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RESEARCH ARTICLE

Effectiveness of calcium carbonate from non-cultivated pearl oyster shells (Pinctada Maxima) in socket preservation

Ainun Isnaeni Ilham, Arni Irawaty Djais*, Asdar Gani, Sri Oktawati, Dian Setiawati, Surijana Mappangara

Department of Periodontology, Faculty of Dentistry, Hasanuddin University, Makassar, Indonesia

Introduction: One of the marine biota that can be used as an alternative for bone regeneration is the non-cultivated pearl oyster shell (*Pinctada maxima*). Calcium carbonate found in the Nacre layer has biocompatible, biodegradable, and osteogenic properties, thereby encouraging bone formation.

Objective: to investigate the impact of calcium carbonate derived from *Pinctada maxima non-cultivated* (PMNC) on socket preservation, focusing on RANKL expression.

Method: Using experimental laboratory research tests and clinical trials with a post-test only control group design. Mandibular right anterior tooth extraction was performed on 27 Cavia Cobaya then divided into three groups, namely the treatment group given calcium carbonate powder of non-cultivated *Pinctada maxima* (PMNC-P), positive control given BATAN xenograft (PC), and negative control not given bone graft (NC). The experimental animals were sacrificed on days 7, 14, and 21 then immunohistochemical examination was performed with the examination indicator being RANKL. The results of the statistical test used the ANOVA test and the Tukey Posthoc test.

Results: RANKL expression decreased significantly in the group PMNC-P and group PC on days 7, 14, and 21 and conversely slightly increased in the negative control group. However, there was no significant difference between the group PMNC-P and group PC.

Conclusion: Calcium carbonate from non-cultivated pearl oyster shells (*Pinctada maxima*) can reduce RANKL expression in bone regeneration.

Keywords: pearl oyster shells (*Pinctada maxima*), xenograft, socket preservation

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Introduction

Dimensional changes in the height and width of the alveolar bone will occur in the first six months after tooth extraction, so it does not support the placement of prostheses or dental implants. The alveolar bone will change due to bone regeneration and physiological resorption then reach 50% of the width of the alveolar ridge in the first year. To maintain the morphology of the remaining ridge and prevent excessive resorption, a socket preservation procedure can be performed. Socket preservation is a surgical procedure that involves adding graft material into the socket as a scaffold for bone regeneration [1–3].

Alternative bone graft materials include xenograft, allograft, and alloplast. Xenograft is obtained from different species, one of which can come from marine biota. In Indonesia marine biota, are easy to find, relatively cheap, and the production process is simple. One of the marine biota that can be used as an alternative for bone regeneration is the pearl oyster shell (*Pinctada maxima*). *Pinctada maxima* has been cultivated in Pangkajene and Kepulauan Regency, South Sulawesi [4–7].

"*Mother of Pearl*" or *Nacre* is a layer found in pearl oyster shells that has a main content of calcium carbonate ($CaCO^3$) which is biocompatible, biodegradable, and osteogenic by activating chemical signals from osteoblasts, thereby encouraging bone formation. Nacre has been shown to facilitate osteoblast proliferation and accelerate the production and mineralization of extracellular matrix. Therefore, pearl oyster shells have the potential to be used as an alternative material that can accelerate the bone regeneration process [7–11].

Pinctada maxima cultivated (PMC) has differences from *Pinctada maxima non-cultivated* (PMNC), namely the quality of nacre is lower because it has through a harvesting process and has an impact on the quality and quantity of aragonite found in shellfish shells, resulting in reduced $CaCO^3$ synthesis [12–14]. Previous studies that have used PMC include Chandha MH et al. conducted research using PMC powder implanted in bone defects in guinea pigs and showed the osteogenic potential of PMC hydroxyapatite with increased expression of *Osteoprotegerin* (OPG) and *Bone Morphogenetic Protein* (BMP2) [7].

The use of bone graft as a filler and scaffold in bone defects supports the remodeling of alveolar bone and helps accelerate the healing process through various stages of osteoblast differentiation. On the other hand, to modulate monocyte differentiation to osteoclasts, osteoblasts will release OPG and *Receptor Activator of Nuclear Factor-Kappa β Ligand* (RANKL).[1] RANK, RANKL, and OPG are 3 important components that are modulated by osteoblasts and influence bone mass density through regulation of osteoblast and osteoclast function. RANKL will bind to RANK, thereby inducing the osteoclastogenic process.

* Correspondence to: Arni Irawaty Djais
E-mail: irawaty.arni@unhas.ac.id

RANK-RANKL binding will initiate the differentiation of osteoclast precursors into mature osteoclasts, stimulate bone resorption capacity, and reduce osteoclast apoptosis. In this process, OPG acts as an inhibitor, preventing RANKL from binding to RANK by binding to RANKL [12,13].

