ORIGINAL ARTICLE

BMP4 Expression Following Stem Cells from Human Exfoliated Deciduous and Carbonate Apatite Transplantation on *Rattus norvegicus*

Tania Saskianti[1*], *Windy Yuliartanti*[1], *Diah Savitri Ernawati*[2], *Chiquita Prahasanti*[3], *Ketut Suardita*[4]

[1]*Department of Pediatric Dentistry,* [2]*Department of Oral Medicine,* [3]*Department of Periodontology,*

[4]*Department of Conservative Dentistry, Faculty of Dental Medicine, Universitas Airlangga*

Abstract:

*Background***:** Alveolar bone defects in children still have a high incidence. Conventional bone graft technique that has been used as a defect therapy is still not effective, so new techniques with tissue engineering approach are needed. Bone Morphogenetic Protein 4 (BMP4) as one of the indicators of osteogenic differentiation has not been widely studied, especially in the transplantation with combination of Stem Cells from Human Exfoliated Deciduous (SHED) and carbonate apatite. *Aim and Objectives:* This research aimed to determine the expression of BMP4 after SHED and carbonate apatite transplantation on *Rattus norvegicus. Material and Methods:* The combinations of SHED and carbonate apatite were transplanted on alveolar bone defects of 4 rats (*Rattus norvegicus*) as the treatment groups and another 4 rats were transplanted with carbonate apatite as the control groups. After 21 days, staining with *Hematoxylin Eosin* (HE) and *Immunohistochemistry* (IHC) BMP4 was performed. *Results:* BMP4 expression in the treatment groups was significantly higher when compared to the control groups. *Discussion:* Carbonate apatite has low crystallization rate and high osteoconductivity that produce more osteoblasts and increased BMP4 expression. *Conclusion:* The transplantation of SHED and carbonate apatite increased BMP4 expression as an indicator of osteogenic differentiation in rats.

Keywords: Bone Morphogenetic Protein 4, Carbonate Apatite, Stem cells from human exfoliated deciduous, tissue engineering

Introduction:

The world of dentistry is closely related to damage or defects of the alveolar bone. Loss of tissue and alveolar bone defects due to trauma, periodontal disease, congenital abnormalities, and craniofacial defects including cleft lip and cleft palate are the world's major problems [1, 2]. Epidemiological studies conducted in Indonesia showed that 0.13% (1 in 750) births each year had cleft palate [3]. Cleft palate is a combination of defects in the soft and hard tissues that affects lips and maxilla. Failures during the facial merging process will interfere skeletal midfacial growth including teeth, speech development, and difficulty in breastfeeding in infants [4].

Tissue engineering technology is a promising thing in the field of dentistry. Tissue engineering is defined as the regeneration of new tissue through a combination of biomaterials and biological mediators such as stem cells [4]. Tissue engineering requires three components: cell, scaffold, and growth factor. Tissue engineering utilizes precursor cells from host, matrix, and growth factor to regenerate lost tissue [5].

Tissue engineering requires an active material component such as Mesenchymal Stem Cell (MSC). Stem Cells from Human Exfoliated Deciduous (SHED) are part of the MSC derived from the pulp tissue of the deciduous tooth that will be shed. SHED is an ideal source of stem cells for

tissue engineering. These stem cells are capable of differentiating into various cells and have greater proliferation and regeneration potential compared with other stem cell sources [6, 7].

In a similar study conducted by Wijayanti in 2013, it has been found that the isolated and cultured pulp cells of deciduous teeth contain stem cell populations by expressing the CD105 marker. According to a similar study conducted by Saskianti in 2014, SHED is known to have good viability potential and proliferation seen from the development of living cell count. In a study conducted by Rizki in 2015 it was also found that at SHED there was expression of BMP receptor, but the study was still a laboratory research and needed further research on SHED interaction with biomaterials [8-10].

The process of bone healing and regeneration in tissue engineering technology involves many molecules including Bone Morphogenetic Proteins (BMPs). BMP is a collection of proteins that belongs to the superfamily of the Transforming Growth Factor β (TGF β) that plays an important role in the formation, maintenance and repair of bone [11]. Some studies revealed that some BMPs, for example BMP4, have an important role in the growth and differentiation of some cells including osteoblasts [5, 12]. BMP4 is an osteoprogenitor that plays a role in increasing Alkaline Phosphatase (ALP) activity [13]. Experimental studies in animal also show the effectiveness of human BMP in bone regeneration [5].

In addition to the SHED and BMP4 components, a biomaterial that acts as a scaffold is required to facilitate cell growth and regeneration [14]. Carbonate apatite is a clinically proven bio-ceramic material that can be a good bone scaffold in regenerative surgery. This material has the same composition as human bone and has the ability to bind with bone and soft tissue, and can stimulate new bone growth [1, 15].

