

# Combined Hydroxyapatite Scaffold and Stem Cell from Human Exfoliated Deciduous Teeth Modulating Alveolar Bone Regeneration via Regulating Receptor Activator of Nuclear Factor-Kb and Osteoprotegerin

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**Submission date:** 08-Dec-2021 11:19AM (UTC+0800)

**Submission ID:** 1724010210


**File name:** Receptor\_Activator\_of\_Nuclear\_Factor-Kb\_and\_Osteoprotegerin.pdf (1.85M)

**Word count:** 4172

**Character count:** 22592

by Chiquita Prahasanti

# Combined Hydroxyapatite Scaffold and Stem Cell from Human Exfoliated Deciduous Teeth Modulating Alveolar Bone Regeneration via Regulating Receptor Activator of Nuclear Factor-Kb and Osteoprotegerin System

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Received: 17 September 2017

Revised: 27 January 2018

Accepted: 04 March 2018

## What's Known

- Stem Cells from Human Exfoliated Deciduous Teeth (SHED) are also an ideal source of stem cells employed in the repair of damaged bone.
- SHED demonstrate adult stem cell characteristics with high proliferation potential, self-renewal capacity, and potential differentiation into various cells with good viability potential and proliferation.

## What's New

- SHED can be attached to and proliferate within a Hydroxyapatite (HA) scaffold.
- HA with SHED increases Osteoprotegerin (OPG) expression and decreases Receptor Activator of NF-Kb ligand (RANKL) expression.
- SHED have good potential as alveolar bone defect regeneration.

## Abstract

**Background:** Tissue engineering using Stem cell from Human Exfoliated Deciduous Teeth (SHED) and a natural biomaterials biomaterial scaffold has become a promising therapy for the alveolar bone defect. The aim of this study was to analyze the Osteoprotegerin (OPG) and Receptor Activator of NF-Kb ligand (RANKL) expression after the application of Hydroxyapatite scaffold and SHED.

**Methods:** A laboratory experimental research with a post test-only control group. 14 male Wistar rats weighing from 260 to 280 g were used as the animal study. The animals were randomly assigned to an experimental group Hydroxyapatite scaffold (group I) and Hydroxyapatite scaffold combined with SHED (group II). The alveolar bone defect in the animal study model was affected by extracting anterior mandible teeth. Immunostaining was performed after 8 weeks in order to facilitate the examination of OPG and RANKL expression. Data were analyzed by independent *t* test. The correlation between OPG and RANKL expression were analyzed using Pearson's correlation test ( $P < 0.05$ ). Statistical analysis was performed using R statistical software version 3.4.0.

**Results:** The independent *t* test showed that the differences were statistically significant. OPG expression in Group I ( $6.0 \pm 1.00$ ) was lower than in Group II ( $11.6 \pm 1.14$ ) ( $P = 0.0004$ ). The independent *t* test showed that the differences were statistically significant. RANKL expression for Group I ( $12.67 \pm 2.08$ ) and Group II ( $4.80 \pm 1.304$ ) showed a statistically significant difference ( $P = 0.0005$ ).

**Conclusion:** Hydroxyapatite scaffold and SHED increase Osteoprotegerin and decrease Receptor Activator of NF- $\kappa$ B Ligand expression with high potential as an effective agent in alveolar bone defect regeneration.

Please cite this article as: Prahasanti Ch, Subrata LH, Saskianti T, Suardita K, Ernawati DS. Combined Hydroxyapatite Scaffold and Stem Cell from Human Exfoliated Deciduous Teeth Modulating Alveolar Bone Regeneration via Regulating Receptor Activator of Nuclear Factor-Kb and Osteoprotegerin System. Iran J Med Sci. 2019;44(5):415-421. doi: 10.30476/IJMS.2019.44962.

**Keywords** • Mesenchymal stromal cells • Tissue engineering • Hydroxyapatites • Osteoprotegerin • RANK ligand

## Introduction

Therapy to repair bone damage can be either resective or reconstructive (regenerative) in nature. Regeneration sometimes

involves the application of a graft as supporting material. Various types of graft have begun to be widely studied and developed rapidly to achieve periodontal tissue regeneration.<sup>1</sup>

A graft constitutes a synthetic or natural material acting as a filler which also serves as a scaffold and is able to initiate the process of bone formation.<sup>2</sup> Osteoblast cells require a scaffold and induction mediator to migrate into defective areas for bone remodeling. Scaffolds play a role in supporting cell attachment and proliferation in the defective area and stabilizing blood clots to prevent tissue damage during the primary phase of regeneration. Ideally, missing or damaged tissues are replaced by biomaterials possessing a high level of biocompatibility. This encourages many clinicians and researchers to apply the concept of tissue engineering using stem cells from human organs and scaffolds derived from natural biomaterials.<sup>3</sup>

