

BUKTI KORESPONDENSI  
JURNAL INTERNASIONAL BEREPUTASI TERAKREDITASI Q4

Judul Artikel : **(C09)** Evaluation of BSP and DMP1 in hydroxyapatite crab shells used for dental socket preservation

Jurnal : Dental Journal (Majalah Kedokteran Gigi)  
Penulis : **Michael Josef Kridanto Kamadjaja\***, Sherman Salim , Wiwik Herawati Waluyo , Tengku Natasha Eleena binti Tengku Ahmad Noor.

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2	Bukti Revisi 1	23 Mei 2022	1-27
3	Bukti Revisi 2	13 Juni 2022	28-30
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9	Bukti accepted	11 Januari 2023	96
10	Bukti published	22 Februari 2023	98

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----- Original message -----

From: michael-j-k-k <michael-j-k-k@fkg.unair.ac.id>  
Date: 7/27/22 12:31 (GMT+07:00)  
To: Jennifer Widjaja <jenniferwidjaja@rocketmail.com>  
Subject: FW: [DJMKG] Editor Decision

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From: Muhammad Dimas Aditya Ari <jurnal@ppjpi.unair.ac.id>  
Date: 7/26/22 14:38 (GMT+07:00)  
To: michael josef kridanto kamadjaja <michael-j-k-k@fkg.unair.ac.id>, sherman salim <sherman.salim@yahoo.com>, wiwik herawati <herawati007w@gmail.com>  
Subject: [DJMKG] Editor Decision

Dear michael josef kridanto kamadjaja, sherman salim, wiwik herawati,

We have reached a decision regarding your submission to Dental Journal (Majalah Kedokteran Gigi), "In vivo evaluation of BSP and DMP-1 in scaffold crab shells-derived hydroxyapatite (Portunus pelagicus) for the purposes of socket preservation".

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To: michael josef kridanto kamadjaja <michael-j-k-k@fkg.unair.ac.id>, josef\_310563@yahoo.com

Subject: [DJMKG] Editor Decision

Dear michael josef kridanto kamadjaja, sherman salim, wiwik herawati,

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Link: <https://e-journal.unair.ac.id/MKG/authorDashboard/submission/36026>

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Subject: [DJMKG] Published article

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We notified that your article with the title:

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## Evaluation of BSP and DMP-1 in hydroxyapatite crab shells for socket preservation

### ABSTRACT

**Background:** Bone resorption as a result of tooth extraction leads to unpredictable bone volume for future prosthetic. Crab shells were promoted as a solution to prevent bone resorption, along with an effort to reduce biological waste. **Purpose:** This study was aimed to analyze the expression of bone sialoprotein and dentine matrix protein-1 in the middle of wound healing process from tooth-extraction sockets after application of scaffold crab shells-derived hydroxyapatite. **Methods:** The subjects (28 *Cavia cobaya*) were divided into two categories: control and treatment group. Control group was left untreated, while treatment group received hydroxyapatite scaffold of *Portunus pelagicus* shell in the tooth socket. The expression of bone sialoprotein (BSP) and dentin matrix protein-1 (DMP1) was subjected to immunohistochemical staining on day 7 and 14. One-way ANOVA and Tukey HSD tests were used to find the group with the most significant difference. **Results:** The highest mean number of BSP and DMP1 was day 14 treatment group, while the lowest was day 7 control group. **Conclusion:** Administering hydroxyapatite scaffold derived from *Portunus pelagicus* shell to the post-extraction sockets has been shown to increase the expression of both BSP and DMP1.

**Keywords:** BSP; Crab Shell; DMP1; Hydroxyapatite; Medicine

### INTRODUCTION

The changes of bone volume after tooth extraction seems to be the physiological consequences, where the ‘unnecessary’ bone which don’t receive strain stimulus will be eliminated.<sup>1</sup> Therefore, the use of bone graft is a solution to maintain the height of the alveolar bone for the future denture prosthesis treatment.<sup>2</sup> The use of bone graft prior to tooth extraction Hydroxyapatite ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})$ ), the most widely used alloplast bone graft material, is well known for its osteoinductive in terms of new bone regeneration for its structure is very close to the bone structure and available on the organic matrix.<sup>3</sup>

*Portunus pelagicus* is one of the leading fishery export commodities in Indonesia. One of the districts that become the largest contributors to these commodities is Lamongan (19.4%).<sup>4</sup> The shells itself contain 40 – 70% calcium carbonate. The right processing of calcium carbonate can be turned into calcium hydroxyapatite that will be useful in osteogenesis. By considering the value of crab shells and the amount of waste from crab shells, a recycling effort is carried out so that the existing waste can be controlled and utilized as well as possible.