In a previous study conducted by Saskianti *et al*. in 2017 [16] stated that combination of SHED and carbonate apatite scaffold has higher proliferation potency compare with SHED and hydroxyapatite. Study by Guan in 2010 [17] declared SHED and Dental Pulp Stem Cell (DPSC) interacted on 2% chitosan scaffold became a good growth medium for SHED and DPSC. Another study conducted by Ellis *et al*. in 1988 [18] mentioned that the biocompatibility of carbonate apatite in in-vivo tests indicated an increase in the number of new bone formed after four weeks.

Several studies on the combination of MSC and scaffold both in vitro and in vivo have been widely practiced. The study included the use of Hydroxyapatite (HA) / chitosan scaffolds in combination with MSC, electrospun collagen nanofiber, honeycomb collagen scaffold, HA / Tri-Calcium Phosphate (TCP) for the treatment of mild / severe defects in dogs, rabbits, or goats [4]. However, tissue engineering research on BMP4 expression as an osteogenic differentiation indicator in combination of SHED and carbonate apatite has not been done. So that, author wanted to know BMP4 expression as indicator of osteogenic differentiation after SHED and carbonate apatite transplantation on *Rattus norvegicus*. The results of this study can be used as preliminary information to determine the potential use of SHED and carbonate apatite as biomaterial candidates in the tissue engineering technique of alveolar bone tissue and the role of BMP4 as an indicator of osteogenic differentiation.

Material and Methods:

This research has been approved by the Ethical Commission of Faculty of Dental Medicine Universitas Airlangga No.187/HRECC.FODM/IX/2017. This study was a quasi experimental post test only control group study with 8 samples of white male Rattus norvegicus, weighing 150-200 g, aged 7-14 days, divided into two groups, control group with carbonate apatite application and treatment group with application of carbonate apatite and SHED.

Stem cell samples from SHED were obtained from Dental Hospital of Universitas Airlangga. The teeth must be in vital condition, no caries, and root resorption is no more than 1/3 of the apical. The SHED sample that has been obtained was then characterized in vitro in the Tissue Bank, Diagnostic Center Building of Dr. Soetomo Regional Public Hospital Surabaya. The pulp tissue taken and was cultured in Dulbeccos Modified Eagle Medium (DMEM, Life Technologies / GIBCO BRL) with the addition of 20% fetal bovine serum (FBS, Biochrom AG, Germany), 5mM L-glutamine (Gibco Invitrogen, USA), 100 U / ml of penicillin-G, 100 µg / ml streptomycin, and 100 µg / ml of kanamycin. Three days later, the medium was disposed to remove the cell part that was not attached to the dish and a new medium was added. At this stage, FGF-2 was added. After the cells were confluent, passaging was done using 0.05% trypsin-EDTA and the cells were rinsed and cultured again in 60- or 100-mm tissue culture dishes (Corning). After the cells were confluent, passaging was done again and the cells were ready to be used. Unused cells were stored in liquid N_2.

The model of alveolar bone defects in rat was made by extracting its anterior mandibular tooth using sterile extraction pliers. A 20 µl SHED suspension at passage 3-5 with a density of 106 cells was added to carbonate apatite then placed in a 24-well tissue culture plate. After 2 hours, 980 µl DMEM was added to each well. Cells were incubated in incubator (CO_2 5% at 37°C). After 3 days, the culture of SHED in carbonate apatite was transplanted on alveolar bone defect of sample's tooth extraction socket. The jaw resection on the socket was done after 21 days after the making and cutting of paraffin block and immunohistochemical process with BMP4 Antibody (3C11C7) NBP2-52424 in Biochemistry Laboratory of Universitas Brawijaya.

Statistical analysis was performed using Statistical Package software for the Social Sciences (SPSS) 23 for windows 8 (SPSS™, Chicago, United State). Measurement results are tabulated according to each group. Data homogeneity was tested using Kolmogorov Smirnov test. If the data is homogeneous, then the Independent T Test statistic with a significance value of 5% was performed.

Results:

BMP4 expression of rat alveolar bone preparation was assesed with immunohistochemical examination using BMP4 Antibody (3C11C7) NBP2-52424 on day 21 after transplantation to detect alveolar bone regeneration. Osteoblasts that expressed BMP4 osteogenic markers changed their color to brown.

The mean value of osteoblasts in the control group was 4±1.8, while in treatment group was 11.2±2.2. The results of Kolmogorov Smirnov Test conducted in both groups obtained $p>0.05$ which means that both data are normally distributed.

