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ORIGINAL RESEARCH

Study of Alveolar Bone Remodeling Using Deciduous Tooth Stem Cells and Hydroxyapatite by Vascular Endothelial Growth Factor Enhancement and Inhibition of Matrix Metalloproteinase-8 Expression in vivo

Tania Saskianti[1], Alexander Patera Nugraha[2], Chiquita Prahasanti[3], Diah Savitri Ernawati[4], Kotaro Tanimoto[5], Wibi Riawan[6], Masami Kanawa[7], Takeshi Kawamoto[8,9], Katsumi Fujimoto[8,10]

[1]Department of Pediatric Dentistry, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia; [2]Department of Orthodontics, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia; [3]Department of Periodontology, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia; [4]Department of Oral Medicine, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia; [5]Department of Orthodontics and Craniofacial Developmental Biology, Graduate School of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan; [6]Biomolecular Biochemistry, Faculty of Medicine, Brawijaya University, Malang, Indonesia; [7]Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima, Japan; [8]Department of Dental and Medical Biochemistry, Graduate School of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan; [9]Writing Center, Hiroshima University, Higashi-Hiroshima, Japan; [10]Department of Molecular Biology and Biochemistry, Graduate School of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan

Correspondence: Tania Saskianti, Department of Pediatric Dentistry, Faculty of Dental Medicine, Universitas Airlangga, Jalan Prof. Dr. Moestopo No. 47, Surabaya, 60132, Indonesia, Tel +62 81 232014445, Email tania-s@fkg.unair.ac.id

Background: Periodontitis progression is characterized by alveolar bone loss, and its prevention is a major clinical problem in periodontal disease management. Matrix metalloproteinase-8 (MMP-8) has been shown to adequately monitor the treatment of chronic periodontitis patients as gingival crevicular fluid MMP-8s were positively associated with the severity of periodontal disease. Moreover, modulating the vascular endothelial growth factor (VEGF) levels in bones could be a good way to improve bone regeneration and cure periodontitis as VEGF promotes endothelial cell proliferation, proteolytic enzyme release, chemotaxis, and migration; all of which are required for angiogenesis.

Purpose: The aim of this study was to determine the effect of hydroxyapatite incorporated with stem cells from exfoliated deciduous teeth (SHED) in Wistar rats' initial alveolar bone remodeling based on the findings of MMP-8 and VEGF expressions.

Methods: A hydroxyapatite scaffold (HAS) in conjunction with SHED was transplanted into animal models with alveolar mandibular defects. A total of 10 Wistar rats (*Rattus norvegicus*) were divided into two groups: HAS and HAS + SHED. Immunohistochemistry staining was performed after 7 days to facilitate the examination of MMP-8 and VEGF expressions.

Results: The independent *t*-test found significant downregulation of MMP-8 and upregulation VEGF expressions in groups transplanted with HAS in conjunction with SHED compared with the HAS group ($p < 0.05$).

Conclusion: The combination of SHED with HAS on alveolar bone defects may contribute to initial alveolar bone remodeling as evident through the assessments of MMP-8 and VEGF expressions.

Keywords: angiogenesis, medicine, osteogenesis, scaffold, tissue engineering

Introduction

Periodontal disease is an infectious and inflammatory condition that damages the teeth's supporting structures through bone resorption and periodontal tissue loss caused by acute (sometimes violent) or chronic inflammation.[1] Periodontal disease may result in edentulism and has been linked to severe systemic disorders, including atherosclerosis, cardiovascular disease, diabetes, and rheumatoid arthritis.[2–5] This may have a direct impact on afflicted individuals' general health,

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social life, and nutritional status, endangering their entire quality of life.[6–9] The global prevalence of periodontal disease is believed to be around 11%, which is the sixth most common human disease with a significant public health burden worldwide.[10] As a response, it is critical to provide a timely and effective therapy for periodontal disease.

The ultimate goal of periodontal therapy is to slow the progression of periodontitis and enhance periodontal tissue regeneration.[11] Scaling and root planing, as well as periodontal surgery for periodontal tissue rebuilding, are the major treatments for periodontal tissue inflammation.[12] However, the clinical outcomes in patients with periodontal disease are not completely satisfactory because the destroyed tissue is not regenerated.[13] The desired therapeutic outcome is a proper regeneration of alveolar bone, root cementum, and periodontal ligaments in the previously damaged periodontium.[14] As a result, various therapeutic options have been proposed, including stem cell–based tissue engineering and regenerative therapy.[15–17]

