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THE EFFECT OF HYDOXYAPATITE XENOGRAFT OF HARUAN FISH (*Channa striata*) BONE ON THE NUMBER OF OSTEOBLAST AND OSTEOCLAST

(*In Vivo* Study On Mandibular Bone of Male Guinea Pigs)

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ABSTRACT

***Background**: Periodontitis is an inflammation of the periodontium tissue caused by certain microorganisms. The condition it self is marked by alveolar bone damage. The repair of damaged alveolar bone requires a process of bone remodeling. To help the process of bone remodeling, a graft material from haruan fish bone is used. The haruan fish bone is a waste from industrial processing crackers that have not been used by the people of South Kalimantan. The fish bone has inorganic substances such as calcium and phosphate. **Objective**: To prove the effect of xenograft hydroxyapatite of haruan fish bone on the number of osteoblast and osteoclast in remodeling process of male marmot bone. **Methods and materials**: This study used a pure experimental study with posttest only control group design. This study used two treatment groups which were the treatment group using hydroxyapatite haruan fish bone and a negative control group using aquades. **Results**: The mean value of osteoblast cell number after given hydroxyapatite of haruan fish bone was 15,72 cells, which was higher than given aquades only with 5,08 cells. While the number of osteoclast after given hydroxyapatite of haruan fish bone was 6,72 cells. It was higher than the given aquades only group which results in 4,04 cells. The Independent T test showed a significant difference ($p<0.05$) between the hydroxyapatite of haruan fish bone group and the negative control group with osteoblast data where $p = 0,000$ and osteoclast data where $p = 0,006$. **Conclusion:** Giving hydroxyapatite ofharuan fish bone can increase the number of osteoblast and osteoclasts in the remodeling process of male marmot bone.*

***Keywords:** Bone remodeling, hydroxyapatite of haruan fish bone, osteoblast cell, osteoclast cell.*

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INTRODUCTION

Periodontitis is an inflammation of the periodontium caused by certain microorganisms, characterized by progressive loss of epithelial attachment, damage in periodontal ligament and alveolar bone destruction also the formation of pocket.[1,2,3] The untreated pocket will result in a gingival recession and a progressive alveolar bone resorption. Those conditions will cause the tooth to come out of its socket.[1,4,5,6]

Over the past decade, surgical methods used to repair alveolar bone damage are GTR / GBR

(*Guided bone regeneration*) .[7,8] The principle of GTR / GBR is using bone graft as scaffolds to stimulate the formation of damaged bone tissue through osteogenesis, osteoinduction and osteoconduction. Bone graft has specific characteristics such as non-toxic, does not cause root resorption (*ankylosis*), stimulates the formation of periodontal ligaments, requires minimal surgical techniques and easy to obtain.[8,9,10]

In general, there are four types of bone graft that have been used to repair bone damage due to periodontal disease, such as autograft, allograft, xenograft, and alloplast.[9] Each bone graft has disadvantages and advantages. Autograft has the advantages of biocompatible, osteoinduction, osteoconduction, and osteogenesis, but has disadvantages of the procedure to take the bone from the body it self and move it to the damaged area, the number is limited and can cause the risk of tissues degeneration. Allograft, alloplast and xenograft have advantages as a graft material which is available in large quantities, and the procedure does not require surgical wound to get the donor material from their body. However, this kind of graft have some disadvantages such as causing autoimmune reaction and the risk of disease transmission. Especially for alloplast graft material, it is known to be relatively expensive.[11.12]

From the various type of bone grafts, xenograft hydroxyapatite is a frequently used graft material. Hydroxyapatite xenograft derived from natural materials, especially animal bones because it has the same inorganic substance structure like human bone former substance such as calcium and phosphate. Today many studies use natural ingredients to repair bone damage, such as research using lemurs bone, cow bones, pig bones, horse bones, shellfish shells and fish scales. Research by Vidyahayati et al (2016) shows that hydroxyapatite may help to increase the number of osteoblasts in bone formation by day 14.[16] In South Borneo, many people make crackers from haruan fish flesh, so haruan fish bone only become a waste and has not been utilized optimally. The content of haruan fish bones such as calcium and phosphate is ideal to assist the process of bone remodeling.[13] The purpose of this study is to prove the effect of xenograft hydroxyapatite haruan fish bone on the number of osteoblast and osteoclasts in the bone remodeling of male marmot.

MATERIAL AND METHOD

This research was pure experimental research with posttest only control group design. The samples used in this study were male marmots (*Cavia cobaya*).

The number of samples were obtained by using Lemeshow formula, which consisted 10 marmots as the total number. The inclusion criteria in this study were male sex, age 2-3 months, and in healthy or normal condition: characterized by movements of marmots such as eating, drinking, no injuries or disabilities. The exclusion criteria in this study were dead marmots and marmots looked ill (inactive motion, do not want to eat, and dull hair / baldness).