Table 1: Mean Values and Number of Osteoblasts on IHC staining

Groups	Mean ± SD	Independent 't' test (p)
Control	4.0 ±1.826	0.002
Treatment	11.25 ±2.217	

p <0.05 there is a significant difference between groups

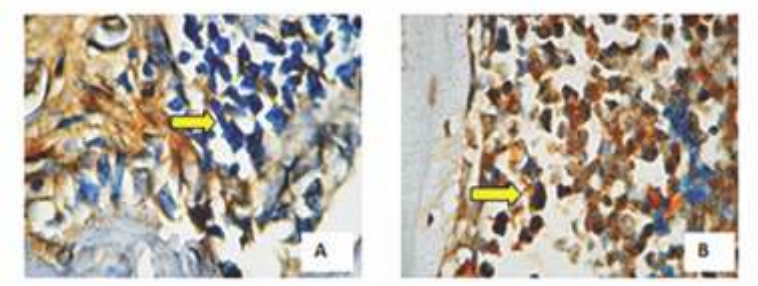

Fig. 1: (A) BMP4 Produced Cells that Identified during the Immunostaining Process (Yellow Arrow) from the Mineralized Alveolar Bone of Rats after SHED and Carbonate Apatite Transplantation for 21 days. (B) Osteoblasts Expressing BMP4 showed a Brown Colour

Independent 't'-test was performed and obtained 0.002 as p value or p <0.05. It can be concluded that there was a significant difference between the control group and the treatment group (Table 1).

Discussion:

The concept of tissue engineering uses stem cells in tissue reconstruction with the aim that the damaged tissues can has its function again. The success of tissue engineering is largely determined by three components: scaffold, cell (in this case stem cells are often be used), and signal molecules [19].

Bone graft as one component in the concept of tissue engineering serves as a substitute for missing micro environments during the process of tissue loss. Xenograft is one of the type of bone grafts that is currently widely developed. The carbonate apatite belong to the xenograft is a bioceramic material that is currently widely studied and developed as the material of choice as a substitute for bone structure due to good biocompatibility to the human body and has the same composition as human bone [20, 21].

GAMACHA contains carbonate apatite as well as a collagen denaturation polymer which serves to accelerate the regeneration process of bone tissue, and has the properties of osteoconduction, osteoinduction, and osteogenesis [21]. In addition, carbonate apatite also has in vivo solubility and low crystallisation rates so that the concentrations of calcium and phosphate which is important for new bone growth will increase. The low level of crystallization will make the process of absorption into the body becomes faster and more easily

integrates with the bone remodeling process so that new bone growth will be faster [21, 22].

Another component in tissue engineering is stem cell. SHED as one of the stem cells that can stimulate new bone growth characterized by an increase in osteoblast expression in immuno-histochemical staining results with osteogenic markers. SHED does not directly differentiate into osteoblasts, but has the ability to stimulate new bone formation. The number of osteoblasts in the treatment group was higher than the control group. This proves that the combination of SHED and carbonate apatite has a better potential in tissue engineering and can be used as an indicator of osteogenic differentiation. In line with the results of similar research conducted by Ishikawa (2010) [23] which proves that when carbonate apatite is implemented in defect of rabbit's tibia with evaluation for 4, 12, and 24 weeks showed that there were new bone formations with ongoing porosity formation, indicating the presence of bioresorpsi materials that are implemented [20, 23]. Another similar study conducted by Surbakti *et al*. (2017) [21] mentions that there is more immature bone formation on carbonate apatite in the healing process of the calvary defect [21]. From the current study, it can be concluded that BMP4 expession in combination of SHED and carbonate apatite is higher compared to control group.