Hydroxyapatite source is abundant, including mammalian bone, marine or aquatic creature, shell sources, plant or algae, and mineral sources. Hydroxyapatite from crab shells

is quite a new idea, the main reason behind this idea was to reduce the biological waste from local environment. The solution that is offered in this study was to reduce biological waste while recycling it into bone graft which is needed to prevent bone resorption.

Bone sialoprotein (BSP), one of the non-collagen protein of extracellular matrix (ECM), is produced by osteoblast and osteoclast cells. The increase in BSP is in line with the increase in the process of bone mineralization. The existence of BSP is influenced by the role of runx 2 and alkaline phosphatase (ALP). The result of the study on mice in the absence of BSP showed, cementum decreased significantly, while long bone length, cortical thinning, and bone formation rates also decreased.<sup>5</sup>

Dentin Matrix Protein (DMP)-1, another non-collagen protein of ECM, expressed by osteoblasts, osteocytes and hypertrophic chondrocyte. DMP1 role in osteogenesis is maturation of odontoblasts and osteoblasts and also mineralization. Research using mice found that deficient in DMP1 revealed severe defects in cartilage formation such as hereditary hypophosphatemic rickets.<sup>6</sup>

BSP is abundantly expressed by osteoblast, especially in sites of primary bone formation. It is also known for its ability to promote osteoblast differentiation and increased production of mineralized matrix.<sup>7</sup> BSP and DMP-1 was observed on day 7 and 14, where the remodeling phase of the wound healing began. This study was aimed to analyze the expression of BSP and DMP1 in tooth extraction sockets after the application of hydroxyapatite scaffold-*Portunus pelagicus* shell derived. BSP was observed in this study in order to track the osteoblast differentiation around extraction sockets, meanwhile DMP1 was observed as a marker for bone ECM protein that conducts bone development.

## **MATERIALS AND METHODS**

Health Research Ethical Clearance Commission (Faculty) has approved this study with certificate number 548/HRECC.FODM/XII/2020.

### **Experimental animal preparations**

The design for this study was post-test only control group design. Twenty-eight male *Cavia cobaya* was the subject, being the number featured in Federer's formula according to a similar study previously conducted by Kresnodi et al.<sup>8</sup> The requirements for the subjects were as follows: adult *Cavia cobaya* (3 – 3.5 months old) in good health weighing 300-350 grams. For one week *Cavia cobaya* were habituated before the experiment was conducted; received standard food pellets and water, and exposed to a 12-hour light/dark cycle. Then these

subjects were randomly assigned to: control group 7 (C7), control group 14 (C14), treatment group 7 (T7), and treatment group 14 (T14). This research has been held from January to May 2020.

### **Hydroxyapatite from crab shell preparation**

Crab shells were obtained from 3-month-old *Portunus pelagicus* on a beach located in Lamongan, Jawa Timur (East Java). These shells were cleaned of soft tissue using distilled water prior to being soaked in a solution of chlorine at a ratio of thirty ml of chlorine to five liters of water. Subsequently, before the samples were dried at room temperature, it was soaked in hydrogen peroxide 3% for 24 hours. The heating process of shell calcination involved: the initial temperature during heating was approximately 50°C with a gradual increase of 5°C/minute to 1,000°C in a furnace. Then the temperature was maintained at a stable 1,000°C for two hours and decreased naturally to approximately 100°C. Scanning electron microscopy with energy-dispersive x-ray (SEM-EDX) was utilized for characterization of hydroxyapatite compounds. This was a process involving the mechanical sifting of powder to produce hydroxyapatite powder with particle size approximately 150-350 µm.<sup>9</sup>

### **Scaffold Gelatin-Hydroxyapatite Manufacture**

Five grams of gelatin was poured slowly into distilled water and mixed at 40°C for one hour. Subsequently, hydroxyapatite (HA)-gelatin composite was produced by adding 1.5 grams of hydroxyapatite powder to the gelatin solution according to the previous research by Kamadjaja *et al.* (2019), stirring it for six hours.<sup>10</sup> The process was continued with centrifugation for ten minutes to isolate the water from the gel. The gel solution was transferred in a mold (2 mm diameter, 5 mm in height), stored in a freezer for 24 hours at a temperature of -80 °C and freeze-dried for 24 hours.<sup>11</sup>

