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Tumor necrosis factor-α and osterix expression after the transplantation of a hydroxyapatite scaffold from crab shell (*Portunus pelagicus*) in the post-extraction socket of *Cavia cobaya*

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ABSTRACT

Background: *Socket preservation using bone graft is one way to minimise resorption and maximise the bone formation process. Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine that affects bone regenerating osteoblast activity, while osterix (Osx) is an osteoblast-specific transcription factor that activates gene receptors during pre-osteoblast differentiation. The hydroxyapatite (HA) scaffold from crab shells (Portunus pelagicus) has osteoconduction properties.* ***Purpose:*** *To analyse the decrease of TNF-α expression and the increase of Osx expression and the correlation between these two in the post-extraction socket after the transplantation of a crab shell HA scaffold.* ***Methods:*** *The lower left incisors of Cavia cobaya (n = 24) were extracted and divided into four groups: the first and second groups were control groups on Day 7 and Day 14 (K7 and K14), the third and fourth groups were treatment groups (P7 and P14). The statistical analysis used was a multivariate analysis of variance (MANOVA) with a significance level of 0.05.* ***Results:*** *A MANOVA test showed that the use of crab shell HA scaffolds led to a significant difference ($p < 0.05$) in TNF-α expression ($p = 0.01$) and Osx expression ($p = 0.01$). A Pearson correlation test result showed a strong inverse correlation between TNF-α and Osx expressions ($p = 0.00$ and $r = -0.78$).* ***Conclusion:*** *The transplantation of HA scaffolds from crab shells can decrease TNF-α expression but increase Osx expression in the post-extraction socket of C. cobayas. Furthermore, an inverse correlation was found between TNF-α and Osx.*

Keywords: *osterix; Portunus pelagicus; scaffold; socket preservation; tumor necrosis factor-α*

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INTRODUCTION

The procedure of denture fabrication needs a healthy alveolar bone and an ideal shape for the retention of the denture. Tooth extraction without follow-up treatment can cause alveolar bone resorption; therefore, the aesthetic aspect and function of retention cannot be achieved. The alveolar bone changes shape not only in a vertical direction, but also in the lingual/palatal direction from the initial position, which causes the alveolar bone to become low, rounded or flat. This phenomenon is called residual ridge resorption.[1] This could be avoided by preserving the socket using hydroxyapatite (HA), which can be obtained from crab shells. HA has been proven to have good biocompatibility and osteoconductive properties, meaning that it is well tolerated by the tissues of the human oral cavity and is able to stimulate osteoblast differentiation.[2]

HA also has the ability to induce mesenchymal cells to differentiate towards osteoblasts, which makes it a scaffold material for bone tissue engineering.[3] HA has long-term biodegradable properties that slow down the bone repair process. A scaffold with a polymer matrix of natural materials, namely gelatine, is needed. In this study, gelatine was chosen as a scaffold material because of its excellent biocompatibility, biodegradability and porosity. HA-gelatine scaffolds are expected to increase the bioactivity,

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biocompatibility and mechanical properties of scaffolds and stimulate the growth of the bone tissue without affecting other mixing substances.[4,5]

The bone-remodelling process that is regulated by inflammatory mediators, osteoclasts and osteoblasts occurs after the procedure of a tooth extraction. Activated macrophage cells (M1) produce tumor necrosis factor-α (TNF-α), which can affect the activity of osteoblasts and osteoclasts through the binding activity of the receptor activator of NF-κB (RANK) and receptor activator of NF-κB ligand (RANKL).[6,7] Osteoblast differentiation is a complex process involving the transcription factor osterix (Osx) for the processes of osteoblast differentiation and bone formation. Osx is an osteoblast-specific transcription factor that activates gene receptors during pre-osteoblast differentiation. It is known that pre-osteoblasts express transcription Osx factors, and their number can affect osteoclasts' activity in bone resorption. Osx is also known as SP7 transcription factor. The absence of Osx disrupts the formation of cortical bone and trabecular bone, both in intramembranous and endochondral ossification.[8]