This study aims to investigate the impact of calcium carbonate derived from PMNC on socket preservation, focusing on RANKL expression.

Methods

This study is a laboratory experimental study with a post-test only control group research design. The study began with the collection of PMNC on Bontosua Island, Pangkajene and Kepulauan Regency, South Sulawesi. Furthermore, the manufacture calcium carbonate powder of non-cultivated *Pinctada maxima* (PMNC-P) at Laboratory of Chemical Engineering, Ujung Pandang State Polytechnic, Makassar. Experimental research on experimental animals was conducted at the Docpet Animal Clinic, Makassar. All stages of this study refer to the ethics published by the Health Research Ethics Commission of the Hasanuddin University Dental and Oral Hospital with number: 0194/PL.09/KEPK FKG-RSGM UNHAS/2024.

PMNC-P is made by calcination method. First, the shell is cleaned and then dried using solar heat for 2 hours and then stored for 1x24 hours at room temperature. The shell is then dried using an oven at a temperature of 110^{o}C and separated from Nacre and then reweighed. The shell is broken into smaller sizes and furnaced for 2 hours. The sample results are then filtered with a size of -60 + 120 mesh to obtain pearl shell powder. The powder is tested using Atomic Absorption Spectrophotometry (AAS) to determine the calcium content. Then the sample is in a 900^{o}C furnace and calcined to obtain the $CaCO^3$ content. The synthesis results are characterized using Fourier Transform Infra Red (FTIR) to see the content and characterization of the compounds formed and contained in the bone graft. Then the Porosity test is carried out.

The subjects of the study were 27 Cavia cobaya guinea pigs that had previously been adapted until they were ready to be treated. The inclusion criteria for the subjects included a body weight of 300-500 grams, age of around 8-10 weeks, male gender, and normal action and activity. The exclusion criteria were if there was a 10% weight loss after a 7-day adaptation period and were given food in the form of pellets.

Guinea pigs received intramuscular injections of anesthesia of the lower right incisor teeth and then extracted. Then the guinea pigs were randomly divided into three groups, namely the negative control group (NC) which was not given bone graft, the positive control group (PC) which was given hydroxyapatite BATAN, and the treatment group which was given non-cultivated pearl oyster shell calcium carbonate powder (PMNC-P) inserted into the socket. After tooth extraction, suturing was performed using absorbable thread (Vicryl 5.0). Euthanasia was performed with an ether chamber on days 7, 14, and 21, namely 3 animals were sacrificed in each group to facilitate tissue sampling. Each tissue was then formed into a slide preparation to be examined through Immunohistochemistry to read RANKL expression. The data obtained were then tested for normality using the Shapiro-Wilk test. The normally distributed data will then be tested using ANOVA. If the ANOVA test obtains a p value <0.05, the data is then subjected to the Tukey posthoc test. The type of data used is primary data, data processing using IBM SPSS Statistics V.21.

Results

Bone graft material from PMNC-P before being applied to experimental animals, the test carried out was the AAS test to see the calcium (Ca) content and the Ca content was obtained at 486,983.49 mg/L. Then the FTIR test was carried out to see the $CaCO^3$ content. Furthermore, a porosity test was carried out to show the percentage of pores formed in PMNC-P and the results obtained were 74,82%.

The FTIR spectrum of the shell samples is in the form of absorbance data at the absorption 4000 to 400 cm^{-1}, as seen in the IR spectrum image (Figure 1). There is a CO_3^{2-} absorption peak at wave numbers 875,71, 1132,25, 1645,33 dan 1751.42 cm^{-1} which is a general characteristic of carbonate ions in $CaCO^3$ and is the basic mode of molecular vibration The Ca-O vibration absorption peak at 669.32 cm^{-1} and C-H vibration at 875,71 cm^{-1} indicate the characteristics of the calcite phase. The C-O functional group is found at 1132.26 cm^{-1}.

Figure 2 shows the results of observations of RANKL expression by immunohistochemical examination with 1000x magnification in the treatment group (PMNC-P), positive xenograft control (KP) and negative control (KN) on days 7, 14, and 21 marked with brownish color on the arrow. Based on these observations, there was a decrease in RANKL expression from days 7, 14 and 21 in the treatment group and positive control.

Table 1 shows a comparison of RANKL expression between the three research groups on days 7, 14, and 21 based on observation time. The results of statistical tests showed that there was a significant difference in RANKL values between the PMNC-P, PC and NC groups with a value of $p<0.05$.