Process of creating xenograft hydroxyapatite of haruan fish bone

The making of xenograft hydroxyapatite haruan fish bone was using *De-proteinized* method. First, the preparation of the sample was done by washing and cleaning the fish bone from the remaining flesh and dirt which left behind. After that, large bones were cut into chips shape using a bone-cutting machine, then the bones were re-washed with high pressure water to remove bone marrow and soft tissue remaining on the bone surface. Furthermore, fat removal was done by extracting the fat contained in the bone using chloroform: methanol (1:1) and the rest of the extracting agent was eliminated with aquadest.

The step was followed by the method of Milling which was the destroying of the bone to obtained a smaller bone with a size of ± 60 Mesh. Then the bone graft making was done by using de-proteinitation method of bone samples. It was resulted from Milling de-proteinitated through a process of hydrolysis using sodium hydroxide (NaOH) with a concentration of 1 M at 70^{o}C for 7 hours. The obtained de-proteinized bone was precipitated for 24 hours, then filtered while getting rinsed with aquades to normal pH. The obtained de-proteinized bone was dried using an oven with 60^{o}C, then filtered with 200 mesh (50-

100nm) strainer. After that the packaging is done with a screw-covered bottle and sterilized with gamma-ray radiation.

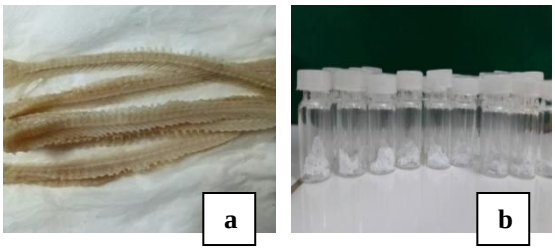

Figure 1. Cleaned Haruan Fish Bone (a) and Hydroxyapatite of Haruan Fish Bone (b).

Treatment of sample

Before conducting the research, the research proposal had been submitted to the Animal Care and Use Committee at the Faculty of Dentistry of Lambung Mangkurat University and was stated as eligible based on the ethical certificate number: 043 / KEPKG-FKGULM / EC / IX / 2017. The samples were grouped with simple random sampling, then divided into 2 groups, each consisted of 5 male marmots. The treatment group was given a haruan fish hydroxyapatite and the negative control group was given distilled water. Before the research began, the marmot was empowered for 12 hours. The randomly-drawn marmot was sedated using 2% lidocaine hcl on the buccal and lingual portion of the mandibular incisor until the marmot was numb. Then, the mandibular central incisor was extracted by using a needle holder, after the extraction, the graft material was inserted into the marmot tooth socket and sutured until the socket closed using 3/0 catgut thread and 16G needle.

The preparation of histopathology sample

Preparation of the samples were made after the cutting of the marmots' mandible jaw. Fixation of the tissue was by soaking it in a container with fixation solvent (*buffer formalin*) for 24 hours. After that the soaking was done by de-calcification solution (Shandon TBD-1 Rapid Decalcifier for 3 days).

The pieces of tissue were made into embedding cassette then rotated by tissue processor approximately 14.5 hours with 10% BNF solvent (I) and then 10% BNF (II) each for 1 hour, 70% alcohol for 1.5 hours, alcohol 80% for 1.5 hours and 96% alcohol for 1.5 hours, absolute alcohol (I) for 1 hour, absolute alcohol (II) for 1 hour, absolute alcohol (II) for 1 hour, xylol (I) and xylol (II) each for 1.5 hour, paraffin liquid (I) for 1 hour and paraffin liquid (II) for 1 hour.

The tissue was inserted into the base mold. Then, filled with liquid paraffin and attached to embedding cassette until cold. The tissue was cut with microtomes. The block was placed in the microtome and then cut as thick as 5µm, the result was placed in the water bath with temperature 37-40^{o}C until no wrinkles and then placed on the object glass. The staining was performed using *Hematoxylin Eosin* (HE) with the following steps: xylol (I) 5 minutes, then xylol (II) for 5 minutes, xylol (III) for 5 minutes, absolute alcohol (I) for 3 minutes, absolute alcohol (II) for 3 minutes, 80% alcohol for 3 minutes, 70% alcohol for 3 minutes, wipe with Aquadest / water, Mayer *Hematoxyll*in for 2-5 minutes, clean back with Aquadest / water, Eosin for 5-10 minutes, alcohol 70% for 3 minutes, 80% alcohol for 3 minutes, absolute alcohol (I) for 3 minutes, absolute alcohol (II) for 3 minutes, xylol (I) for 5 minutes, xylol (II) for 5 minutes, xylol (III) for 5 minutes, xylol (IV) for 5 minutes, and mounting.

The observation of osteoblast and osteoclast and cell counting

Observation was done by using a light microscope (*Olympus, United States*) completed with a digital camera with 1000 times magnification. Calculation of the number of osteoblast and the specified osteoclast presented in all of the outer boxes was done in the determined of tooth extraction area and performed in five view fields. Before the movement of the site, the last osteoblast and osteoclast cell area at the previous site were observed to avoid recurring calculations. The results of the calculation were summed and calculated to obtain the average value for one research subject.