Bone remodelling is a process that occurs over time. In this process, old bone is removed, which is also known as the bone resorption process, and new bone is added, which is also known as the bone formation process. The cycle of normal bone remodelling can only happen if the bone resorption and bone formation processes occur in a coordinated manner, and this depends on the activation of osteoclasts and osteoblasts. The osteogenic response occurs between Day 7 and Day 25, while bone remodelling and maturation occur between Day 14 and Day 35. Therefore, the examination of TNF-α and Osx expressions in this study was carried out on Day 7 and Day 14.[9,10] Based on this background, this study's aim is to analyse the decrease in TNF-α expression and the increase in Osx expression and the correlation between the two in the post-extraction sockets of *Cavia cobaya* that had been given HA scaffolds derived from *Portunus pelagicu*s.

MATERIALS AND METHODS

This research received an ethical permit from the Faculty of Dental Medicine, Universitas Airlangga, no. 547/HRECC.FODM/VIII/2019. The first step was to prepare HA powder from crab shells. Crab shells were purchased from the Pabean market in Surabaya. They were then cleaned using distilled water (Otsuka®, Lawang, Indonesia) and submersed in a chlorine solution (5 litres of water: 30 ml chlorine). The submersion was continued with 3% H_2O_2 (OneMed®, Sidoarjo, Indonesia) for 24 hours, and then the shells were dried at room temperature. The shell calcination process was carried out with a furnace at 1000°C for approximately 2 hours. The characterisation of HA compounds was done using a scanning electron microscope-energy dispersive X-ray (SEM-EDX; Inspect™ S50 type TP 2017/12, FEI Company, Hillsboro, OR, USA) with a size of ± 150 µm.[11]

After the HA powder was created from the crab shells, the scaffold was prepared. Five grams of gelatine (Sigma-Aldrich®, St. Louis, MO, USA) were added to distilled water (Otsuka®, Lawang, Indonesia) at 40°C and stirred for 1 hour. An HA-gelatine scaffold was made by adding 1.5 grams of crab shell HA powder to 0.5 grams of gelatine solution, stirring for 6 hours, and then centrifuging (Hettich®, Tuttlingen, Germany) for 10 minutes to separate the water and the gel. The gel solution was placed on a custom resin acrylic mould (Ortho Resin®, England) with a diameter of 5 mm, a height of 2 mm and a pore size of 150 µm. It was then put in a freezer with a temperature of -80°C for 24 hours. Furthermore, freeze drying (Christ® Beta 1-8 LSCplus, Osterode am Harz, Germany) was carried out for 24 hours.[5,12]

For the experimental animals, male *C. cobaya* (n = 24) aged 3–3.5 months that had a body weight of 300–350 grams and were healthy, with no injuries or disabilities, were obtained from the Biochemistry Laboratory of the Faculty of Medicine at Universitas Airlangga. Each group of *C. cobaya* was placed in a cage measuring 50 x 70 x 50 cm and placed in a room with sufficient airflow and light. Food was given *ad libitum,* with an emphasis on foods that contain a great deal of crude fibre, tubers, corn and other greens, every morning and evening. Mineral water was provided in 300 ml bottles that were equipped with small pipes. The experimental animals were allowed to adapt for three days to obtain good general health and adaptation to the environment. The cage was placed in a place that was shady but got enough sunlight in the morning. The cage was placed a bit away from noise so that the experimental animals could be calm. The cages were placed in a dry place so as to prevent disease. The cages were free from the direct influence of strong winds, rain and scorching sun. The experimental animals were weighed to ensure they met the sample criteria.[13] The left lower incisors of *C. cobaya* were extracted with a sterile needle holder, and the animals divided into four groups. Each group contained six samples. Group I was a control group that received no treatment until Day 7 (K7). Group II was a control group that received no treatment until Day 14 (K14). Group III was a treatment group in which the extraction socket was given a crab shell HA scaffold until Day 7 (P7). Group IV was a treatment group in which the extraction socket was also given a crab shell HA scaffold until Day 14 (P14). The concentrations of the active ingredient processed from crab shells were 1.5 grams of HA and 0.5 grams of gelatine.

