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Research Report

Effects of *Anadara granosa* shell combined with *Sardinella longiceps* oil on oesteoblast proliferation in bone defect healing process

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ABSTRACT

Background: *Alveolar bone damage is the most common case in dentistry. One way to fix the bone damage is by using bone graft. Anadara granosa shell is a potential bone substitute since it is rich in calcium which can be processed into hydroxyapatite. The addition of Sardinella longiceps oil rich in omega-3 can modulate inflammation, thus accelerating the healing process.* ***Purpose:*** *This study aimed to determine effects of application of Anadara granosa shell combined with Sardinella longiceps oil on osteoblast proliferation in the healing process of bone defects.* ***Method:*** *The subjects were 32 male rats type Wistar divided into 4 groups (n = 8). Making defect was performed on the right bone of the femurs with a half of the diameter of round Mcisinger® Germany bur sized 18. The first group (K) is a negative control group that was not given anything. The second group (AG) was given Anadara granosa pasta. The third group (AM10) was given Anadara granosa pasta combined with 10% Sardinella longiceps oil. And, the fourth group (AM30) was given Anadara granosa pasta combined with 30% Sardinella longiceps oil. Next, preparations and animal euthanasia were performed on the 7th day after the treatment. The number of osteoblasts then was measured after making preparations for HPA with Hematoxylin eosin staining (HE). Afterward, tabulation of data followed by statistical analysis of Anova and HSD Tukey was carried out.* ***Result:*** *The average number of osteoblasts in Groups K, AG, AM10, and AM30 was 19.00, 34.63, 33.50, and 38.50. The results of Anova test showed a significant difference ($p<0.05$). Similarly, the results of Tukey-HSD test also showed significant differences ($p<0.05$) between Group K and all other groups (AG, AM10, and AM30). Nevertheless, there were no significant differences between Group AG and Groups AM10 and AM30, as well as between Group AM10 and Group AM30.* ***Conclusion:*** *The application of the combination of Anadara granosa shell and Sardinella longiceps oil can not increase the proliferation of osteoblasts in the healing process of bone defects.*

Keywords: *Anadara granosa; sardinella longiceps oil; bone graft; osteoblasts; bone healing*

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INTRODUCTION

Bone damage usually can occur because of trauma, tumor, congenital abnormalities, infection, inadequate prosthesis, and systemic disease.[1] In dentistry, alveolar bone damage as the highest prevalence of bone damage is commonly caused by periodontal disease. Periodontal disease is an oral and dental problem with the prevalence of the disease reached 96.58% in Indonesia.[2] In addition, complications after tooth extraction due to sizeable trauma can cause damage to the alveolar bone.[3]

Naturally, abnormal condition can occur in the body. For instance, bone defects can be cured by mechanical balance in the body. This process is known as bone healing. The process of bone healing plays an important role after the treatment in dentistry.[4] Generally, bone healing requires a long period to get back on the normal state.[5] The bone tissue healing process is generally the same as wound

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healing process in soft tissue, but the difference is related to physiological ability to repair the hard tissues during the healing process.[6]

The bone healing process begins with the formation of hematoma and the response of inflammatory cells lasting for 24-48 hours followed by reparative phase occurred in the first few days before the inflammation is reduced, and then last for several weeks, resulting in the development of tissue repair. In this phase, pluripotential mesenchymal cells start to form other cells, such as fibroblasts, chondroblasts, and osteoblasts.[5]

Osteoblast activity appears first at reparative phase. Osteoblasts will be seen in the cortex of the bone, a few millimeters from the defect area.[7] Osteoblasts function as the main cells in the bone healing process. Osteoblasts then will produce, secrete, depose, and mineralize bone matrix.[8] Based on these functions, it is necessary to increase the activity of osteoblasts to accelerate the bone healing process. A small number of osteoblastic activities occur continuously in all the living bone tissue (about 4 percent of all the bone surfaces in adults at various times), resulting in the least number of new bone formed constantly.[9]

