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Original article

Evaluation of BSP and DMP1 in hydroxyapatite crab shells used for dental socket preservation

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ABSTRACT

Background: *Bone resorption due to tooth extraction leads to unpredictable bone volume for future prosthetics. Crab shells were promoted as a solution to prevent bone resorption, along with an effort to reduce biological waste.* ***Purpose:*** *This study aimed to analyze the expression of bone sialoprotein (BSP) and dentine matrix protein-1 (DMP1) in the wound healing process in tooth-extraction sockets after applying a crab shell-derived hydroxyapatite scaffold.* ***Methods:*** *The subjects (28 Cavia cobaya) were divided into control and treatment groups. The control group was left untreated, while the treatment group received a hydroxyapatite scaffold of Portunus pelagicus shell in the tooth socket. The expression of BSP and DMP1 was determined by immunohistochemical staining on days 7 and 14. One-way analysis of variance and Tukey's honest significance difference test were used to find the groups with the most significant difference.* ***Results:*** *The highest mean expression of BSP and DMP1 was in the day 14 treatment group, while the lowest was in the day 7 control group.* ***Conclusion:*** *Administering hydroxyapatite scaffold derived from the Portunus pelagicus shell to the post-extraction sockets increased the expression of both BSP and DMP1.*

Keywords: *BSP; crab shell; DMP1; hydroxyapatite; medicine*
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INTRODUCTION

The changes in bone volume after tooth extraction seem to be physiological consequences, where 'unnecessary' bone which does not receive strain stimulus is eliminated.[1] Therefore, a bone graft is the solution to maintain the height of the alveolar bone for future denture prosthesis treatment.[2-4] Hydroxyapatite is the most widely used alloplastic bone graft material. It is well known for its osteoinductive properties in new bone regeneration since its structure is very close to normal bone and is available on the organic matrix.[5,6]

Portunus pelagicus, a crab species, is one of Indonesia's leading fishery export commodities. The shells of the crab contain 40–70% calcium carbonate. Suitably processing the calcium carbonate can turn it into calcium hydroxyapatite, which is helpful in osteogenesis. Considering the value of crab shells and the amount of waste they generate, a recycling effort is carried out so that the existing waste can be controlled and utilized as well as possible. Lamongan district in Indonesia is one of the largest contributors to these commodities (19.4%).[7]

Abundant hydroxyapatite sources include mammalian bone, marine or aquatic creatures, shell sources, plant or algae, and mineral sources.[8] Hydroxyapatite from crab shells is a new idea. The main reason behind this idea was to reduce the biological waste in the local environment. The solution offered in this study was to minimize biological waste while recycling it for use in the bone graft needed to prevent bone resorption.

Bone sialoprotein (BSP), one of the non-collagen proteins of extracellular matrix (ECM), is produced by osteoblasts and osteoclasts. The increase in BSP corresponds to the increase in bone mineralization. The

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existence of BSP is influenced by the role of runt-related transcription factor 2 and alkaline phosphatase.[9] A study on mice showed that cementum decreased significantly in the absence of BSP. Long bone length and bone formation rates also decreased with cortical thinning.[10]

Dentin matrix protein-1 (DMP1) is another non-collagen protein of ECM, expressed by osteoblasts, osteocytes, and hypertrophic chondrocytes. The role of DMP1 in osteogenesis is the maturation of odontoblasts, osteoblasts, and mineralization. Studies have found that DMP1-deficient mice revealed severe defects in cartilage formation, such as in hereditary hypophosphatemic rickets.[11]

Osteoblasts abundantly express BSP, especially at sites of primary bone formation. BSP is also known for its ability to promote osteoblast differentiation and increased production of mineralized matrix.[12] High levels of BSP and DMP1 were observed on days 7 and 14 when the remodeling phase of wound healing begins. This study aimed to analyze the expression of BSP and DMP1 in tooth extraction sockets after applying a *P. pelagicus* shell-derived hydroxyapatite scaffold. BSP expression was studied to track the osteoblast differentiation around extraction sockets, while DMP1 was observed as a marker for bone ECM protein responsible for bone development.

MATERIALS AND METHODS

The Health Research Ethical Clearance Commission of the Faculty of Dental Medicine, Universitas Airlangga, approved this study with certificate number 548/HRECC. FODM/XII/2020. The design for this study was a post-test-only control group design. Twenty-eight male *Cavia cobaya* (guinea pigs) were the subjects (the number featured in Federer's formula according to a similar study previously conducted by Kresnoadi et al.).[13] The requirements for the subjects were as follows: adult *Cavia cobaya* (3–3.5 months old) in good health, weighing 300–350 grams. The *C. cobaya* were habituated for one week before the experiment was conducted. They received standard food pellets and water and were exposed to a 12-hour light/dark cycle. These subjects were then randomly assigned to the following groups: control group 7 (C7), control group 14 (C14), treatment group 7 (T7), and treatment group 14 (T14). This study was carried out from January to May 2020.

