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Research Report

Grafting effectiveness of *Anadara granosa* shell combined with sardinella longiseps gel on the number of osteoblast-osteoclast cells

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ABSTRACT

Background: *Bone grafts derived from Anadara granosa shells contain calcium carbonate that possesses bone-healing properties. The combination of Sardinella Longiceps fish oil, containing EPA and DHA, and Anadara granosa shells was assumed to regulate the number of osteoblasts-osteoclasts during the bone-healing process.* ***Purpose:*** *This study aimed to determine the effectiveness of Anadara granosa shell grafts, combined with Sardinella Longiceps fish oil, in the bone-healing process by observing the ratio of osteoblasts-osteoclasts in Rattus novergicus rats.* ***Methods:*** *The Wistar rat subjects (n = 25) were divided into five groups, namely: one untreated group (control), one group treated with bone grafts derived from Anadara granosa shells (P1), and the other three groups treated with a combination of Anadara granosa shells and Sardinella longiceps fish oil at concentrations of 10%, 20%, and 30% (P2, P3, and P4). Then, a wound equivalent in size to half the diameter of a round bur (±1.5mm) was intentionally inflicted on the right femur of all the subjects. The rats were subsequently sacrificed on day 14, their femur in the transversal side being cut before HE staining was completed. Thereafter, the ratio of osteoblasts to osteoclasts was measured by means of a light microscopy. The data was subsequently analyzed using one-way ANOVA.* ***Results:*** *The average number of osteoblasts in all research groups increased, viz: 9.420±0.8044 for control group (K), 12.080±0.79811 for group P1, 20.020±0.7190 for group P2, 25.940±0.7197 for group P3, and 36.280±0.9985 for group P4. Similarly, the number of osteoclasts in all groups subject to analysis also increased, namely: 1.73±0.098 for group K, 2.19±0.305 for group P1, 1.60±0.088 for group P2, 1.60±0.724 for group P3, and 1.80±1.302 for group P4. Moreover, the results of the One-way Anova test confirmed that there were no significant differences in osteoclasts between all research groups ($p>0.05$). The results of the one-way ANOVA and LSD tests confirmed there to be significant differences ($p<0.05$) between group K and other treatment groups (P1, P2, P3, and P4).* ***Conclusion:*** *The grafts derived from the combination of Anadara granosa shells and Sardinella longiceps gel can induce the production of osteoblasts, but not in the numbers necessary during the healing processin the femurs in Rattus novergicus rats.*

Keywords: *Bone graft; Anadara granosa; Sardinella longiceps; osteoblasts; osteoclasts*

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INTRODUCTION

In dentistry, the main causes of bone damage are predominantly those of tooth extraction (90%), trauma, and conditions, such as cysts or tumors affecting the jaw. Post-extraction, the alveolar bone will undergo an anatomical change in its shape involving several stages. This condition can, in turn, cause the jawbone to shrink and become thin and brittle, thus facilitating fractures, reducing the success of other dental treatments, and decreasing the function of mastication and food digestion.[1,2]

Damage to the alveolar bone is a post-tooth extraction complication potentially impacting negatively on an individual's health, while also having an undesirable

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aesthetic effect. The damage can, nevertheless, be corrected by bone grafts that serve to restore the contours of bones or stimulate the formation of new bone. Bone grafting is a surgical procedure whereby missing bone is replaced with substitutes either taken from the patient's own body or which are artificial, synthetic or naturally occurring. Bone grafting is feasible since bone tissue demonstrates the ability to regenerate effectively, given the availability of space for bone growth. Bone formation can then occur owing to several factors influencing the acceleration of healing. Bone graft is one option to precipitate the bone healing process, incorporating the use of a material that promotes reconstruction, stabilizes the structure and bonding of bones, and stimulates the process of osteogenesis and the healing of large bone defects.[3–5]

The bone graft material normally utilised is derived from non-metallic synthetic materials obtainable from ceramic materials (potassium), composites, and polymers. This material must be biocompatible and osteoconductive and can also be fused with bone, thereby enhancing the bone regeneration process.[4] In general, bone grafts can be classified into four common types, namely: autograft, allograft, xenograft, and alloplast. Xenograft involves the use of bone derived from donors drawn from species other than that of the recipient, such as *Anadara granosa* shells.[5]

Anadara granosa shell is of a type commonly found in both East and Southeast Asia and contains red blood pigment/hemoglobin enabling the shell to exist in conditions with relatively low levels of oxygen.[6,7] *Anadara granosa* shell is also classified as a mineral since biopolymer composites consist of between 95% and 99% $CaCO_3$ in the form of aragonite crystals.[8] Unfortunately, *Anadara granosa* shells are relatively little used, usually being thrown away and allowed to break up naturally.