Bone formation, moreover, can occur due to several factors affecting the acceleration of healing process, such as use of bone graft. Bone graft has been widely used as a substituting bone material, especially in periodontal therapy.[10] Graft tissue including bone, has been widely used until now. In general, there are two main functions of bone graft for recipients' bone, namely to encourage osteogenesis (bone formation) and to provide mechanical support in the framework of the recipients.[11]

Bone graft, furthermore, is used to stimulate bone healing and stabilize the dimensions of the alveolar bone in the field of dentistry.[12] Bone graft is also considered as an appropriate action to increase the height of the alveolar crest, remodel jawbone, transfer microvascular free tissue, and reshape the alveolar crest.[13]

In addition, xenograft is one form of bone grafts that has biocompatible properties in humans and has higher osteoconductive properties than alloplast. Additionally, xenograft can provide a suitable medium for osteogenesis by bone marrow cells.[14] Currently, xenograft materials widely used are derived from bone bovine.

The main compound used in bone graft is calcium phosphate as the major component of bone mineral constituent. The calcium phosphate compound is an inorganic material that has bioactive and biocompatible properties. Calcium phosphate compound used as bone graft can be in a crystalline phase or in an amorphous phase, namely tricalcium phosphate (Ca3 (PO4) 2) and hydroxyapatite (CA10 (PO4) 6OH2).[15] The form of the most stable calcium phosphate is hydroxyapatite (HA). HA is not only considered as a bioactive ceramic material with high bioaffinity as well as non-corrosive, non-toxic and biocompatible properties to the human body, but also as one of the calcium phosphate crystals that give the tough nature in bone.[16]

One of the natural ingredients found as a valuable economic resource, which generally has not been utilized is *Anadara granosa*. *Anadara granosa* is mostly used as food rich in protein. Meanwhile, the shell is discarded into the waste. Several researches actually have been conducted to increase the additional value of *Anadara granosa*. For instance, *Anadara granosa* shells containing a lot of calcium are used for bone healing process. *Anadara granosa* shells also contain other minerals, such as CaC (98.7%), Mg (0.05%), Na (0.9%), P (0.02%) and other elements (0.2%).[1]

Similarly, a research conducted by Hafisko *et al*.[18] showed that *Anadara granosa* shells contains CaCO3 that can be converted into more biocompatible hydroxyapatite compounds than calcium carbonate alone, so the process is more osteoconductive conditioned by inducing BMP-2. BMP-2 can increase the differentiation of periosteal cells, derivatives of mesenchymal stem cell (MSC) in the formation of chondroblasts and osteoblas.[19] Thus, the use of HA bone graft can effectively improve bone healing.

Another stimulus to accelerate the bone healing process is omega-3. Some studies suggest that omega-3 can reduce the production of proinflammatory cytokines and eicosanoids by inhibiting the metabolism of arachidonic acid (AA), the substrates of eicosanoids (prostaglandin). Omega-3 may also alter the inflammatory gene expression through transcription factor activity, therefore, omega-3 can be potentially considered as anti-inflammatory.[20]

High omega-3 diet, lead to an increase in both activity of serum isoenzyme of alkaline phosphatase (ALP), an enzyme marker of osteoblast activity, and activity of osteoblasts.[21] High omega-3 diet can also lower the production of prostaglandin E2 (PGE2).[22] PGE2 and proinflammatory cytokines are mediators which play an important role for the occurrence of bone resorption. High omega-3 diet, consequently, can result in a decrease in the formation and activity of osteoclasts, which serve to bone resorption.[23]

Fish species in Indonesia mostly consisted of fish oil is *Sardinella longiceps*. Fish oil derived from *Sardinella longiceps* contains a lot of omega-3, namely 13.70% eicosapentaenoic acid (EPA) and 8.91% docosahexaenoic acid (DHA). *Sardinella longiceps* is a type of pelagic fish (containing oil) distributed in all waters in Indonesia. The largest number of *Sardinella longiceps* is found in the Strait of Bali, around Muncar near Banyuwangi (East Java).[24]

The use of *Sardinella longiceps* oil as a therapy in wound healing has already been proven by Wijaya[25] who observed the healing of cut wound in Wistar rats using ointment of *Sardinella longiceps* oil as the test material. The research also shows that at concentrations of 10%, *Sardinella longiceps* oil can make cut wound cover 100% within 7 days. Finally, based on the description above, this research aimed to to know effects of application of *Anadara granosa* shell combined with *Sardinella longiceps* oil on osteoblast proliferation in the healing process of bone defects.