The combination HA-gelatine scaffold was implanted in the *C. cobaya* socket, and then the post-extraction wound was sutured in the control groups and the treatment groups with Polyamide monofilament suture thread, DS 12 3/8 c, 12 mm, 6/10 meth, 0.7 sterile (Braun Aesculap®, Melsungen, Germany).[14]

For the tissue samplings, Groups I and III were terminated on the 7th day after extraction to observe TNF-α

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and Osx expressions. Groups II and IV were terminated on Day 14 after extraction to compare the total TNF-α and Osx expressions on Day 7 and Day 14. Termination was carried out using ketamine (Pfizer®, NY, USA) at a dose of 0.2 ml. The mandible was removed.[15]

The preparations of *C. cobaya* mandibular tissue were decalcified with Ethylenediaminetetraacetic acid for 60 days. After that, they were processed using a paraffin-embedding technique, and the samples were then sliced in a mesial-distal direction with a thickness of 4 µm. Evaluation of the calculation of the number of TNF-α and Osx expressions was carried out with immunohistochemical methods using TNF-α and Osx monoclonal primary antibody and secondary antibody, catalog: Anti-Osx antibody (F-3): sc-393325, Anti-TNF-α antibody (52B83): sc-52746 (Santa Cruz Biotechnology, Inc, Dallas, TX, USA); each of the antibodies had 1:100 concentration. Visualisation of immunohistochemical results was conducted with CRF^{tm} Anti-Polyvalent HRP Polymer (DAB) Lab Pack, CPP-125 (ScyTek Laboratories, Inc, West Logan, UT, USA) by counting the amount of TNF-α expressed by the cytoplasm of macrophage cells that had a brownish colour at the base of the extraction socket, and whether the macrophages exhibited an amoeboid, elongated spindle-like, or round shape depending on their lamellipodial extensions.[16] Also, the amount of Osx transcription factor expressed by the cytoplasm of osteoblasts that had a brownish colour at the base of the extraction socket was measured. In terms of morphology, osteoblasts are cuboidal cells that are found at the interface of newly synthesised bone and are strongly basophilic in their cytoplasm.[17] Observations and counts of TNF-α and Osx expressions were carried out using a microscope (Nikon® E100) and Sony® α 7 as a camera attachment with 1000x magnification and 20 fields of view, and they were done by one person.

Statistical analysis was conducted using Statistical Package for Social Science (SPSS) software version 21 (SPSS Inc, Chicago, IL, USA). When examining the research data, the first step was to test for normality using the Shapiro-Wilk test. After the normality test was carried out, the research data were then processed for homogeneity using Levene's test. A multivariate analysis of variance (MANOVA) test was conducted to analyse TNF-α and Osx expressions in the post-extraction sockets of *C. cobaya* after the transplantation of HA scaffolds from crab shells. Analysis was then continued using the Tukey honest significant different method to detect the differences in the parameters of each group. A Pearson correlation test was performed to determine the correlation between the TNF-α and Osx expressions with a significance level of $p < 0.05$.

RESULTS

The histological appearance of the osteoblasts and osteoclasts after the transplantation of an HA scaffold from crab shells (*P. pelagicus*) in the post-extraction sockets of *C. cobaya* can be seen in Figures 1 and 2. According to the statistical analysis all of the data were normally distributed and come from a homogenous population ($p > 0.05$). The transplantation of an HA scaffold from crab shells (*P. pelagicus*) showed the lowest mean TNF-α expression in the control group on Day 14 (K14). In the control and treatment groups, the amount of TNF-α expression continued to decrease from Day 7 to Day 14. However, when the two treatment groups (P7 and P14) were compared, there was no significant decrease in TNF-α expression from Day 7 to Day 14. A MANOVA test showed that the transplantation of the HA scaffold from crab shells (*P. pela*gicus) had the effect of decreasing TNF-α expression, with a significant difference in each group (Table 1). The histological appearance of the TNF-α in the osteoblast can be seen in Figure 3.

