

### Evaluation of osteogenic properties after application of hydroxyapatite-based shells of *Portunus pelagicus*

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#### ABSTRACT

**Background:** After tooth extraction, the socket leaves a defect on the alveolar bone. The administration of shell crab-derived hydroxyapatite maintains bone dimensions that are important for achieving successful prosthodontic treatment. **Purpose:** The aim of the study was to determine the osteogenic properties, such as the number of osteoclasts, osteoblasts and osteocytes, after the application of hydroxyapatite-based shell crab in the post-extraction sockets of Wistar rats. **Methods:** There were two groups: the control group (K) and the treatment group (T). Wistar rats were randomly divided into control and treatment groups. After tooth extraction, hydroxyapatite gel derived from *Portunus pelagicus* shells was applied to the tooth sockets of Wistar rats. Observations and calculations of osteoclasts, osteoblasts and osteocytes were carried out on the 14<sup>th</sup> and 28<sup>th</sup> days under a light microscope with 400 times magnification. Statistical analysis was performed using one-way ANOVA. **Results:** There was a significant difference ( $p < 0.05$ ) between the K14 and P14 groups, K28 and P28 groups, K14 and K28 groups, and P14 and P28 groups. The results indicated that there were significant differences between groups of variables. **Conclusion:** The application of shell crab-derived hydroxyapatite (*Portunus pelagicus*) was able to decrease the number of osteoclasts and increase the number of osteoblasts and osteocytes.

**Keywords:** hydroxyapatite; *Portunus pelagicus*; osteoblasts; osteoclasts; osteocytes

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#### INTRODUCTION

Tooth extraction is the process of removing the tooth from the alveolar bone when teeth are unable to withstand further treatment.<sup>1</sup> The post-extraction socket healing process leaves an alveolar defect. Along with the growth of bone in post-extraction sockets, there is also a process of resorption on the alveolar ridge.<sup>2</sup> There is a decrease in the buccolingual dimensions, as well as a decrease in the apicoronal dimensions of the alveolar ridge, as is often found after tooth extraction.<sup>3</sup> Reduction of the alveolar ridge can interfere with prosthodontic treatment. Resorption can lead to a loss of aesthetic and function, which can be harmful when paired with dental implants, especially in the anterior maxilla.<sup>4</sup>

Bone resorption after tooth extraction would cause difficulties for implant placement. This can be overcome by preserving the socket. There are several methods that

can be used to minimise the occurrence of bone resorption. Among them is the use of demineralised freeze-dried bone allograft (DFDBA), Bioglass and hydroxyapatite (HA), which has been used in the form of both a resorbable and non-resorbable membrane.<sup>5</sup>

Calcium phosphate bioceramics, such as HA, are popular materials for bone reconstruction.<sup>6</sup> HA bioceramic materials form up to 70% of bone structure. HA can be produced synthetically from chemical reagents or can be synthesised from natural resources through hydrothermal transformation and the high-temperature calcination of bones.<sup>7</sup> Raw HA biomaterials are easily available and are abundant in Indonesia. Among the abundant raw materials is shell crab, which is one of Indonesia's export commodities. Indonesia exports 604,215–625,000 tons of crab without shells per year.<sup>8</sup> Flower crabs (*Portunus pelagicus*) has been a mainstay of Indonesia's export commodities to various countries around the world.<sup>8</sup> Crab shells contain calcium

carbonate ( $\text{CaCO}_3$ ), which can be processed further into HA [ $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$ ]. HA's structure is identical to that of human bone, which renders it a potential source of synthetic bone for bone grafts. In the field of dentistry, bone grafts are used to increase the alveolar ridge height, remodel the jawbone, transfer tissue free of microvascular problems and re-establish the alveolar crest.<sup>8</sup>

HA has osteoconductive properties and can stimulate mesenchymal cells to proliferate and differentiate in the bone regeneration process. Porous HA forms a strong bond between the bones, accelerating the process of vascularisation. The porosity of the bone graft increases the osteoconductive properties and the colonisation of osteoblasts, facilitates the penetration of osteoblast cells and provides a medium for osteoblasts to attach to.<sup>9</sup> Osteoblasts and osteocytes secrete osteoprotegerin (OPG), which acts as a binder for the RANKL receptor and decreases the differentiation of osteoclasts.<sup>10</sup> OPG has been shown to function as an inhibiting factor for osteoclastogenesis *in vivo* and *in vitro*.<sup>11</sup> This study aimed to determine the effect of HA crab shell on the number of osteoclasts, osteoblasts and osteocytes in the tooth sockets of Wistar rats.

