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Flower Crab Shell (*Portunus pelagicus*) Hydroxyapatite Increased Osteoblast Cells, TNF-α, and IL-6 in Rabbit with Femoral Defect

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ABSTRACT

Bone grafts are commonly used to support fracture healing, while hydroxyapatite (HA), which is generally derived from bovine sources, remains costly and limited in production in Indonesia. Therefore, alternative biomaterials with comparable efficacy are needed. Flower crab shell (Portunus pelagicus), which contains high calcium carbonate levels and is abundantly available in Indonesian waters, has potential as a synthetic HA source. This study aimed to evaluate the effect of flower crab shell-derived HA on bone healing in rabbits with femoral defects. The research employed an experimental post-test only control group design using 12 male New Zealand rabbit aged 6–12 weeks and weighing 2.5–3 kg. The animals were randomly divided into three groups: control without HA, bovine HA treatment, and flower crab shell HA treatment. A femoral defect measuring 5 mm in diameter and 5 mm in depth was surgically created in each rabbit, followed by implantation of the respective graft materials in the treatment groups. Six weeks after surgery, bone healing was evaluated through histopathological examination of osteoblast cells and immunohistochemical analysis of TNF-α and IL-6 expression. The results demonstrated significant differences among groups, indicating that both bovine HA and flower crab shell HA accelerated bone healing compared to the control group. Flower crab shell-derived HA showed similar or superior efficacy to bovine HA by increasing osteoblast cell numbers as well as TNF-α and IL-6 expression during the bone healing process.

Keywords: Bone graft, flower crab shell, hydroxyapatite, IL-6, osteoblast, TNF-α

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BACKGROUND

Previous studies have demonstrated that hydroxyapatite (HA) is an effective biomaterial for bone regeneration because of its osteoconductive and biocompatible properties (Blom et al., 2017). Most commercially available HA is derived from bovine sources and has been widely used to enhance osteoblast proliferation and accelerate fracture healing (Roberts & Rosenbaum, 2012). However, bovine-derived HA has several limitations, including relatively high production costs, limited local availability in Indonesia, and dependence on imported raw materials (Egol et al., 2015). Previous investigations have also explored the utilization of marine waste materials, such as crab and seashell waste, as alternative calcium sources for HA synthesis because of their high calcium carbonate content and environmental availability (Harsini & Oryan, 2018). Several studies reported that HA synthesized from crustacean shells possesses favorable physicochemical characteristics, including high purity and smaller particle sizes, which may improve cellular attachment and biomaterial absorption (Vecchio et al., 2007).

Despite these findings, research evaluating the biological effectiveness of flower crab shell-derived HA in vivo remains limited, particularly in relation to bone healing mechanisms involving inflammatory cytokines and osteoblast activity (Bhatt & Rozental, 2012). Earlier studies primarily focused on material characterization, physicochemical analysis, or indirect biomarkers of bone regeneration, while comprehensive evaluation using histopathological and immunohistochemical parameters in femoral bone defects has rarely been performed(Tempo.co, n.d.). Therefore, a clear research gap exists regarding the comparative efficacy between flower crab shell-derived HA and conventional bovine HA in stimulating osteoblast formation and regulating pro-inflammatory cytokines such as TNF-α and IL-6 during bone healing (Yanuar et al., 2009). This study addresses that gap by investigating the biological response and regenerative potential of flower crab shell HA in an experimental rabbit femoral defect model(Menteri Kelautan dan Perikanan Republik Indonesia, 2016).

The use of New Zealand rabbit as the experimental animal model was considered appropriate because rabbits possess bone remodeling and fracture healing characteristics that are relatively comparable to humans, particularly in cortical bone regeneration and inflammatory response during bone repair (Sholichah et al., 2013). In addition, rabbits have sufficient femoral bone size to allow standardized defect creation and graft implantation procedures while remaining manageable for laboratory handling and postoperative observation(Hu et al., 2017). Male rabbits were selected to minimize hormonal variations that could influence bone metabolism and healing outcomes (Sholichah et al., 2013). Consequently, the rabbit femoral defect model provides relevant biological and translational insight into the potential clinical application of flower crab shell-derived hydroxyapatite as an alternative biomaterial for human bone graft therapy.

METHODS

Animal Models and Surgical Procedures

This experimental research was conducted with a "post-test only control group" design from June to July 2022 at the Semarang Animal Centre Veterinary Clinic, Diponegoro University Veterinary Laboratory, Semarang and “Desa Wisata Lembah Kalipancur” Animal Clinical Laboratory, Semarang. The experiments were conducted following the institutional guidelines, and the protocol was approved by the Health Research Ethics Committee of the Faculty of Medicine Diponegoro University (Protocol Numbers: 14/EC/H/FK-UNDIP/I/2023).

