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RESEARCH ARTICLE

Application of Hydroxyapatite scaffold from *Portunus pelagicus* on OPG and RANKL expression after tooth extraction of *Cavia cobaya*

Michael Josef Kridanto Kamadjaja[1], Sherman Salim[1], Birgitta Dwitya Swastyayana Subiakto[2]
[1]Departement of Prosthodontic, Faculty of Dental Medicine, Universitas Airlangga, Surabaya - Indonesia.
[2]Resident of Prosthodontic, Faculty of Dental Medicine, Universitas Airlangga, Surabaya - Indonesia.
*Corresponding Author E-mail: **michael-j-k-k@fkg.unair.ac.id**

ABSTRACT:

Objective: This study was to determine OPG and RANKL expression after hydroxyapatite (HA) scaffold from crab shells *(Portunus pelagicus)* application in tooth socket of *Cavia cobaya*. **Methods:** This study was a post-test only control group design. Twenty four *Cavia cobaya* was divided into 4 groups. The lower left incisor was extracted and given a combination of HA and gelatin scaffold. Experimental animals were sacrificed on the 7th and 14th day. The amount of OPG and RANKL expression was calculated under a light microscope at 1000x magnification. The statistical analysis was done by One Way ANOVA Test and Tukey HSD. **Results:** Compared to other groups, the lowest and the highest level of OPG and RANKL were in P14 group. **Conclusion:** HA scaffold from crab shells (*Portunus pelagicus*) can increase OPG expression and decrease RANKL expression in the process of regenerating alveolar bone after tooth extraction.

KEYWORDS: OPG, RANKL, crab shell, socket preservation, scaffold, Medicine.

INTRODUCTION:

Tooth extraction is a common procedure performed in the field of dentistry that can cause trauma to the bone and surrounding tissues. That trauma causes inflammation which leads to alveolar bone resorption. If this condition does not resolved immediately, it can affect the making of dentures. Therefore, socket preservation is needed to prevent alveolar bone resorption. Hydroxyapatite (HA) scaffold, which has biocompatible, osteoconductive, and osseointegration properties, can increase bone regeneration process[1].

Socket preservation is a way to prevent alveolar bone resorption due to trauma post extraction. The trauma post extraction causes inflammation, which causes alveolar bone resorption and unfitting dentures. Bone damage caused by tooth extraction can be treated using bone grafts to maintain the dimensions of the alveolar bone[2]

Hydroxyapatite (HA) is the main mineral found in bones and teeth. HA crystals has the same composition as bones. This material is biocompatible, osteoconductive, and can be merge with bone so that it can enhance the process of bone regeneration. Hydroxyapatite being bioactive in nature is highly compatible with the tissues and bones and has a very wide spectrum of activity to treat highly infectious disease such as cancer.[1,3]

Portunus pelagicus contains calcium carbonate in large quantities, which is 40-70% of the crab shells. Calcium carbonate is a source of calcium which can be used as a HA synthesis material. HA biomaterials consist of bioactive components that are compatible with bones and teeth. This allows the use of crab shells as an alternative bone substitute biomaterial based on the similarity of chemical composition.[4-6]

After tooth extraction, a bone remodeling process occurs which is regulated by osteoclasts and osteoblasts. It is known that osteoblasts express Osteoprotegerin (OPG). OPG is a natural inhibitor which inhibit the interaction of Receptor Activator of Nappar-Nuclear Factor Kappa-B Ligand (RANKL) and Receptor Activator of Nappar-Nuclear Factor Kappa-β (RANK). So OPG and RANKL play an important role in the process of osteogenesis. This research is to prove that HA scaffold from crab

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shell *(Portunus pelagicus)* can influence OPG and RANKL expression in socket post-tooth extraction of *Cavia cobaya.*

MATERIAL AND METHOD:

This research was conducted in accordance with the ethics governing experimental use of animal subjects as approved by Health Research Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga, No. 040/HRECCFODM/II/2019.

Subjects:

This research was a true experimental study. 24 male, three months old, and 300-350 grams in weight of *Cavia cobaya* was the subject of this research. Subjects were divided into control group and treatment group, each of groups consist of six subjects. The treatment groups were given 1.5 gr of HA Scaffold and 0.5 gr of Gelatin.

