Induction of Angiogenesis Process in Mandible Using Anadara granosa Shell Graft
(Experimental Laboratory Study on Rattus norvegicus)

Widyastuti1*, M Rubianto2, Soetjipto3
1Department of Periodontia, Faculty of Dentistry, University of Hang Tuah, Indonesia
2Department of Periodontia, Faculty of Dentistry, Airlangga University, Indonesia
3Department of Biochemistry, Faculty of Medicine, Airlangga University, Indonesia

*widyastuti@hangtuah.ac.id

Abstract. Alveolar bone is a highly vascularized organ and angiogenesis plays an important role in osteogenesis. Vascular endothelial growth factor (VEGF) is an essential mediator during the process of angiogenesis and osteogenic potential, fibroblast growth factor (FGF-2) either synergistically with VEGF, act on early stages of induction by stimulating fibroblast and osteoblast proliferations. A. granosa shell graft (AG) has osteoconductivity so it can be a scaffold to attract pluripotent cells that support bone healing process. Purpose: To improve bone blood flow and bone remodeling in mandible using A. granosa shell graft for accelerate osteogenesis. Methods: Total of 18 male rats Rattus norvegicus aged 6 months with body weight 250-300 grams were randomly divided into 3 groups with 6 rats each group. All samples got its mandibular right incisors extracted, curettage and irrigation. The group consist of: treatment group with AG, treatment group with xenograft, control group untreated graft. After one week rats were sacrificed for FGF-2 and VEGF analyzed with One-way Anova. Results: AG shell graft containing 44% calcium carbonate, 33% calcium hydroxide and 23% calcium phosphate could increase the expression of FGF-2 and VEGF (p<0.05) at 7th days. Conclusions: AG shell graft has potential for accelerate angiogenesis process in the mandible with increased FGF-2 and VEGF.

Keywords: bone graft, healing, FGF-2, VEGF, angiogenesis

1. Introduction
Chronic inflammation of periodontal tissue is caused by complex subgingival biofilms containing several periodontopathogens. Biofilms generally contain commensal anaerobic gram-negative bacteria and opportunistic pathogens, including Porphyromonas gingivalis. The response to periodontal pathogens is polymorphonuclear cells (PMNs) releasing reactive oxygen species (ROS), such as superoxide, protease and other factors that can damage host tissue. This molecule causes further oxidative damage to the gingival tissue, periodontal ligaments and causes alveol bone resorption also increases the amount of proinflammatory production of cytokines that cause damage, such as interleukin (IL) -1ß, IL-6 and tumor necrosis factor (TNF-α) which can improve gingival crevicular fluid (GCF).[1] According to Nobre & Malo (2017) of 22,009 patients, the number of periodontitis patients was 17.6% and the implant peri was 13.9%.[2] This shows that patients with moderate to severe periodontitis and dental implant patients who require regenerative therapy used large bone grafts. Besides, preprosthetic therapy using graft materials is needed for correction of bone defects in dental implants.

Various materials and techniques are used as regenerative therapy for alveol bone damage caused by periodontitis, other bone defects and acceleration of healing process after tooth extraction...
with graft, guided tissue regeneration (GTR), growth factors or substances that play a role in growth, substitution and differentiation periodontal cells. The alveol bone augmentation process which is often used to regenerate periodontal damage, includes osteoconductive and osteoinductive ingredients to stimulate new bone formation and regeneration. Graft is a part of a network that is taken from one place and transplanted to another place, both in the same and different individuals. The goal is to repair a bone defect by returning contours caused by illness, accident, or growth and development anomalies. Bone graft is a widely used choice to repair alveol bone damage which is an important part of the periodontal tissue regeneration process. In general, bone graft procedures, such as autograft, allograft and xenograft are used to replace or repair damaged bone tissue.[3; 4; 5]

*Anadara granosa* (AG) is a type of bivalvia that lives on the bottom of the sea and has a characteristic that is covered by two pieces of shell that can be opened and closed because there is a joint in the form of elastic hinges which are liaison.[6; 7] AG shell mineral composition is almost the same as coral, so it is thought that it can be used as an alternative biomaterial for bone substitution in bone defects based on mineral content composition, which is similar to bone grafts from coral are useful for bone defect therapy.[8; 9] Corals that are often used come from the type of *Porites sp.* which has similarities with cancellous bone mechanically. Shell contain high calcium carbonate, show biocompatibility, osteoconduction and biodegradability. Although not osteoinductive or osteogenic, coral grafts act as carriers of growth factors followed by cell attachment, growth, dissemination and differentiation.[6; 10]