Among mesenchymal stem cells (MSCs) from dental tissue, human exfoliated deciduous tooth cells (SHEDs) are prominent.[18] Dental stem cells were initially isolated from the dental pulp of permanent teeth (DPSC) and then from the dental pulp of deciduous teeth (SHED).[19] Miura et al were the first to successfully employ SHED in vivo in conjunction with a scaffold for bone tissue building applications. Other research showed that SHED and human DPSC transplantation in the calvaria of immunodeficient mice resulted in nearly the same quantity of new bone formation as human bone marrow MSC transplantation.[20] As they originate from a more immature subpopulation than permanent teeth, SHED have a higher proliferation rate and differentiation potential since they can differentiate into neural cells, adipocytes, osteoblasts, and odontoblasts.[19] In addition, SHED are capable of spontaneously producing large volumes of bone in vivo.[19,21] Because of the ease of availability, SHED are excellent source of stem cells.

In addition to the source of the stem cell, other aspects are critical for successful tissue engineering, such as the biomaterial to be selected as a scaffold and the correct linkage between them.[22] To regenerate the bone tissue defect, the selected biomaterial must allow cells to migrate, proliferate, and differentiate into bone cells, but local angiogenesis is also required to provide the necessary nutrients and environmental factors for correct bone tissue development.[23] Hydroxyapatite (HA) is a frequently used biomaterial for constructing a scaffold. When utilized as a bone graft, HA, a key mineral component of human hard tissue that is widely used clinically to repair alveolar bone defects, is a bioactive material that also exhibits osseointegration, osteoconduction, and osteogenesis characteristics.[19,24] However, little research exists on the initial alveolar bone-remodeling ability of HA as a scaffold material used as therapy along with the use of SHED as an osteoinductive substance in alveolar bone defects.

Matrix metalloproteinase-8 (MMP-8) and vascular endothelial growth factor (VEGF) are involved in regenerative therapy with transplanted SHED in alveolar bone defects. In this study, SHED was combined with a hydroxyapatite scaffold (HAS) and transplanted onto rat models with alveolar bone defects to demonstrate the potential effects of these incorporated materials on initial bone remodeling by evaluating MMP-8 and VEGF expressions. Because of its high level of expression from neutrophils, MMP-8 plays a role in initiating collagen degradation in the extracellular matrix during embryogenesis, bone healing, and bone regeneration, as well as reflecting the inflammatory response in the first wound repair stage.[25–29] Moreover, angiogenesis is controlled by a number of growth factors, most notably VEGF, which is produced by inflammatory and stromal cells that are recruited to the site of the bone injury to promote blood vessel formation. Because of its primary ability to stimulate neovascularization, VEGF is of special importance in bone regeneration.[30–32] Thus, the aim of this study is to investigate the effect of both HA with SHED on MMP-8 and VEGF expression in the alveolar defects of Wistar rats (*Rattus norvegicus*).

Materials and Methods

Ethical Approval

The Universitas Airlangga, Faculty of Dental Medicine ethics committee granted ethical approval for both human sampling and animal experiments (171/HRECC.FODM/VIII/2017).

Study Design

This was an experimental laboratory study with a posttest-only control group design. The sample size was calculated using the minimal sample size formula. The sample count was five experimental animals in each group (N=10, n=5). Each group's sample was selected at random by assigning a tag number to each experimental animal and selected blindly.

Cell Culture

The SHED was collected from deciduous teeth that met the following criteria: #83 and #73 deciduous teeth that were free of cavities, had no root resorption confirmed by apical radiography, and had a vital and intact pulp. Healthy deciduous teeth were taken from a healthy 9-year-old male child who was undergoing orthodontic treatment at the Universitas Airlangga Dental Hospital, Surabaya, Indonesia. Patient confidentiality was protected, and a signed informed consent from the patient's parents was acquired.

The SHED was isolated using the same protocol as previously described.[33] The stemness of the SHED was confirmed by cluster of differentiation (CD) 105 (+) and CD 45 (-). The medium was changed every four days to remove the detached cell from the culture plate, and the cells were maintained for four passages. To remove debris, the cells were washed with a phosphate buffer saline. To separate the cells and transfer them to a larger culture plate, trypsin-EDTA 0.05% was used. The SHED cells in the four passages were prepared for the next step of the investigation after they attained 70–80% confluence.[33–35] A 20-mL suspension of the SHED at passage four to five with a density of 10^6 cells was seeded into HAS (bio hydrox hydroxyapatite, Biomaterial Center Dr. Soetomo Tissue Bank) before being placed in a 24-well tissue culture plate and prepared for the experimental group. The dose was determined using the data from a prior in vivo investigation, which reported 10^6 cells per sample.