Crab shells were obtained from a 3-month-old *P. pelagicus* on a beach in Lamongan, East Java. These shells were cleaned of soft tissue using distilled water before being soaked in a chlorine solution with a ratio of thirty ml to five liters of water. The samples were soaked in hydrogen peroxide 3% for 24 hours before being dried at room temperature. The heating process of shell calcination was as follows: the initial temperature during heating was approximately 50°C with a gradual increase of 5°C/minute to 1,000°C in a furnace. The temperature was then maintained at a stable 1,000°C for two hours and decreased naturally to approximately 100°C. A scanning electron microscope with energy-dispersive X-ray was utilized to characterize hydroxyapatite compounds. The process involved the mechanical sifting of powder to produce hydroxyapatite powder with a particle size of approximately 150–350 μm.[14]

Five grams of gelatin were poured slowly into distilled water and mixed at 40°C for one hour. The hydroxyapatite-gelatin composite was produced by adding 1.5 grams of hydroxyapatite powder to the gelatin solution and stirring it for six hours, as described previously by Kamadjaja et al.[15] The process was continued with centrifugation for ten minutes to isolate the water from the gel. The gel solution was transferred to a mold (2 mm in diameter and 5 mm in height), stored in a freezer for 24 hours at a temperature of -80 °C, and freeze-dried for 24 hours.[16]

The *C. cobaya* were injected 20 mg per 300 mg body weight of ketamine intramuscularly (Kepro, ZA, Denmark) for sedation and anesthesia. Before tooth extraction, the left mandibular incisive tooth area was debrided. Then, tooth extraction was done carefully using a sterile needle holder to prevent root fracturing. The sockets of the control group members (C7 and C14) were left untreated. Those in the treatment groups (T7 and T14) were administered up to 1 ml of the gelatin-hydroxyapatite scaffold, depending on the volume of the tooth socket. Simple suturing was used in all groups using polyamide monofilament DS 12 3/ 8c, 12 mm, 6/10 met, 0.7 (Braun VetCare SA, Rubi, Spain).[13]

The *C. cobaya* were sacrificed on days 7 and 14 by administering a lethal dose of ketamine (Kepro, ZA, Denmark). The mandibles of the *C. cobaya* were cut medio-sagitally. The mandibular samples were fixed with a 10% formalin buffer for 24 hours at 80°C and decalcified with 2% nitric acid. Dehydration was then performed using graded alcohol concentrations (decreasing from 100% to 70%), followed by clearing in xylol and embedding in paraffin. Paraffin blocks were cut to a thickness of four microns and placed in an object glass.[17]

The tissue deparaffinization process was completed using a solution of xylol, ethanol, and alcohol. Processing of the tissues was continued with a 3.3'-diaminobenzidine (DAB) staining kit (Pierce™ DAB Substrate Paint Kit 34002, Thermofisher™, Massachusetts, United States). The tissues were incubated at room temperature with primary antibodies to BSP (Santacruz Biotech, cat#SC7360) and DMP1 (Santacruz Biotech, cat#sc-73633). After adding the DAB buffer solution, the antibody complex was observed under a light microscope (Nikon Eclipse E 100, Japan). The observation area was specified as the apical third of the socket.[17]

The IBM Statistical Package for the Social Sciences, Statistics for Windows, version 24.0. (Armonk, NY: IBM Corp) was used in this study. The results were shown as means and standard deviations. The one-sample Kolmogorov Smirnov test, the Levene's Test, one-way

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analysis of variance, and Tukey's honest significance difference test were utilized to study the differences between groups.

RESULTS

The statistical analysis demonstrated a significant difference ($P < 0.05$) between the control and treatment groups. The control group (C7) expressed a significant difference compared with the two treatment groups (T7 and T14) ($P < 0.05$). Similarly, the control group (C14) also showed a significant difference compared with the 14-day treatment group (T7 and T14) ($P < 0.05$) (Tables 1 and 2, and Figure 1). A surge of BSP was observed in the post-extraction sockets in more than half the subjects in the treatment groups from day 7 to day 14. The highest BSP expression was observed in the treatment group on day 14, while the lowest was in the control group on day 7. Figure 1 shows the expression of BSP as indicated by the arrows. Osteoblasts that synthesized BSP are marked by brown tinting of their cells. Osteoblast cells in the matrix near the lining cells appear as cuboidal or polygonal cells.

