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THE ENHANCEMENT OF BONE DEFECT HEALING BY THE APPLICATION OF HYDROXYAPATITE EXTRACTED FROM INDONESIAN LIMESTONE

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ABSTRACT : The purpose of this to examine the effect of hydroxyapatite extracted from Indonesian limestone on bone defects healing by means of the number of osteoblasts, fibroblasts and osteoclasts. This study design is an true experimental with randomized post-test only control group design. Femurs of eighteen *Cavia cobaya* were drilled to make bone defects and divided into 3 groups; group 1 (G1): defects without bone graft treatment, group2 (G2): bone defects were filled with bovine hydroxyapatite and group 3 (G3): bone defects were filled with Indonesian limestone. After 14 days, the animals were sacrificed and femurs were extracted. The number of osteoblast, fibroblast and osteoclast were documented by histologically. The analysis of variance (ANOVA) continued by Least Significant Different (LSD) were conducted to analyze the data with p-value < 0.05 considered as statistically significant. The number of osteoblasts were higher in G3 than that in group G1 ($p < 0.05$) but not with group G2 ($p > 0.05$). The number of osteoclasts were significantly lower in group G3 compared with group G1 and G2 ($p<0.05$). The number of fibroblast were higher in G3 than that in group G1 and group G2 ($p<0.05$). Hydroxyapatite from Indonesian limestone enhanced the bone deffect healing through the enhancement of osteoblasts, fibroblasts and reducement of osteoclasts number.

Key words : Indonesian limestone, hydroxyapatite, osteoblasts, regenerative medicine, osteoclast.

INTRODUCTION

Bone defects are often occur in dentistry as a result of trauma, infection, surgery or congenital musculoskeletal disorders. The healing of bone defects requires the process of reconstruction of bone tissue and this process requires a complex and long procedure (Blumenfeld *et al*, 2002). Most bone defects can heal spontaneously because of bone regeneration process. However, the healing process can not occur in large bone defects (Clements *et al*, 2008).

Bonegraft is a temporary material for bone growth and it provides a special environment and architecture that is important for bone remodeling. Biomaterials in the form of bonegraft are often used as therapeutic materials for bone defects as these materials are able to fill bone defects to assist the reconstruction of bone defect (Li *et al*, 2015; Prahasanti *et al*, 2020). At present, the gold standard treatment for bone defect regeneration is autograft (Liu *et al*, 2008). However it is difficult to get donor supply and autograft also cause donor site pain and haemorrhage (Oest *et al*, 2007).

Hydroxyapatite (HAp) ($Ca_{10}(PO_4)_6(OH)_2$) has been considerably used for bone regeneration (Nugraha *et al*, 2019). HAp is calcium phosphate which is the most abundant inorganic component in human bones (Oshikawa and Myoui, 2005). HAp is the most commonly biomaterial used for artificial bone due to its biocompatible and osteoconductive characteristics (Schicker *et al*, 2006; Bang *et al*, 2014; Sari *et al*, 2020). HAp has been shown to increase osteoblast cell division to form bone. HAp can also increase fibroblasts, which are cells derived from mesenchymes that play a role in maturing osteoblast cells. In addition, HAp can affect the process of osteoclastogenesis to absorb osteoclast cells so that bone formation can occur optimally (Zhurong *et al*, 2015; Winkler *et al*, 2018).

HAp can be produced from chemical synthesis and can also be isolated from natural sourcessuch as mammals, marine, shell, plants, algae and from mineral sources (Akram *et al*, 2014). The advantages of HAp from natural sources is inexpensive and uncomplicated.Limestone is a natural mineral which can be used to produce hydroxyapatite. Deposition of animal skeleton or eksoskeleto, foraminifera or algae contained lots of calcium carbonate ($CaCO_3$). Indonesian limestone has

enormous potential and can be found in almost every province in Indonesia. Indonesian limestone contains between 40-45% calcium oxide (CaO), which is a main component of HAp ceramics (Habibie *et al*, 2017).