Alveolar Bone-Defective Animal Model Preparation

Ten healthy, three-month-old male Wistar rats (*R. norvegicus*) of approximately 150–250 grams body weight were obtained from the Research Center of the Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia. Five samples were randomly allocated to one of two groups: HAS and HAS + SHED.

To minimize animal suffering, all experimental procedures involving animals were carried out in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.[35] Because the animal models originated from different places, they were acclimatized for a week at a temperature of 21–23°C with controlled humidity (50 ± 5%) in a 12-hour artificial light cycle (8 am to 8 pm) to let them adjust to the environment. All of the rats were placed in polycarbonate cages sized 0.90 m × 0.60 m × 0.60 m. Furthermore, all animal models were fed a regular pellet diet and given free access to water, and the husk was replaced every three days. Food consumption and fecal parameters of all animal models were routinely inspected and observed.[36] Following the induction of an alveolar bone defect by extracting the rat's mandibular incisor, samples from the HAS and HAS + SHED groups were transplanted into the affected area. A 5.0 suture monofilament was utilized to initiate the interrupted suture's repair of the incision following transplantation.[37]

All animal models were euthanized seven days after the transplantation to analyze early alveolar bone remodeling. Euthanasia was performed via an overdosed rodent anesthesia, an intravenous injection of 100 mg/kg BW (Pentobarbital, PubChem, USA). This method of euthanasia was selected to alleviate any pain caused by the euthanization process. The affected alveolar bone samples were collected for histological investigation following the animal trial. Using sterile sharp surgical scissors (metzenbaum scissors fine tips, no cat. 3565, Medesy, Maniago, Italy) and a tweezer (Tweezer de bakey mini, no cat. 1007/10-TO, Medesy, Maniago, Italy), the animal model's head was cut from the back, exposing the anterior mandible and allowing the afflicted alveolar bone sample to be obtained. All of the animals were examined for any signs of general toxicity, such as edema and their body weight was assessed using a digital scale (ZB22-P, Zieis®, USA). A single blind observer performed all of the measurements. Finally, the affected tissue was removed and fixed in a 10% neutral buffer formalin solution.

Tissue Embedding, Sectioning, and Processing

The sample was decalcified and submerged in 10% EDTA (Ajax Finechem, Thermo Fisher Scientific, Taren Point, Australia; cat no. 17,892). The samples were then processed overnight (Leica TP1020, USA) before being embedded in molten paraffin wax (Leica HistoCore Arcadia H - Heated Paraffin Embedding Station, USA). A 5 m rotary microtome (RM2235, Leica, USA) was used to cut the sections. Flattened paraffin ribbons were collected onto polysine microscope slides (Thermo Scientific) and dried at 60°C for 16 hours (Sakura Heater, Tokyo, Japan).[38]

Immunohistochemistry Staining

A 3.3'-diaminobenzidine stain kit (DAB; cat no. D7304-1SET, Sigma Aldrich, US) was used for immunohistochemistry staining. This study used a 1:500 dilution of VEGF antibody monoclonal (AbMo; cat. no sc-7269) and MMP-8 (cat. no sc-514803; Santa Cruz BiotechnologyTM, US). Using a Nikon H600L light microscope (Japan) at 400× magnification, two observers manually counted and examined the number of VEGF expressions in the periodontal tissue in five fields of view. Each marker was also magnified by 1000× for context (Nikon, Japan).[38]

Statistical Analysis

To analyze the data in this study, the Statistical Package for Social Science (SPSS) 20.0 version (IBM corporation, Illinois, Chicago, United States) software was utilized. A *t*-test ($p < 0.05$) was used to compare the significant differences in VEGF and MMP-8 expressions across the groups.

Results

To examine whether SHED + HAS affected MMP-8 and VEGF expression after transplantation, immunohistochemical staining was performed on day 7. The number of MMP-8–expressing cells in the HA + SHED group was significantly lower than those in the HAS group ($p < 0.05$; see Figure 1, Table 1). Meanwhile, the number of VEGF-positive cells in the HA + SHED group was significantly higher than those in the control group (see Figure 2, Table 1).