### **Experimental animal study**

*Cavia cobaya* were injected 20mg of ketamine intramuscularly (Kepro, ZA, Denmark) per 300mg of body weight in terms of causing sedation and an anesthetized state. Before tooth extraction, the left mandibular incisive tooth area was debrided. Then, tooth extraction was done carefully using a sterile needle holder in order to prevent root fracturing. After that, the sockets of the control group members (C7 and C14) were left untreated, while those of the



treatment groups (T7 and T14) were administered as much as 1 ml using a blunt-tip syringe, filled with scaffold gelatin-hydroxyapatite according to tooth socket's volume. Simple suturing is used in all groups using polyamide monofilament DS 12 3 / 8c, 12 mm, 6/10 met, 0.7 (Braun VetCare SA, Rubi, Spain).<sup>8</sup>

### **Immunohistochemical staining**

*Cavia cobaya* were sacrificed on day 7 and 14 through the administering of lethal dose of ketamine (Kepro, ZA, Denmark). The mandibles of *Cavia cobaya* were cut median-sagittally with the mandibular samples being fixed with 10% formalin buffer for 24 hours at 80°C and decalcified with 2% nitric acid. Dehydration was then performed by means of 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.<sup>12</sup>

Tissue deparaffinization process was completed using a solution of xylol, ethanol, and alcohol. The tissues were continued to processed 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 BSP (Santacruz Biotech, cat#SC7360) and DMP1 (Santacruz Biotech, cat#sc-73633). After the addition of the DAB buffer solution, antibody complex was observed under a light microscope (Nikon Eclipse E 100, Japan). The observation area was specified at the apical third of the socket.<sup>12</sup>

### **Statistical analysis**

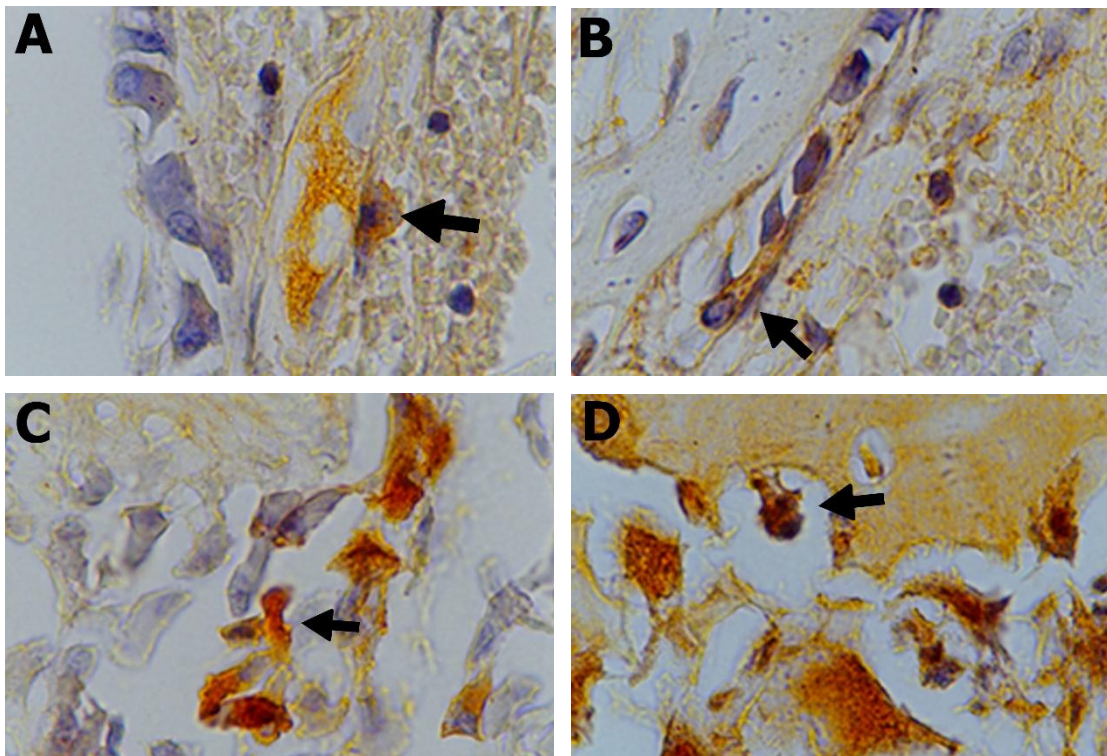
Statistical Package for the Social Sciences Software (SPSS) edition 24.0 (SPSS™, Chicago, Illinois, United States) was used in this study with the results showed as mean and standard deviation. One-Sample Kolmogorov Smirnov, a Levene's Test, a one-way analysis of variance (ANOVA) test, and a Tukey HSD Test were utilized as a method to exactly determine the differences between groups.

