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Research Article

Flower Crab Shell (Portunus Pelagicus) Hydroxyapatite Increased Osteoblast Cells, Tnf-A, And Il-6 In Rabbit with Femoral Defect: Experimental Animal Studies

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Abstract

A fracture is a breakdown of the structural continuity of the bone, with the healing process requiring a balance between biological and biomechanical stability through immobilization. Modern internal fixation techniques as a means of immobilization can interfere with the effectiveness of bone synthesis, leading to nonunion or failure of the bones to join. Therefore, a bone graft is needed to support the fracture healing process. A bone graft is any substance implanted in the bone to help the bone heal. One of the widely used bone grafts is hydroxyapatite (HA), a major mineral component of bone that makes up about 50% of the bone weight and is biocompatible.

Keywords: bone graft; hydroxyapatite; flower crab shell; osteoblast; tnf-α; il-6

Introduction

A fracture is a breakdown of the structural continuity of the bone, with the healing process requiring a balance between biological and biomechanical stability through immobilization.[1] Modern internal fixation techniques as a means of immobilization can interfere with the effectiveness of bone synthesis, leading to nonunion or failure of the bones to join.[2] Therefore, a bone graft is needed to support the fracture healing process.[3] A bone graft is any substance implanted in the bone to help the bone heal.[4] One of the widely used bone grafts is hydroxyapatite (HA), a major mineral component of bone that makes up about 50% of the bone weight and is biocompatible.[5,6] Hydroxyapatite is mostly produced from bovine, which tends to cost more and has limited production in Indonesia[7], so an alternative synthetic source with similar efficacy is needed. One alternative to hydroxyapatite is the flower crab shell, which has a high calcium carbonate content, around 40-70%. The flower crab (Portunus pelagicus) is easy to find in almost all Indonesian waters. Crab production data in Indonesia from 2005-2014 obtained an estimated resource potential of 37,911 tons/year was obtained. [8] If the flower crab shells represent 50% of the body's weight, [9] then the estimated total shell waste each year is around 18,956 tons, indicating the presence of abundant flower crab shells in Indonesia. The Faculty of Mechanical Engineering, Diponegoro University, and CBIOM3S (Center for Biomechanics, Biomaterials, Biomechatronics, and Biosignal Processing) of Diponegoro University succeeded in making HA flower crab shells with high purity and smaller particle size than HA bovine. This smaller size provides more surface area for macrophage attachment, resulting in faster inflammation and absorption. [10] Histopathological assessment is the most accurate test to analyze the reparative and regenerative processes of the bone healing process, which can be done by analyzing the number of osteoblast cells. Osteoblasts are cells derived from mesenchymal progenitor cell differentiation and play a role in the synthesis of the bone matrix, which will gradually mineralize and turn into bone components of osteocytes. This differentiation can be triggered by an inflammatory reaction during bone fracture. [11,12] Within this cascade are several regulatory factors, cytokines and hormones, and an osteoconductive extracellular matrix that interacts with several cell types. TNF-α and IL-6 cytokines are known to reflect the bone healing process, where an increase in these cytokines can promote osteogenic differentiation and affect osteoclastogenesis, thereby increasing the bone healing process. [13] This study aims to determine the effect of HA flower crab shells on the process of bone healing in rabbit femur defects as assessed by expression levels of proinflammatory cytokines Il-6, TNF-α, and the number of osteoblast cells.

Methods:

Animal Models and Surgical Procedures

This experimental research was conducted with a post-test-only control group design from February to March 2023 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) and fully compliant with ARRIVE guidelines.

Based on the resource equation method, twelve male New Zealand rabbits (Oryctolagus cuniculus) pure strains aged 6-12 weeks and weighing 2.5-3 kilograms were used as experimental animal models. Twelve rabbits that met the inclusion criteria were housed at 28,0±2,0 oC room temperature 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 the adaptation period to prevent disease. Each of the rabbits was then separated into one cage and randomized by the researchers using a computer program into three groups; 4 rabbits in the 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 the injection of enrofloxacin (5 mg/KGB) intravenously (IV) into the lateral auricular vein in the ear as a 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 the T1 group, the defect was filled with bovine HA; in the T2 group, it was filled with the HA of flower crab shells. After the procedure, the incision wound was well-sutured. All rabbits were observed for activity and signs of inflammation every 12 hours after surgery for 6 weeks and were given dexamethasone IM (2 mg/KGB) and enrofloxacin IM (5 mg/KGB) antibiotics every 24 hours for three days after surgery. Rabbits that died during treatment or suffered postoperative infection were excluded.