In addition, according to previous research, the presence of nano-calcium carbonate ($CaCO_3$) crystals derived from shells can be used in bone tissue engineering since they possess the potential to mimic the original composition, structure, and bone properties.[9] The investigation referred to also showed that an increase in osteogenic activity of alkaline phosphatase may accelerate the differentiation of mesenchymal stem cells. Therefore, the activity of alkaline phosphatase is considered to be an indicator, or bone marker, that signifies the presence of mineralized bone and promotes the formation of HA in osteoblast matrix vesicles by releasing it into the extracellular matrix and increasing osteoblast cell differentiation. Subsequently, calcium carbonate stimulates macrophages in areas containing defects, with the macrophages, together with inflammatory cells, strengthening angiogenesis processes.[10]

On the other hand, fish oil is rich in omega-3 polyunsaturated fatty acid (PUFA), composed of eicosapentaenoid acid (EPA) and docohexaenoic acid (DHA), which plays an important role in maintaining human health.[11] Similarly, *Sardinella longiceps* fish oil contains n-3 PUFA, 13.70% (EPA), and 8.91% DHA.[12] The high levels of EPA and DHA contained in *Sardinella longiceps* fish oil possess the potential to regulate the formation and activity of osteoblasts and osteoclasts. Meanwhile, omega-3 PUFA has the ability to act as a vasoconstrictor and platelet aggregation secreting growth factors, such as VEGF, that directly affect the formation of new blood vessels.[13] Previous research confirmed that omega-3 PUFA is also able to increase mediators of bone and tooth formation.[14]

The study reported here aimed to evaluate the effectiveness of bone graft derived from a combination of *Anadara granosa* shells and *Sardinella longiceps* fish oil gel by measuring the number of osteoblasts and osteoclasts during the bone healing process in animal subjects.

MATERIALS DAN METHOD

The research was truly experimental in character with a completely randomized design. Thus, animal subjects used in both the control and treatment groups were randomly selected and consisted of two-month old, male Wistar rats weighing between 150 and 357 grams. The total number of animal subjects used as samples was 25, divided into five groups. The research was then conducted following approval from the Ethical Commission for Animal Subjects Faculty of Dentistry, Universitas Hang Tuah, Surabaya No.193/KEPK/IX/2016.

The bone graft was prepared by collecting, boiling and cleaning *Anadara granosa* shells taken from seaweed waste.[15] The shell powder was then sterilized by the application of gamma-ray radiation at BATAN's Jakarta laboratory. Thereafter, 0.2 grams of *Sardinella longiceps* fish oil was mixed with 2 ml of 10% gelatin to produce 10% *Sardinella longiceps* gel. In addition to producing *Sardinella longiceps* gel at concentrations of 20% and 30%, 0.4 grams and 0.6 grams of *Sardinella longiceps* fish oil were mixed with 2 ml of 10% gelatin.

The experimental procedure which the animal subjects underwent began with seven days of acclimatization. The Wistar rats were then divided into five groups, namely: one group not receiving treatment (control/K), one group treated with bone grafts derived from *Anadara granosa* shells (P1), and the other three groups administered a combination of *Anadara granosa* shells and *Sardinella longiceps* fish oil at concentrations of 10%, 20%, and 30% (P2, P3, and P4) respectively. Following the acclimatization process, surgery was performed on the dextral side of the subjects' femurs along the lines of the procedure performed by Fleckhell.[16]

The Wistar rats were anesthetized by the administering of ketamine and xylazine intramuscularly at a dose of 0.11 mL/100 gr BM.[17] Once unconscious, subjects' fur in the area where the defect had been induced was shaved using a Gillete razor. 10% povidine iodine was applied to the area for five minutes as an antiseptic.[18] A two-centimetre long incision was made using a One Med Indonesia surgical