Wound and bone healing is a complex process, consisting of varied interactions between different types of cells, protein structures, growth factors, cytokines, proteinases and mediators. Healing of periodontal tissue after graft implantation, can achieve the regeneration process when both tissues are re-formed through the process of angiogenesis and osteogenesis. The inflammatory response is very important in initiating and forming the two processes. VEGF is an important mediator during the process of angiogenesis. Osteoprogenitor cells that responded to growth factors including VEGF, FGF which will be released during remodeling as a grafting response.[11; 12; 13;14]

Fibroblast growth factors have anabolic effects on bone by mitogen osteoblasts, endothelial vascular cells and fibroblasts.[15] FGFs are considered as potent regulators in cell growth and wound healing. The FGF family has the same amino acid sequence in bone produced by various cells including osteoblasts, macrophages and endothelial cells and in active form of extracellular bone matrix. FGFs act by autocrine and paracrine as mitogens in many cell types, while also being involved in a number of other cellular processes, including angiogenesis, wound healing and cell differentiation. Other expressions of FGFs show that these growth factors are involved in the regulation of condrogenesis and bone osteogenesis, FGF-2, also known as bFGF. FGF-2 alone is unable to stimulate ectopic bone formation, but plays an important role in the regulation of normal bone healing, and stimulates mitogenesis and chemotaxis of periodontal ligament cells. FGFs increase osteoblast proliferation and indirectly increase collagen production by differentiating osteoblasts. bFGF is able to stimulate endothelial cell migration and periodontal ligaments and the proliferation of the dentine surface.[12; 16] FGF expression increased during the first 4 days, to reach the peak in the second week.[17]

Vascular endothelial growth factor in the last two decades the results of the study show as a key regulator of physiological and pathological angiogenesis, because it stimulates the formation of endothelial cell proliferation, stimulates angiogenesis, vasodilation and increases vascular permeability, which is a key factor in the first phase of fracture and bone regeneration.[15] The same thing also mentioned that osteogenesis and angiogenesis are two very close correlations during the process of growth, development, bone remodeling and repair.[14]

2. Experimental Methods

2.1. Experimental Design

The design of this research was post test only control group design. The initial step of this study started with 18 male *Wistar* rats were divided into three groups, each group consists of 6 rats. All experimental rats will be adapted, adjusted, and kept in a same environment for one week. Day 8 all animal experiments mandibular incisive were extracted. Graft was not applied on C group’s defect. The A group defect was applied with *A. granosa shell graft* while B group was applied with xenograft.
2.2. Preparation and applied A. granosa shell graft

First anesthesia with ketamine 0.01mL and xylazine 0.1mL mixed and injected with a dose of 0.11mL / 100gr BW on os femur dextra of the rats intramuscular.[18] After the rats began to unconsciously, then we can start the mandibular incisive extraction procedure. 10% povidine iodine antiseptic was given on the area around the defect for five minutes. Next step is making a defect by extracting the mandibular incisive, curettage and irrigation. Preparation of the graft using dappen glass upside down and mix the graft material and blood of rat in the back side of the dappen glass. Stir graft material and blood of rat until they are well mixed. Shift the particles material graft and the blood of rats in the defect using the excavator and condensed until the surface is solid enough, then close the defect surface with suturing. After implantation was done and do suturing to socket margin, we inject of 0.1 cc / 100gr BW novalgin analgesic to control swelling and pain.[19; 20] Ethical clearance of the study should be obtained from Faculty of Dentistry Airlangga University with the number 013/HRECC.FODM/I/2017.

After seven days, the rats were sacrificed by decapitation. The mandible were dissected out by surgically, and rinsed with physiologis saline. FGF-2 and VEGF expression as angiogenesis marker were examined with immunohistochemistry method. The research was analyzed by one way ANOVA.