Table 2 shows that the comparison of RANKL expression between PMNC-P and NC groups on day 7, day 14 and day 21 was significantly different on day 14 and day 21 ($p<0.05$). The comparison of RANKL expression between PMNC-P and PC groups on day 7, day 14 and day 21 was not significantly different ($p>0.05$). RANKL expression in each group is also shown in Figure 2 below.

Based on the graphic image shown in Figure 3, mean expression of RANKL in immunohistochemical observations on days 7, 14, and 21, the PC group and the PMNC-P

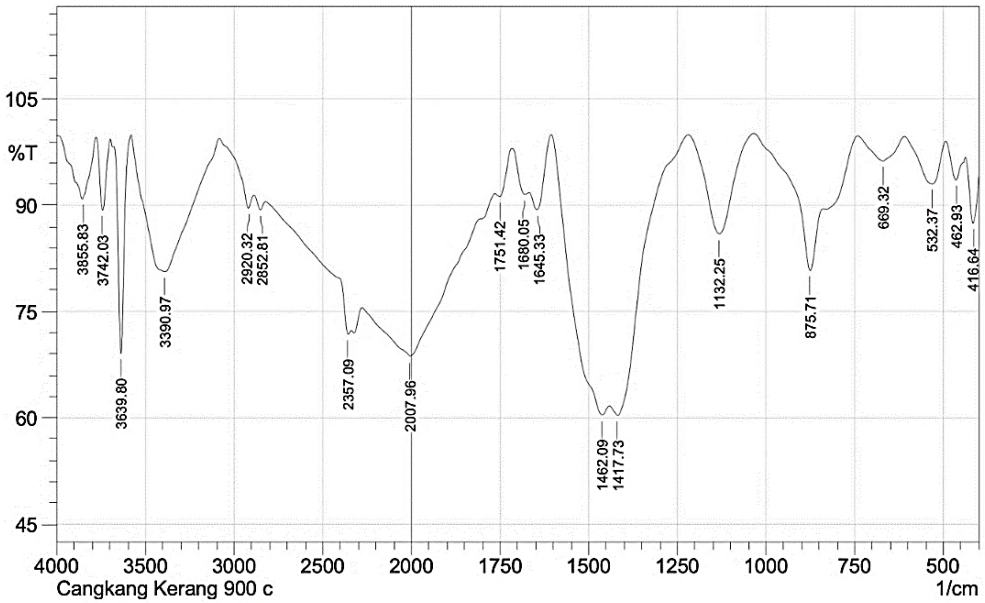

Fig. 1. The FTIR Spectrum of Non-Cultivated Pearl Oyster Shell Calcium Carbonate Powder

