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Research Report

Effectiveness of *Anadara granosa* shell-*Stichopus hermanni* granules at accelerating woven bone formation fourteen days after tooth extraction

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ABSTRACT

Background: *Post-extraction complications can cause alveolar bone resorption. Hydroxyapatite-tricalcium phosphate (HA-TCP) is one potential bone graft material that can be synthesized from Anadara granosa shell. Another biomarine, Stichopus hermanni, contains hyaluronic acid which can accelerate bone formation on the fourteenth day.* ***Purpose:*** *This study aims to prove the effectiveness of Anadara granosa shell-Stichopus hermanni granules in weaving bone formation fourteen days after tooth extraction.* ***Methods:*** *Twenty-five male Wistar rats were divided into five groups. Their lower left incisor was extracted with gelatin being administered to the control group (C) and granule scaffold derived from Anadara granosa (AG) shell and Anadara granosa shell-Stichopus hermanni at concentrations of 0.4%-0.8%-1.6% (AGSH1-AGSH2-AGSH3) to the treatment group. This study developed a HA-TCP synthesized from Anadara granosa combined with whole Stichopus hermanni to create granule scaffolds by means of a freeze-dried method. The jaw was removed on the fourteenth day post-tooth extraction. Observation of HPA involved the use of an Image Raster®. The resulting data was subjected to analysis by ANOVA and tukey-HSD tests (p<0.05).* ***Results:*** *Data showed the mean of C=0.157±0.078; AG=1.139±0.371; AGSH1=1.595±0.291; AGSH2=1.740±0.308; and AGSH3=1.638±0.286. Statistical analyses showed significant differences in the woven bone area (mm^2) between C and the treatment groups AG;AGSH1;AGSH2; AGSH3; and between AG and the AGSH2 groups.* ***Conclusions:*** *Scaffold granules from Anadara granosa shells and Stichopus hermanni effectively accelerate the bone formation process with the most effective being Stichopus hermanni at a concentration of 0.8%.*

Keywords: Anadara granosa; bone formation; granule scaffold; Stichopus hermanni; tooth extraction

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INTRODUCTION

In 2013, Indonesia's National Basic Health Research Body produced a missing teeth (MT) index of 2.9, indicating that of the permanent teeth extracted, 290 tooth roots per 100 people remained in the jaw.[1] Following tooth extraction, the alveolar bone is gradually resorbed and offset by a remodeling process which still results from vertical to palatal resorption.[2] Schropp *et al.* (2003),[3] estimated that two-thirds of hard and soft tissue changes occur within the initial three months. In another study, alveolar bone resorption occurred in the vertical and horizontal directions ranging between 40-60% during a 2-3 year period.[4] This post-extraction process will continue in an integrated manner ultimately resulting in disruption to the stomatognathic system.[5]

One week after extraction, the blood clots with which the socket had initially been filled were almost completely replaced by granulated tissue. This process was followed by an inflammatory response capable of triggering proliferation which was characterized by the presence of growth factors. These played a role in new blood vessel formation which, in turn, increased the number of fibroblasts and pre-osteoblasts and promoted their differentiation into mature osteoblasts.

These osteoblasts will appear in the bone cortex surrounding the defect site. Mature osteoblasts synthesize bone matrix, especially type I collagen, and regulate newly-formed bone mineralization.[6,7] In this phase, granulation tissue and provisional matrix (PM) are filled with mesenchymal cells, collagen fibers, and blood vessels. Within two weeks, woven bone had begun to form in the apical region of the tooth socket.[8,9]

Therapy to stimulate bone formation and regeneration of bone damage due to tooth extraction generally utilizes bone graft[10] which must have a physical structure and properties similar to bone in order for it to accelerate the healing process. Bone grafts must satisfy certain conditions, firstly, that they are biocompatible or acceptable to the body; secondly, that they facilitate bone formation through a mechanism that contains bone-forming cells (osteogenesis); thirdly, that they serve as a scaffold for bone formation (osteoconduction); and, lastly, that they contain bone-inducing ingredients (osteoinduction).[11]