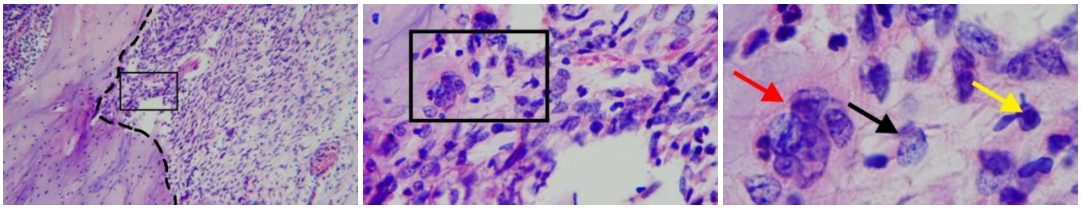

Figure 1. The histological appearance of the bottom of the extraction socket in the control group under 100x, 400x and 1000x magnification. Red arrow = osteoclast, black arrow = osteoblast, and yellow arrow = macrophage.

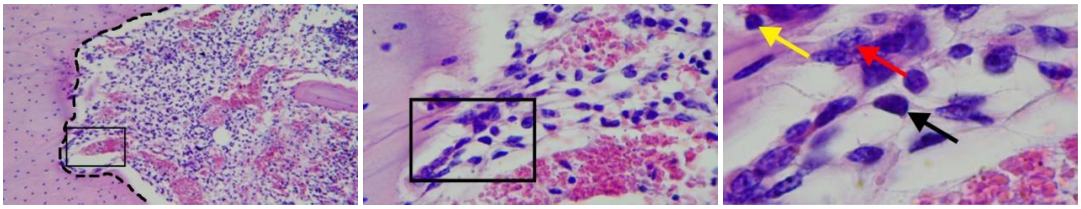

Figure 2. The histological appearance of the bottom of the extraction socket in the treatment group under 100x, 400x and 1000x magnification. Red arrow = osteoclast, black arrow = osteoblast, and yellow arrow = macrophage.

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Table 1. Mean of expression of TNF-α

Parameter	Control groups (Mean ± SD)		Treatment groups (Mean ± SD)		P (MANOVA $p < 0.05$)
	K7	K14	P7	P14	
TNF-α	15.54 ± 2.460	13.68 ± 2.857	6.86 ± 2.300	6.25 ± 2.092	0.001*