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MATERIALS AND METHOD

This research was a true experimental research with completely randomized design. It means that the control group and the selection of experimental animals in the treatment groups were randomly conducted. Experimental animals used in this research were 32 male rats aged 5 months old and weighed 200-250 grams.

Tools used in this research, moreover, were animal cages, storage tubes for femurs' right bones, scissors, scalpels, anatomical tweezers, resparatorium, scales for animals, food and beverage, microtome for cutting, light microscopy, glass objects, glass cover, bottles, shelves for painting, syringes 3cc, round bur (straight and piece) of Mcisinger® Germany sized 18, micromotor 1200 rpm, fur shaver, probe for eating, dappen Glass, as well as separating disc. Meanwhile, materials used were *Anadara granosa* powder processed by Hafisko *et al.* with Nano size.[18] *Sardinella longiceps* oil processing in Banyuwangi, membranes, sewing silk thread, needles, cotton or tissue, rat food, distilled water to drink those rats replaced every today, 10% buffered formalin, ketamine hydrochloride, xylazine hydrochloride, 10% povidine iodine, 30%, 50%, 70%, 80%, and 96% alcohol, novalgin, as well as 10% EDTA.

Before conducting the research, *Anadara granosa* shell pasta (AG), a combination of *Anadara granosa* and 10% *Sardinella longiceps* oil (AM10), and a combination of *Anadara granosa* and 30% *Sardinella longiceps* oil (AM30) were made. AG shell pasta was made from 5 grams of *Anadara granosa* shell powder mixed with glycerin to obtain 1 ml of pasta. The pasta was mixed and condensed until well blended and homogeneous, and viscosity values obtained were quite thick, so easy to apply. Preparations of AM10 and AM30 then were made by mixing 5 grams of *Anadara granosa* powder, 0.1 ml and 0.3 ml of *Sardinella longiceps* oil, and glycerin to obtain 1 ml of pasta.

All those rats were acclimatized for one week. They were kept in cages with a size of 40 cm (L) x 30 cm (L) x 14cm (T). Afterwards, they were divided into 4 groups, namely the negative control group (C), the group AG using *Anadara granosa* pasta, the group AM10 using *Anadara granosa* pasta combined with *Sardinella longiceps* oil 10%, and the group AM30 using *Anadara granosa* pasta combined with *Sardinella longiceps* oil 30%. Each group consisted of 3-4 rats. Then those were given food in a small container every morning, afternoon, and evening, while drink was supplied in bottles of 300 ml equipped with a small pipe filled with cooked water (Ad libitum).

Bone defects then were performed on the femurs' right bone with the following procedure. First, anesthetics was performed using 1 mL of ketamine mixed with 0.5 mL of xylazine, then injected with a dose of 1.5 mL / 100 g BM on the femurs' right bone intramuscularly.[26] Second, after they began to be unconscious, fur in part to be done the defect was sheared. Third, 10% povidine iodine was applied on the area around the defect for five minutes.[27] Fourth, the soft tissue (skin and muscle) around the defect area was incised about 2 cm using a scalpel and removed using a periosteal elevator. After finding femurs' right bone, a hole defect then was made using bone bur (straight and piece) sized half of the diameter of round Mcisinger® Germany bur sized 18.