HAp has been synthesized from various sources such as bovine and fish bone, but the burning process of these bones causes environmental and economical problems. The process produces odorous gas, the price is quite expensive, and the quality and quantity is not stable (Manalu *et al*, 2015). To overcome this problem, researchers from *Balai Besar Keramik* (BBK)Indonesia have successfully synthesized HAp in the form of bone ash from Indonesian limenstones as natural resources (Wahyudi *et al*, 2016). HAp derived from Indonesian limestone has good level of purity and resembles the composition of calcium phosphate in bones so that has the potential to be applied in the field of dentistry as a bonegraft material for repairing bone defects due to trauma or bone resorption (Wahyudi *et al*, 2016). Until now, the study that examine the potential effect of HAp extracted from Indonesian limestone for bone substitute is still limited. Thus, the aim of this study was to examine the effect of HAp extracted from Indonesian limestone on bone defects healing towards the number of osteoblasts, fibroblasts and osteoclasts.

MATERIALS AND METHODS

This study was designed as a randomized post-test only control group. This research has received the approval of the the Health Research Ethical Clearance Commission, Faculty of Dental Medicine Universitas Airlangga (No. 341/HRECC.FODM/VI/2019).

A total of 18 male Cavia cobaya, 3 months old, weighing 500 g were used in the study and divided into three groups, each group consisted of six C. cabaya. All animals were housed in a cage in standard environmental conditions with 12 hours light and 12 hours dark cycle. All animals were provided with standard commercial diet and distilled water *ad libitum*. They were acclimatized for 5 days before the start of experiment.

All animals in each group were anaesthetized with ketamine (30 mg/kg BW) and a wound was made by performing drilling on the right femur of animal. The defect was made as deep as 2mm with a diameter of 2mm. Group 1 (G1) was the negative control group that the defect was not applied with bonegraft. Group 2 (G2) was the positive control group that the defect was applied with bovine HAp (BATAN, No. BX-G / 11-17 / 03-110). Group 3 (G3) was a group that the defect was applied with HAp from Indonesian limestones (*Balai Besar Keramik* (BBK) Hydroxyapatite, Indonesia). Those of all groups were sutured using 3/0 black absorbable silk. On day-14, animals were euthanized using carbon dioxide gas inhalation and the femurs were harvested for histological examination. The resected femurs were fixed using 4% paraformaldehyde, then were demineralized. Histological sections that embedded with parafin were stained with hematoxylin and eosin to calculated the number of osteoblast, fibroblast and osteoclast. Histological sections were observed using light microscope (Olympus BX41 series, Japan) at x 1000 magnification, at 5 different visual fields. Images were taken using digital camera (DP-70, software Olysia, Japan). The analysis of variance (ANOVA) continued by Least Significant Different (LSD) were conducted to analyze the data with p-value < 0.05 considered as statistically significant.

RESULTS

The statistical tests using one way ANOVA showed that there was significant difference of the number of osteoblasts between groups with the p-value = 0.000 ($p < 0.05$). The application of HAp extracted from Indonesian limestone in G3 showed significantly increase the number of osteoblast compared to the negative control group (G1), but not to G2 (Fig. 1). The microscopic images of osteoblast at 400x magnification of on day 14 have shown in Fig. 2.

There was significant difference of the number of fibroblasts between groups with the p-value = 0.003 ($p < 0.05$). The application of BBK HAp (G3) showed a significantly increase the number of fibroblast compared

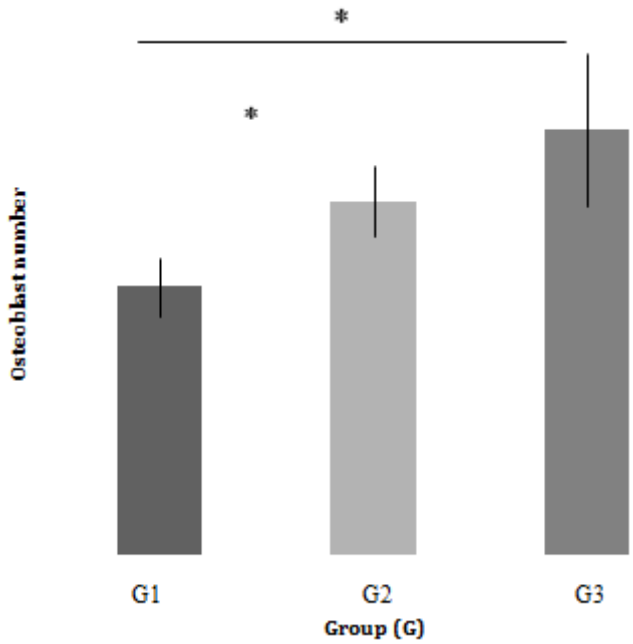

Fig. 1 : The mean and standard deviation of osteoblast number on day 14. *significant difference between two groups ($p < 0.05$).