* Significant at $p < 0.05$

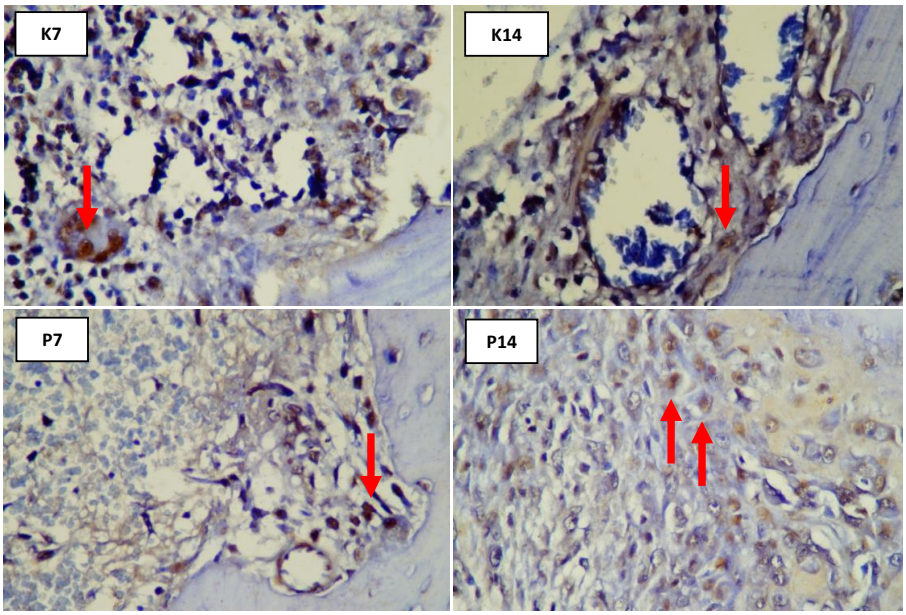

Figure 3. The histological appearance of TNF-α (indicated by red arrows) in the post-extraction socket under 1000x magnification. K7 = control group without treatment until Day 7; K14 = control group without treatment until Day 14; P7 = treatment group in which the extraction socket was given a crab shell HA scaffold until Day 7; P14 = treatment group in which the extraction socket was also given a crab shell HA scaffold until Day 14.

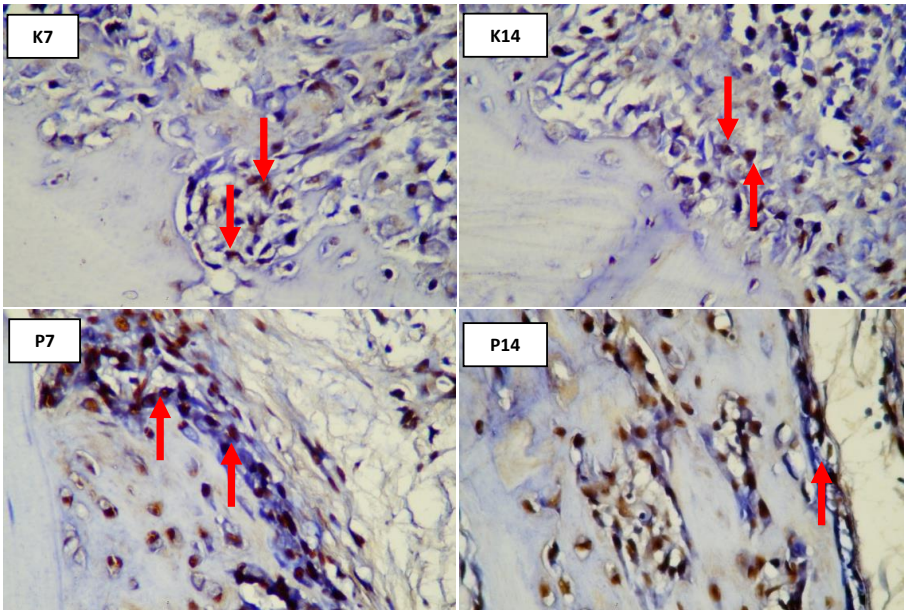

Figure 4. The histological appearance of the Osx (indicated by red arrows) in a post-extraction socket under 1000x magnification. K7 = control group without treatment until Day 7; K14 = control group without treatment until Day 14; P7 = treatment group in which the extraction socket was given a crab shell HA scaffold until Day 7; P14 = treatment group in which the extraction socket was also given a crab shell HA scaffold until Day 14.

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The histological appearance of the Osx in the osteoblast can be seen in Figure 4. The transplantation of an HA scaffold from crab shells (*P. pelagicus*) showed the highest mean Osx expression in the control group on Day 7 (K7). In the control and treatment groups, the amount of Osx expression continued to increase from Day 7 to Day 14. A MANOVA test showed that the transplantation of an HA scaffold from crab shells (*P. pelagicus*) had an effect on the increase in Osx expression with significant differences in each group (Table 2).