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knife, before the removal of soft tissue (skin and muscle) by means of an Osung periosteal elevator. Defects as deep as a half the diameter of the bur (1.5mm) were produced on the dextral and lateral areas of the femur using a German-made, size 18, Mcisinger® round bur, together with a straight hand piece. After defects in the femur had formed, all research groups received different types of treatment. Then, the application of membrane was performed. Each surgical procedure invariably culminated in suturing to close skin wounds and cover soft tissue.[19] The subsequent administering of novalgin consisted of a dose 0.09 cc/200 gr BM, before the antibiotic interflox was given as a dose of 0.1 cc/100 gr BM for three days. In short, this procedure was usually required in order to control inflammation and pain.[20]

At this point, the rats were euthanized using ether before their femur was taken from the dextral side on day 14.[17] The femur specimens collected were hulled in order to access the bone after grafting by cutting it with a separating disc, and then inserting it into 10% formalin buffer solution. This was intended to keep the tissue from decomposing and hardening, as well as to increase the affinity of the tissue against the paint.[19] After the process of tissue fixation, a decalcification process was induced by means of administering ethylene diamine tetra acid (EDTA) over two months. The femur specimens collected were then subjected to sagittal piece preparations before haematoxylin eosin (HE) staining was carried out. Thereafter, the number of osteoblasts and osteoclasts in the defect areas was measured with a light microscope (Olympus® CX21, Japan) at 400X magnification. The data obtained was analyzed to obtain details of data distribution and data summary in order to clarify the results. The hypothesis was then tested using a parametric statistical test, a one-way ANOVA test and, finally, a least significance difference (LSD) test.

RESULTS

Clinically, the defects in the dextral and lateral sides of the femurs of Group P1 subjects were slightly closed due to a less than optimal process of bone formation. There were also significant differences in the number of osteoblasts between group P1 and groups P2, P3, and P4. group P1 had fewer osteoblasts than groups P2, P3, and P4.

The histological features indicated significant differences in the number of osteoblasts and osteoclasts located at the defect sites in all research groups (Figures 1 and 2). The results also revealed that the average number of osteoblasts in all research groups increased, viz: 9.420 ± 0.8044 for group K, 12.080 ± 0.79811 for group P1, 20.020 ± 0.7190 for group P2, 25.940 ± 0.7197 for group P3 and 36.280 ± 0.9985 for group P4. Similarly, the average number of osteoclasts in all research groups also increased, 1.73 ± 0.10 for group K, 2.19 ± 0.305 for group P1, 1.60 ± 0.09 for group P2, 1.60 ± 0.07 for group P3, and 1.80 ± 0.302 for group P4. Moreover, the results illustrated the morphologies of osteoblasts and osteoclasts in the defect sites of all research groups.

In Figure 2, the lowest number of osteoblasts was found in group K, while the highest was that of group P3. Similarly, the average number of osteoclasts showed almost the same results. Furthermore, the statistical test results using SPSS 18.0 confirmed the data as being both normally distributed (p>0.05) and homogeneous (p=0.324) with a significance level of 0.05. Furthermore, the one-way ANOVA test results indicated a significant difference in the presence of osteoblast indicators, but not those of the osteoclast variety. Another further statistical test, a LSD test, was then performed in order to compare osteoblast indicators between one group and another (Table 1). The

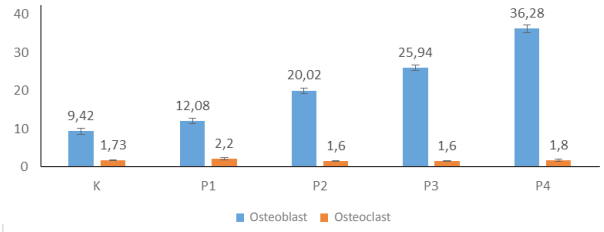

Figure 2. The average number of osteoblasts and osteoclasts in each research group (n=5).

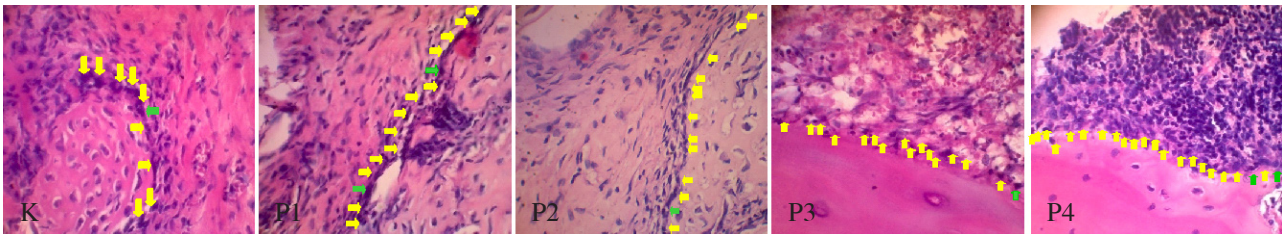

Figure 1. The histologic features of osteoblasts in each research group.