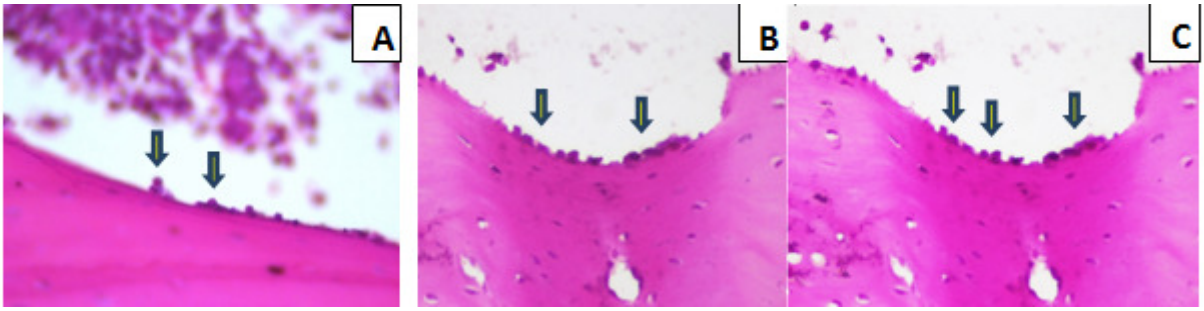

Fig. 2 : The microscopic expression of osteoblast (blue arrow) on day 14 in group G1 (A), G2 (B) and G3 (C).

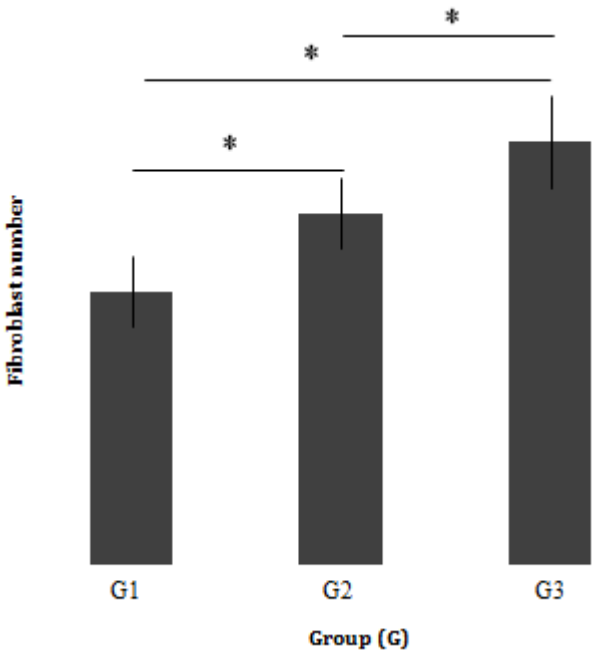

Fig. 3 : The mean and standard deviation of fibroblast number on day 14. *significant difference between two groups ($p < 0.05$).

to G1 and G2 (Fig. 3). Fig. 4 showed the microscopic images of fibroblast at 400x magnification on day 14.

There was significant difference of the number of osteoclast between groups with the p-value = 0.000 ($p < 0.05$). The application of BBK HAp (G3) showed a significantly increase the number of osteoclast compared to G1 and G2 (Fig. 5). Fig. 6 showed the microscopic images of fibroblast at 400x magnification on day 14.