## MATERIALS AND METHODS

This study was approved by the Institutional Health Research Ethical Clearance Commission with certificate number 177/HRECC.FODM/VII/2018. An HA powder was made from crab shell by soaking the shell in water (ratio 3:20) for 15 minutes. The powder was immersed in chlorine and dissolved in water (10 ml of chlorine was used for 20 litres of water). It was soaked for 5 minutes. The calcination process was carried out by heating the material in a furnace at an initial temperature of  $\pm 50^\circ\text{C}$ , which slowly increased by  $5^\circ\text{C}/\text{minute}$  until the temperature reached  $1000^\circ\text{C}$ . It was maintained at this temperature for two hours. The HA powder was made into a gel by adding carrageenan powder and water with a ratio of 6:3:2, then mixed and heated slowly at  $70^\circ\text{C}$  for 10 minutes to form a gel compound.

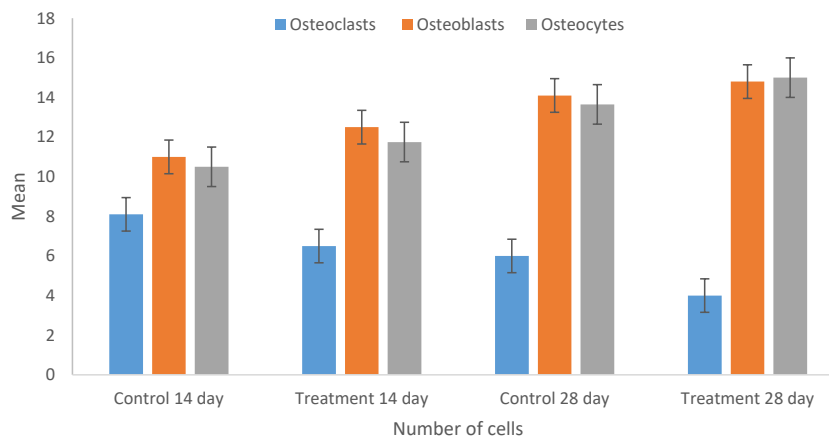
The experimental subjects were 36 Wistar rats divided into four subgroups. Each group consisted of nine Wistar rats: control group at 14 days (K14), control group at 28 days (K28), treatment group at 14 days (P14) and treatment group at 28 days (P28). The rats were sedated with 10% ether. Each had its mandibula left incisor extracted using sterile forceps. For the treatment groups, HA gel was applied to the sockets, which were then sutured with silk thread 3/0. Meanwhile, the sockets of the control groups were sutured without the application of HA gel.

All the Wistar rats were sacrificed on the 14<sup>th</sup> and 28<sup>th</sup> days. Rats were euthanised using ketamine at a lethal dose (66–88 mg/kg of body weight). The mandible was cut and immersed in a 10% formaldehyde solution for at least 24 hours. Decalcification was performed using ethylenediaminetetraacetic acid. The tissue then underwent dehydration and was stored at  $60^\circ\text{C}$  for some time before being submerged in liquid paraffin. The paraffin blocks containing tissue were then cut using a microtome machine (3–4 $\mu\text{m}$ ).

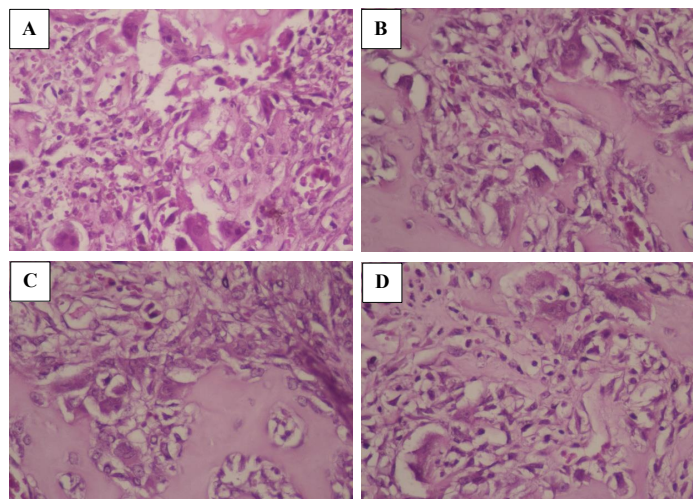
The slices of tissue were then inserted into a water bath ( $30\text{--}40^\circ\text{C}$ ). The tissue pieces were carefully attached to a glass object and 1–2 drops of albumin were added to the tops of the tissue pieces. After that, the glass object was heated with a hot plate at a temperature of  $30\text{--}40^\circ\text{C}$ . Haematoxylin and eosin staining was then used to measure the number of osteoclasts, osteoblasts and osteocytes. This observation was carried out under a light microscope with 400 times magnification. Statistical analysis was performed using one-way ANOVA with a p value of  $<0.05$ .