Note: K (negative control), P1 (group with the administration of calcium powder derived from *Anadara granosa* shells), P2 (group with the administration of HA powder derived from *Anadara granosa* shells and 10% *Sardinella longiceps fish oil*), P3 (group with the administration of HA powder derived from *Anadara granosa* shells and oil 20% *Sardinella longiceps fish oil*), and P4 (group with the administration of HA powder derived from *Anadara granosa* shells and 30% *sardinella longiceps fish oil*). Yellow arrows indicate the presence of osteoblasts, while osteoclasts are indicated by green arrows.

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Table 1. Results of the Post-Hoc LSD test for osteoblast

Average		Mean difference (I-J)	P valvue
Group (I)	Group (J)		
K	P1	-2.3400*	.048
	P2	-11.2800*	.000
	P3	-16.2000*	.000
	P4	-26.5400*	.000
P1	P2	-8.9400*	.000
	P3	-13.8600*	.000
	P4	-24.2000*	.000
P2	P3	-4.9200*	.000
	P4	-15.2600*	.000
P3	P4	-10.3400*	.000

Note: This table shows the comparison of the mean number of osteoblasts between groups. The average number of osteoblasts in group P> group K. The higher the concentration of group P, the greater the average number of osteoblasts.

results of this LSD assay showed that there were significant differences between one group and another ($p<0.05$).

DISCUSSION

Anadara granosa shells combined with *Sardinella longiceps* gel at concentrations of 10% and 30% did not effectively induce osteoblast proliferation, although at these concentrations there was a tendency to increase osteoblast proliferation.[20] Therefore, this research used *Sardinella longiceps* fish oil of 10% and 30% concentrations as a reference, while also employing the gel form to improve the attachment of the test materials to bone defects.

The animal subjects used in this research were Wistar rats since they not only possessed certain characteristics relatively similar to those of humans but also demonstrated similarities to the physiological aspects of human metabolism.[21] This research specifically used Wistar rat femurs since they possess a trabecular bone formation that is well developed in the cortical bone. This is identical to the alveolar bone in Wistar rats, allowing it to be used as a model to study the regeneration of the jawbone.[22] Furthermore, male Wistar rats were selected as samples for this research based on the consideration of their lacking or having relatively little estrogen, as well as their enjoying more stable hormonal conditions. Stress levels in females are also known to be higher than those of males.[23] Besides, the effects of estrogen in suppressing osteoclastic activity are known to occur indirectly through their action on osteoblastic receptors. One of the cytokines produced by osteoblasts, transfoming growth factor-β (TGF-β), can even be suppressed by estrogen. In fact, TGF-ß plays a role in osteoclast differentiation.[24]

The normal bone healing process begins with inflammation since the cells of first defense, such as leukocytes, lymphocytes, monocytes, and macrophages are activated. Macrophages are phagocytic cells produced in bone marrow that play an important role in inflammation, while they also remove cytokines consisting of proinflammatory, anti-inflammatory, and growth factors. The macrophages will then trigger the secretion of pro-inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6) as inflammatory mediators to strengthen the immune response and increase metabolic processes.[25] The presence of proinflammatory cytokines will, as a result, increase the production of preosteoclasts so that receptor activator of nuclear factor kappa (RANK) are also widely produced. RANK will subsequently bind to receptor activators of nuclear factor kappa ligand (RANKL), thus inducing osteoclasts to increase bone damage.[26]

The proliferative or reparative phase begins when the inflammatory phase releases the cytokines and growth factors, resulting in the proliferation of fibroblasts to form extracellular matrix and calcium salts through an attachment, thus forming a woven bone.[27] This can be seen in group K, a group with normal bone healing process, in which the osteoblasts remained visible, although without any treatment. Osteoblasts are known not only to take various forms, from cuboid to cylindrical within the basophil cytoplasm, but also to be visible around the osteoidous layer where new bone is formed. Meanwhile, the osteoclasts are known to have very large branched shapes with multiple cores.[28] In group K, defects were not clinically closed since the process of bone formation was undetected.