DISCUSSION

This study analyzed the effect of aplication of HAp derived from Indonesian limestones to the bone healing process toward the expression of osteoblast, fibroblast and osteoclast. HAp have similar composition to bone mineral and thermodynamically stable in body fluid, so that HAp have been used as biomaterial for bone defect healing (Sadat-Shojai *et al*, 2013; Hendi, 2017). In addition, application of HAp for bone healing did not induce toxicity and inflammation response (O'Hare, 2010). HAp can be extracted from mineral sources e.g limestone. Limestone that found in Indonesia has a potential as bone substitute biomaterial because it contains between 40-45% CaO, the main component of HAp (Wahyudi *et al*, 2016).

In this study, aplication of HAp extracted from Indonesian limestone (BBK Hydroxyapatite) showed a

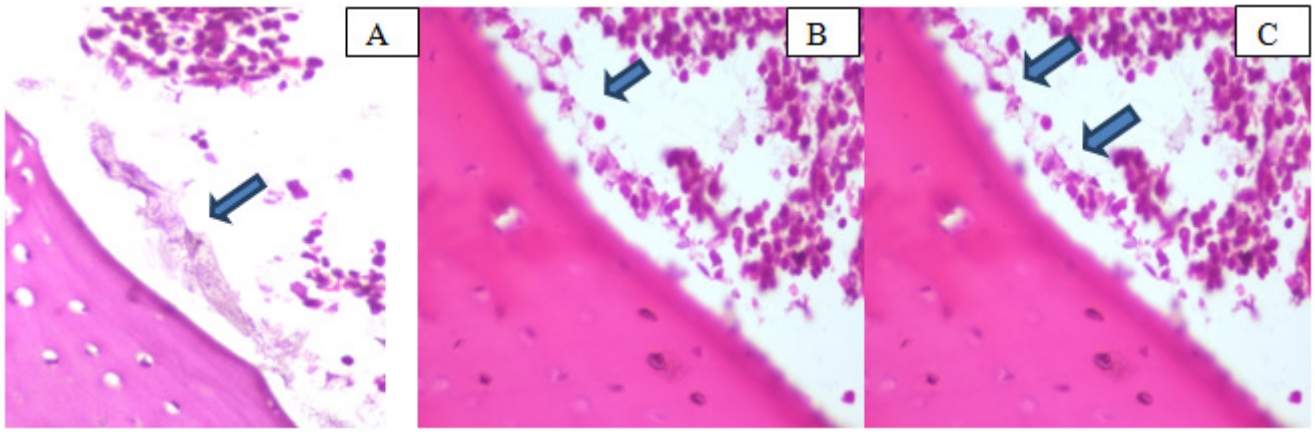

Fig. 4 : The microscopic expression of fibroblast (blue arrow) on day 14 in group G1 (A), G2 (B) and G3 (C).

higher number of osteoblasts compared to other groups. This probably due to the ratio of calcium (Ca) to phosphorous (P) of the HAp. BBK Hydroxyapatite have Ca/P ratio of 1.64 that is a similar with native human bone (Hermanto *et al*, 2017). BBK Hydroxyapatite was

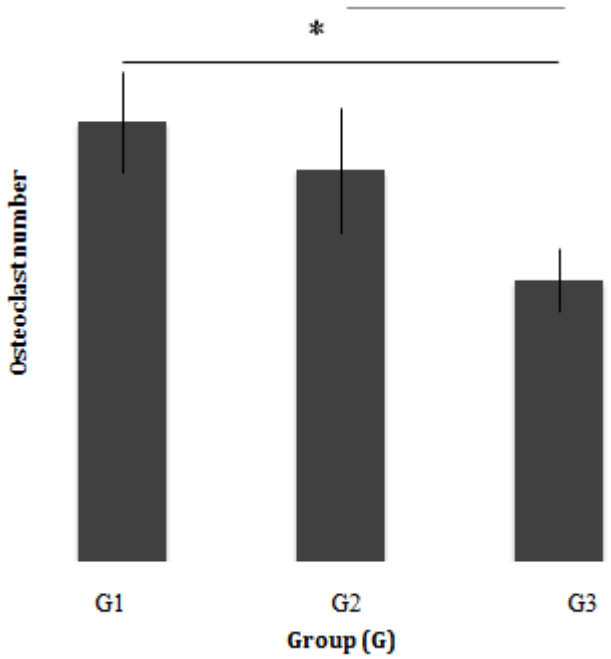

Fig. 5 : The mean and standard deviation of osteoclast number on day 14. *significant difference between two groups ($p < 0.05$).

differentiation are also induced by Hap (Zhang *et al*, 2009).