BioHydrox® products made from bovine bone are biomaterials that have the same microarchitecture and mineral composition as human bone does.<sup>4</sup> Hydroxyapatite (HA) is one of the materials classified as bioactive in nature and has osteointegrative, osteoconductive, osteoinductive, and osteogenetic properties when used as a bone graft.<sup>5</sup>

Stem Cells from Human Exfoliated Deciduous Teeth (SHED) are also an ideal source of stem cells employed in the repair of damaged tooth structure and bone regeneration while also demonstrating the ability to differentiate into osteoblasts. SHED demonstrate adult stem cell characteristics with high proliferation potential, self-renewal capacity, and potential differentiation into various cells.<sup>6, 7</sup> A previous study conducted by Puspitasari and others confirmed that SHED possessed good viability potential and proliferation by observing the development of living cell counts. Similarly, the potential for stem cell differentiation of SHED was proved to be largely positive.<sup>8</sup>

A previous study conducted by Iohara described an innovative dental treatment employing tissue engineering technology. It showed that dentin regeneration using Dental Pulp Stem Cells (DPSCs) combined with Bone Morphogenetic Protein-2 could stimulate odontoblastic cell differentiation as a means of forming new dentin.<sup>9</sup> Studies conducted by Sloan and Smith confirmed that DPSCs played a role in the regeneration and repair of damaged dentin.<sup>10</sup>

Bone quality can be established based on its size and shape, remodeling process, mineral composition, and collagen content.<sup>11</sup> In the constantly ongoing remodeling process,

the cells playing a role include progenitor cells, osteoblast, osteocytes, and osteoclasts.<sup>12</sup> Under normal circumstances, the remodeling process will demonstrate a balance between resorption and bone formation while, under pathological conditions, bone resorption is more dominant leading to pathological bone destruction. Walsh and Choi (2104) and Gibertoni and others, highlighted the main lines defining the remodeling process of bonding as being Osteoprotegerin (OPG), Receptor Activator of NF- $\kappa$ B (RANK), and Receptor Activator of NF- $\kappa$ B ligand (RANKL) initiated by osteoblasts and osteoclasts.<sup>13, 14</sup> OPG and RANKL will induce processes referred to as osteogenesis and osteoclastogenesis.<sup>14, 15</sup> OPG and RANKL expression biomarkers can be used at the initial stage in the bone formation process marking to assess the effectiveness of treatment outcome. OPG protects the skeleton from excessive bone resorption by binding to RANKL and preventing it from binding to its receptor, RANK. Thus, RANKL/OPG ratio is an important determinant of bone mass and skeletal integrity.<sup>16</sup> The aim of this study was to analyze the OPG and RANKL expression combination HA with SHED.

#### Material and Methods

This study received approval through an ethical clearance letter from the Faculty of Veterinary Medicine, Universitas Airlangga 042/HRECC.FODM/IV/2017. This study was conducted according to the guideline of animal ethics and welfare.<sup>17</sup> The rats were housed at 21–23 °C and under controlled humidity (50±5%) for less than a 12-hour artificial light cycle (7 am to 7 pm). All the animals had access to water and the AIN93M diet 1 week before the commencement of the study for adaptation.

This research was experimental in nature, incorporating post-test-only control group design. 14 male Wistar rats weighing from 260 to 280 g were used as the animal study model in this research. The samples were divided randomly into two groups. The animals were randomly assigned to an experimental group HA+SHED (Group II) and control group HA (Group I). Sample stem cells were isolated from SHED according to certain inclusion criteria such as the presence of vital conditions, the absence of caries, and root resorption of primary teeth being more than 1/3 of the tooth root or intact. Pulp tissue was placed in the medium within a 15 ml conical tube within a cool box before immediately being sent to the Tissue Bank at the Diagnostic Center, Dr. Soetomo Surabaya General Hospital. Pulp tissue was subsequently



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cultured in Dulbeccos Modified Eagle Medium (DMEM®, Life Technologies, Gibco BRL™, USA) with the addition of 20% fetal bovine serum (FBS, Biochrom AG®, Germany), 5mm L-glutamine (Gibco Invitrogen®, 25, USA), 100 U/ml penicillin-G, 100 ug/ml streptomycin, and 100 ug/ml kanamycin (Gibco Invitrogen®, 25, USA). After 3 days, the medium was disposed of in order to remove that part of the cell not attached to the dish and transfer it to a new medium. At this stage, Fibroblast Growth Factor-2 was added. The confluent passage was performed using 0.05% trypsin-EDTA before the cell was washed and cultured a second time in either a 60 mm or 100 mm tissue culture dish. After the cells had reached the point of confluence, they were ready to be used for further research. Any cells not used immediately must be stored in liquid N<sub>2</sub>.<sup>8</sup>