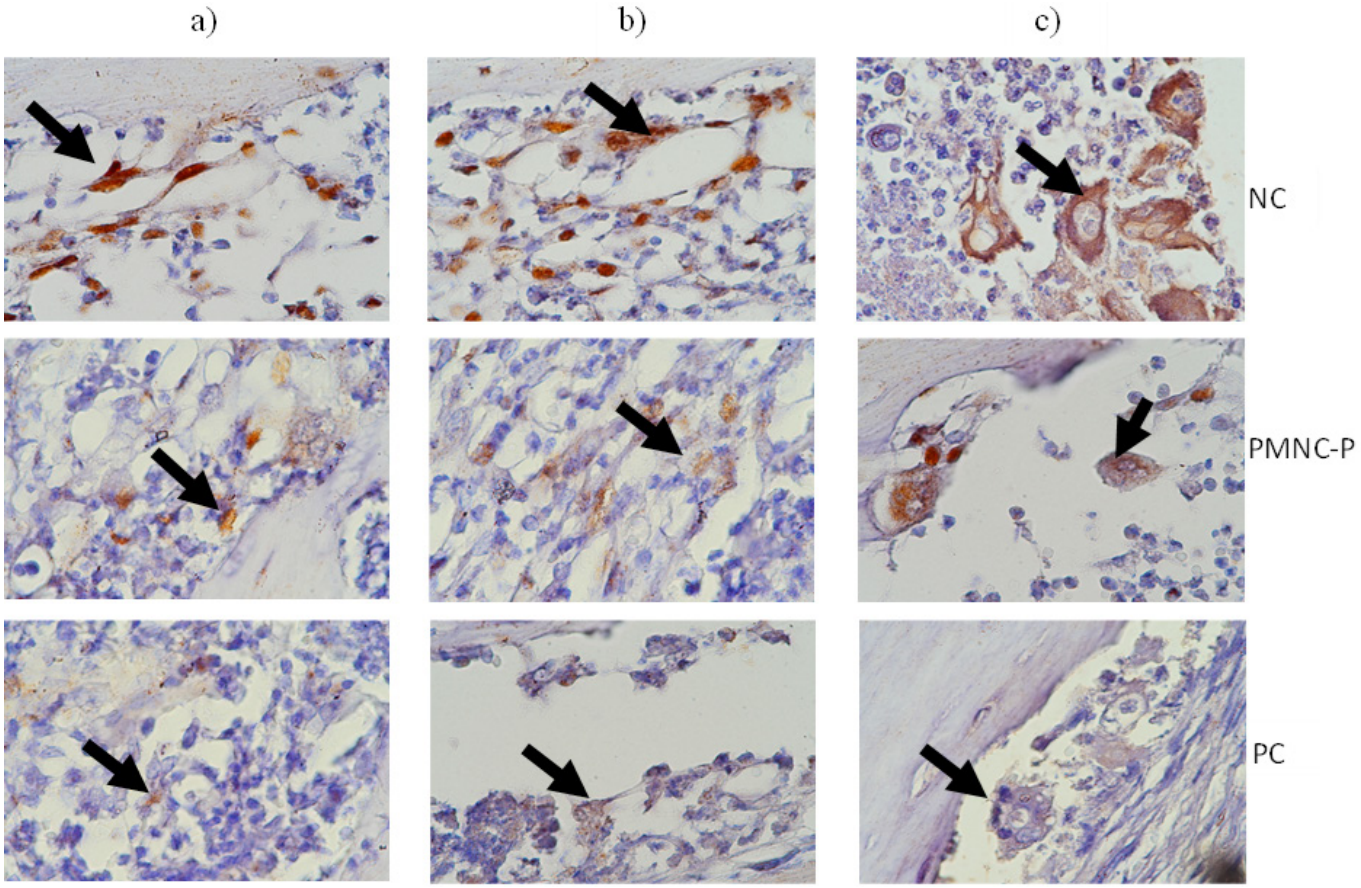

Fig. 2. The results of observations of RANKL expression by immunohistochemical (IHC) examination in the incisor tooth sockets of guinea pigs after not being given bone grafts/negative control (NC); administration of calcium carbonate treatment of non-cultivated Pinctada maxima (PMNC-P); xenograft/positive control (PC): a) on the 7th day; b) the 14th day; c) the 21st day

Table 1. Comparison of RANKL between Groups

Group	Day-7		Day-14		Day-21	
	Mean	SD	Mean	SD	Mean	SD
PMNC-P	8.00	1.00	6.67	1.53	2.67	1.53
PC	6.67	2.08	4.67	1.53	2.33	1.53
NC	9.33	1.53	10.67	2.08	12.67	2.52
p-value	0.205		0.014*		0.001*	

*Significant One way anova (P<0,05); Treatment group of calcium carbonate powder non-cultivated Pinctada maxima (PMNC-P); Positive control group of xenograft (PC); Negative control group not given bone graft (NC)

Table 2. Posthoc LSD Test of RANKL Expression between Groups

Group	Day-7			Day-14			Day-21		
	Mean	SD	p-value	Mean	SD	p-value	Mean	SD	p-value
PMNC-P	8.00	1.00	0.346	6.67	1.53	0.030*	2.67	1.53	0.001*
NC	9.33	1.53		10.67	2.08		12.67	2.52	
PMNC-P	8.00	1.00	0.346	6.67	1.53	0.207	2.67	1.53	0.838*
PC	6.67	2.08		4.67	1.53		2.33	1.53	

*Significant posthoc LSD (P<0,05); Treatment group of calcium carbonate powder non-cultivated Pinctada maxima (PMNC-P); Positive control group of xenograft (PC); Negative control group not given bone graft (NC)

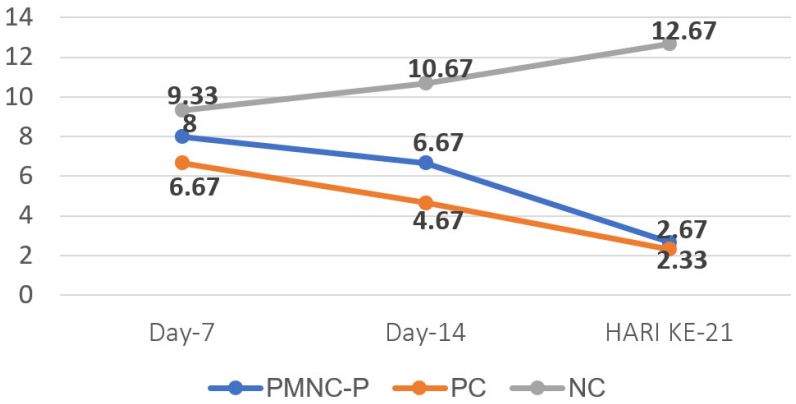

Fig. 3. Comparison graph of RANKL expression between groups on days 7, 14, and 21. Treatment group of calcium carbonate powder non-cultivated Pinctada maxima (PMNC-P); Positive control group of xenograft (PC); Negative control group not given bone graft (NC)

group descriptively experienced a decrease in RANKL expression. This is different from the expression of immunohistochemical observations of RANKL on days 7, 14, and 21 in the NC group.