Twelve male New Zealand rabbits (*Oryctolagus cuniculus*) aged 6-12 weeks and weighed 2.5-3 kilograms were used as experimental animal models. Twelve rabbits that met the inclusion criteria were then adapted and given food and drink ad libitum for one week. The rabbits were given vitamin ADE (0.1 mL/KGB IM), vitamin B complex (0.1 mL/KGB IM), and ivermectin (0.4 mg/KGB IM) during adaptation period to prevent disease. Each of the rabbit were then separated into one cage and randomized into three groups; 4 rabbits in control group (C), 4 rabbits in bovine HA graft treatment (T1), and 4 rabbits in flower crab shell HA graft treatment (T2).

The surgery involved injection of enrofloxacin (5 mg/KGB) intravenously (IV) into the lateral auricular vein in the ear as prophylactic antibiotic. Ketamine (25 mg/KGB) and acepromazine (0.3 mg/KGB) were administered intramuscularly (IM) in the longissimus dorsi caudal muscle as the anesthetic. An incision was made at the shaved and disinfected surgical area until we reached the periosteum of the femur. A drill was used to create a 5 mm diameter and 5 mm deep defect at the lateral aspect of the distal femoral metaphysis. In group T1, the defect was filled with bovine HA; in group T2 it was filled with HA of flower crab shells. After the procedure, the incision wound was sutured. All rabbits were observed for 24 hours after surgery and were given dexamethasone IM (2 mg/KGB) and enrofloxacin IM (5 mg/KGB) antibiotics every 24 hours for three days after surgery.

Hydroxyapatite Preparation

We calculated that 300 mg of HA would be required to implant a tubular defect 5 mm in diameter and 5 mm in depth. HA required was calculated as mentioned below, with m = HA mass, p = HA density (3.18 g/cm3), V = volume HA = defect volume, r = defect diameter (0.25 cm), and t = defect depth (0.5 cm):

$$m = p\ x\ V = p\ x\ \pi r2t$$

Outcome Evaluation

The center of the treatment area of the necropsied rabbit femur was made of slides for histopathological and immunohistochemistry assessments at 6th week postoperatively. The bone healing process is measured based on the number of osteoblast cells, TNF-α, and IL-6 expression. For assessing the osteoblast cells, the slides were stained with Hematoxylin Eosin and the histopathologic examination was carried out using a light microscope with 400x magnification in 5 fields of view. While the expression of TNF-α and IL-6 was calculated based on the percentage of positively-stained cells for each field of view. An Anatomical Pathology specialist from Gadjah Mada University, Yogyakarta, performed both assessments.

Data Analysis

The normality of the data distribution was analyzed using the Shapiro-Wilk test. Normally distributed data were analyzed with parametric statistical analysis ANOVA and Post Hoc test to assess the differences between each group. Otherwise, the Kruskal-Wallis and Mann-Whitney tests were used. Data was analysed with SPSS ver.25 software for Windows 10, with the result is considered significant if $p < 0.05$.

RESULTS

During the study period, one rabbit in each group died or dropped out, with a total of 3 rabbits, which was suspected due to complications of postoperative infections.

Osteoblast Cell Histopathology

The highest mean value of osteoblasts was found in group T1 (HA bovine) with a mean of 52.00 ± 20.66, followed by group T2 (HA flower crab shell) with a mean of 20.33 ± 7.51, and lastly group C (control) with a mean of 6.33 ± 4.04 (Figure 1). The osteoblast cell data were normally distributed, with the One-Way Anova test showed a significant difference between each group (Table 1). The results of the LSD Post Hoc test showed that there was a significant difference between the control group and the T1 group (HA Bovine) and between the T1 group (HA Bovine) and the T2 group (HA flower crab shell). Comparison between the control and the T2 group (HA flower crab shell) showed no significant difference (Table 2).