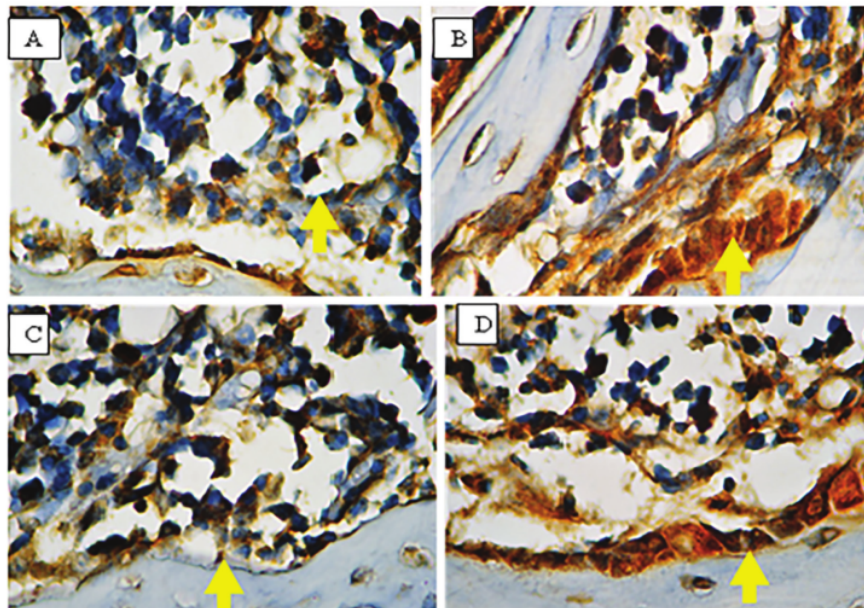
The alveolar bone defect model was induced in Wistar rats as the animal study model by extracting the mandible anterior teeth using sterile needle holder clamps. 20 mL suspension of SHED at passage 3-5 with a density of 10<sup>6</sup> cells was added to HA before being placed in a 24-well tissue culture plate. After 2 hours, 980 µl Dulbecco's Modified Eagle Medium (DMEM) (Gibco™, Thermo Fisher Scientific, Waltham, MA USA) was added to each well. In addition, the cells were incubated in the incubator (CO<sub>2</sub>

5% at 37 °C). Media culture was replaced every 3 days after which the culture in the 26 HA+SHED was transplanted to the alveolar bone defect. Immunostaining was performed after 8 weeks to examine OPG and RANKL expression using a marker kit (Sigma Aldrich™, Germany).<sup>8</sup>

Data were analyzed by independent *t* test to compare the treatment and control group. The correlation between OPG and RANKL expression were analyzed using Pearson's correlation test. The P value < 0.05 was considered to be significant. Statistical analysis was performed using R statistical software version 3.4.0.

## Results

SHED can be attached to and proliferate within an HA scaffold. An Immunohistochemistry (IHC) examination using anti-OPG monoclonal antibodies was observed. The IHC result of OPG expression in the control and treatment groups on Day 7 can be seen in figure 1. In the present study, the result of OPG expression in the treatment group was higher than that of the control group. The mean±SD of OPG expression was 6.0±1.00 and 11.6±1.14 for the control and treatment groups, respectively. The mean of OPG expression between group samples showed a significant difference between control



**Figure 1:** Immunohistochemical staining shows OPG Expression (1000× magnification). A positive reaction produced a brown color in the cytoplasm due to antigen (OPG) (yellow arrow) with monoclonal antibodies (anti OPG); (A) Expression of OPG in osteoblast on Group I day 7, (B) Expression of OPG in osteoblast on Group II day 7; (C) Expression of OPG in osteoblast on group I day 14, (D) Expression of OPG in osteoblast on group II day 14.

and intervention groups. Independent *t* test showed that the differences between the mean of OPG expression between group samples were statistically significant (table 1).

The IHC result of RANKL expression in the control and treatment groups on day 7 and 14 can be seen in figure 2. Moreover, RANKL expression in the treatment group was lower than that of the control group. The mean of RANKL expression in control group was  $12.67 \pm 2.08$  while it was  $4.80 \pm 1.30$  for the treatment group. The mean RANKL expression between the groups showed a significant difference (table 2). The OPG expression had a strong reverse significantly significant correlation with RANKL expression ( $r = -0.912$ ,  $P = 0.0016$ ).

