Enhancement of Osteogenesis Using a Combination of Hydroxyapatite and Stem Cells from Exfoliated Deciduous Teeth

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Enhancement of Osteogenesis Using a Combination of Hydroxyapatite and Stem Cells from Exfoliated Deciduous Teeth

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Abstract

Periodontal regeneration, especially regeneration of alveolar bone, remains difficult to achieve with the result that alternative therapies for achieving periodontal regeneration are still lacking. Tissue engineering using stem cells and biomaterial scaffold represents an alternative for solving this problem. Stem cells from human exfoliated deciduous teeth (SHED) are one source of adult stem cells which can proliferate and differentiate into many types of specific cells. Hydroxyapatite (HA) constitutes a bioactive scaffold possessing osseointegration, osteoconduction and osteogenesis properties when used as bone graft.

The aim of this study was to analyze osteocalcin (OCN) and osteopontin (OPN) expression after the application of a combination of HA and SHED to alveolar bone defects. Eight experimental animals were divided into two groups. The first group was transplanted with HA only, while the second was transplanted with HA/SHED. The expression of OCN and OPN was examined 21 days later by means of immunohistochemistry analysis.

The analysis result of an independent T-test with significance value p= 0,0041 < 0.05 showed a significant difference between the two groups. The HA/SHED group showed a significantly higher expression of OCN and OPN than that of the HA only group. Transplantation of a combination of HA and SHED onto alveolar defects increases the expression of OCN and OPN.

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Introduction

Periodontal disease is one affecting the oral cavity that causes damage to tooth-supporting tissues consisting of periodontal ligament, cementum and alveolar bone. If not treated appropriately, periodontal disease can cause bone destruction which may eventually culminate in premature tooth loss.[1] Several forms of therapy have been proposed to regenerate periodontal tissue, including guided tissue regeneration, bone grafting, growth factor application and host modulation factors.[2]

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Periodontal regeneration, in particular the complete and functional regeneration of alveolar bone, remains difficult to achieve. Thus, alternative therapies for achieving periodontal regeneration remain indispensable.[3]

Stem cells derived from the teeth show considerable promise for the treatment of alveolar bone regeneration because they are isolated from the tissue that is often discarded in the dental clinic and experiences lower morbidity compared with non-tooth stem cells. Stem cells derived from the pulp may express osteogenic markers such as alkaline phosphatase (ALP), type I collagen, osteocalcin (OCL) and osteopontin (OPN).[4] Dental pulp stem cells (DPSC) are multipotent and capable of differentiating into chondrocytes, adipocytes, osteoblasts/osteocytes, myocytes, neuronal cells and cardiomyocytes.[5] DPSC can be used for the regeneration of periodontal and bone tissue as they have the potential to form tissues such as

bone.[6]

Stem cells from exfoliated deciduous teeth (SHED) are isolated from deciduous dental pulp tissue. Like DPSCs, SHED demonstrate the capacity to differentiate into osteogenic, adipogenic, chondrogenic and myogenic cells.[7] SHED possess higher proliferative capacity and expression of bFGF and BMP-2 genes compared to bone marrow mesenchymal stem cells (BMMSCs) and DPSCs. Therefore, SHED is a suitable cell for bone regeneration treatment.[8]

Scaffold constitutes an important component of tissue engineering which serves as a platform suitable for cell proliferation, growth and differentiation. Scaffolds such as HA, tricalcium phosphate, biphasic calcium phosphate, calcium silicate and bioactive glass are widely used in bone regeneration. These materials are chemically and structurally similar to the original bone, possess high mechanical strength and are resistant to deformation.[9] HA, a major mineral component of human hard tissue often applied clinically to replenish alveolar bone defects[10], is one of the materials classified as bioactive which demonstrates osseointegration, osteoconduction and osteogenesis properties when used as bone graft.[11]

Osteoblasts play a very important role in the process of bone regeneration.[12,13] OPN, ALP and OCL are early osteoblast markers that participate in controlling osteoblast function and extracellular mineralization of bone matrix (ECM). Upregulation of OPN, ALP and OCN correlates directly with osteoblast differentiation.[14] In this study, HA and SHED were combined and transplanted onto alveolar bone defects. The aim of this study was to analyze OPN and OCN expression after the application of a combination of HA and SHED on alveolar bone defects.

Materials and methods

Study Design

This in vivo study was designed as a post test only control group.