In the first group, the holes then were not given any materials because it was used as a negative control (C). In the second group, the holes were given *Anadara granosa* pasta (AG). In the third group, the holes were given AM10, while in the fourth group, the holes were given AM30. After filling, the holes were covered with membrane materials and sewed to cover the soft tissue and the skin on the femurs' right bone, so the graft materials would not be wasted (out of the defect areas). [28] Analgesics then was given one hour after the surgery, ie novalgin® at a dose of 175 mg / kg dissolved in saline to control swelling and pain.[29]

Afterward, making preparations and animal euthanasia were performed on the 7th day after the treatment. Those rats were sacrificed first with ether, and the tissues of the femurs' right bone were removed. The tissues then were peeled to take the bone part after bone grafting by cutting with a separating disc, and put in 10% buffered formalin solution so that the tissues would not be rot and hard, and the affinity values of tissue against staining material increased.[27] After tissue fixation process, decalcification process was conducted using EDTA for 2 months. Specimens of the femurs were made in the form of a sagittal slice preparation with HE staining. The number of osteoblasts in the defect areas then was observed using light microscope with a magnification of 400x. Next, the acquired data were tabulated. And, several statistical tests were performed using a parametric test, oneway Anova test, followed by Tukey-HSD test.

RESULTS

The results of the histological examination showed that there were osteoblasts in the defect areas of all the research groups (Figure 1). The calculation results, moreover, showed that the average number of osteoblasts in group K was 19.00 ± 1.74, in group AG was 34.63 ± 1.94, in group AM10 was 33.50 ± 2.94, while in group AM30 was 38.50 ± 2.28. Figure 2 shows that the lowest average of osteoblast count was found in the control group, while the highest average of osteoblast count was found in group AM30.

The results of the statistical test using SPSS 18.0, moreover, indicated that the data were normally distributed with ($p > 0.05$) and homogeneous ($p = 0.324$). The results of the statistical test using one way Anova test, furthermore, showed that there was a significant difference. Another statistical test, Tukey-HSD test, was then performed to compare one group to another group.

In addition, the results of Tukey HSD test showed that there was a significant difference between group K and the treatment groups using bone graft. There was no significant difference between group AG and both group

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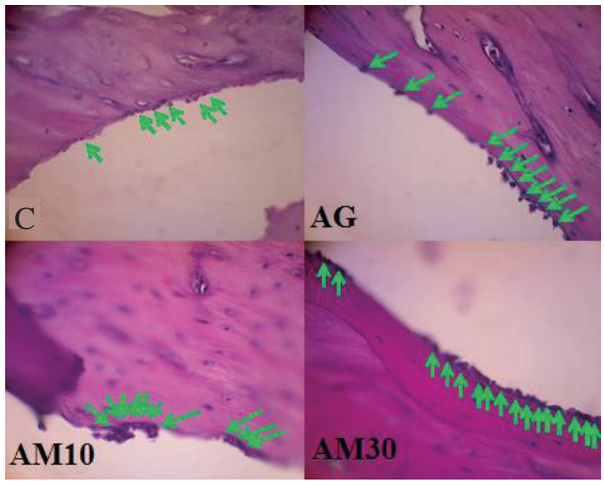

Figure 1. Histological results of osteoblasts in each group.
Group C (negative control), group AG using *Anadara granosa* pasta, group AM10 using *Anadara granosa* pasta combined with *Sardinella longiceps* oil 10%, and group AM30 using *Anadara granosa* pasta combined with *Sardinella longiceps* oil 30%. The presence of osteoblasts was indicated by green arrows.

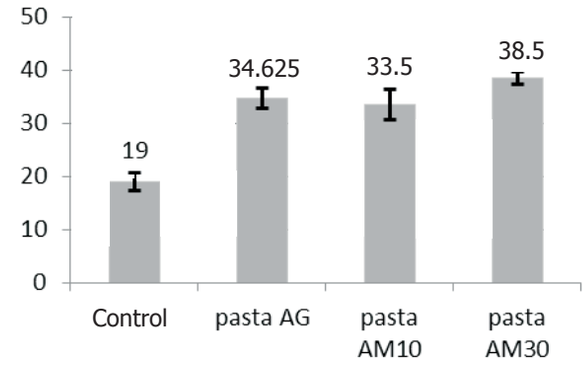

Figure 2. The average number of osteoblasts in each group

Table 2. The results of Tukey-HSD test

Group	AG	AM10	AM30
C	.030*	.049*	.005*
AG	-	0.997	.883
AM10	-	-	.781

AM10 and group AM30 as well as between group AM 10 and group AM30.