3. Results and Discussion

The aim of this study is to investigate mandible angiogenesis by A. granosa shell graft application. The result in this experiment showed that FGF-2 and VEGF expression (figure 1-2)

**Figure 1**: Microscopic photos of FGF-2 expression: Anadara granosa group (A), xenograft (positive control) group (B) and negative control group (C). At the tip of the arrow, there are brown macrophages in the cytoplasm with 400x magnification.

| Groups     | n  | FGF-2 |   |   |   | p     |
|------------|----|-------|---|---|---|-------|
| A: A. granosa | 6  | 12.33  | 2.81 | 9 | 16 | 0,000* |
| B: Xenograft | 6  | 9.33   | 1.37 | 7 | 11 |       |
| C: Control  | 6  | 5.50   | 1.52 | 4 | 8  |       |

* The significance on α=0.05 (Oneway Anova)

* Different superscripts show that there are differences between groups (Multiple comparisons Tukey HSD)

**Figure 2**: Microscopic photos of VEGF expression: Anadara granosa group (A), xenograft (positive control) group (B) and negative control group (C). At the tip of the arrow, there are brown macrophages in the cytoplasm with 400x magnification.
Table 2. Different test of VEGF expressions on day 7

| Groups       | n  | VEGF Mean | SD   | Minimum | Maximum | P       |
|--------------|----|-----------|------|---------|---------|---------|
| A: A. granosa| 6  | 12.83a    | 1.94 | 10      | 15      | 0,000*  |
| B: Xenograft | 6  | 9.17ab    | 3.37 | 3       | 13      |         |
| C: Control   | 6  | 5.67b     | 1.86 | 3       | 8       |         |

* The significance on α=0.05 (One way Anova)

Different superscripts show that there are differences between groups (Multiple comparisons Tukey HSD)

Table 1 shows the FGF-2 expressions from each treatment group. A. granosa shell grafts group (A) have not significant difference with xenograft group (B) and significant difference with negative control (C). Table 2 shows the VEGF expressions from each treatment group. A. granosa shell grafts group (A) have significant difference with xenograft group (B) and negative control (C). Xenograft group (B) have significant difference with negative control (C).

**Discussion.** AG shell graft used in this study is a graft that has been processed with high temperature and in powder form with a size of 150 µm with SEM tests and examinations, can function as osteoconduct and vascularization. Vascularity is an important factor in optimally supporting blood vessel supply to maintain the survival of osteogenic cells.[21] The shell graft AG has a composition consisting of 44% CaCO3 calcium carbonate, 33% calcium hydroxide Ca(OH)2 and 23% calcium hydrogen phosphate CaHPO4 from material identification tests or XRD. XRF test results of graft material derived from AG shells which are processed with high temperatures have a content of 98.24% calcium.

The results of the 7th day study showed a significant increase in FGF-2 in the xenograft group and AG shell graft compared to the graftless control group. The mean group of AG shell grafts is higher than the xenograft group. Increased FGF-2 through pro-regenerative macrophage bone remodeling pathways and the occurrence of hematoma, which induces it. FGF-2 is the initial phase of angiogenesis and neovascularization in the process of bone healing. Through increased FGF-2, activates BMP-2, differentiation and proliferation of osteoblasts and release of osteogenic factors in later stages.[20; 22; 23] Growth factors that play a role in bone formation are fibroblasts and osteoblast proliferation, extracellular matrix deposition, mesenchymal cell differentiation and vascular proliferation. The complexity of regulation of bone induction is reflected in the specificity of growth factors during the initial phase of bone regeneration. Platelet derived growth factor and FGF act at an early stage of induction to stimulate fibroblasts and osteoblast proliferation. In contrast, BMPs act at the next osteoinduction stage with mesenchymal cell differentiation and vascular proliferation.[23]

The xenograft and AG groups had higher FGF-2 than controls, because cellular migration and growth factors to the recipient side were associated with more than 30% regeneration and the magnitude of bone density on the side of the graft.[23] The results of the VEGF study experienced a significant increase on the 7th day of the AG shell group compared to the control group without grafts and xenograft groups. This is because FGF-2 induces VEGF and VEGF as key regulators of the angiogenesis-osteogenesis process. So that the average VEGF results have the same trend as FGF-2. VEGF has the dual role of regulating osteoblast differentiation and the release of osteogenic factors. In periodontal VEGF tissue contributes to healing and ossification roles.[12; 22; 24] The increase of FGF-2 and VEGF in the AG shell group on day 7 was due to the faster absorption of calcium carbonate grafts compared to the xenograft group consisting of hydroxyapatite (HA) so that the initial resorption process was slower.[23]