### Discussion

In this study, based on the statistical test, it was found that there was a strong relationship in OPG/RANKL ratio. The increased OPG ratio compared to RANKL shows SHED's ability to stimulate OPG to bind to RANKL, thus inhibiting osteoclastogenesis. The increased expression of OPG is supported by the research undertaken by Walsh and Choi. Belibasakis asserted that OPG expression increase due to an elevated level of TGF- $\beta$ 1 and RUNX2.<sup>13,18</sup> TGF- $\beta$ 1 induced OPG expression in OPG cells. The osteoblasts produce OPG and RANKL. The higher the number of osteoblasts, the greater the resulting

Table 1: OPG expression in the control (group I) and treatment group (group II)

Group	Mean $\pm$ SD	P value
I (Treatment)	11.6 $\pm$ 1.14	0.0004*
II (Control)	6.0 $\pm$ 1.00	

Statistical tests used independent *t* test, \*Significant  $P < 0.05$

Table 2: RANKL expression in the control (group I) and treatment group (group II)

Group	Mean $\pm$ SD	P value
I (Treatment)	4.80 $\pm$ 1.14	0.0005
II (Control)	12.67 $\pm$ 1.00	

Statistical tests used an independent *t* test, \*Significant  $P < 0.05$

increase in OPG and RANKL expression.<sup>11, 19, 20</sup>

RANKL and OPG play an important role in bone remodeling, modulation, and osteoclasts differentiation. Osteoclast maturation will produce a mature osteoblast so that the bone remodeling can work well. The balance between OPG-RANKL ratios plays an important role in maintaining the homeostasis of phosphorus and calcium. The remodeling process is maintained by the formation of bone matrix through osteoblast and bone resorption by osteoclast.<sup>21</sup>

OPG and RANKL can be detected in the gingival tissue as well as biological fluids such as saliva, serum, and gingival cervical fluid. In

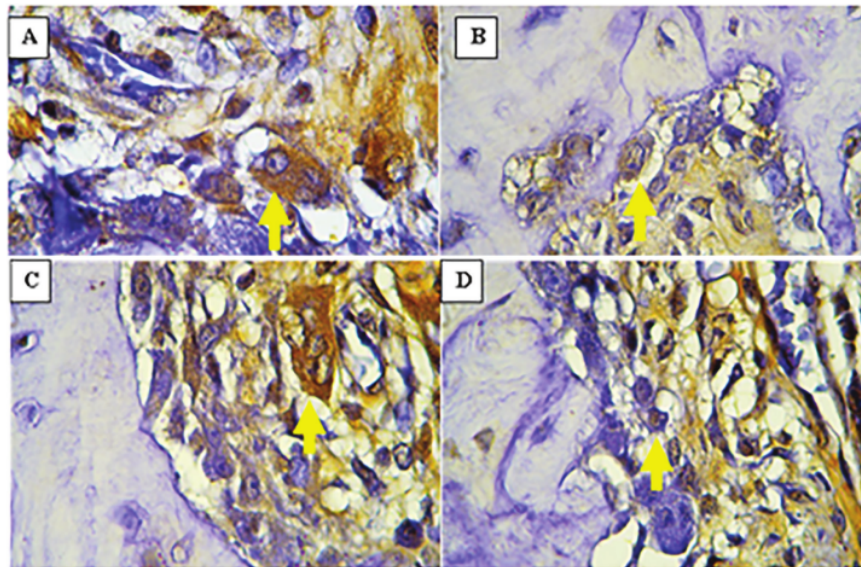


Figure 2: Immunohistochemical staining shows RANKL Expression (1000 $\times$  magnification). A positive reaction produced a brown color in the cytoplasm due to antigen (RANKL) (yellow arrow) with monoclonal antibodies (anti RANKL); (A) Expression of RANKL in osteoblast on Group I day 7, (B) Expression of RANKL in osteoblast on Group II day 7; (C) Expression of RANKL in osteoblast on group I day 14, (D) Expression of RANKL in osteoblast on group II day 14.