Isolation of SHED

Human dental pulp tissue was obtained with informed consent from patients undergoing extraction for orthodontic reasons or persintence tooth at the Department of Pedodontic, Faculty of Dental Medicine, Universitas Airlangga, Surabaya Indonesia. The pulpal tissue was separated from teeth and subjected to primary culturing via the outgrowth method.[12] In brief, after disinfection the teeth were mechanically fractured and the dental pulp was gently isolated. The pulp tissue was rinsed in Dulbeccos Modified Eagle Medium (DMEM®, Life Technologies, Gibco BRL™, USA) suplemented with 20% fetal bovine serum (FBS, Biochrom AG®, Germany), 5mm L-glutamine (Gibco Invitrogen®, USA), 100 U/ml penicillin-G, 100 ug/ml streptomycin dan 100 ug/ml kanamycin (Gibco Invitrogen®, USA) and minced into fragments of 1-2 mm^3 before being placed in a 37°C humidified tissue culture incubator at 5% CO2 for four days. The culture medium was changed every 3 days with the addition of 10 ng/ml fibroblast growth factor-2 (FGF-2). When reaching 80% confluence, cells were harvested by using 0.05% trypsin-EDTA solution (Sigma-USA) and sub-culture being carried out for the experiments. SHED between passage 2 and 4 were used for the experiments.

In vivo transplantation, histological analysis and immunohistochemical staining

The in vivo procedures were performed in accordance with approved by Health Research Ethical Clearance Commission Faculty of Dental Medicine, Universitas Airlangga No.171/HRECC.FODM/VIII/2017). Eight white male Rattus norvegicus, weighing 150-200 g, aged 7-14 days, were used in this study. Two groups were established, the HA only and HA/SHED group. An alveolar bone defect model was created by extracting the mandibular tooth using sterile extraction pliers under general anesthesia.

20 µl SHED suspension of 10^6 cell density was added to the HA scaffold (BioHydrox, Tissue Bank of Dr. Soetomo Hospital, Indonesia). The mixture was placed in a 24-well tissue culture plate. After 2 hours, 980 µl DMEM was added to each well and were incubated in 5% CO_2 at 37°C. After 3 days, the SHED culture in HA was transplanted (HA/SHED group) to the alveolar bone defect as described previously.[13] HA alone were placed as a negative control.

Three weeks after the transplantation, rats were sacrificed for immunohistochemistry (IHC) analysis. *Hematoxyilin eosin (*HE) staining was performed to examine the osteoblasts numbers. Immunohistochemical staining was performed to examine osteoblast marker: osteopontin (OPN) and osteocalcin (OCN) expression using a marker kit (Sigma Aldrich™, Germany). The staining results were observed

and photographs were taken under using phase contrast microscope (CKX41, Olympus, Japan) at a magnification of 1000X.

Statistical Analysis

Data was analyzed by independent t-test to compare the treatment and control groups. A p-value <0.05 was considered to be significant. Statistical analysis was performed using R statistical software version 3.4.0.

Results

To detect alveolar bone regeneration after transplantation with HA only and HA/SHED, osteoblast numbers and OCN/OPN expression examination were performed. With HE staining, the osteoblast can be seen in Figure 1.

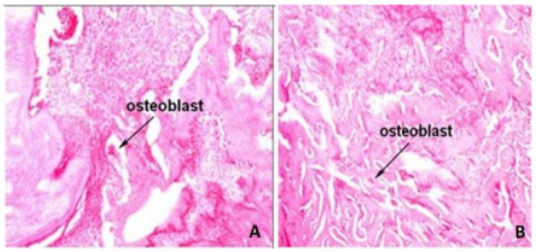

Figure 1. HE staining shows osteoblast (black arrow; 1000x magnification) in : A) the HA only group and B) the HA/SHED group.

There were significant differences (p <0.05) in osteoblast numbers between the HA only and HA / SHED groups (Figure 2).

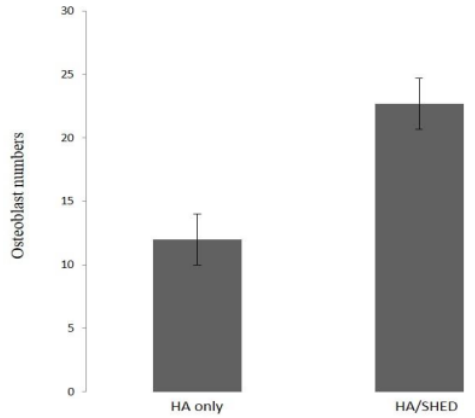

Figure 2. Osteoblast numbers in the HA only and HA/SHED group.

IHC examination showed the OCN and OPN expression in the HA only and HA/SHED groups (Figure 3 and 4).

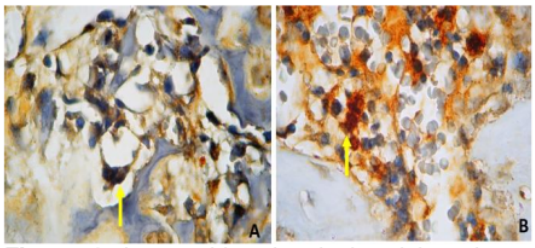

Figure 3. Immunohistochemical staining shows OCN expression (yellow arrow; 1000x magnification) in A) the HA only group and B) the HA/SHED group.