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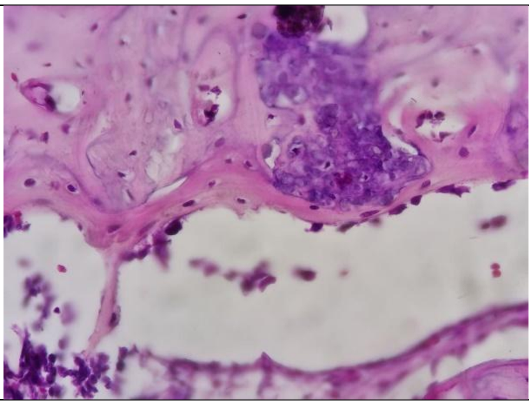
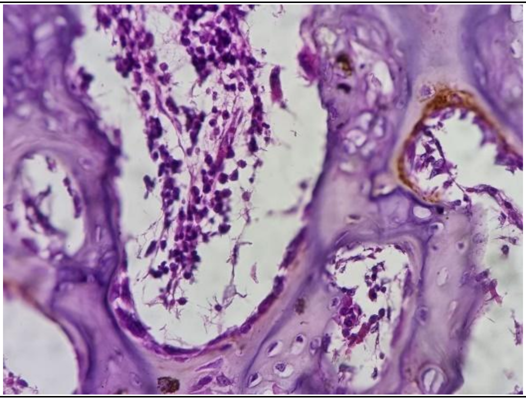
Figure 1. Histopathology examination of Osteoblast Cell

Control



T1 Group (HA Bovine)



T2 Group (HA flower crab shell)

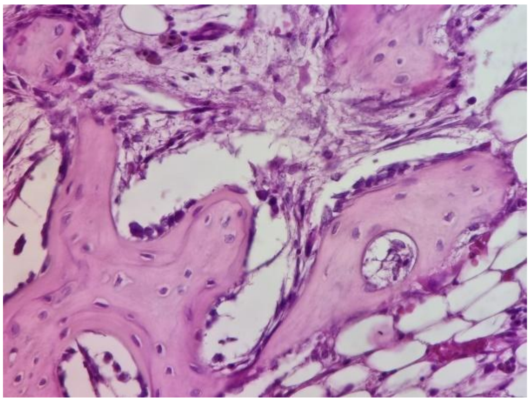


Table 1. One-Way Anova test for Osteoblast Cell Histopathology

Osteoblast	N	Mean ± SD	p	Levene
C	3	6,33 ± 4,04	0,012§*	0,137
T1	3	52,00 ± 20,66		
T2	3	20,33 ± 7,51		

Description : * Significant (p < 0,05); § One Way Anova

Table 2. Post Hoc LSD test for Osteoblast Cell Histopathology

Osteoblast	T1	T2
C	0,005*	0,232
T1	–	0,024*

Description : * Significant (p < 0,05)

IL-6 Expression

The highest IL-6 expression was found in group T2 (HA flower crab shell) with a mean of 81,67 ± 18,93, followed by group T1 (HA bovine) with a mean of 76,67 ± 22,09, and group C (control) with a mean of 5,00 (Figure 2). The data were not normally distributed, and the Kruskal-Wallis test showed a significant difference between each group (Table 3). The results of the Mann-Whitney test showed that there was a significant difference between the control and the T1 group (HA Bovine) and between the control and the T2 group (HA flower crab shell). Comparison between the T1 group (HA Bovine) and the T2 group (HA flower crab shell) showed no significant difference (Table 4).

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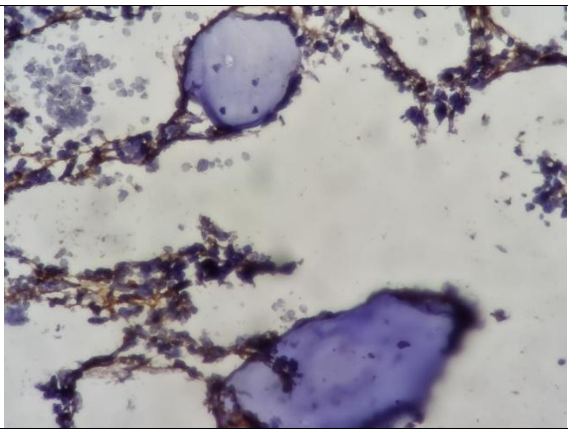
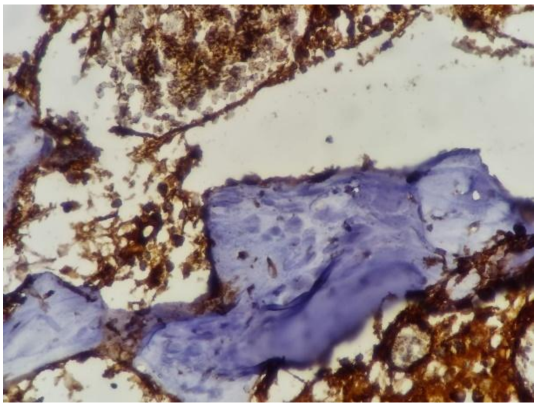
Figure 2. The expression of IL-6

