RESEARCH ARTICLE

Bioscrew of Green Mussel (*Perna viridis*) Shells-derived Hydroxyapatite, Polylactic Acid and Polycaprolactone Increases Procollagen 1 Intact N-Terminal Propeptide and Alkaline Phosphatase in Rabbit Model with Bone Defect

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Abstract

BACKGROUND: Hydroxyapatite (HA) is essential for bone regeneration and healing. Green mussel (*Perna viridis*) shell is a potential choice for preparing HA because they are abundant, widely available, have a smaller particle size, and have a higher HA content. Rather than using HA in powder or granule form, bioscrew has been fabricated as a composite of green mussel shell-derived HA, polylactid acid and polycaprolactone. However, its dynamic bone healing process has not been clearly disclosed, therefore Procollagen 1 Intact N-Terminal Propeptide (P1NP) and bone Alkaline Phosphatase (ALP) were investigated.

METHODS: Male New Zealand white rabbits (*Oryctolagus cuniculus*) were used as animal model. The rabbits were anesthetized and prepared for surgery. A standardized defect was created in the metaphyseal region. For the treatment group, the defect was filled with a bioscrew implant, whereas the control group did not receive any implant. At week-2, -4 and -6 post-surgery, about 3 mL of blood was collected from rabbits' marginal ear vein to collect blood serum. The serum was used to quantify P1NP and ALP levels using Enzyme-linked Immunosorbent Assay (ELISA). Data of P1NP and ALP levels were then statistical analyzed.

RESULTS: P1NP level of the treatment group was significantly (p<0.05) higher than the one of control group since the first monitor, at week-2. At the next monitor (week 4 and 6), P1NP levels of the treatment group were also significantly (p<0.05) higher than the ones of control group. In accordance with the P1NP results, the ALP level of the treatment group was significantly (p<0.05) higher than the one of control group.

CONCLUSION: Since bioscrew of green mussel shell-derived HA, PCL and PLA could increase the PINP associated early matrix synthesis, and ALP associated with later-stage mineralization, it can be concluded that bioscrew of green mussel shell-derived HA, PCL and PLA can be a promising material to promote bone repair.

KEYWORDS: bone, HA, green mussel, bioscrew, P1NP, ALP, PCL, PLA

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Introduction

Indonesia has the most significant incidence of fractures in Southeast Asia. The main causes are falls and traffic accidents, and in some cases caused by particular diseases.(1,2) Fracture healing requires basic biological requirements, which are a combination of mechanical stability due to proper fixation, synthesis, adequate bone angiogenesis, osteoclast and osteoblast, growth factors, and contact between broken bone fragments.(3,4) Materials made from polymers have a lower elastic property than human bone, so they can be combined into a biocomposite to have biological, mechanical and physical properties similar to human bone. Hydroxyapatite (HA) is one of the polymers in the form of calcium phosphate compounds that are stable and used as biomaterials for bone replacement.(5)

Recent studies have highlighted the ability of biological and natural materials on inducing bone formation (6,7), as well as creating scaffolds, such as biosilica-based composites from marine sponges namely *Melophlus sarasinorum* and *Xetospongia testudinaria* that immensely increased osteogenic differentiation and bone regeneration (8). Similar studies with marine-derived HA xenografts have shown increased osteoblast growth and higher levels of osteocalcin expression, with other findings offers insights into the molecular pathways regulating bone resorption and formation.(9-12) Additionally, *in vivo* experiments indicate that interventions using biomaterials can significantly improve bone turnover by boosting osteoblast activity and lowering osteoclast activity.(13) Alkaline phosphatase (ALP) has been shown as a reliable marker of osteoblast activity as its upregulations reflects increased bone formation.(14) Additionally, adding bioactive compounds has been shown to improve bone remodeling by increasing ALP activity and stimulating osteoblast growth.(15)

Green mussel *(Perna viridis*) is a promising source of HA due to their abundance, availability, smaller particle size, and higher HA content.(16) Green mussels shell-derived HA can be combined as biocomposites, which consist of a combination of two or more different materials in the form of microscopic units that have different chemical and physical properties from each other. The biocomposite used in this study was the combination of polylactid acid (PLA), polycaprolactone (PCL), and HA. PLA and PCL composites have biocompatibility, biodegradability, and non-toxic properties.(17-19)

Although previous studies have explored HA derived from green mussel shells for bone formation, this study was conducted to introduce a distinct and innovative approach by integrating HA into a fully functional bioscrew rather than using it in powder or granule form. Bioscrew was fabricated as a composite of HA, PLA and PCL. This material was used to mimic bone structure and to increase functional properties in superior fixation, gradual biodegradation, and enhanced biological responses. To understand its dynamic bone healing process, the bone formation markers, including Procollagen 1 Intact N-Terminal Propeptide (P1NP) and ALP were investigated at multiple time points.

