis

All data were presented as the means \pm SD, and statistical analysis was carried out by one-way ANOVA followed by the least significant difference (LSD) multiple-comparison test. All statistical analyses were performed with GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA); $P < 0.05$ was considered to be statistically significant.

Results

SHED local administration promotes bone regeneration in mice periodontitis

To assess the therapeutic effects of SHED on periodontitis, we established a mice periodontitis model using orthodontics ligation wire (Fig 1a and b). The cultured

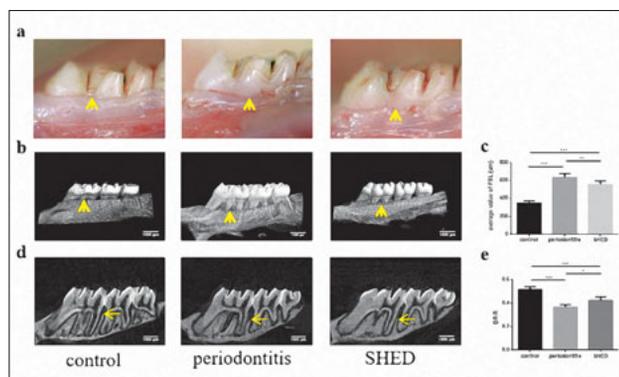
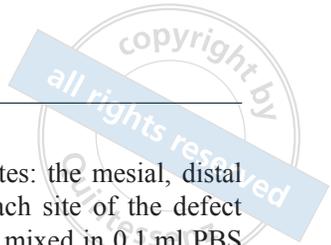


Fig 2 The oral and the micro-CT examinations showing the effect of SHED administration. **(a)** Intra-oral photographs 2 weeks after SHED transplantation, showing better gingival tissue regeneration in the SHED administration group than in the periodontitis group. The arrow indicates gingival margin. **(b)** Micro-CT 3D imaging showing better bone tissue regeneration in the SHED group than in the periodontitis group. The arrow indicates the alveolar ridge crest. **(c)** The average value of probing bone loss (PBL) was lower in the SHED group than in the periodontitis group. **(d)** Micro-CT 2D imaging was used to assess intrabony defects. The arrow indicates the alveolar ridge crest. **(e)** The bone regeneration rate (BRR) was higher in the SHED group than in the periodontitis group. BRR was calculated as [apex-cusp distance/apex-alveolar ridge distance] x 100%. The data are expressed as the mean ± SEM (n = 5), *P < 0.05, **P < 0.01, ***P < 0.001. CT, computed tomography.

SHED was administrated at 3 sites: the mesial, distal and the middle of the molar. Each site of the defect was injected with 1×10^6 SHED mixed in 0.1 ml PBS to treat periodontitis (Fig 1c). The PBL was evaluated by measuring the distance between the CEJ and alveolar bone crest at 5 sites in each defect, in particular in the middle of: the mesial root, distal root, middle root, and mesial and distal furcation (Fig 1d). The BRR were calculated by measuring the AC and the AR distances (Fig 1e). SHED exhibited a fibroblastic morphology with a bipolar spindle shape (Fig 1f). The proliferation rate and multi-lineage differentiation capacity were also analysed and SHED showed osteogenic and adipogenic differentiation under conditional induction (Fig 1 g and h). SHED also showed a rapid proliferation rate (Fig 1i).

Two weeks after administering SHED, the maxillae samples were harvested and the oral examination showed that the periodontitis group presented gingiva injury on the site of the wire ligation, and the SHED local injection was able to restore the gingiva injury (Fig 2a). Micro-CT 3D imaging showed that the SHED group showed a greater alveolar bone regeneration than the periodontitis group, but a lower regeneration than the control group (Fig 2b and c). The micro-CT 2D imaging showed more bone formation in the group injected with SHED compared with the periodontitis group; however, the bone formation did not reach normal levels (Fig 2d and e). Furthermore, the histopathologic analysis showed that the periodontitis group showed significant bone loss compared with the control group, whereas the SHED local injection partially restored the alveolar bone height between first and second molars (Fig 3).