Control	T1 Group (HA Bovine)
	

T2 Group (HA flower crab shell)

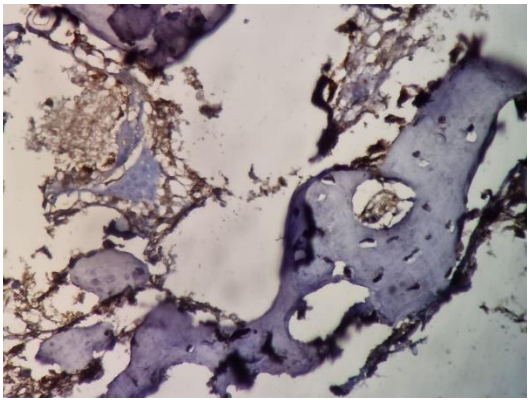


Table 3. Kruskal-Wallis test for Osteoblast Cell Histopathology

IL-6	N	Mean ± SD	p
C	3	5,00	0,050‡*
T1	3	76,67 ± 22,09	
T2	3	81,67 ± 18,93	

Description : * Significant (p < 0,05); ‡ Kruskal-Wallis

Table 4. Mann Whitney test for IL-6 Expression

IL-6	T1	T2
C	0,034*	0,037*
T1	–	0,487

Description : * Significant (p < 0,05)

TNF-α Expression

The highest TNF-α expression was found in group T2 (HA flower crab shell) with a mean of 90,00 ± 8,66, followed by group T1 (HA bovine) with a mean of 78,33 ± 12,58, and group C (control) with a mean of 6,67 ± 2,89 (Figure 3). The data were not normally distributed, and the Kruskal-Wallis test showed a significant difference between each group (Table 4). The results of the Mann-Whitney test showed that there was a significant difference between the control and the T1 group (HA Bovine) and between the control and the T2 group (HA flower

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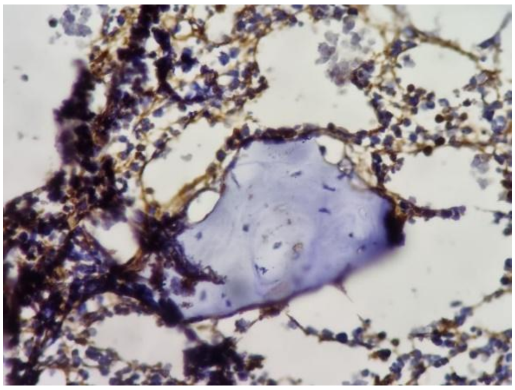
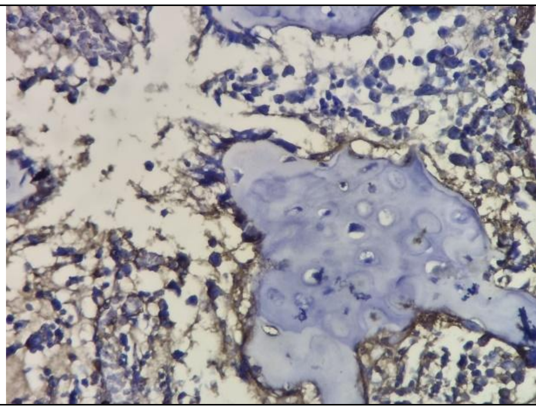
crab shell). Comparison between the T1 group (HA Bovine) and the T2 group (HA flower crab shell) showed no significant difference (Table 6).

Figure 3. The expression of TNF-α

Control



T1 Group (HA Bovine)



T2 Group (HA flower crab shell)

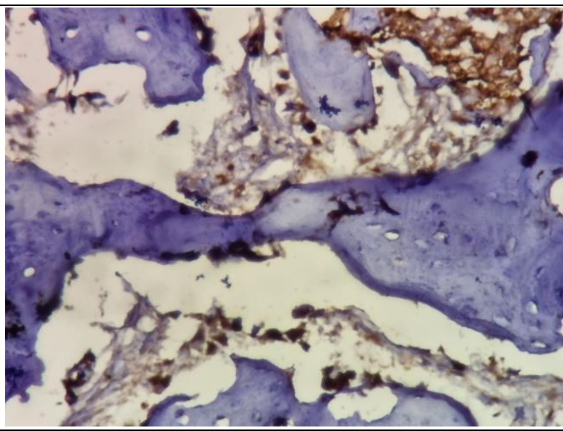


Table 5. Kruskal-Wallis test for TNF-α expression

TNF-α	N	Mean ± SD	p
C	3	6,67 ± 2,89	0,042‡*
T1	3	78,33 ± 12,58	
T2	3	90,00 ± 8,66	