## RESULTS

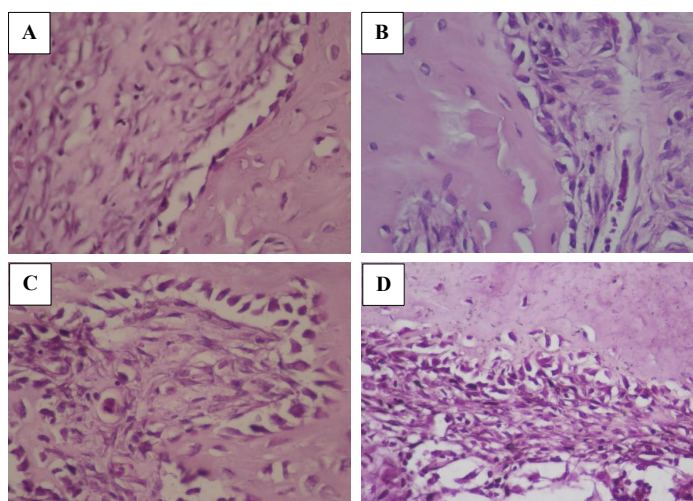
The measurements of the osteoblasts, osteoclasts and osteocytes on the 14<sup>th</sup> and 28<sup>th</sup> days from all groups can be seen in Figure 1. Histological imaging of the osteoblasts, osteoclasts and osteocytes can be seen in Figures 2, 3 and 4. The data was analysed using the Kolmogorov-Smirnov test and Levene's test, and the results showed that the data were normally distributed ( $p>0.05$ ) and homogenous ( $p>0.05$ ).



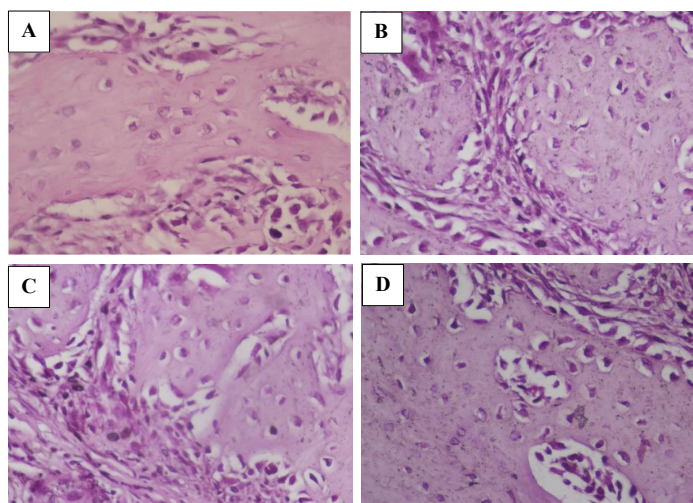
**Figure 1.** Diagram of the mean number of osteoclasts, osteoblasts and osteocytes from the control and treatment groups.



**Figure 2.** A histological view of the osteoclasts on the 14<sup>th</sup> (A) and 28<sup>th</sup> (B) days of the control group and the 14<sup>th</sup> (C) and 28<sup>th</sup> (D) days of the treatment group.



**Figure 3.** A histological view of the osteoblasts on the 14<sup>th</sup> (A) and 28<sup>th</sup> (B) days of the control group and the 14<sup>th</sup> (C) and 28<sup>th</sup> (D) days of the treatment group.



**Figure 4.** A histological view of the osteocytes on the 14<sup>th</sup> (A) and 28<sup>th</sup> (B) days of the control group and the 14<sup>th</sup> (C) and 28<sup>th</sup> (D) days of the treatment group.

**Table 1.** This data represents the p value between each group of all-day treatment. All groups showed significant differences ( $p < 0.05$ )

	K14	K28	P14	P28
K14		0.044	0.000	0.000
K28			0.024	0.000
P14				0.024
P28				

After the homogeneity test was carried out, a significance test was conducted using the one-way ANOVA test. The results showed that there were significant differences between groups of variables ( $p < 0.05$ ). A post hoc Tukey test was also conducted to determine the significance of the number of osteoclasts, osteoblasts and osteocytes in each study group. The significant differences between each group can be seen in Table 1.