Discussion

Periodontitis progression is characterized by alveolar bone loss. A range of treatment techniques have been proposed, including bone grafts, directed tissue regeneration, root conditioning, enamel matrix derivatives, and a combination of the above procedures. Among these attempts, an unequivocal success of these treatments has not been found. Novel technologies based on tissue engineering (using stem cells and scaffolding) may emerge as possible therapies.[1]

In this study, the animal experiment was done in seven days to analyze the early markers of alveolar bone remodeling via the expressions of VEGF and MMP-8. This experimental work supports the idea that SHED seeded in HAS could decrease the number of biomarker expressions for detecting alveolar bone destruction (such as MMP-8 expression) in bone defects after seven days when compared with the HAS group. Due to their role in the pathological breakdown of the extracellular matrix (ECM) within periodontal tissues, several pieces of evidence show that the active MMP-8 (collagenase-2) derived from neutrophils is the most critical mechanism in the tissue destruction associated with periodontal disease. Pathogens in dental plaque can trigger host cells to increase MMP-8 release, which is one of the indirect causes of tissue damage that occurs in periodontitis.[39,40] A high level of MMP-8 in the HAS group could be explained by an increased immune response to the presence of the scaffold as a foreign object. A significant decrease in MMP-8

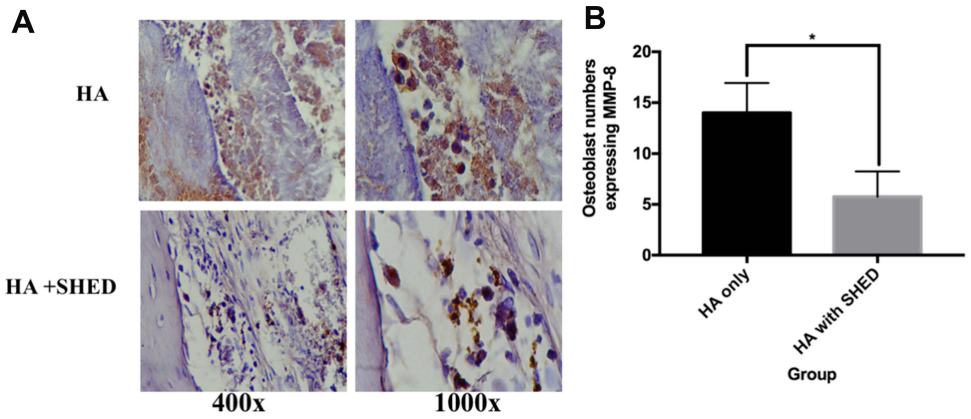

Figure 1 Histological sections of periodontal tissues from Wistar rats (*R. Norvegicus*). (**A**) A positive reaction of MMP-8 in cytoplasm was shown in a brown color (black box) under a 400× and 1000× magnification using a light microscope following immunohistochemistry staining with antibody monoclonal (AbMo) and DAB (**A**). (**B**) The number of osteoblasts expressing MMP-8 in the alveolar bone of the rats was compared. * = significant between groups ($p < 0.05$).

Table 1 The Expression of MMP-8 and VEGF in Osteoblast in the Periodontal Tissue Afflicted Area of Wistar Rats (*R. norvegicus*); n=5

Group	MMP-8	VEGF
	Mean ± SD	
HA	14 ± 1.472	4.5 ± 0.6455
SHED + HA	5.75 ± 1.25	15.5 ± 0.6455
p-value	0.0053*	0.00001*

Note: *information: independent *t*-test was used with significant difference at p<0.05.

expression was noted in the HAS + SHED group compared with the HAS group ($p < 0.05$). This result supports the theory that the SHED as an MSCs lineage may play a role in supporting the immunomodulation towards an inflammatory response suppression. Similar findings by Mauney et al and Rahyussalim et al showed that when MSCs were induced for osteogenic differentiation, their expressions of MMP-1 and MMP-8 decreased. MMP-8, a collagenase that degrades collagen, was regulated to ensure the greatest possible ECM environment and structural formation after osteogenic differentiation.[27,41]

Ceramic scaffolds such as HAS offer the greatest promise for stem cell–based bone engineering due to its high cell adhesion and proliferation,[42,43] and is also essential in promoting SHED proliferation and differentiation.[35] Furthermore, using HA as a biodegradable scaffold provides skeletal support for osteogenic cell development during the early stages of bone repair. When SHED was seeded in an HA scaffold, the VEGF angiogenesis markers expressing cells significantly increased compared with those in the HAS group ($p < 0.05$). This could be explained by the fact that HA is a porous bioceramic that permits the formation of capillaries and other blood vessels. Due to their ease of vascularization and high oxygen permeability, the pores of an HA scaffold aid in osteogenesis.[44,45] SHED, in addition to its ability to differentiate into osteoblasts, may also differentiate into vascular endothelial cells.[46] Angiogenesis and osteogenesis are very strongly linked. Angiogenesis is required to sustain and maintain bone formation and maintenance. Blood vessels also serve as a network of communication for bones and surrounding tissues.[47,48] Cetinkaya et al showed that VEGF expression was greatly elevated throughout the healing stage of periodontal disease.[49] Further, the study showed that VEGF expression was more connected with the non-inflammatory component of the enlarged tissue than with the inflammatory component as there was a clear positive association between the number of blood vessels and VEGF expression only in the healing group. These findings could suggest a relationship between VEGF production and vascularization in the resolution of inflammation and the spontaneous healing of periodontal tissues.