Procedure for making HA from crab shells[4]

Portunus pelagicus shells were cleaned using distilled water and removed from soft tissue. It was soaked using a chlorine solution with a ratio of 30 ml of chlorine and 5 liters of water, followed by 3% H_2O_2 for 24 hours and dried at room temperature. The process of shell's calcination was carried out at 1000℃ for approximately 2 hours. Characterization of HA compounds was done using scanning electron microscope-energy dispersive X-ray (SEM-EDX) (Tescan, Kohoutovice, Czech Republic). The powder shifting process was then carried out using a shifting machine to obtain HA powder crab shell measuring approximately 150µm.

Procedure for Making Gelatin-HA Scaffold[7,8]

500mg of gelatin was added to the distilled water and stirred at 40°C for 1 hour. The composition of HA-gelatin was made by adding 1.5 gr of HA powder into the gelatin solution and followed by stirring for 6 hours and centrifuged for 10 minutes. The gel solution was placed in a mold (Ø2mm x 5mm) that has been provided and put into freezer (-80°C) for 24 hours. Freeze drying was done for 24 hours.

Treatment Procedure:

Cavia cobaya was anesthetized with ketamine 60mg/kg (Pfizer®, New York, United States body weight intramuscularly. Extraction of the lower left incisor was carried out carefully using a sterile needle holder in a unidirectional motion so that the tooth root did not fracture. The socket was irrigated with a sterile aquades solution, applied with a HA-gelatin gel, and sutured with polyamida monofilament sewing thread (DS 12 3/8 c, 12 mm, 6/10 meth, 0.7 sterile Braun Aesculap)[9]

Tissue Sampling Preparation:

The termination was carried out using ketamine 100mg/cc injection intramuscularly (Pfizer®, New York, United States) at a dose of 0.2 cc. The mandibles were dissected at intervals of 7 and 14 days after extraction, fixated in 10% formalin buffer for 24 hours at room temperature ,and decalcified with EDTA for 60 days. The mandibles around the lower left incisor was cut into small, rectangular shape. The samples were dehydrated using alcohol, underwent clearing- embedding-deparaffinization process, dipped into xylene for 5 minutes, given malinol before being covered with object glass, and dripped with Canadian balm[10]

Interpretation of Immunohistochemical Results[10]

Each tissue sample was cut into 4-µm-thick sections, then detected by immunohistochemical examination to observe the OPG and RANKL expression. OPG expression was determined based on the presence of cells with brownish color. The area observed was the apical third of the socket. The results of each calculation were written on a worksheet and measured for average value. Total amount calculation of OPG and RANKL expression was carried out under a light microscope at 1000x magnification (Leica α6000, Wetzlar, Germany)[10]

Statistical Analysis:

This research was analyzed using Statistical Package for the Social Sciences Software (SPSS) 17.0 edition (SPSS™, New York, USA). A normality test was conducted with One Sample Kolmogorov-Smirnov. In addition to the normality test, a homogeneity test was also carried out with the Levene's Test. One-way analysis of variance (ANOVA) test was carried out to determine the group differences.

RESULTS:

In Figure 1, it appears that the lowest OPG number was in K7 group. This number was lower than in K14 group. The highest amount of OPG was seen in P14 group. The number of OPG expressions on the 14th day was more than on the 7th day.

Meanwhile in Figure 2, it appears that the lowest RANKL number is in P14 group, which was treated with HA scaffold from a *Portunus pelagicus* shell and was observed on the 14th day. This amount is lower than in P7 group. The highest RANKL was in K14 group, which is without being treated and was observed on the 14th day. The number of RANKL on the 14th day was more than on the 7th day.

From the Kolmogorov-Smirnov test, the p value of OPG expression in control group was 0.958 ($p > 0.05$) which means the data from the entire sample was normally distributed. Similarly, the results of the data from the entire sample of the treatment group have a value of $p = 0.508$ ($p > 0.05$) which means the data from the whole sample is normally distributed.

The results were then processed for homegeneity test using Levene's Test. From the homogeneity test, the p value was 0.350 (p> 0.05) for OPG and 0.206 (p> 0.05) for RANKL, which means the data is homogeneous.

From one-way ANOVA test, it was found that the p value of OPG and RANKL was 0,000 (p <0.05). There were significant differences between the expressions of OPG and RANKL of each groups.

There were significant differences (p <0.05) between all groups. There were significant differences (p <0.05) between K7, K14, and P7 groups; meanwhile P7 and P14 were not statistically significant difference. Image of OPG expression in the extraction socket by immunohistochemical examination under a light microscope can be seen in Figure 2.