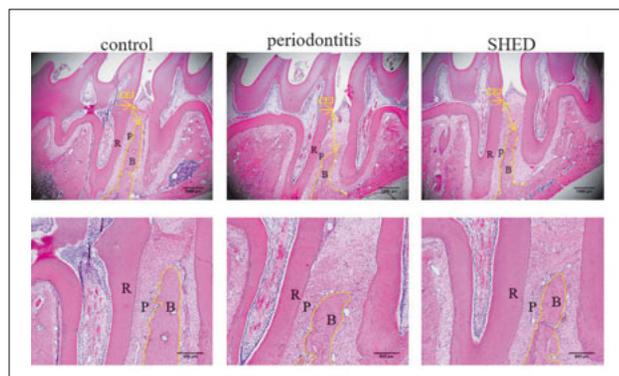


Fig 3 Histopathologic assessment of periodontal tissue regeneration by haematoxylin and eosin (HE) staining. HE staining showed new periodontal tissue regeneration in the periodontal defects of the SHED group, while bone and periodontal ligament fiber shortages remained the same in the periodontitis group. The alveolar bone height in the SHED group was also much larger than in the periodontitis group. R, root; B, bone; P, periodontal ligament; CEJ, cemento-enamel junction.

SHED administration inhibited the infiltration of inflammatory cells in mice periodontitis

It has been reported that inflammatory cells play an important role in the development of periodontitis¹⁰. In the present study, the inflammatory cells infiltration was analysed using immunohistochemical staining, and the results showed that there were more CD4⁺ T cells present in the periodontitis group than in the control group; thus, the SHED solution was capable of reducing the number of CD4⁺ T cells (Fig 4a and b). Furthermore, the number of IFN-γ-positive and TNF-α-positive cells were greater in the control group; this is because these cytokines were reduced by the SHED administration in the SHED group (Fig 4c to f). Since IFN-γ and TNF-α are largely produced by T-helper 1 (Th1) T cells¹¹, the results indicate that the SHED administration may have attenuated periodontitis via inhibiting the Th1 cells.

Fig 4 The SHED administration reduced the expression of inflammatory factors and T lymphocytes in periodontal tissues. **(a)** Immunohistochemical staining showed that the CD4⁺ positive cells were mainly distributed in the periodontal tissue of the periodontitis group and the SHED group. **(b)** The CD4⁺ positive cells were significant less in the SHED group than in the periodontitis group. **(c)** Immunohistochemical staining showed the INF- γ positive cells were mainly distributed in the periodontal tissue of the periodontitis group and SHED group. **(d)** The INF- γ positive cells were significantly less in the SHED group than in the periodontitis group. **(e)** Immunohistochemical staining showed that the TNF- α positive cells were mainly distributed in the periodontal tissue of periodontitis group and the SHED group. **(f)** The TNF- α positive cells were significantly less in the SHED group than in the periodontitis group. The arrow indicates positive cells. The data are expressed as the mean \pm SEM (n = 3), *P < 0.05, **P < 0.01, ***P < 0.001. R, root; B, bone; P, periodontal ligament.

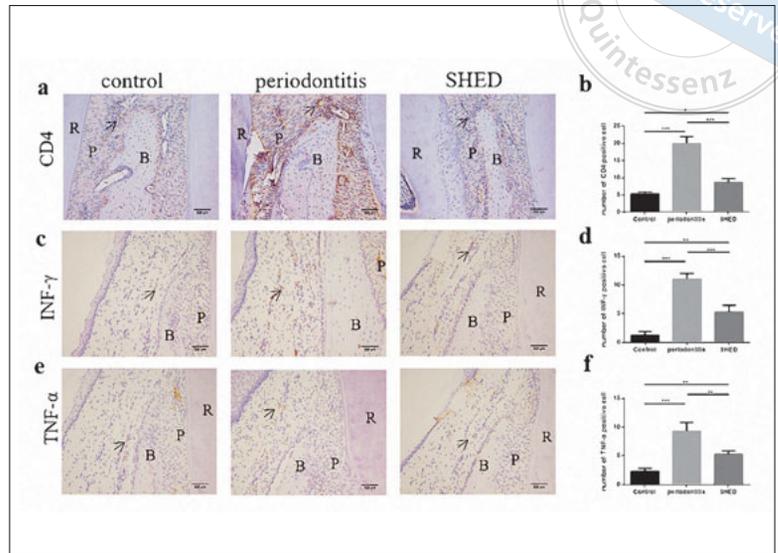
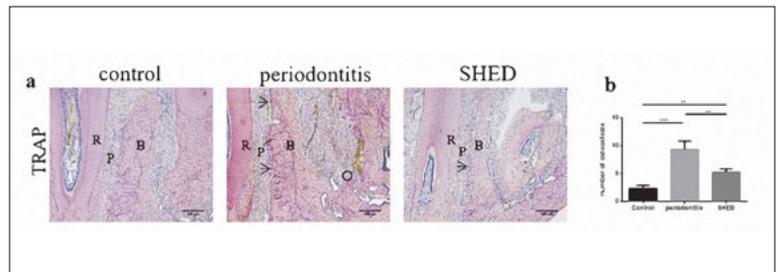