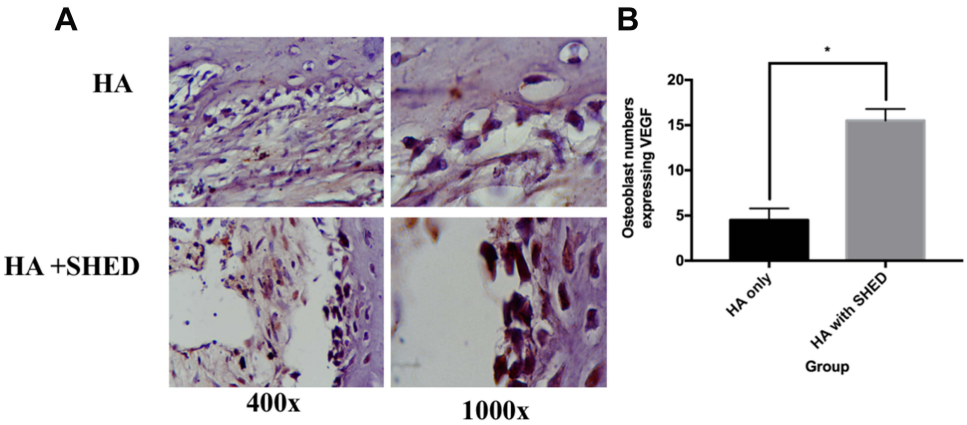

Figure 2 Histological sections of periodontal tissues from Wistar rats (*R. Norvegicus*). (**A**) A positive reaction of VEGF in cytoplasm was shown in a brown color (black box) under a 400× and 1000× magnification using a light microscope following immunohistochemistry staining with antibody monoclonal (AbMo) and DAB (**A**). (**B**) The number of osteoblasts expressing VEGF in the alveolar bone of the rats was compared. * = significant between groups ($p < 0.05$).

The VEGF expressed by osteoblasts is important in supporting bone regeneration during inflammation and maintaining bone hemostasis. VEGF plays crucial roles in some phases of the bone-remodeling process. A previous study showed that VEGF depletion in osteoblasts inhibits the bone-remodeling process. Macrophages, as inflammatory cells, require VEGF to promote their migration during inflammation phase. Adequate VEGF levels or expressions are necessary in maintaining angiogenesis and osteogenesis in the bone-defective area.[50] In the alveolar bone-defective area, the microenvironment was hypoxic. In addition, VEGF and stem cell migration was regulated by the condition of hypoxia. The vascularization supports bone development and the proliferation of osteoblast cells.[51]

SHED showed a prominent ability to differentiate into osteogenic and odontogenic lineage in vitro.[33] Regenerative therapy using SHED and HAS can regenerate alveolar-defective animal models by increasing VEGF expression and decreasing MMP-8 expression. Compared with DPSCs, SHED showed both a higher capacity to increase osteoblast markers related to osteoblastic differentiation and expressed higher levels of alkaline phosphatase (ALP), Col I and osteocalcin (OCN) compared with DPSCs.[52] The stemness and multipotency of SHED was maintained by some growth factor, such as basic fibroblast growth factor and VEGF.[53]

The limitations of this study were that the observations and evaluations were performed seven days post transplantation of SHED seeded in HAS on the animal model, and only an immunohistochemical examination was performed. Further studies are necessary to evaluate the changes in the alveolar bone and periodontal tissue post transplantation of SHED seeded in HAS in the alveolar bone defect in animal models. With a longer observation time, further studies using other methods, such as quantitative reverse transcription polymerase chain reaction (RT-qPCR) or the Western blot analysis, could be conducted to estimate the expression of bone molecular markers. Future studies are also required to confirm the effective dose of the selected biomaterials when they are ready to be applied in clinical human studies.

Conclusion

The expression of VEGF increases significantly with treatment of SHED seeded in HAS, whereas MMP-8 expression in the alveolar bone decreases in SHED seeded in HAS, as observed immunohistochemically.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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