Many different cells are involved in the bone healing process including fibroblast. In our study, the number of fibroblasts were higher in Group 3 compared to control group. This condition probably because BBK Hydroxyapatite is an ideal scaffold for fibroblasts. A good scaffold should provide an appropriate environment for attachment, growth and differentiation of the cells. Moreover, Ca^{2+} and PO_4^{3-} also play a role in fibroblast cell division. These cells originate from the mesenchyme which assist in maturation of osteoblast cells. The osteoblast maturation controlled by osteorix and runt-related transcription factor-2 (RUNX2) (Zhang *et al*, 2009; Nugraha *et al*, 2018; Sitasari *et al*, 2020). Moreover, fibroblast which express same markers as mesenchymal stem cell can differentiate into adipocytes, chondrocytes and osteoblasts so that fibroblast has important function for bone regeneration (Tonnesen *et al*, 2000; Sabatini *et al*, 2005; Denu *et al,* 2016; Lorenz *et al*, 2008; Hanson *et al*, 2010; Hisham *et al*, 2019).

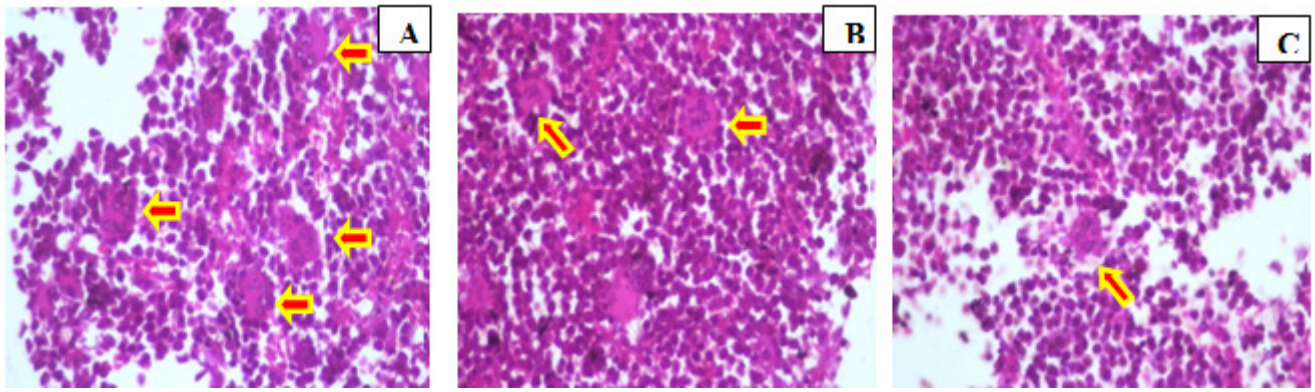

Fig. 6 : The microscopic expression of osteoclast (red arrow) on day 14 in group G1 (A), G2 (B) and G3 (C).

produced using calcination and precipitation method that cause Ca/P ratio was high. The isolated Hap may affect the higher rasio of Ca/P ratio (Wahyudi *et al*, 2016; Mohd Pu'ad *et al*, 2019).

Calcium ions (Ca^{2+}) promotes mature bone cells by the nitric oxide formation and induces growth of bone precursor cells (Foreman *et al*, 2005). Ca^{2+} stimulates the pathway of osteoblastic bone synthesis by activating ERK1/2. The osteoblast lifespan increased by Ca^{2+} through the activation of PI3K/Akt pathways (Liu *et al*, 2008; Danciu *et al*, 2003). Phosphorous in the form of phosphate (PO_4^{3-}) controlsthe osteoblast proliferation and maturation through the activation of IGF-1 and ERK1/2 pathways, and enhancement of BMPs expression (Julien *et al*, 2009; Tada *et al*, 2011). Osteoblasts usually attach more and spread better on osteoinductive material such as HAp, and secretion some markers of osteoblastic

Osteoclasts have important functions in the bone healing process. Osteoclasts affect bone regeneration by it is function in the degradation (Narmada *et al*, 2019; Hernawan *et al*, 2020). The bone grafting materials such as calcium phosphate, HAp and some polymers (Detsch and Boccaccini, 2015 ; Schilling *et al*, 2004). We found that the number of osteoclast was lower in G3 than that in G1 group on day 14. This is probably due the effect of phospate that release by HAp. The signaling between RANK-RANKL affected by the negative feedback by Phosphate.The reducement of RANKL/OPG by phosphate can ameliorate osteoclastogenesis and bone resorption (Mozar *et al*, 2008; Zhang *et al*, 2011).