RESULT

Based on the result of the research, mean value of the osteoblast and osteoclast in all treatment group are as follow:

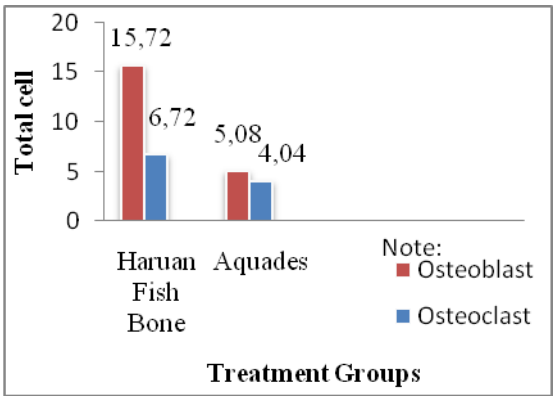

Figure 2. Mean value of Osteoblast and Osteoclast cell in male marmot.

Figure 2 above shows that the mean value of osteoblast and osteoclast in the haruan fish bone hydroxyapatite treatment group is higher than the aquades treatment group.

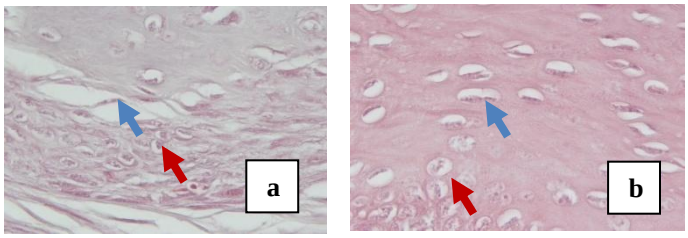

Figure 3. Histopathology View of Osteoblast Cell (Blue arrow) and Osteoclast Cell (Red arrow) (a) Group Given Hydroxyapatite of Haruan Fish Bone (b) Negative Control Group Given Aquades Observation by *Olympus* Light Microscope with 1000x Magnification.

Table 1. The result of *Shapiro-Wilk*normality test

Treatment	Osteoblast cell Significance (*)	Osteoclast cell Significance (*)
Hydroxyapatite of haruan fish bone	0,150*	0,387*
Aquades	0,814*	0,544*

***=** Data distributed normally (p>0,05)

Data analysis in this research used *Shapiro-Wilk* normality test. It obtained a significant value of p>0,05 for both osteoblast and osteoclast data groups, which means that data were distributed normally, so it is eligible to perform the *independent T test* parametric test.

Tabel 2. *Independent T test* result

Treatment	Total of Osteoblast cell Mean ± SD	Sig.	Total of Osteoclast cell Mean ± SD	Sig.
Hydroxyapatite of haruan fish bone	15,72 ± 3,26	0,000*	6,72 ± 1,30	0,006*
Aquades	5,08 ± 0,22		4,04 ± 0,65	

*=There is significant difference (p<0,05)

Based on *Independent T test* results in the table above, it can be seen that the significance of osteoblast and osteoclasts has p-value<0,05. It can be concluded from the results of the Independent T test that there is a significant difference in the number of osteoblasts and osteoclasts between the haruan fish bone hydroxyapatite group and aquades group. The average osteoblasts and osteoclasts cells were higher in the haruan fish bone hydroxyapatite group compared with the group given distilled water.

DISCUSSION

The results showed that osteoblast number were higher than osteoclast after the 14th day of haruan fish bone hydroxyapatite application on male marmots. The increase of osteoblast cells in male marmots on day 14 is caused by the osteoblast which are actively working to repair damaged bone by forming collagen and non-collagen proteoglycans and regulate the mineralization process between calcium and phosphate during the reversal phase. This phase is known to have the peak levels of osteoblast cells.[14,15] Similar with the study of Vidyahayati et al (2016) which proved that the use of hydroxyapatite can aid the process of bone remodeling. It is characterized by an increase in the number of osteoblast cells by day [14.16]

This study is also supported by a research from Ismardianita et al (2017) by using animals sample which proved the increase of osteoblast cell number by day 14. It is also supported by a research by Salim et al (2015) which says that the process of resorption and bone formation in marmots takes about 2-4 weeks.[11,17] In this study, the decrease in osteoclast cell number on day 14 is due to the inactivation of osteoclast to resorb bone during reversal phase. It is also supported by Delaisse (2014) research that says the decrease in the number of osteoclast during reversal phase is caused by the activation of osteoblast to repair bone damage that has been resorbed by osteoclast.[18]

This research also proves the increase in osteoblast and osteoclasts number after given haruan fish bone hydroxyapatite which is higher when compared with aquades treatment group on male marmots tooth sockets. This happens because the hydroxyapatite of haruan fish bone has osteoconduction properties that function as a scaffold. This scaffold allows blood vessels into the damaged bone tissue, which stimulate the formation of new bone through the inter-porosity of haruan fish bone graft. This inter-porosity is produced by crystal nanoparticles from the graft materials of haruan fish bone with size about 50-100nm. This is supported by a research from Guarnieri et al (2017) which states that hydroxyapatite serves as a scaffold and has interporosity to facilitate the cell transfer for new bone formation.[19] It can be concluded that giving haruan fish bone hydroxyapatite increases the number of osteoblast and osteoclasts in the bone remodeling of male marmots.

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