Other growth factors that play an important role in remodeling and bone homeostasis are IL-1 and TNF-α. At the molecular level, cytokines that modulate development have been identified and include FGF and TGFβ. FGF-2 has implications for the control and differentiation of angiogenesis, wound healing and tissue repair. FGF-2 in the extracellular matrix, stimulates osteoblast proliferation and TGFβ1 production. Given the involvement of FGF-2 in differentiation, growth and improvement and the dependence of these related processes on adequate blood supply, FGF-2 is a potent angiogenesis modulator. FGF-2 stimulates endothelial cell migration, proliferation and angiogenesis and plays a role in blood vessel development. In addition, FGF-2 induces VEGF so that the VEGF-
induced angiogenesis process depends on FGF-2. As a key regulator of the angiogenesis-osteogenesis process, VEGF has a dual role to regulate osteoblast differentiation and release of osteogenic factors. VEGF can increase vascular tissue around bone defects for healing and increase osteogenesis by inducing neovascularization and direct effects on bone cells through regulation of OPG / nuclear factor betta kapa ligand (RANKL) receptor activators, alkaline phosphatase, osteopontin and osteocalcin.[22; 24; 25; 26]

Vascular endothelial growth factor is produced by osteoblast precursors in the pericondrium and chondrocytes and induces osteoblasts to migrate in the ossification center along with blood vessels, osteoclasts and hematopoietic stem cells. VEGF activates osteoblasts which stimulate angiogenesis, differentiation of osteoblasts and bone formation.[22; 27]

4. Conclusion
AG shell graft has potential for accelerate angiogenesis process in the mandible with increased FGF-2 and VEGF.

References
[1] Helieh SO dan Puleo DA. 2011. Review article : Animal models for periodontal disease. Hindawi Publishing Corporation, *Journal of Biomedicine and Biotechnology*. Article ID 754857. p.1-9
[2] Nobre MA & Maló P. 2017. Prevalence of periodontitis, dental caries, and peri-implant pathology and their relation with systemic status and smoking habits: Results of an open-cohort study with 22009 patients in a private rehabilitation center. *J of Dentistry* Volume 67. p. 36-42
[3] Newman MG, Takei HH, Klokkevold PR, Carranza FA; 2012; Clinical Periodontology, 11th edition, St Louis: Saunders.
[4] Wikesjö UM, Susin C, Lee J, Dickinson DP, Polimeni G. 2012. Oral Wound Healing. Chapter 10 : Periodontal regeneration, experimental Observations-clinical consequences. *Cell Biology and Clinical Management*. John Wiley & Sons.Inc. p.243-5
[5] Kumar P, Vinitha B, Fathima G. 2013. Bone grafts in Dentistry. *J Pharm Bioallied Sci* Volume 5(1). p.125-7
[6] Hazmi AJA, Zuki ABZ, Noordin MM, Jalila A, Norimah Y. 2007. Mineral composition of the cockle (Anadara granosa) shells of west coast of Peninsular Malaysia and it’s potential as biomaterial for use in bone repair. *Journal of Animal and Veterinary Advances*. Volume 6 (5). p. 591-94
[7] Mohamed M, Yusup S, Maitra S. 2012. Decomposition study of calcium carbonate in cockle shell. *Journal of Engineering Science and technology*. Volume 7 (1). p.1-10
[8] Vuola J. 2001. Natural coral and hydroxyapatite as bone substitute. Department of Plastic Surgery, *Helsinki University Central Hospital. Academic Dissertation*. p.7-8
[9] Zakaria ZAB, Zakaria N, Kazim Z. 2004. Mineral Composition of the Cockle (Anadara granosa) Shells, Hard Clamp (Meretix meretix) Shells and Corals (Porites spp) :A comparative study. *J of Animal and Veterinary Advances* 3(7). p.445-7
[10] Ige OO, Umoru LE, Aribio S. 2012. Review article : Natural Products : A Minefield of biomaterials. *ISRN Materials Science*. Article ID 983062. p.1-20
[11] Tommila M. 2010. Granulation Tissue Formation. The effect of hydroxyapatite coating of cellulose on cellular differentiation. Review of the Literature. p.47-51
[12] Pisoschi C, Stanciulescu C, Banita M. 2012. Growth factors ang connective tissue homeostatis in periodontal disesase. Available from httpcdn.intechopen.compdfs26477InTech. p.55-80 (diakses 20 Juni 2016)
[13] Sandor GKB, Carmichael RP, Ylikontiola LP, Jan A, DuVal MG, Clokie CML. 2012. Chapter 15 Healing of Large Dentofacial Defects. *Oral Wound Healing : Cell Biology and Clinical Management*. Edited by Hannu Larjava. John Wiley & Sons.Inc. p.347-92
[14] Yang Y, Tan Y, Wong R, Wenden A, Zhang L, Rabie ABM. 2012. Review The role of vascular endothelial growth factor in ossification. *International Journal of Oral Science* Volume 4. p. 64-8
[15] Kini U & Nandeesh BN. 2012. Physiology of Bone Formation, Remodelling and Metabolism. Fogelman et al eds. Radionuclide and hybrid bone imaging. Springer-Verlag Berlin Heidelberg. p.29-55