The application of HAp extracted from Indonesian limestone as graft perhaps inhibits bone resorption by osteoclasts.Moreover, when the body is traumatized, it will experience an inflammatory phase. To overcome the

inflammation, the body will respond to secrete macrophages 2 (M2) which will release proinflammatory mediators such as IL-10, IL6, TNF-α and IL-1. At this stage, osteoclasts will decrease and osteoblast cells will increase so that the healing process occurs optimally (Zhang *et al*, 2009).

CONCLUSION

Hydroxyapatite extracted from Indonesian limestone enhanced the bone deffect healing by incresing the number of osteoblasts and fibroblasts and reducing the number of osteoclasts.

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REFERENCES

Akram M, Ahmed R, Shakir I, Ibrahim W A W and Hussain R (2014) Extracting hydroxyapatite and its precursors from natural resources. *J. Mater. Sci*. **49**, 1461–1475.

Bang L T, Long B D and Othman R (2014) Carbonate hydroxyapatite and siliconsubstituted carbonate hydroxyapatite: Synthesis, mechanical properties and solubility evaluations. *The Scientific World J*. **19**, |Article ID 969876 | https://doi.org/10.1155/2014/969876

Blumenfeld I, Srouji S, Lanir Y, Laufer D and Livne E (2002) Enhancement of bone defect healing in old rats by TGF-b and IGF-1. *Exp Gerontol*. **37**, 553, e65.

Clements J R, Carpenter B B and Pourciau J K (2008) Treating segmental bone defects: a new technique. *J. Foot Ankle Surg*. **47**, 350, e6.

Danciu T E, Adam R M, Naruse K, Freeman M R and Hauschka P V (2003) Calcium regulates the PI3K-Akt pathway in stretched osteoblasts. *FEBS Lett*. **536**, 193–197.

Denu R A, Nemcek S, Bloom D D, Goodrich A D, Kim J and Mosher D F (2016) Hematti P. Fibroblasts and Mesenchymal Stromal/Stem Cells Are Phenotypically Indistinguishable. *Acta Haematol.* **136**, 85–97.

Detsch R and Boccaccini A R (2015) The role of osteoclasts in bone tissue engineering. *J. Tissue Eng. Regen. Med.* **9**, 1133–1149.

Foreman M A, Gu Y, Howl J D, Jones S and Publicover S J (2005) Group III metabotropic glutamate receptor activation inhibits Ca2+ influx and nitric oxide synthase activity in bone marrow stromal cells. *J. Cell Physiol*. **204**, 2, 704–713.

Habibie S, Wargadipura A, Riban D, Herdianto N, Riswoko A, Nikmatin S and Clarke S (2017) Production and Characterization of Hydroxyapatite Bone Substitute Material Performed from Indonesian Limestone. *Int. J. Biomed. Engineering and Sci*. **4**(1), 11-23.

Hanson S E, Kim J, Johnson B H, Bradley B, Breunig M J, Hematti P and Thibeault S L (2010) Characterization of mesenchymal stem cells from human vocal fold fibroblasts. *Laryngoscope* **120**, 546–551.

Hendi A A (2017) Hydroxyapatite based nanocomposite ceramics. *J. Alloy. Comp*. **712**, 147–151.

Hermanto E, Rima P, Asri C and Kevin A (2017) Grafting Effectiveness of Anadara Granosa Shell Combined With Sardinella Longiseps Gel On the Number of Osteoblast-Osteoclast Cell. *Dental J.* (Majalah Kedokteran Gigi) **50**, 30, 138- 143.