The control group (C7) demonstrated a significant difference as compared with the treatment groups (T7 and T14) ($P < 0.05$). Similarly, the control group (C14) showed a significant difference compared with the treatment groups (T7 and T14) ($P < 0.05$) (Tables 1 and 2). There was an increase in the amount of DMP1 expression in the post-extraction socket in most of the subjects in the treatment

Table 1. Mean expression of BSP and DMP1 on days 7 and day 14

Group	Day	BSP			DMP1		
		Σ Samples	Mean	Standard Deviation	Σ Samples	Mean	Standard Deviation
Control	7	7	6.14	1.773	7	6.43	3.359
	14	7	7.86	1.952	7	8.43	1.902
Treatment	7	7	12.29	1.976	7	12.43	1.718
	14	7	14.43	2.507	7	14.71	2.812
Total		28	10.18	2.052	28	10.5	2.447

Table 2. Tukey's honest significance difference test results. C7: control group on day 7, C14: control group on day 14, T7: treatment group on day 7, T14: treatment group on day 14

Group	BSP				DMP1			
	C7	C14	T7	T14	C7	C14	T7	T14
C7			*	*			*	*
C14			*	*			*	*
T7	*	*			*	*		
T14	*	*			*	*		

The asterisk (*) symbol represents groups with significant differences ($P < 0.05$).

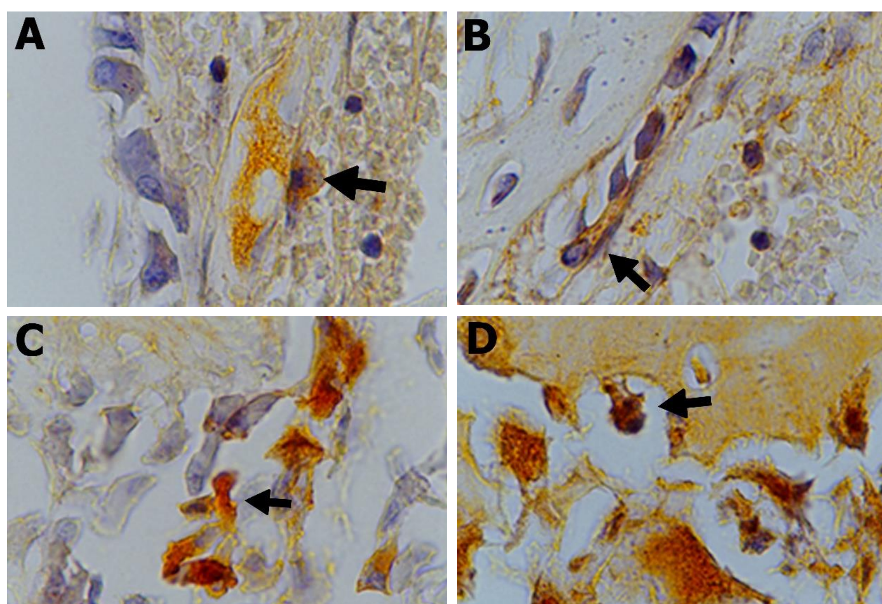

Figure 1. The black arrows point to the expression of BSP observed beneath a 1000X magnification light microscope. A: The control group on day 7 (C7). B: The control group on day 14 (C14). C: Tooth extraction and crab shell hydroxyapatite application on day 7 (T7). D: Tooth extraction and crab shell hydroxyapatite application on day 14 (T14).

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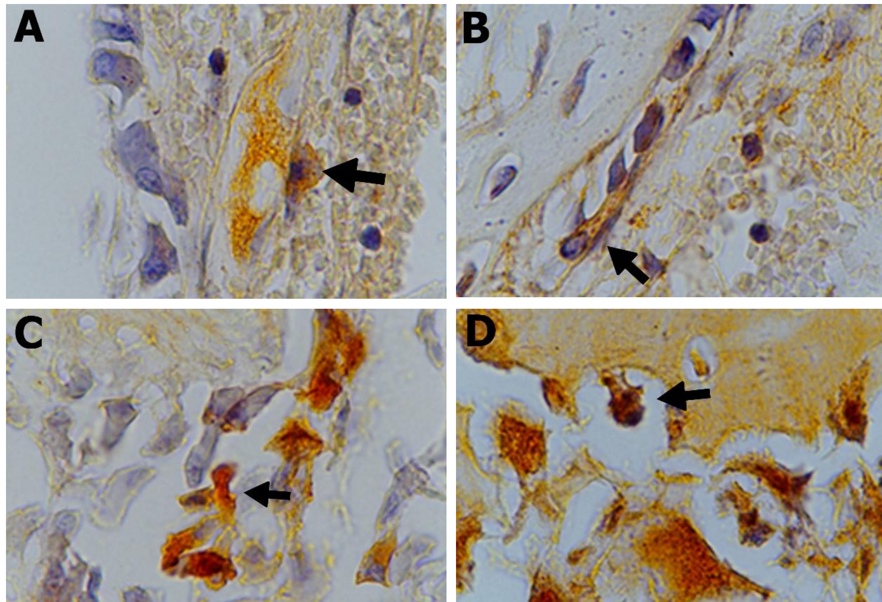