Fig 5 The SHED administration reduced osteoclastogenesis in rat periodontitis. **(a)** More osteoclasts were observed in the periodontitis group than in the SHED group as shown by the tartrate-resistant acid phosphatase (TRAP) staining. **(b)** The SHED administration had significantly reduced the number of osteoclasts. The arrow indicates osteoclasts positive cells. The data are expressed as the mean \pm SEM (n = 3), *P < 0.05, **P < 0.01, ***P < 0.001. R, root; B, bone; P, periodontal ligament.



SHED treatment reduced osteoclasts formation in mice periodontitis

In the next step, the osteoclasts formation was analysed by TRAP staining, and the results showed that there was more formation of TRAP-positive osteoclasts in the periodontitis group compared with the control group (Fig 5). Thus, the SHED treatment could reduce osteoclast formation, and therefore, reduce bone absorption.

Discussion

Periodontitis is one of the most prevalent inflammatory diseases; its clinical manifestation is the loss of periodontal support tissue, and if left untreated, it will eventually lead to tooth loss and affect the patients' quality of life¹. Traditional treatment methods for the restoration of periodontal structures have been based on scaling, open flap debridement, guided tissue regeneration, and local administration of anti-inflammatory drugs

or various growth factors. However, these procedures limit the potential for regenerating the periodontal support tissue^{12,13}. Previous studies have shown that stem cells are effective in the treatment of periodontitis, not only due to their capacity to regenerate different types of tissues, but also due to their paracrine potential, and ability to secrete large quantities of growth factors and anti-inflammatory cytokines, such as the transforming growth factor-beta (TGF- β) and interleukin (IL)-10, which play an important role in systemic and local immunomodulation^{14,15}. Stem cells can be delivered to periodontal tissues via both biomaterial-free and biomaterial-based methods¹⁶⁻¹⁸. Previous studies have shown that local injections with a mesenchymal stem cell (MSC) suspension using a rat periodontitis model can repair tissue defects^{4,19,20}. The injected cells can be administered directly to defective tissues using a minimally invasive surgery. Therefore, in the present study, we used this method to administrate SHED into the rat periodontal tissue.



SHED can be easily acquired from primary teeth and have low immunogenicity. Furthermore, SHED have greater proliferation ability *in vitro* compared with BMSCs and PDLSCs, and are capable to generate robust amounts of bone *in vivo*⁵. Therefore, in the present study, we choose SHED for treating the rat periodontitis.

The regenerative effects of a local injection of SHED in periodontitis were investigated. We found that the SHED delivery alleviated inflammation and promoted periodontal regeneration. Previous studies that evaluated chronic periodontitis in humans and in experimental animal models reported high levels of TNF- α in periodontal gingival tissue and crevicular fluid^{21,22}. Higher levels of TNF- α are commonly associated with an increase in large multinucleated osteoclasts and a reduction in the alveolar bone volume²³. The SHED administration regulated the periodontitis microenvironment by decreasing the expression of the inflammatory factors TNF- α and IFN- γ and inhibited osteoclast activation. Previous studies have shown that the reduction of IFN- γ and TNF- α concentrations markedly improves MSC-based bone regeneration^{24,25}. These results indicate that there is a relation between the inhibition of TNF- α and IFN- γ and periodontal regeneration, and support the results obtained here: the promotion of periodontal regeneration by inhibiting the inflammatory reaction produced by TNF- α and IFN- γ . However, further studies are required to clarify whether the inhibition of TNF- α and IFN- γ is the main mechanism that regulates the SHED-induced periodontal regeneration.

Conclusion

Overall, the results obtained here indicate that the local injection of SHED in a rat periodontitis model promotes alveolar bone regeneration and reduces the inflammatory response. The treatment effects of SHED were possibly attributable to their ability to inhibit the inflammatory factors TNF- α and INF- γ . The SHED administration comprises an easy and non-invasive therapy for treating periodontitis.

Conflicts of interest

The authors reported no conflicts of interest related to this study.

Author contribution

Drs Yi Qiang QIAO and Ling Su ZHU contributed to the collection and assembly of data, data analysis and interpretation, and manuscript drafting. Dr Ting ZHANG contributed to the collection of data and data interpretation. Dr Rui Li YANG and Prof. Yan Heng ZHOU contributed with the overall design of the study, critical editing of the manuscript and financial support. All authors read and approved the final manuscript.

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