DISCUSSION

Animals used in his research were male Wistar rats since there is no hormonal influence that can affect healing mechanisms. Decalcification process in Wistar rats is faster than in other experimental animals. A rat femur also has certain characteristics that are relatively similar to humans, for instance, histologically epicondyle of the femurs has trabecular bone growing well below the cortical bone, resulting in providing a substantial model for dentistry.[30, 31]

Moreover, improvements in bone defect healing process involve a complex physiological sequence. Once damage occurs, hematoma will appear around the site of trauma triggering fibrous tissues to form a lesion area and to develop into callus.[32] In some ways, the bone will activate all intraosseous and periosteum osteoblasts maximally in the defect areas. Number of new osteoblasts are formed from progenitor cells as bone stem cells in the surface of the tissue covering the bones, called as bone membrane.[9]

Bone graft as a substituting bone material actually has been widely used because of its osteoconductive properties.[10] Contribution of the graft begins during the osteoconductive process, namely to make framework as the bone matrix in the recipient tissue. This is followed by stimulation of bone formation during bone healing.[11] Hydroxyapatite possess good biocompatibility and bioactivity. Continually, HA will improve osteoconductive process. The use of HA bone graft can effectively improve bone healing caused by bone defect. The addition of HA bone graft can also attach bioactivity and osteoconductivity in making improvements during the mineral phase.[33]

Based on crystallography and chemical properties, HA approaches bone structure, thus providing an inorganic component in the form of crystalline hydroxyapatite together with sodium, magnesium, carbonate, and structural skeleton.[34] Similarly, the results of this research showed a significant difference between the negative control and the treatment groups AG, AM10, and AM30.

Omega-3 is one of eicosanoids rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA and DHA play a role in generating Resolvins and related compounds, such as protectins through pathways that involve cyclooxygenase and lipoxygenase enzymes. Resolvin E1, resolvin D1, and D1 protectin inhibit transendothelial migration of neutrophils, thereby preventing the infiltration of neutrophils at sites of inflammation. Resolvin D1 inhibits the production of IL-1β, while protectin D1 inhibit the production of TNF and IL-1β. Therefore, resolvins and related compounds play a very important role in stopping the ongoing inflammatory process and in limiting tissue damage.

In addition, EPA and DHA in *Sardinella longiceps* oil can also inhibit inflammatory mediators, such as IL-6, IL-8, and TNF-α. *Sardinella longiceps* oil actually can decrease the activation of NFκB more than corn oil.[35] Decreasing in inflammation products can reduce the ratio of RANKL/OPG. OPG protects bone skeleton by preventing bond between RANKL and RANK as pre osteoclast receptors, thereby suppressing the formation of osteoclasts

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and activating more osteoblasts then leading to new bone formation.[36]

Although there was statistically no significant difference, the average number of osteoblasts in the groups using the combination of *Anadara granosa* shell pasta and *Sardinella longiceps* oil was higher than in the group using *Anadara granosa* shell powder only. This may be due to the non-optimal physicochemical and biomaterial properties of the preparation materials used. Thus, further researches need to be improve bone graft preparations. The tendency of increase in the number of osteoblasts with higher concentration may be the reason for increasing the concentration of *Sardinella longiceps* oil added to *Anadara granosa* shell powder. Finally, it can be concluded that the application of a combination of *Anadara granosa* shell and *Sardinella longiceps* oil can not increase the proliferation of osteoblasts in the healing process of bone defects.

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