On the other hand, group P1 was a group given bone grafts derived from *Anadara granosa* shells containing calcium carbonate. This material was expected to accelerate the bone healing process since calcium carbonate serves as a skeleton in bone formation, improves the wound healing process and acts as a mineral reservoir that helps in new bone formation.[29] Bone grafts derived from *Anadara granosa* shells is beneficial in the treatment of bone defects.[29] The structure of *Anadara granosa* shells is generally similar to cancellous bone and demonstrates bone-like mechanical properties. The shells are also known to contain high concentrations of calcium carbonate which has biocompatible, osteoconductive, and biodegrable properties. Moreover, the shells act as an adequate carrier for growth factors and enable cell attachment, cell growth, cell spread, and cell differentiation.[30] However, *Anadara granosa* shell powder can trigger an inflammatory response and increase the number of osteoclasts. Therefore, group P1 had a higher number of osteoblasts compared to that of group K (Table 1). Nevertheless, the number of osteoclasts in group P1 tended to be descriptively higher than in group K, although there was no statistically significant difference ($p>0.05$).

Group P1 was treated only with bone graft derived from shells in the form of a brittle powder. This made it rather difficult for bone to be formed based on the required

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substituting graft material. The powder used for the graft on the damaged bone usually suffers from weakness that does not promote stable grown as a graft.[31]

Furthermore, groups P2, P3, and P4 were given bone grafts at concentrations of 10%, 20% and 30%, derived from a combination of *Anadara granosa* shells containing calcium carbonate and *Sardinella longiceps* gel containing omega-3. *Anadara granosa* shells, can be selected as bone replacement biomaterials since they are known to contain calcium carbonate compounds.[31] CaCO3 crystals derived from shellfish can facilitate osteoblast proliferation, differentiation and adhesion. The osteogenic activity of alkali phosphatase can also be enhanced by CaCO3 crystals. Increased osteogenic activity of alkali phosphatase can then increase the differentiation of mesenchymal cells.[9]

Sardinella longiceps fish oil contains 12.5% n-3 PUFAs. n-3 PUFAs consist of EPA and DHA and are capable of producing resolvin that can serve as an anti-inflammatory mediator.[32] Resolvin promotes the elimination of inflammation by creating a macrophage 2 (M2) phenotype which releases high levels of IL-10 inhibiting TNFα, IL-6, and IL-1.[32] These proinflammatory cytokines then trigger the formation of RANKL in osteoblasts that can bind to RANK in the pre-osteoclast to differentiate into osteoclasts.[33] As proinflammatory cytokines decrease, the number of osteoclasts is also expected to decline, while that of osteoblasts is anticipated to increase. Proinflammatory cytokines can directly stimulate osteoblast apoptosis and its precursors, or do so indirectly by stimulating Fas expression of potential proapoptotic mediators.[33] The bone graft used in this research was derived from a combination of *Anadara granosa* shells and *Sardinella longiceps* gel of three different concentrations in order to determine the most effective in the bone healing process. Although within the investigation reported here the number of osteoclasts in all research groups was largely similar, the results of the one-way ANOVA test highlighted a significant difference in the number of osteoblasts. This suggests that osteoclasts might be used as the only indicator. Therefore, the osteoblast-osteoclast ratio needs to be monitored during the healing process since it is considered an appropriate indicator of healing or bone formation.[34]

From a clinical perspective, in groups P2, P3, and P4 the defects were effectively, although not optimally, closed. Closure defects were due to the administration of bone grafts derived from the combination of *Anadara granosa* shells and *Sardinella longiceps* fish oil gel. Effective material attachment to the defects was usually expected to accelerate the bone healing process.[35] Thus, the provision of bone graft derived from the combination of *Anadara granosa* shells and *Sardinella longiceps* gel in this research was expected to accelerate the healing process since it could serve as a scaffold for new bone formation.[35] The stimulation of bone grafts was then expected to improve the cellular biology activity by analyzing the osteoclast ratio (Figure 2). During the bone healing process, bone graft usually stimulates and triggers osteoblast proliferation, before migrating to the defect site.[33] It can be concluded that bone grafts derived from the combination of *Anadara granosa* shells and *Sardinella longiceps* gel can successfully generate osteoblasts and osteoclasts indicating a more effective healing process. The most effective concentration of *Sardinella longiceps* gel used was 30%.

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