[16] Kempen DHR, Creemers LB, Alblas J, Linchun-Lu, Verbout AJ, Yaszemski MJ, Dhert WJA. 2010. Growth factor interactions in bone regeneration. Tissue Engineering Part B. Volume 6 (6), p. 551-61

[17] Planell JA, Best SM, Lacroix D, Merolli A. 2009. Bone Repair Biomaterials. 1st edition. Elsevier, p.89-92

[18] Rokn AR, Khodadoostan MA, Ghahroodi AR, Motahary P, Fard MJK, De Bruyn H, Afzalifar R, Soolar E, Soolari A. 2011. Bone Formation with Two Types of Grafting Materials: A Histologic and Histomorphometric Study. The Open Dentistry Journal Volume 5. p.96-104

[19] Schimidlin PR, Nicholls F, Kruse A, Zwahlen RA, Weber FE 2011. Evaluation of In Situ Hardening Calcium Phosphate Bone Graft Substitutes. Clin Oral Implants Res. Volume 24(2), p.149-57

[20] Sutherland D & Bostrom M. 2005. Grafts and Bone Graft Substitutes. Bone and Repair: Biology and Clinical Applications. Edited by Lieberman JR & Friedlaender GE. Humana Press Inc, Totowa,NJ. p.133-51

[21] Zhang X, Awad HA, O’Keefe RJ, Guldberg RE, Schwarz EM. 2008. A Perspective Engineering Periosteum for Structural Bone Graft Healing. Symposium: New Approaches to Allograft Transplantation. Clin Orthop Relat Res Volume 466. p.1777-87

[22] Hu K & Olsen BR. 2016. Osteoblast-derived VEGF regulates osteoblast differentiation and bone formation during bone repair. Jci.org Volume 126(2). p.509-24

[23] Sheikh Z, Sima C, Glogaur M. 2015. Bone replacement materials and techniques used for achieving vertical alveolar bone augmentation. J Materials Volume 8. p.2953-93

[24] Saadeh PB, Mehrara BJ, Steinbrech DS, Spector JA, Greenwald JA, Chin GS, Ueno H, Gittes GK, Longaker MT. 2000. Mechanism of fibroblast growth factor-2 modulation of vascular endothelial growth factor expression by osteoblastic cells. J Endocrinology. Volume 141(6), p.2075-83

[25] Yang Y, Tan Y, Wong R, Wenden A, Zhang L, Rabie ABM. 2012. Review The role of vascular endothelial growth factor in ossification. International Journal of Oral Science Volume 4. p. 64-8

[26] Kon T, Cho TJ, Aizawa T, Yamazaki M, Nooh N, Graves D, Gerstenfeld LC, Einhorn TA, 2001. Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. J Bone Miner Res. Volume 16(6).p.1004-14

[27] Hu K & Olsen BR. 2016. The roles of vascular endothelial growth factor in bone repair and regeneration. HHC Public Access 2016 October; 91. p. 30-8

Acknowledgment
The research was supported by a grant from Fundamental Research Program, funded by Ministry Education and Culture Indonesia FY 2015-2016