Methods

Animal Preparation and Ethical Approval

Forty healthy, male, 6–12 months, 2.5-3.0 kg, New Zealand white rabbits (*Oryctolagus cuniculus*) were selected and acclimatized for 7 days under standard laboratory conditions (temperature 22–25°C, 12-hour light/dark cycle, and relative humidity of 50–60%). The rabbits were provided with standard pelleted feed and water *ad libitum.* The rabbits were randomly assigned using a computer-generated sequence to one of the study groups. The study was conducted between September 2023 and August 2024 at the Animal Center in Semarang and the Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Diponegoro. All animal procedures adhered strictly to national and institutional standards for ethical animal care, applying the principles of the 3Rs (Replacement, Reduction, and Refinement). Ethical approval for the study was obtained from the Health Research Ethics Committee, Faculty of Medicine, Diponegoro University (Approval No. 110/EC-H/KEPK/FK-UNDIP/IX/2023).

Bone Defect Model and Implant Surgical Procedure

As preoperative prophylaxis, rabbits were administered intravenously with 5 mg/kg enrofloxacin through the lateral auricular vein. Anesthesia was conducted with intramuscular administration of 10–40 mg/kg ketamine combined with 0.5–1 mg/kg acepromazine into the caudal longissimus dorsi muscle. After skin preparation and aseptic draping, a longitudinal incision was made along the lateral femoral shaft. A standardized defect, 4 mm in diameter and 4 mm deep, was created in the metaphyseal region using a sterile drill bit at pressures below 180 MPa. Micro-computed tomography was used for accurate femoral landmarks and dimensions.(20) For the treatment group, the defect was filled with a bioscrew implant, whereas the control group did not receive any implant (Figure 1). Bioscrew implant

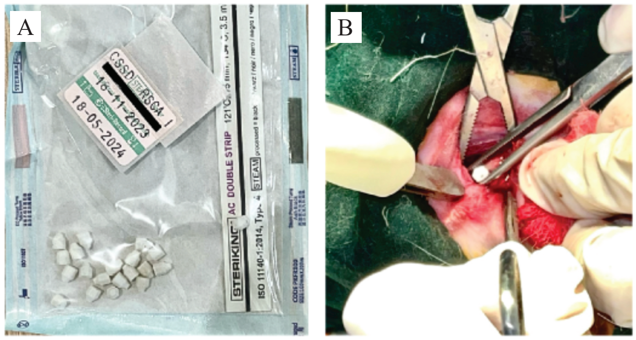

Figure 1. Bioscrew design and implantation in the rabbit femur. A: Macroscopic view of the bioscrew. B: intraoperative photograph showing the placement of the screw into the femoral bone of the rabbit.

used in this study was a composite material of 80.75% PLA, 14.25% PCL, and 5% green mussel shells-derived HA.(14) After surgery, the rabbits were observed for 24 hours to assess for signs of pain, distress, surgical site infection, and procedural complications. Postoperative care included oral administration of 2 mg/kg carprofen and 5 mg/kg enrofloxacin, was performed. Then, the rabbits were returned to standard housing conditions and monitored for 2, 4 and 6 weeks. Daily observation was performed to monitor appetite, wound healing, and mobility. At study completion, humane euthanasia was carried out with a ketamine overdose followed by cervical dislocation, in full adherence to The American Veterinary Medical Association (AVMA) and institutional ethical guidelines.

Blood Collection and Preparation

At week-2, -4 and -6 post-surgery, rabbits' marginal ear vein was disinfected and warmed to improve venous access. About 3 mL of blood was collected using 23G butterfly needle and serum separator tube. The blood was centrifuged at 3000 rpm for 10 minutes to collect the blood serum.

P1NP Enzyme-linked Immunosorbent Assay (ELISA)

Serumal P1NP level was quantified using Rabbit P1NP ELISA Kit (Cat. No. MBS017936; MyBioSource, San Diego, CA, USA), which was a sandwich ELISA kit with detection range of 6.25 ng/mL-200 ng/mL and the sensitivity level of 1 ng/mL. P1NP ELISA was performed according to the manufacturer's protocol. Briefly, antibody specific for P1NP was pre-coated on the strip plate. Standards and serum were pipetted into the wells. Horseradish Peroxidase (HRP)-conjugate reagent was added to the wells and incubated for 60 min. After washing, chromogen was added to the wells and incubated for 15 min. After adding stop solution, the color was developed in proportion to the amount of P1NP bound from blue to yellow. The color intensity was measured immediately using a microplate reader at the absorbance at 450 nm.

ALP ELISA

For quantification of ALP, Rabbit ALP ELISA Kit (Assay Genie, Dublin, Ireland) was used. It was a Sandwich ELISA kit as well with detection range of 0.156-10 ng/mL and the sensitivity level of 0.094 ng/mL. ELISA was performed according to the manufacturer's protocol. Briefly, standards and 1:200 diluted serum were added in to each well of the 96-well plate precoated with capture antibody for rabbit ALP. After washing, the biotin conjugated antibody was added in to each well as the detection antibody. After washing off unbound conjugates, HRP-Streptavidin was added. After a third washing, 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added. After adding acidic stop solution, blue color product turned yellow. The color intensity was measured immediately using a microplate reader at the absorbance at 450 nm.