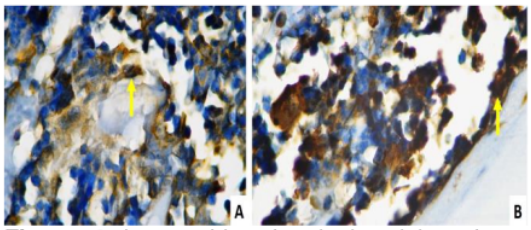

Figure 4. Immunohistochemical staining shows OPN expression (yellow arrow; 1000x magnification) in: A) the HA only group and B) the HA/SHED group.

The t-test showed that there were significant differences (p <0.05) in OCN and OPN expression between the HA only and HA / SHED groups (Tables 1 and 2).

Group	Mean±SD	p-value
HA only	7.0±2.00	0.004*
HA/SHED	14.2±2.28	

Table 1. OCN expression in HA only group and HA/SHED group.
*significant p<0.05

Group	Mean±SD	p-value
HA only	1.7±0.58	0.036*
HA/SHED	16.0±1.58	

Table 2. OPN expression in HA only group and HA/SHED group.
*significant p<0.05

Discussion

HA is the most widely used calcium phosphate biomaterials for research and clinical

use that has a composition and structure similar to natural bone mineral. HA binds chemically directly to bone when implanted.[14] HA is most often used for bone regeneration and osteogenic diferentiation due to its osteoconductive and osteoinductive properties.[6] Biomaterials can be combined with stem cells, delivering transplanted cells to guide the spatially complex process of tissue formation and stimulating osteoconduction, osteoinduction and osteogenesis. In order to both sustain and modulate cell behavior, a scaffold must have biocompatibility, suitable surface texture and chemistry for osteogenic cellular proliferation and differentiation as well as mechanical properties.[15] SHED is a promising source of MSCs to be used in tissue bioengineering studies due to their multi differentiation potential and the ability to form engineered bone grafts and also differentiate into vascular cells.[16] SHEDs have the capacity to differentiate into osteogenic, adipogenic, chondrogenic and myogenic cell lineages.[10] SHED is promising for bone regeneration therapy, where fresh and cryopreserved SHED can improve the calvaria critical size bone defects in immunocompromised mice.[17] SHED demonstrated the ability to form engineered bone grafts and also differentiate into vascular cells. Moreover, SHED exposes osteogenic markers such as ALP, type I collagen, OCN and OPN.[16, 18]

In this study, in the HA only group osteoblasts are present in the defect area and also the detected expression of OCN and OPN. This is probably due to the migration of mesenchymal stem cells from the internal environment of the host to the porosity of the HA scaffold.[19] The porosity of the scaffold provides a substrate for cell adhesion and a three-dimensional space for bone tissue formation at the time of implantation.

In the HA/SHED group, the bone regeneration process is more pronounced as can be seen from the greater number of osteoblasts than in the HA only group. The combination of HA and SHED possibly change the nature of HA which was originally osteoconductive to become osteoinductive. This is probably due to the contribution of mesenchymal stem cells from the host and SHED present in the HA scaffold. HA combined with SHED is able to induce more osteoprogenitor cells, resulting in pre-osteoblasts that will transform into osteoblasts. Mangano et al in their study demonstrated that a porous natural HA (Biocoral scaffold) could support differentiation of DPSCs into osteoblasts, forming a biocomplex of Biocoral, ECM and differentiated cells.[20]

HA is a porous bioceramic that allows the growth of capillaries and other blood vessels. The relatively large pores of HA scaffold support osteogenesis due to easier vascularization and high oxygen permeability.[21,22] In addition to having the ability to differentiate into osteoblasts SHED can also differentiate into vascular endothelial cells.[23] The relationship between angiogenesis and osteogenesis is very close. Angiogenesis is a prerequisite to support and maintain bone development and maintenance. The blood vessels also function as a communication network for skeletal and surrounding tissues.[24,25]

The differentiation of stem cells into osteoblasts is largely controlled by transforming growth factor-beta, bone morphogenic protein, Smad and p38 MAPK and Runx2 signaling pathways. Osteoblasts, derived from the differentiation process of stem cells, play a very important role in the process of bone formation.[26, 27] This osteoblast then begins the process of forming new bone by removing osteoid, which secretes type I collagen, OCN and OPN. Osteoid undergoes a mineralization process within which, at this stage, ALP can be detected.[28-31]

In this study, OCN and OPN expression occurred to a greater degree in defects that were transplanted with HA/SHED compared to HA alone. OPN and OCN are early osteoblast markers that play a role in controlling osteoblast function and ECM. Increased OPN and OCN correlate directly with osteoblast differentiation.[29, 30, 32] In HA/SHED combinations, the entire surface and porosity of HA contain both stem cell and SHED host stages, resulting in new bone matrix deposition throughout the HA scaffold so that OCN and OPN expression are higher.

Conclusions

A combination of HA and SHED enhanced osteogenesis by increasing the expression of OCN and OPN.

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Declaration of Interest

The authors of this manuscript declare no conflicts of interest in this article.

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