16 their study, Belibasakis and Bostanci stated that the ratio of OPG/RANKL in periodontitis patients showed an increased ratio of OPG/RANKL. The balance of OPG/RANKL ratio showed success in the treatment of periodontitis. Increased RANKL or decreased OPG expression will lead to bone resorption.<sup>18</sup> OPG expression was higher than the RANKL expression treatment group that showed no bone resorption occurred in our study. In a previous study conducted by Lapin on a periodontitis patient, OPG expression decreased that showed the severity of the disease. OPG released by osteoblasts and inhibits bone resorption inhibits osteoclast differentiation and activity, by binding to RANKL receptors. A balanced ratio between OPG/RANKL is a protective property against bone loss.<sup>13, 19, 20</sup>

Bone graft is a material used for augmentation and stimulation of new bone formations in certain cases of bone defect.<sup>22</sup> Based on the graft materials ability in bone regeneration, the use of autograft is golden standards because it comes from the patient's bone. Technological advancements allow for the development and combination of bone graft materials and SHED. The combination of bone graft and SHED was used in this study. The most famous bone graft biomaterial was HA scaffold because of its osseointegration, osteoconduction, osteoinduction, and osteogenesis properties. Progenitor cells infiltrate porosity in HA which then proliferates and forms new bone on the surface of the graft material with subsequent replacement or combination with HA material. The properties of osteoinduction HA are derived from the microporous and macropore surface. The presence of a microporous surface provides an interconnection between the macropores that support the occurrence of interstitial fluid circulation along the matrix. After the graft is applied to the bone defect, the HA granules will dissolve to form a biological apatite layer of HA. This coating will produce a chemically hospitable environment and possesses a high affinity that can induce mesenchymal differentiation of cells into osteogenic cells.<sup>23</sup>

HA scaffold has a porous matrix whose size may vary and depend on the volume of scaffold produced. The advantages of HA scaffold include its allowing the cells to move through existing pores as well as creating pore conditions for nutrient transport, tissue infiltration, and vascularization.<sup>6</sup> Pores in HA can bind strongly to bone tissue due to the structure of HA with regular porosity being similar to that of natural bone tissue. The pores have an open structure with the result that the biocompatible surface provides ideal conditions for cell growth and tissue differentiation.<sup>4, 5, 7, 21</sup>

HA contains calcium and phosphate, potentially increasing the affinity of bone tissue, resulting in an ion exchange in calcium phosphate and the interaction between calcium and cell surfaces. The ion exchange takes place on the surface of the adjacent phosphate compound of the surrounding water (liquid phase) through the surfaces of  $\text{Ca}^{2+}$ ,  $\text{PO}_4^{3-}$ , and existing impurities such as  $\text{CO}_3^{2-}$ ,  $\text{Cl}^-$  or  $\text{F}^-$ . In addition, the ion exchange is also bound to fibronectin protein which is fundamental in the SHED attachment process. When SHED interact with HA, it will stick to it and obtain nutrients from the suspension medium, resulting in SHED growth and proliferation.<sup>3, 12, 24</sup>

Ions can enter on membranes that are hydrophobic in nature. The cell membrane is coated by a skeleton in the cytoplasm rendering the organelle crescent-like cilia or filipodia which expands the surface area in order to increase the nutrient absorption by the cell. The surface area in hydroxyapatite is cuboid leading to greater nutrient absorption and promotion of cellular activity which, in turn, result in more attachment and SHED proliferation.<sup>4, 5, 11, 22</sup>

In addition to the HA contained in human hard tissues, ions such as carbonate, sodium, and magnesium are also present in them.<sup>4, 5</sup> The results of this study proved that HA scaffolding could lead to attachment, with SHED proliferation occurring even though variation in the number of SHED existed within the scaffold. HA Scaffold can be used as a carrier for application of SHED and also increasing cell growth and regenerating the damaged tissues such as bone. SHED proliferation in scaffolds can be used as an alternative to osteogenic tissue engineering. SHED can induce bone formation with nerve marker expression. It demonstrates an encouraging potential for clinical application.<sup>7</sup> While SHED cannot directly differentiate into osteoblasts, they possess the capacity to stimulate new bone formation by means of an osteoinductive template in order to obtain osteogenic cells. This suggests that primary teeth not only provide a guide for permanent tooth eruption but also involve bone formation during this process.<sup>8, 25</sup>

## Conclusion

36 Hydroxyapatite scaffold and Stem cells from Human Exfoliated Deciduous Teeth increase OPG and decrease RANKL expression via regulating OPG-RANKL system that has a high potential to be used as an effective alternative tissue engineering biomaterial for alveolar bone defect regeneration.

#### Acknowledgement

The authors would like to thank the Universitas Airlangga (UNAIR), Faculty of Dental Medicine and Dr. Soetomo Surabaya General Hospital. This research was supported by Airlangga University (Hibah Mandat Research Grants, 2016).

**Conflict of Interest:** None declared.

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