Figure 2. The black arrows point to the expression of DMP1 beneath a 1000X magnification light microscope. A: The control group on day 7 (C7). B: The control group on day 14 (C14). C: Tooth extraction and crab shell hydroxyapatite application on day 7 (T7). D: Tooth extraction and crab shell hydroxyapatite application on day 14 (T14).

groups between days 7 and 14. The highest level of DMP1 expression was detected in the treatment group on day 14, while the lowest occurred in the control group on day 7. The expression of DMP1 in the histological field is shown by the arrows (Figure 2). Osteoblasts that synthesized BSP are marked by brown tinting of their cells. Osteoblast cells in the matrix near the lining cells appear as cuboidal or polygonal cells.

DISCUSSION

The groups with the hydroxyapatite scaffold from the crab shell demonstrated a significant amount of BSP and DMP1 expressions on the 14th day. The highest BSP and DMP1 expressions were found in the hydroxyapatite scaffold group on day 14.

The increase in the amount of BSP and DMP1 is due to the role of hydroxyapatite in regulating bone formation. As per this theory, hydroxyapatite progressively reduces the activity of osteoclasts, decreasing bone resorption activity. On the other hand, adding hydroxyapatite can also improve the formation and differentiation of osteoblasts. Osteoblasts play a role in adhering and developing effectively within bone defects, resulting in the stability of the wound due to cartilage (soft callus). In the later stage, the soft callus becomes a hard callus (bone).[18]

Bone sialoprotein and DMP1 are members of the small integrin-binding ligand N-glycosylated family, secreted into the ECM during bone formation. Mineralization of ECM facilitates the deposition of hydroxyapatite. Although BSP can be found at the onset of bone formation, excess expression of BSP in osteoblasts appears to increase during mineralization.[9-11] BSP can also trigger hydroxyapatite crystal nucleation and osteoblast differentiation.[19] As observed in this study, levels of BSP rose significantly in the T7 and T14 groups. BSP increases slowly because the proliferation process is ongoing and the mineralization process is imperfect; hence the actual value of their increase is of no great significance. Consequently, it is sufficient to conduct regular inspections until the 7th day, and further examination up to and including the 14th day is unnecessary.

In addition to BSP, DMP1 is a fossilized acid ECM that binds to hydroxyapatite and mediates cell attachment through the arginyl-glycyl-aspartic acid domain. The existence of DMP1 is closely related to osteocytes and pericytes.[20] In DMP1, bone is processed into fragments of 37 kDa derived from the N-terminal for growth and proliferation, and fragments of 57 kDa derived from the COOH-terminal (containing peptide a*cidic serine aspartate-rich* matrix extracellular phosphoglycoprotein-*associated)* of the calcification and ossification zone.[9-11] Therefore, the expression of DMP1 rose significantly in the T7 and T14 groups compared with their C7 and C14 counterparts. However, this increase was not significant, suggesting that DMP1 increases slowly since the proliferation is ongoing while the mineralization process is imperfect. Therefore, analysis up to and including the seventh day is sufficient, with further examination until the 14th day being redundant.

A previous similar study using *P. pelagicus* shell in the tooth socket after tooth extraction examined its effect on tumor necrosis factor-alpha, osterix, receptor activator of nuclear factor kappa-B ligand, and osteoprotegerin.[21,22] Its effect on bone matrix mineralization has not yet been

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explored. This stage is essential, particularly in future bone maturation and strength. In this study, BSP and DMP1 were increased in the treatment group from day 7 to day 14. The mean expression of the positive cells from the treatment group was significantly higher than in the control group, proving that the *P. pelagicus* shell induces bone matrix mineralization and density. This study could be refined more effectively later, given the exceptionally significant role of hydroxyapatite scaffold derived from crab shells in wound healing. Furthermore, this research was limited and needed another marker to confirm the bone regeneration process. From the discussion above, it can be concluded that applying a hydroxyapatite scaffold derived from the *P. pelagicus* shell to the post-extraction sockets increases the expression of both BSP and DMP1 as documented immunohistochemically, *in vivo*.

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