The results of the correlation test show that the mean TNF-α expression is inversely proportional to the Osx expression ($p = 0.00$ and $r = -0.78$), which means there is a strong negative correlation. This shows that an increase in the expression of Osx causes a decrease in the expression of TNF-α (Table 3).

A comparison of the inflammation value and the apposition value shows that the apposition value is more dominant. The expression of Osx, which is a marker of apposition, is inversely proportional to the expression of TNF-α as a marker of inflammation. Increasing the expression of Osx will decrease the expression of TNF-α (Figure 5).

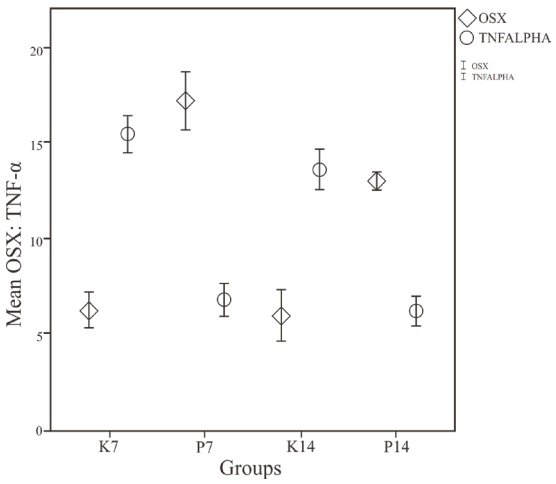

Figure 5. Graph of TNF-α and Osx expressions.

DISCUSSION

HA is an inorganic biomaterial that composes about 67% of the mineral content in bone. HA is used because of its excellent biocompatibility to hard tissue in humans because chemically and physically, its mineral content is the same as bones and teeth in humans. The formation of chemical bonds with good tissue provides an advantage in the clinical application of HA as a bone substitute material.[18]

HA has been known to regenerate bone by conduction or by working as a scaffold to fill bone defects. HA is one of the materials that has osseointegration properties: osteoconduction, osteoinduction and osteogenesis; therefore, it can be used for a bone graft. The definition of osteoconduction is that in the HA function as a scaffold, it is able to induce and stimulate mesenchymal stem cells and osteoblasts to proliferate and differentiate in the formation of new bone or in the process of bone regeneration. Meanwhile, gelatine is derived from collagen and has good biological properties. Because the main organic portion of hard tissue is made of collagen, it has potential medical applications. Among the advantages of gelatine are ductility and high efficiency, which can facilitate manufacturing.[18–21] In this study, the variables that are focused on are TNF-α and Osx, and the correlation between them. By studying these variables, inflammation and apposition in post-extraction sockets can be compared.

The research results of the biomarker TNF-α showed a significant difference between the control group (K7 and K14) and the treatment group (P7 and P14) after the transplantation of an HA scaffold from crab shells (*P. pelagicus*). The correlation test results showed that the transplantation of HA caused an increase in Osx expression and a decrease in TNF-α expression. These results are in accordance with the research hypothesis, which stated that the transplantation HA scaffold from crab shells (*P. pelagicus*) would decrease TNF-α expression in the post-extraction sockets.

In this research, we used a combination of HA and gelatine. Although the gelatine in this study was used only as a binding agent, we can further improve the effectiveness

Table 2. Mean of expression of Osx

Parameter	Control groups (Mean ± SD)		Treatment groups(Mean ± SD)		P (MANOVA $p < 0.05$)
	K7	K14	P7	P14	
Osx	6.29 ± 2.44	6.00 ± 3.54	17.29 ± 4.04	13.07 ± 1.18	0.001*

* Significant at $p < 0.05$

Table 3. Correlation test results of TNF-α and Osx expressions

Control variables			Osx	TNF-α
Groups	Osx	Correlation	1.000	-.779
		Significance (2-tailed)	.	.0000
		df	0	25
	TNF- α	Correlation	-.779	1.000
		Significance (2-tailed)	.000	.
		df	25	0