Description : * Significant ($p < 0,05$); ‡ Kruskal-Wallis

Table 6. Mann Whitney test for TNF-α expression

TNF-α	T1	T2
C	0,046*	0,043*
T1	–	0,178

Description : * Significant ($p < 0,05$)

DISCUSSION

Bone healing is a complex, multifactorial process involving many components. This study aims to study the effect of administrating hydroxyapatite made from flower crab shells on bone healing in rabbits with femoral defect. The bone healing was assessed by the number of osteoblast cells, IL-6 levels, and TNF-α levels, which was evaluated 6 weeks post intervention.

Osteoblast cells are one of the components of the histological structure of bone which has a function in the formation of organic components of the bone matrix and the regulation of bone metabolism. In the process of bone remodeling, osteoblasts express receptor nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) (Ariani, 2019). (Ngatidjan & Morita, 2012) This study demonstrated that the use of bovine HA and flower crab shell HA could produce a greater number of osteoblasts in bone defects compared to the group that did not receive HA. Descriptively, the use of bovine HA resulted in a higher number of osteoblast cells compared to the HA of green mussel shells.

This finding is in line with research by Liang et al, where the administration of HA can facilitate the healing and formation of bone tissue by increasing the number of osteoblast cells and osteoblastic differentiation of stem cells (Kanakaris et al., 2015). (Strier et al., 2011) A study by Kamadjaja et al explained that HA administration of crab shells increased the expression of OPG and decreased the expression of RANKL, an indirect biomarker of osteoblast cells, which indicates regeneration activity of alveolar bone after tooth extraction. (Giannoudis et al., 2014) HA has structural characteristics similar to inorganic components found in bones and teeth, with osteoconductive and osteoinductive properties that play a role in triggering tissue repair and regeneration processes. HA can promote the adhesion and proliferation of osteoblastic cells to bone surface, by providing a scaffold framework that becomes a medium for osteoblastic cells to attach and carry out their proliferative and differentiation functions in bone healing(Giannoudis et al., 2014).

TNF-α and IL-6 are pro-inflammatory cytokines that play an important role in the immune response and bone metabolism(Fillingham & Jacobs, 2016). Both are known to increase the activation of macrophages and antigen presentation and regulate immunity through various mechanisms(Sohn & Oh, 2019). IL-6 is a cytokine that influences osteoblast and osteoclast differentiation activity and plays a role in inducing the phases of bone formation and resorption that depend on the microscopic environment. (Lamsihar Manalu et al., 2015)This study showed that the use of HA bovine and HA of green mussel shells resulted in higher expression of TNF-α and IL-6 compared to the group that did not receive HA. There was no difference between the bovine HA group and the HA crab shell group, indicating that the two types of HA produced a similar increase. However, descriptively, the HA of flower crab shells resulted in higher expression of TNF-α and IL-6 than HA bovine (Nadra et al., 2008).

The results in this study align with research by Nadra et al., which showed that HA crystals can stimulate TNF-α secretion by macrophages, which will lead to NF-kappa B activation and is influenced by the size of the particles and their pores. (Friedrich et al., 2021) The study by Freidrich et al also found a similar finding, where the use of HA nanoparticles increased the release of TNF-α and IL-6 in murine macrophages. Although not known with certainty, the release of cytokines in response to pro- and anti-inflammatory agents can be modulated by the presence of HA nanoparticles. (Harmer et al., 2019)The differentiation and adhesion of osteoblast cells to HA are known to be enhanced by the presence of the cytokines TNF-α and IL-6. TNF-α is known to play a role in fracture healing, which can increase the migration and recruitment of osteoblast cells and pro-osteogenic effects. Meanwhile, the presence of IL-6 in HA administration was found to promote osteogenic differentiation and reduce osteoclastogenesis, thereby increasing bone formation (Oryan et al., 2015).

CONCLUSION

Hydroxyapatite from the flower crab shell had a similar or better efficacy with the HA bovine, which both could increase the number of osteoblast cell, TNF-α and IL-6 expression in bone healing process.

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Authors Contribution

ABZ: Planned the study, collected the data, performed the analysis, and wrote the manuscript; SB, AP, MM, RN: critically revised the draft for important intellectual content and finally approved the manuscript. All authors read and approved the final manuscript.

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