There were significant differences (p <0.05) between P7 and K14, P14 group; while P7 and K7 group were not statistically significant difference. There were significant differences (p <0.05) between K14 and P7 group, meanwhile, the p value between K14 and K7, P14 group was less than 0.05 so there were no significant differences. There were significant difference (p <0.05) between P14 and K7, P7 group. Image of RANKL expression in the extraction socket by immunohistochemical examination under a light microscope can be seen in Figure 3.

DISCUSSION:

Crab shell of *Portunus pelagicus* contains much amount of calcium carbonate ($CaCO_3$) (approximately 40-70%), calcium (19.97%) and phosphorus (1.81%). This composition varies depending on the species. Calcium is a mineral that needed by the body in amounts of more than 100 mg per day[4,11,12]

This study used *Cavia cobaya* as experimental animals. *Cavia cobaya* was chosen as experimental animal because it has similar physiology to human. The extraction was carried out on each *Cavia cobaya* group. In the control group, the socket was only sutured, dissected, and examined on the 7th and 14th day. Meanwhile, socket in the treatment group was given HA scaffold, sutured, dissected, and examined on the 7th and 14th day.

Based on the research data, the lowest average OPG was found in K7 group, which is the control group and observed on the 7th day, while the highest average OPG number was in P14 group, which is the treatment group that given HA scaffold from crab shell (*Portunus pelagicus*) and observed on the 14th day. Meanwhile, the lowest mean number of RANKL was at P14 group, and the highest was observed at K14 group.

Grafting in the post-extraction socket with HA can limit the process of normal resorption because of the ability of HA to maintain the space needed for new bone formation. HA is an inorganic biomaterial, consists of 67% mineral content in bone. HA was used because of its excellent biocompatibility in humans. The mineral content is also the same as bones and teeth in humans chemically and physically. HA scaffold has also high osteoconduction properties[13,14]. The nature of osteoconduction in the form of macroporosity and microporosity in HA allows vascularity, oxygen transport, nutrient transfer, and migration and binding of osteogenic cells from the host[15,16]

HA minerals have high affinity properties to bind osteoinductive cytokines so that bound cytokines will stimulate mesenchymal stem cells contained in the HA mineral pores. These cytokines will differentiate into osteoblasts, then ion exchange occurs in calcium phosphate and interactions between calcium and cells. The formation of chemical bonds with good tissue gives advantages in clinical application of HA as a bone replacement material[15,17]

According to Adrianto (2011), osteoconduction of HA stimulates stem cells and osteoblasts to proliferate and differentiate into the formation of new bone or bone regeneration processes[18,19]. This is in line with the results above that the highest OPG mean and lowest RANKL are in the P14 group, which is the group that has been given with HA scaffold from crab shells (*Portunus pelagicus*) and observed on the 14th day.

According to Vieira et al. (2015), osteoclasts accumulate at the apex of the alveolar bone in the first week after extraction.[20] In the process of fibroblast, OPG is stimulated to inhibit the interaction of RANKL and RANK and triggers FGF2 which plays a role in the process of osteoblast cell proliferation and differentiation. The results of these studies are likely to occur due to increased activity and differentiation of osteoblasts on the 14th day compared to the 7th day.

The results of this study are in accordance with research conducted by Ma (2016) on rat femoral bones[21] In Ma's study, the application of HA on rat femoral bones on day 14 was known to be able to increase OPG expression in the treatment group compared to the control group on day 7. The high amount of OPG in this study shows that HA can trigger osteoblasts to secrete OPG more than during normal healing without treatment.

The increase in OPG expression on the 14th day was also supported by the study of Huang *et al* (2014), which stated an increase in OPG on the 14th day was caused by an increase in TGF-β1 expression. TGF-β1 play a role to

increase OPG expression in osteoprogenitor cells. OPG acts as a RANKL receptor that competes with RANK to avoid interactions with RANK, resulting in inhibition of osteoclastogenesis. OPG inhibits osteoclast activity by binding directly to RANKL, through interactions with receptors in the early stages of osteoclast formation.[22]

The decrease in RANKL itself is thought to be roled by HA which is able to regulate bone remodeling. HA will progressively reduce the production of prostaglandins and inflammatory cytokines namely IL-1, IL-6 and TNF-α.[23] This is caused by the inhibition of serotonin and histamine release and prostaglandin synthesis. Decreased prostaglandin and inflammatory cytokines cause the formation and activity of osteoclasts disrupted thereby reducing the level of bone resorption. In addition, HA can also increase the formation and differentiation of osteoblasts in the process of bone formation.[24]

Ma (2016) said that osteoblasts are cells that produce OPG and RANK, in other words the higher the osteoblasts the more the expression of OPG and RANK. OPG produced by osteoblasts has the duty to protect the occurrence of resorption by binding to RANKL so that there is no RANKL-RANK bonding. It can be concluded that the RANKL-OPG ratio plays an important role in the process of osteogenesis and osteoclastogenesis[21].