Hermawan R W, Narmada I B, Djaharu'ddin I, Nugraha A P and Rahmawati D (2020) The Influence of Epigallocatechin Gallate on the Nuclear Factor Associated T Cell-1 and Sclerostin Expression in Wistar Rats (*Rattus novergicus*) during the Orthodontic Tooth Movement. *Research J. Pharm. Tech*. **13**, 4, 1730-1734.

Hisham P B B M, Narmada I B, Alida A, Rahmawati D, Nugraha A P and Putranti N A (2019) Effects of Vitamin D in Alveolar Bone Remodeling on Osteoblast Numbers and Bone Alkaline Phosphatase Expression in Pregnant Rats During Orthodontic Tooth Movement. *J. Orofac. Sci.* **11**, 79-83.

Julien M, Khoshniat S, Lacreusette A, Gatius M, Bozec A, Wagner E F, Wittrant Y, Masson M, Weiss P, Beck L, Magne D and Guicheux J (2009) Phosphate-dependent regulation of MGP in osteoblasts: role of ERK1/2 and Fra-1. *J Bone Miner Res*. **24**, 11, 1856–1868.

Li Y, Chen S K, Li L, Qin L, Wang X L and Lai Y X (2015) Bone defect animalmodels for testing efficacy of bone substitute biomaterials. *J. Orthop.Transl*. **3**(3), 95–104.

Liu D, Genetos D C, Shao Y, Geist D J, Li J, Ke H Z, Turner C H and Duncan R L (2008) Activation of extracellular-signal regulated kinase (ERK1/2) by fluid shear is Ca2+– and ATP-dependent in MC3T3-E1 osteoblasts. *Bone* **42**(4), 644–652.

Lorenz K, Sicker M, Schmelzer E, Rupf T, Salvetter J, Schulz-Siegmund M and Bader A (2008) Multilineage differentiation potential of human dermal skin-derived fibroblasts. *Exp. Dermatol.* **17**, 925–932.

Manalu J L, Soegijono B and Indrani D J (2015) Characterization of Hydroxyapatite Derived from Bovine Bone. *Asian J. Applied Sci*. **03**, 758-765.

Mohd Pu'ad N A S, Koshy P, Abdullah H Z, Idris M I and Lee T C (2019) Syntheses of hydroxyapatite from natural sources. *Heliyon*. **5**(5), e01588.

Mozar A, Haren N, Chasseraud M, Louvet L, Mazière C, Wattel A, Mentaverri R, Morlière P, Kamel S, Brazier M, Mazière J C and Massy Z A (2008) High extracellular inorganic phosphate concentration inhibits RANK–RANKL signaling in osteoclast-like cells. *J. Cell Physiol*. **215**, 47–54.

Narmada I B, Husodo K R D, Ardani I G A W, Rahmawati D, Nugraha A P and Iskandar R P D (2019) Effect of Vitamin D during Orthodontic Tooth Movement on Receptor Activator of Nuclear Factor Kappa-B Ligand Expression and Osteoclast Number in Pregnant Wistar Rat (*Rattus novergicus*). *JKIMSU* **8**(1), 38-42.

Nugraha A P, Narmada I B, Ernawati D S, Dinaryanti A, Hendrianto E, Ihsan I S, Riawan W and Rantam F A (2018) Osteogenic potential of gingival stromal progenitor cells cultured in platelet rich fibrin is predicted by core-binding factor subunit-α1/Sox9 expression ratio (*in vitro*). *F1000 Research* **7**, 1134.

Nugraha A P, Rezkita F, Putra K G, Narmada I B, Ernawati D S and Rantam F A (2010) Triad Tissue Engineering: Gingival Mesenchymal Stem Cells, Platelet Rich Fibrin and Hydroxyapatite Scaffold to ameliorate Relapse Post Orthodontic Treatment. *Biochem. Cell. Arch*. **19**(2), 3689-3693.

O'Hare P, Meenan B J, Burke G A, Byrne G, Dowling D and Hunt J A (2010) Biological responses to hydroxyapatite surfaces

deposited via a co-incident microblasting technique. *Biomaterials* **31**, 515–522.

Oest M E, Dupont K M, Kong H J, Mooney D J and Guldberg R E (2007) Quantitative assessment of scaffold and growth factor-mediated repair of critically sized bone defects. *J. Orthop. Res*. **25**(7), 941-950.