## DISCUSSION

Tooth extraction is the most common procedure in the field of dentistry. The response to the body's normal healing process after tooth extraction often causes significant bone resorption.<sup>12</sup> After tooth extraction, the alveolar bone is gradually absorbed by the body. Then, a remodelling process occurs, which results in a decrease in the dimensions of the alveolar bone. The vertical plane decreases and tends to be more palatal than its original position.<sup>13</sup>

The bone remodelling process consists of several phases, beginning with the activation phase. The activation phase involves the recruitment and activation of osteoclast monocyte-macrophage precursors from the circulation, resulting in the interaction of osteoclast precursor cells and osteoblasts. Then, during the resorption phase, osteoclasts begin to dissolve the mineral matrix and decompose the osteoid matrix.<sup>12</sup> The resorption phase is dominated by osteoclasts. Next comes the recovery phase, in which the transition from bone resorption to bone formation occurs. Bone absorbed in the resorption phase contains various mononuclear cells, including monocytes, osteocytes released from the bone matrix and preosteoblasts, which function to begin the process of new bone formation. In the formation phase, osteoblast cells are released on the surface to begin bone formation.<sup>13</sup> The process is completed by the mineralisation phase, which begins 30 days after osteoid deposition.<sup>14</sup>

To maximise bone regeneration after tooth extraction and minimise the occurrence of bone resorption, the socket is filled with bone graft material. When filling the socket, actions that could cause trauma to the bone should be avoided, thereby reducing the occurrence of buccal, lingual and ridge alveolar resorption.<sup>12</sup>

Calcium phosphate bioceramics, such as HA, are popular materials for bone reconstruction. Bioceramic HA material forms up to 70% of the bone structure. HA

is effectively used to replace part or all of the bone tissue. It can be used as a bone filling material. HA can produce a physicochemical interaction between ceramics and bone tissue, thus encouraging the binding and growth of new tissue.<sup>15</sup>

The HA in this study was made from crab shell, which was first made into a HA powder using a furnace, then converted into a crab shell-based HA gel. The crab shell-based HA gel used in this study contained 87.11% HA.

The results showed a significant difference in the number of osteoclasts on the 14<sup>th</sup> and 28<sup>th</sup> days. This was because, on the 14<sup>th</sup> day, the resorption phase was dominated by osteoclasts. Osteoclasts need 2–4 weeks for the remodelling cycle to complete bone resorption. Meanwhile, on the 28<sup>th</sup> day, there was a decrease in the number of osteoclasts due to the commencement of the initial stage of the recovery phase. It was found that the number of osteoclasts on day 14 was higher than the number of osteoclasts on day 28 in both the control groups and the treatment groups. The results also showed that there was a decrease in the number of osteoclasts in the treatment group when compared with the number of osteoclasts in the control group on the 14<sup>th</sup> and 28<sup>th</sup> days. This indicates that the administration of crab shell-based HA can reduce the number of osteoclasts in sockets after extraction.

The number of osteoblasts in P14 and P28 was higher than the number of osteoblasts in K14 and K28. No significant differences were found between P14 and P28. This is because, on the 28<sup>th</sup> day, an insignificant number of osteoblasts were formed due to the continuation of osteoblast cells in the maturation phase forming osteocytes for apoptosis.<sup>16</sup>

Figure 1 also shows that there were significant differences between K14 and P14 and K28 and P28. This is because HA gel can trigger osteocytes to differentiate, so there is an increase in the number of osteocytes. However, the differences were only significant between the K14 and P14; the differences between K28 and P28 were not significant. This was due to osteocyte apoptosis occurring after a period of 10 to 14 days. Osteocyte apoptosis plays a key role in activating the bone remodelling mechanism.<sup>17</sup> Although the P28 showed the highest number of osteocytes, maximum cell growth actually occurred before the 28<sup>th</sup> day. Thus, the number of osteocytes did not increase much between days 14 and 28. This is because crab shell-based HA has osteoconductive and osteoinductive properties, facilitating the growth of new bone tissue in the gap between mineral particles in HA. Adding crab shell-based HA particles can significantly reduce the number of osteoclasts. The formation of an apatite layer on the surface of a biomaterial has the ability to bind living bones. The potential for the osteoinductive properties of HA has been confirmed in previous studies. Furthermore, the administration of HA is found to deposit a higher number of collagen fibres around the HA particles.<sup>18</sup>

HA can bind to bone tissue and provide a specific biological response that can stimulate osteoblast cells to form

new bone tissue and help the bone regeneration process.<sup>18</sup> Combined with osteoconduction, it can increase osteoblast attachment. The activation of osteoblasts and osteocytes can produce OPG.<sup>19</sup> OPG is one of the main factors in regulating osteoclast differentiation. OPG is found to inhibit the spontaneous induction of bone absorption.<sup>20</sup> OPG is a feed receptor for RANKL and competes with RANK to bind RANKL. As a result, OPG can be an effective inhibitor for osteoclast cell maturation and osteoclast cell activation. When the bone resorption phase by osteoclasts is complete, the resorbed bone cavity contains various mononuclear cells, including monocytes, osteocytes released from the bone matrix and preosteoblasts, which function to initiate new bone formation. In conclusion, the administration of HA-based shell crab to Wistar rats after tooth extraction can reduce the number of osteoclasts and increase the number of osteoblasts and osteocytes.

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