## **RESULTS**

### **BSP expression**

The statistical analysis demonstrated significant difference ( $p < 0.05$ ) between the control and treatment groups. The Control group (C7) expressed a significant difference to

the two treatment groups (T7 and T14) ( $p < 0.05$ ), as was also the case with the control group (C14) compared to the 14-day treatment group (T7 and T14) ( $p < 0.05$ ) (Table 1 and 2, Figure 1). A surge amount of BSP was observed in the post-extraction sockets in more than half of treatment groups from day 7 to day 14. The most BSP expression was observed in the treatment group on day 14, while the smallest expression was in the control group on day 7. The expression of BSP is indicated by the arrows in Figure 1. Osteoblasts that synthesized BSP are marked by brown tinting of their cells. **Osteoblast cells located in the matrix near lining cells, appeared as cuboidal or polygonal cells.**



**Figure 1.** The expression of BSP observed beneath a 1000x magnification light microscope is pointed by the black arrows. 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 on day 14 (T14).

### **DMP1 expression**

The control group (C7) demonstrated a significant difference with the treatment groups (T7 and T14) ( $p < 0.05$ ). This was also the case with the control group (C14) compared to the 14-day treatment group (T7 and T14) ( $p < 0.05$ ) (Table 1 and 2). There was a gain in the amount of DMP1 expressions in the post-extraction socket in the greater part of treatment groups between days 7 and 14. The biggest level of DMP1 expression was detected in the treatment group on day 14, while the smallest 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 located in the matrix near lining cells, appeared as cuboidal or polygonal cells.

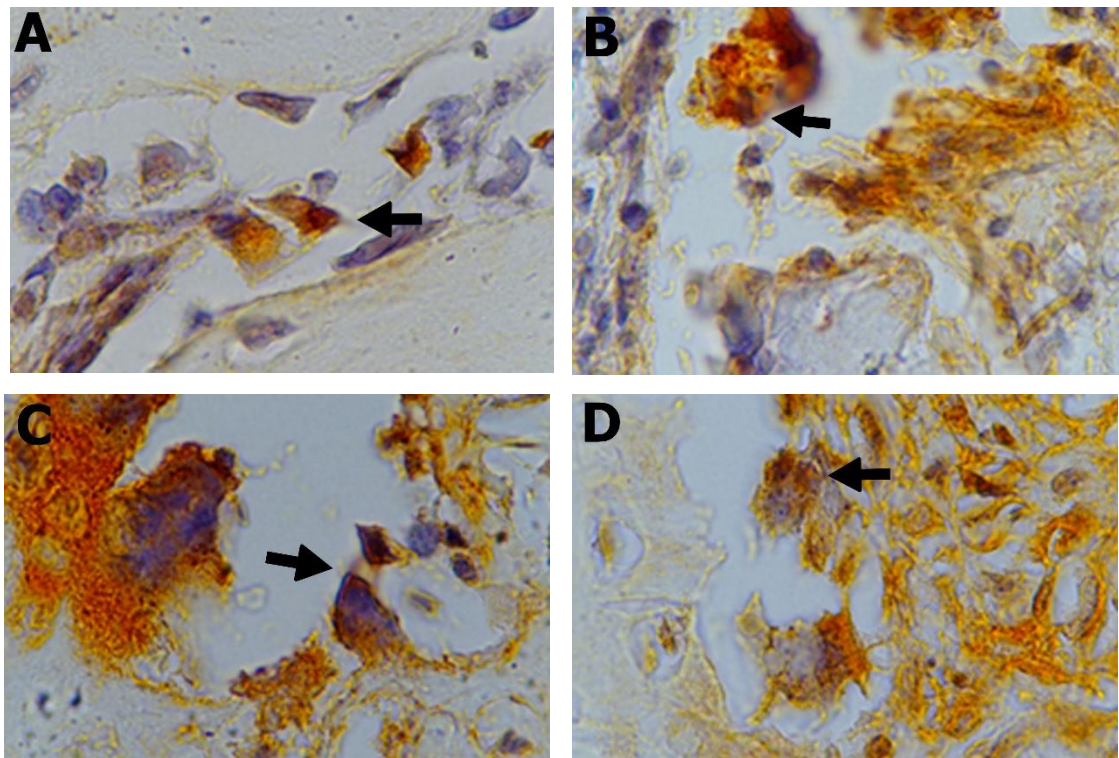


Figure 2. Expression of DMP1 beneath a 1000x magnification light microscope is pointed by the black arrows. A: The control group on day 7 (C7). B: The control group on day 14 (C14). C: Tooth extraction and crab shell hydroxyapatite application on day 7 (T7). D: Tooth extraction and crab shell hydroxyapatite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

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

Group	Day	BSP			DMP1		
		$\Sigma$ Samples	Mean	Standard Deviation	$\Sigma$ 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 HSD test results. C7: control group on day 7 observation, C14: control group on day 14 observation, T7: treatment group on day 7 observation, T14: treatment group on day 14 observation.