References

1. Ferdiansyah DR, Fedik AR, Aulani'am. Regeneration on massive bone defect with bovine hydroxyapatite as scaffold mesenchymal stem cell. *JBP* 2011; 13(3):179-95.
2. Abou Neel EA, Wojciech C, Vehid MS, Hae-Won K, Jonathan CK. Tissue Engineering In Dentistry. *J Dent* 2014; 42: 915-28
3. Godfrey K. Epidemiology of Congenital Cleft Lip and Palate. In: Integrated Treatment of Cleft Lip and Palate Seminar. Semarang Indonesia 1994.
4. Zuk, PA. Tissue Engineering Craniofacial Defects with Adult Stem Cells? Are We Ready Yet? *Pediatric Res* 2008; 5(63): 478-86
5. Sheikh Z, Mohammad AJ, Nader H, Raheel H. Bone Regeneration Using Bone Morphogenetic Proteins and Various Biomaterial Carriers. *Materials* 2015; 8(4):1778-816.
6. Chen VJ, Smith LA, Peter X. Bone Regeneration on computer- designed nano-fibrous scaffolds. *Biomaterials* 2006; 27(21): 3973-9.
7. Sharma K, Husain SY, Das P, Hussain M, Syed MA. Regenerative Potential of Mesenchymal Stem Cells: Therapeutic Applications in Lung Disorders. In: Pham P. (eds) Liver, Lung and Heart Regeneration. Stem Cells in Clinical Applications. Springer, Cham 2017: 77-117.
8. Wijayanti TP. Characterization of Primary Teeth Stem Cell with Modification in Culture Technique and Isolation. Thesis. Surabaya: Faculty of Dental Medicine Universitas Airlangga 2013.
9. Saskianti T, Budiyanto ARP, Suhariadji FX. Modifikasi Kultur Sel Pulpa Gigi Sulung menggunakan Enzym Trypsin. Modified Culture of Primary Teeth Pulp Cell. Surabaya: Faculty of Dental Medicine Universitas Airlangga 2014.
10. Rizki BS. Sekresi Bone Morphogenetic Protein Receptor II in Stem Cell Human Exfoliated Deciduous and Periodontal Ligament Stem Cells as a Determinant of Osteogenic Potential. Thesis. Surabaya: Faculty of Dental Medicine Universitas Airlangga 2015.
11. Zhou H, Qian J, Wang J, Yao W, Liu C, Chen J, Cao X. Enhanced bioactivity of bone morphogenetic protein-2 with low dose of 2-N-6-O-sulfated chitosan in vitro and in vivo. *Biomaterials* 2009; 30(9): 1715-24
12. Istiati S. Regeneration and Healing in Dentistry. Jakarta: Sagung Seto 2013.
13. Cheng H. Osteogenic activity of the fourteen types of human bone morphogenetic protein (BMPs). *J Bone Joint Surg* 2003; 85(8):1544-52

14. Hardhani PR, Lastianny SP, Herawati D. The Effect of Platelet-Rich Plasma Addition On Bone Transplant To Osteocalcin Level in Gingival Sulcus Fluid of Infrabony Pocket Therapy. Jurnal PDGI 2013; 62(3): 75-82.
15. Isyaturrodliyah, A. Fabrication of Porous Hydroxyapatite Scafflod with Polymer Hydroxy Ethyl Cellulose and Poly Vinyl Alcohol. 2013. Available from http://etd.repository.ugm.ac.id/index.php Accessed on 12 March 2017
16. Saskianti T, Ramadhani R, Budipramana ES, Pradopo S, Suardita K. Potential Proliferation of Stem Cell from Human Exfoliated Deciduous Teeth (SHED) in Carbonate Apatite and Hydroxyapatite Scaffold. *JIDMR* 2017; 10(2): 350-53
17. Guan Z, Kamolmatyakul Suttatip, Shi Shuwen. Chitosan as scaffolds for DPSC and SHED. 2010. Available from https://www.researchgate.net/publication/266772398_Chitosan_as_scaffolds_for_DPSC_and_SHED Accessed on 12 March 2017
18. Ellis LG, Carter JM, Natiella JR, Feathersone JD, Nelson DG. Quantitative analysis of early in vivo tissue response to syntethic apetite implans. *J Biomed Mater Res* 1988; 39(2): 197-207
19. Mahanani ES, Indra B, Ika DA. Human mesenchymal stem cell behavior on synthetic coral scaffold. *Key Engineering Materials* 2016; 696:205-2011
20. Zakaria MN, Arief C. An Introduction to carbonate apatite as a biocompatible material in dentistry. Pertemuan Ilmiah Tahunan 8 Prodi Kedokteran Gigi Unjani 2016; 10:80-84
21. Surbakti A, Maximillan CO, Eko P. Comparison between Carbonate Apatite and Hydroxyapatite on Closure Process of Kalvaria Defect Using Plasma Rich Platelets. 2017. Available at https://ejournal.unsrat.ac.id/index.php/biomedik/article/download/15967/15482 Accessed on Juny 09 2017
22. Ana IK, Shigeki M, Kunio I. Engineering of carbonate apatite bone substitute based on composition-transformation of gypsum and calcium hydroxide. *Engineering Scientific Research* 2010; 2: 344-52
23. Ishikawa K. Bone substitute fabrication based on dissolution-precipitation reactions. *Materials* 2010; 3:1138-55

[*]***Author for Correspondence:*** *Tania Saskianti, Department of Pediatric Dentistry, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia. Email: tania-s@fkg.unair.ac.id*
Tel/Fax: +62315020255/+6231.5020256