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):

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

Outcome Evaluation

The center of the treatment area of the necropsied rabbit femur was made of slides for histopathological and immunohistochemistry assessments at the 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. In this study, the investigators were only aware of the sample identity after the data were collected.

Data Analysis

The normality of the data distribution was analyzed using the Shapiro-Wilk test. Normally distributed data were analyzed with parametric statistical analysis One-Way ANOVA and Post Hoc test to assess the differences between each group. Otherwise, the Kruskal-Wallis and Mann-Whitney tests were used. Data were analyzed with SPSS ver.25 software for Windows 10, with the result 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 due to complications of postoperative infections. The baseline data are presented in Table 1.

Osteoblast Cell:

The highest mean value of osteoblasts was found in the T1 group with a mean of 52.00 ± 20.66, followed by the T2 group with a mean of 20.33 ± 7.51, and lastly the C group with a mean of 6.33 ± 4.04 (Figure 1). The osteoblast cell data were normally distributed, with the One-Way ANOVA test showing a significant difference between each group (Table 2). The results of the LSD Post Hoc test showed a significant difference between the C group and the T1 group and between the T1 group and the T2 group. Comparison between the C and T2 groups showed no significant difference (Table 3).

IL-6 Expression

The highest IL-6 expression was found in the T2 group with a mean of 81,67 ± 18,93, followed by the T1 group with a mean of 76,67 ± 22,09, and the C group 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 2). The Mann-Whitney test results showed a significant difference between the C group and the T1 group, and also between the C group and the T2 group. Comparison between the T1 and T2 groups showed no significant difference (Table 3).

TNF-α Expression

The highest TNF-α expression was found in the T2 group with a mean of 90,00 ± 8,66, followed by the T1 group with a mean of 78,33 ± 12,58, and the C group 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 2). The Mann-Whitney test results showed a significant difference between the C group and the T1 group, and also between the C group and the T2 group. Comparison between the T1 and T2 groups showed no significant difference (Table 3).

Discussion:

Bone healing is a complex, multifactorial process involving many components. This study aims to see how hydroxyapatite generated from flower crab shells affects bone repair in rabbits with femoral defects. 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 forming organic components of the bone matrix and regulating bone metabolism. In the process of bone remodeling, osteoblasts express receptor NF-κB ligand (RANKL) and osteoprotegerin (OPG). [14,15] This study demonstrated that using bovine HA and flower crab shell HA could produce more osteoblasts in bone defects compared to the group that did not receive HA. Descriptively, using bovine HA resulted in a higher number of osteoblast cells than 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.[16] 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.[17] HA has structural characteristics similar to inorganic components found in bones and teeth, with osteoconductive and osteoinductive properties that trigger tissue repair and regeneration processes. HA can promote the adhesion and proliferation of osteoblastic cells to the 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. [18-20] TNF-α and IL-6 are pro-inflammatory cytokines that play an important role in the immune response and bone metabolism. Both are known to increase the activation of macrophages and antigen presentation and regulate immunity through various mechanisms. 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. [21] 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.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-κB ligand activation and is influenced by the size of the particles and their pores. [22] 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. [23] 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. [24] The experimental model of the fracture healing process in rabbit femur can mimic the clinical condition of patients with fractures, as well as the interventions performed on the condition with adjusted doses. This model can be a useful method to evaluate the effectiveness of interventions, as researchers evaluate the levels of pro-inflammatory cytokines that affect the fracture healing process.The limitation of this study is that it did not assess other variables that influence the healing of bones such as osteoclast cells, NF-κB ligands, and osteoprotegerin. Furthermore, a larger sample size should be used for this investigation

Conclusion:

The efficacy of hydroxyapatite from flower crab shell was comparable to that of HA bovine, which both could increase the number of osteoblast cells, TNF-α, and IL-6 expression in the bone healing process.

Conflicts of interest: the authors have no conflict of interest

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