Groups	BSP				DMP1			
	C7	C14	T7	T14	C7	C14	T7	T14
C7			*	*			*	*
C14			*	*			*	*
T7	*	*			*	*		
T14	*	*			*	*		

Asterisk (\*) symbol represented groups with significant differences ( $p < 0.05$ ).

## DISCUSSION

Scaffold hydroxyapatite from the crab shell group demonstrated significant amount of BSP and DMP1 expressions on the 14th day. The most dominant BSP and DMP1 expressions were found in the scaffold hydroxyapatite of the crab shell group on day 14.

The increase in the amount of BSP and DMP1 is due to the role of hydroxyapatite in regulating bone formation. Within this theory, hydroxyapatite will progressively reduce the activity of osteoclasts, which means decreasing bone resorption activity. In the other hand, the addition of hydroxyapatite can also improve the formation and differentiation of osteoblasts. Osteoblasts play a role to adhere and develop effectively within bone defects, resulting the stability of the wound by cartilage (soft callus). In later stage, soft callus became hard callus (bone).<sup>13</sup>

BSP and DMP1 are both members of the SIBLING family, secreted into the extracellular matrix (ECM) during the process of bone formation. Mineralization of ECM facilitates the deposition of hydroxyapatite. At the first onset of bone formation, BSP can be found, while excess expression of BSP in osteoblasts appears to increase during mineralization.<sup>6</sup> BSP also can trigger hydroxyapatite crystal nucleation and osteoblast differentiation.<sup>14</sup> As observed during this study, levels of BSP significantly increased in the T7 and T14 groups. However, the value of their increase in both cases is of no great significance. This means that the presence of BSP increases slowly because the proliferation process remains ongoing and the mineralization process is imperfect. Consequently, it is sufficient to conduct regular inspections until the 7th day, further examination up to and including the 14th day is unnecessary.

In addition to BSP, DMP1 is also a fossilized acid extracellular matrix protein (ECM) which closely binds to hydroxyapatite and mediates cell attachment through the RGD (Arg-

Gly-Asp) domain. Existence of DMP1 is closely related to osteocytes and pericytes.<sup>15</sup> In DMP1, bone is processed into two fragments, namely; fragments of 37 kDa derived from nh3 terminal for growth, proliferation, and fragments of 57 kDa derived from the COOH-terminal (containing peptide ASARM) of the calcification and ossification zone.<sup>6</sup> Therefore, the DMP1 was able to rise significantly in the T7 and T14 groups when compared to their 7C7 and C14 counterparts. However, a comparison of the two indicates that this increase is not significant. This means that the amount of DMP1 increases slowly because the proliferation process is still ongoing, while the mineralization process is imperfect. Therefore, analysis up to and including the seventh day is sufficient with further examination continuing until the 14th day being redundant.

The previous similar study about usage of *Portunus pelagicus* shell in tooth socket after tooth extraction was examining the effect of that on tumor necrosis factor-alpha, osterix, RANKL, and OPG<sup>16,17</sup>. The effect on bone matrix mineralization have not yet been explored. This stage is important, particularly in future bone maturation and strength. BSP and DMP1 in this study was increased in the treatment group from day 7 to day 14. The mean of positive cells from the treatment group was significantly higher than the control group. This can be proof that the *Portunus pelagicus* shell induces bone matrix mineralization and bone density.

This study could be refined more effectively at a later date, given the exceptionally significant role of scaffold hydroxyapatite derived from crab shell in wound healing. Furthermore, this research was of limited time span and needed another marker to confirmed the bone regeneration process. From the discussion above, it can be concluded that the application of hydroxyapatite scaffold derived from *Portunus pelagicus* shell to the post-extraction sockets has been shown to increase the expression of both BSP and DMP1.

## ACKNOWLEDGEMENT

None.

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Kepada: michael-j-k-k@fkg.unair.ac.id; josef\_310563@yahoo.com

Tanggal: Jumat, 3 Februari 2023 pukul 13.45 WIB

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Tanggal: Jumat, 3 Februari 2023 pukul 13.45 WIB

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Dear Michael Josef Kridanto Kamadjaja, Sherman Salim, Wiwik Herawati, Tengku Natasha Eleena binti Tengku Ahmad Noor,

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Tanggal: Kamis, 25 Agustus 2022 pukul 09.23 WIB

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