Discussions

The effect of calcium carbonate from PMNC as a material that can regenerate bone on RANKL expression after tooth extraction can be seen in this study. Various biomaterials and procedures with different functions such as osteoconduction and osteoinduction have been used to maintain alveolar bone volume [14–16]. The use of bone grafts provides good results in bone formation through increasing OPG expression and decreasing RANKL expression as an indicator of bone formation. RANKL plays an important role in osteoclastogenesis. The balance of the RANK–RANKL–OPG system is very important in physiological bone metabolism [17,18].

Table 1 showed a significant difference in the average value of RANKL expression between the PMNC-P, PC, and NC groups on days 7, 14, and 21. This is due to the treatment of PMNC-P in the post-tooth extraction socket as an osteoconductive material as a scaffold for bone growth and high biocompatibility by increasing interaction with osteoblasts. This is supported by research by Djais et al. which reported that bone formation can be seen through increased OPG expression and decreased RANK expression. The bone remodeling process requires a balance of osteoblast and osteoclast activity. If RANKL expression is higher, bone resorption occurs, conversely if OPG expression is higher, bone formation occurs [11,19].

Remodeling is the final phase of wound healing, starting on day 21 and continuing for up to 1 year. The normal bone remodeling cycle requires bone resorption and bone formation [20–22]. This study is also in line with the study of Chanda et al. which showed an increase in OPG on day 14 to day 21 with the addition of PMC hydroxyapatite to femur defects. This suggests that the PMC hydroxyapatite component affects OPG formation faster than physiological healing or negative control groups. The presence of OPG expression affects osteoclast production and inhibits bone resorption in the bone remodeling cycle [7]. Similar things were also reported in the study of Soekobagiono et al. which showed a decrease in RANKL on day 7 to day 30 with the addition of xenograft in socket preservation. The results of the treatment showed a tendency to decrease the amount of RANKL expression on day 30 when compared to the results on day 7 [17].

Table 2 shows that the comparison of RANKL expression between PMNC-P and PC groups on day 7, day 14 and day 21 did not differ significantly ($p>0.05$). This shows that RANKL expression in both groups is almost the same, namely decreased RANKL expression, which can also be seen in Figure 2 and Figure 3. The cause of decreased RANKL expression is because the administration of PMNC-P and the administration of BATAN bone graft in the positive control group (PC) as a bone structure material causes an increase in osteoblast activity and a decrease in osteoclast activity. Meanwhile, RANKL expression in the NC group experienced a slight increase in osteoclasts due to the physiological process in the bone remodeling cycle. This study is in line with the study of Yonatasya et al which showed that $CaCO^3$ compounds from the synthesis of blood clam shells affect the healing process of socket wounds after tooth extraction on day 7 and day 14. The calcium compound that is often used as a bone graft is $CaCO^3$ due to its flexible, biodegradable, and osteoconductive properties. The combination of cal-

cium compounds in the form of hydroxyapatite, tricalcium phosphate, and calcium carbonate in a bone graft is expected to help accelerate the healing process of socket wounds [23,24].

The limitations in this study are the limited number of samples and the form of calcium carbonate preparations in this study, so further research is needed with a larger number of samples so that it can further strengthen the results of previous studies.

Conclusion

Socket preservation action using calcium carbonate powder from non-cultivated pearl oyster shells (*Pinctada maxima*) can reduce the growth of osteoclast cells which is indicated by a significant decrease in RANKL expression in bone regeneration.

Authors' contribution

AII (Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Validation; Visualization; Writing – original draft; Writing – review & editing)
AID (Formal analysis; Investigation; Writing – review & editing)
AG (Formal analysis; Investigation; Writing – review & editing)
SO (Methodology; Supervision; Writing – original draft; Writing – review & editing)
DS (Methodology; Supervision; Writing – original draft; Writing – review & editing)
SM (Methodology; Supervision; Writing – original draft; Writing – review & editing)

Conflict of interest

None to declare.

Ethics approval

0194/PL.09/KEPK FKG-RSGM UNHAS/2024.

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