Oshikawa H and Myoui A (2005) Bone tissue engineering with porous hydroxyapatite ceramics. *J. Artificial Organs* **8**, 131–136.

Prahasanti C, Nugraha A P, Saskianti T, Suardita K, Riawan W and Ernawati D S (2020) Exfoliated Human Deciduous Tooth Stem Cells Incorporating Carbonate Apatite Scaffold Enhance BMP-2, BMP-7 and Attenuate MMP-8 Expression During Initial Alveolar Bone Remodeling in Wistar Rats (*Rattus norvegicus*). *Clinical, Cosmetic and Investigational Dentistry* **12**, 79–85.

Sabatini F, Petecchia L, Tavian M, Jodon de Villeroche V, Rossi G A and Brouty-Boye D (2005) Human bronchial fibroblasts exhibit a mesenchymal stem cell phenotype and multilineage differentiating potentialities. *Lab. Invest.* **85**, 962–971

Sadat-Shojai M, Khorasani M T, Dinpanah-Khoshdargi E and Jamshidi A (2013) Synthesis methods for nanosized hydroxyapatite with diverse structures. *Acta Biomater*. **9**, 7591–7621.

Sari D S, Maduratna E, Ferdiansyah, Latief F D E, Satuman Nugraha A P, Sudiana K and Rantam F A (2019) Osteogenic Differentiation and Biocompatibility of Bovine Teeth Scaffold with Rat Adipose-derived Mesenchymal Stem Cells. *Eur. J. Dent*. **13**(2), 206-212.

Schicker M, Seitz H, Drosse I, Seitz S and Mutschler W (2006) Biomaterials as scaffold for bone tissue engineering. *Eur. J. Trauma* **32**(2), 114–124.

Schilling A F, Linhart W, Filke S, Gebauer M, Schinke T, Rueger J M and Amling M (2004) Resorbability of bone substitute biomaterials by human osteoclasts. *Biomaterials* **25**, 3963–3972.

Sitasari P I, Narmada I B, Hamid T, Triwardhani A, Nugraha A P and Rahmawati D (2020) East Java green tea methanolic extract can enhance RUNX2 and Osterix expression during orthodontic tooth movement *in vivo*. *J. Pharm. Pharmacogn. Res.* **8**(4), 290–298.

Tada H, Nemoto E, Foster B L, Somerman M J and Shimauchi H (2011) Phosphate increases bone morphogenetic protein-2 expression through cAMP-dependent protein kinase and ERK1/2 pathways in human dental pulp cells. *Bone* **48**(6), 1409- 1416.

Tonnesen M G, Feng X and Clark R A F (2000) Angiogenesis in Wound Healing. *J. Investigative Dermatology Symposium Proceedings* **5**, 40-46

Wahyudi K, Edwin F and Sofiyaningsih N (2016) Sintesis danKarateristik Bone Ash dari Bahan Alam. *Jurnal keramik dan gelasIndonesia* **25**(2), 46-58.

Winkler T, Sass F A, Duda G N and Schmidt-Bleek K (2018) A review of biomaterials in bone defect healing, remaining shortcomings and future opportunities for bone tissue engineering: The unsolved challenge. *Bone Joint Res.* **7**(3), 232-243.

Zhang L, Hanagata N, Maeda M, Minowa T, Ikoma T, Fan H and Zhang X (2009) Porous hydroxyapatite and biphasic calcium phosphate ceramics promote ectopic osteoblast differentiation from mesenchymal stem cells. *Sci Technol Adv Mater*. **10**, 025003.

Zhang R, Lu Y, Ye L, Yuan B, Yu S, Qin C, Xie Y, Gao T, Drezner M K, Bonewald L F and Feng J Q (2011) Unique roles of phosphorus in endochondral bone formation and osteocyte maturation. *J. Bone Miner. Res*. **26**, 1047–56.

Zhurong T, Xiangfeng L, Yanfei T, Hongsong F and Xingdong Z (2015) The material and biological characteristics of osteoinductive calcium phosphate ceramics. *Regenerative Biomaterials* **5**(1), 43–59.