Statistical Analysis

After Saphiro-Wilk test, when the data were normally distributed, independent t-tests and repeated ANOVA were used. An Independent t-test was conducted to test the treatment and control groups, while repeated ANOVA was conducted to compare between weeks in each group. When the data were not normally distributed, Mann Whitney and Friedman tests were used. Differences were considered significant if $p<0.05$.

Results

After surgery, the rabbits of both treatment and control groups, did not show any sign of pain, distress, surgical site infection, and procedural complications.

Bioscrew implant increased P1NP level

P1NP level of the treatment group was significantly ($p<0.05$) higher than the one of control group since the first monitor, at week-2 (Table 1). At the next monitor (week-4 and -6), P1NP levels of the treatment group were also significantly ($p<0.05$) higher than the ones of control group. However, the P1NP levels of treatment group at week-2, -4 and -6 were not significantly increase along with the period. Similar to the treatment group, the P1NP levels of control group at week-2, -4 and -6 were also not significantly increase along with the period.

Table 1. P1NP level of treatment and control groups.

Assessment Time	P1NP Level (nmol/L)		*p*- value
	Treatment Group	Control Group	
Week-2	322.93±10.56	265.35±55.69	0.025[a,*]
Week-4	353.78±47.11	284.75±51.44	0.036[b,*]
Week-6	350.93±33.00	283.27±53.38	0.025[b,*]
p- value	0.069[d]	0.899[c]	

[a]Mann-Whitney; [b]Independent-t; [c]Repeated ANOVA; [d]Friedman.

Bioscrew implant increased ALP

In accordance with the P1NP results, the ALP level of the treatment group was significantly (p<0.05) higher than the one of control group (Table 2). However, at week-4, ALP level of the treatment group was not significantly higher than the one of control group. Later on, at week-6, ALP level of the treatment group was again significantly higher than the one of control group. The ALP level of both treatment and control groups at week-2, -4 and -6 were also not significantly increase along with the period.

Discussion

Bone healing can be influenced by several factors, including the presence of an osteoconductive matrix, which facilitates mineralization and supports the attachment of osteoconductive and osteogenic cells at the fracture site.(21) Bone tissue consists of approximately 69% minerals, 22% organic matter, and 9% water, with HA accounting for nearly 50% of its mineral fraction, making it crucial for bone regeneration and repair.(18) Additionally, PLA and PCL used in the composite demonstrate favorable properties such as biocompatibility, biodegradability, and non-toxicity.(17,18)

Bone formation levels tend to be expected or slightly increased in the bone healing process due to the influence of HA content, which can work as a scaffold in bone. The content of PLA and PCL in bioscrews can increase bone formation and decrease inflammatory cells *in vivo*, compared to standard polymer materials. PLA and PCL can also improve strength retention, bone-bonding potential, and pH buffering during the fracture bone healing period.(21-23)

P1NP and ALP are important biomarkers to evaluate bone healing which could be measured from the blood sample.(19,24) Significant higher P1NP and ALP levels of the treatment group than the ones of control group were obtained since the first monitor at week-2, suggesting that bioscrew of green mussel shell-derived HA, PCL and PLA could have an efficacy in accelerating bone healing. However, the P1NP and ALP levels of the treatment group at week-2, -4 and -6 were not significantly different along with the period, suggesting that the effectiveness of HA administration is consistent throughout weeks-2, -4, and -6.(23)

Conclusion

Since bioscrew of green mussel shell-derived HA, PCL and PLA could increase the PINP associated early matrix synthesis, and ALP associated with later-stage mineralization, it can be concluded that bioscrew of green mussel shell-derived HA, PCL and PLA can be a promising material to promote bone repair.

Table 2. ALP level of treatment and control groups.

Assessment Time	ALP Level (µg/dL)		*p*- value
	Treatment Group	Control Group	
Week-2	152.96±16.19	100.79±39.81	0.014[b,*]
Week-4	124.09±48.12	129.35±54.73	0.749[a]
Week-6	164.89±26.74	101.47±41.19	0.025[a,*]
p- value	0.301[c]	0.614[c]	

[a]Mann-Whitney; [b]Independent-t; [c]Repeated ANOVA.

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Authors Contribution

KDA, EAN, MH, and RN were involved in concepting the research and performed the data acquisition along with DRP. Data analysis was done by YN, KCT, DRPR and FS. KCT, DRP, FM and FS aided in result interpretation and manuscript preparation. KCT and FS designed the figures and tables. All authors contributed in providing critical revision of the manuscript.

Conflict of Interest

The authors declare no conflicts of interest or competing interests related to the content of this manuscript.

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