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of this treatment by adding silver. As demonstrated in previous studies, which used a combination of HA and silver to reduce the release of nitric oxide and decrease the secretion of IL-1 and TNF-α in cells that were stimulated with Lipopolysaccharide (LPS), LPS stimulates monocytes/macrophages through TLR4, resulting in the activation of a series of signalling events that potentiate the production of inflammatory mediators.[22,23] There is a correlation between the particle size of HA and the secretion of TNF-α. A previous study showed that a smaller HA particle size (especially a diameter of 1-2 μm and a pore size of 10-50Å) stimulates a higher macrophage TNF-α secretion. In this study, we used an HA particle size of 150-350 μm; therefore, this material exhibited a lower macrophage TNF-α secretion. The mechanism of this result is not yet understood, but it requires the internalisation of crystal microaggregates into phagocytic vacuoles and the recruitment of protein kinase C to the vacuole membrane.[24]

At 10 days post-extraction, TNF-α expression was almost entirely absent, which means that inflammation was not prolonged. TNF-α plays an important role in bone resorption. Several previous studies have shown that TNF-α increases because the host response is stimulated by plaque, bacterial products and increased osteoclast activity, consequently accelerating bone resorption and periodontal destruction. In other words, a decrease in TNF-α can reduce the host response, reducing the expression of cytokines that stimulate bone resorption, resulting in less bone loss.[25] The acute inflammatory response peaks in the first 24 hours, although the proinflammatory molecules later also play an important role in regeneration, while TNF-α concentrations peaked in the first 24 hours and returned to baseline within 3 days post-trauma.[26] A previous study investigated the potential impact of TNF-α on the teeth of Zucker diabetic fatty rats with diabetes and periodontitis, which was induced by ligature for 7 days. The results of the study indicated that the TNF-α from the test group reached a peak on the third day, and TNF-α expression decreased gradually on the following days. Therefore, it is advisable to conduct research with TNF-α biomarkers, and further examinations need to be added on Day 1 and Day 3.[27]

In contrast, in the study with the Osx biomarker and the data from Day 7 and Day 14, the highest mean number of Osx expression was found in the treatment group on Day 7, but this number began to decline on Day 14. This is because Osx is a transcription factor that is important for osteoblast differentiation in the early stages, but it inhibits osteoblast differentiation at a late stage. However, the function of Osx at the late stage of osteoblast differentiation is not fully elucidated; instead, runt-related transcription factor 2 (RUNX2) plays an important role in inhibiting osteoblast differentiation at a late stage.[28,29] However, there was a significant difference between the control groups (K7 and K14) and the treatment groups (P7 and P14). HA is able to stimulate osteoblast differentiation; HA made from crab shells (*P. pelagicus*) also contains a high level of calcium carbonate ($CaCO_3$) of around 40–70%. Calcium carbonate has properties that are easily absorbed by tissues, easily biodegradable and osteoconductive; as a result, it can support the process of forming new bone.[2,30] These results are in accordance with the research hypothesis, which stated that the transplantation of an HA scaffold from crab shells (*P. pelagicus*) would increase Osx expression in the post-extraction sockets.

In previous studies, the transplantation of an HA scaffold from crab shells (*P. pelagicus*) showed positive results for reducing the osteoclasts' number and increasing the osteoblasts' number in the post-extraction socket when examined on Days 14 and 28. It can increase the expression of Osteoprotegerin (OPG), Osteocalcin, collagen type 1 and transforming growth factor-β1 (TGF-β1) and decrease RANKL expression.[31–36] In conclusion, the transplantation of an HA scaffold from crab shells (*P. pelagicus*) can reduce TNF-α expression in the post-extraction socket on the Day 7. There was an inversely proportional correlation between TNF-α and Osx after providing an HA scaffold from crab shells (*P. pelagicus*). However, for further studies that evaluate TNF-α biomarkers, it is important to add silver and also to include examinations on Day 1 and Day 3.

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