CONCLUSION:

The administration of HA scaffold from crab shells (*Portunus pelagicus*) can increase OPG expression and decrease RANKL expression in the regeneration of socket bone after tooth extraction of guinea pigs (*Cavia cobaya*) on the 7th and 14th days.

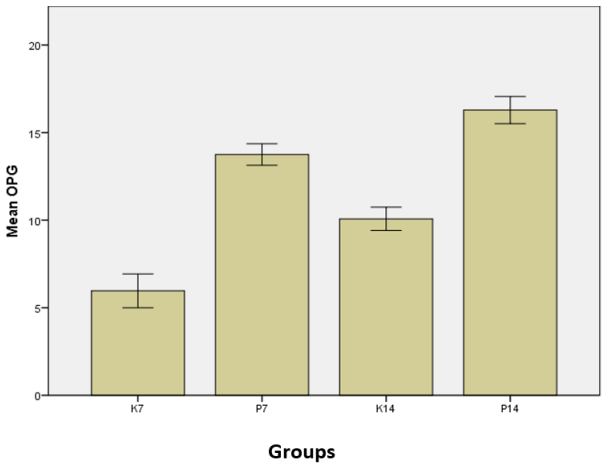

Figure 1. Bar chart of OPG expression mean.

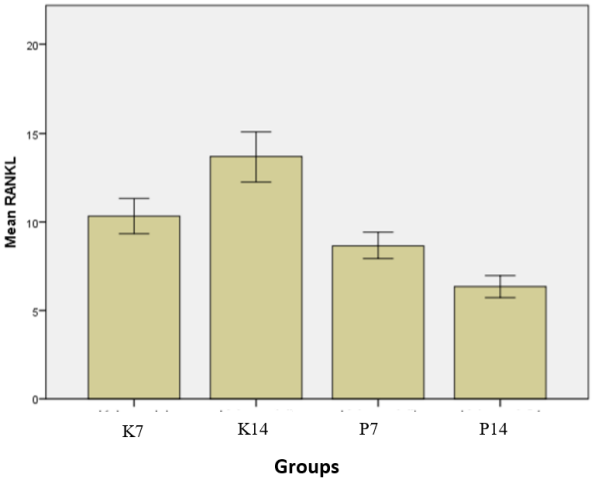

Figure 2. Bar chart of RANKL expression mean.

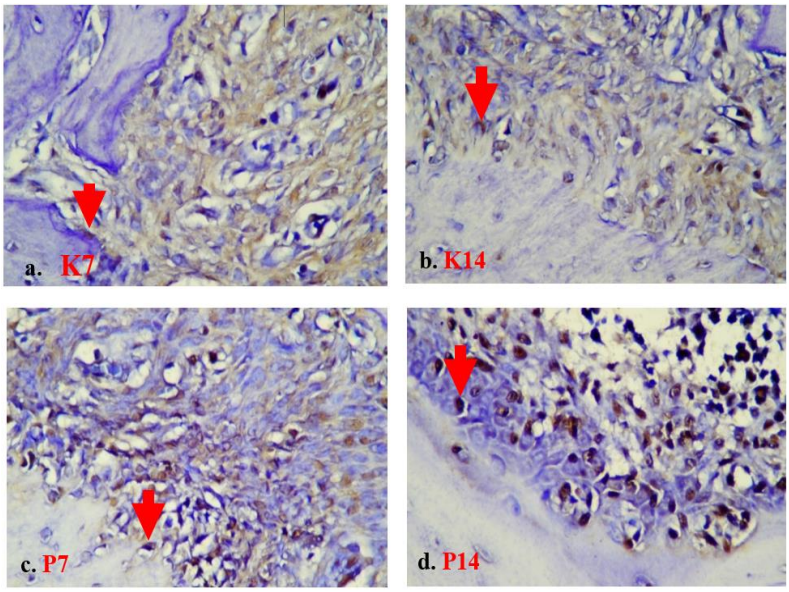

Figure 3 The arrows show a description of the OPG expression on the *Cavia cobaya* extraction socket group: (a) in the control group and observed on the 7th day, (b) in the control group and observed on the14th day, (c) in the treatment group and observed on the 7th day, (d) in the treatment group and observed on the14th day.

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