The basic ingredient of bone graft is calcium phosphate (HA) which plays a role in bone mineralization. Research has been carried out on ceramic materials with HA/TCP combination at different ratios. A study conducted by Kim *et al.* (2017),[12] indicated a good HA/TCP ratio of 70:30, while other analyses have posited the HA/TCP ratio to be one of 60:40 [13]. This does not constitute a significant difference with the HA/TCP ratio of 80:20 identified by the biomaterial test. One natural ingredient with the potential for use as a candidate bone substitute material is that of blood clam shells (*Anadara granosa*). The compound found in blood vessels was extracted from cleaned restaurant waste containing 98.8% $CaCO_3$.[14] In the research conducted by Sari (2018),[15] after implementing a series of synthesizing processes using the hydrothermal method, it was found that the HA and TCP content were 72% and 21% respectively, with the remaining 7% being accounted for by calcium carbonate.

In certain cases, the use of calcium phosphate compounds as bone graft requires the addition of polymeric material to the bone graft structure. AH polymer, a naturally-occurring, hydrophilic, nonimmunogenic material, is found in the cytoplasm of osteoprogenitor cells[16] and plays a complex role in cell adhesion, cell proliferation and cell movement. In the extracellular matrix, AH represents a framework that triggers post-injury tissue recovery, suppresses anti-inflammatory activity, modulates tissue hydration, osmotic balance and collagen recognition and contractions during the repair process.[17] AH in the form of hydrogel can reduce inflammatory cells on day 7 and increase bone density and blood vessel length on day 14. This study identified a microscopic change in the inflammatory process and angiogenesis on the seventh day of the wound healing process.[18] Research using AH at a concentration of 0.8% suggests that it accelerates bone regeneration through chemotaxis, proliferation and differentiation of mesenchymal cells.[19] *Stichopus hermanni*, a natural resource found on the island of Raas, in Sumenep Regency,

is high in hyaluronic acid (AH) component (75.7%), and collagen (29.47%).[15]

Granule is one of the scaffolds, small in size and porous in nature, that provides room for cells to attach to bone and grow into new bone tissue.[20] Granule constitutes gelatin derived from collagen in the skin, bones and connective tissue of animals that has been hydrolyzed by an acid or base. Hyaluronic acid-rich gelatin with *Stichopus hermanni* acts as a binding material enabling the graft to turn into a hydrogel which will subsequently undergo a freeze-drying process to produce granular formations.[21]

This study seeks to explore the potential role of natural wealth in the form of marine life present in Indonesian waters to produce innovations in bone substitute materials. These would constitute a combination of HA-TCP synthesized *Anadara granosa* shell and *Stichopus hermanni* polymer at various concentrations in granular form within bone formation after tooth extraction on the fourteenth day.

MATERIALS AND METHODS

This research constitutes a complete randomized design involving the use of Wistar strain Rattus novergicus which was issued ethical approval number 002/HRECC.FODM/ I/2018 by the Faculty of Dental Medicine, Universitas Airlangga, Surabaya. Preparation for the study began with the production of a graft from an *Anadara granosa* (AG) shell synthesized in the form of HA-TCP. Blood clam shells (*Anadara granosa*) were boiled, cleaned, crushed and sifted through a 100 mesh to produce smaller particles. AG shell powder containing up to 1M and 0.6M of ammonium dihydrogen phosphate ($NH_4H_2PO_4$) solution mixed with a magnetic stirrer was transferred to the reactor which had been heated in an electric oven at a temperature of 200^oC for 12 hours. The results obtained were cooled at room temperature, repeatedly washed with distilled water, separated and showed normal pH (7). The latter was washed with methanol to limit the agglomeration of HA particles during the process of drying. The sample was dried in an electric oven at 50^oC for four hours before being sintered at $900°C$ for three hours to remove impurities and increase its crystallinity.[15]

Preparation of other *Stichopus Hermanni* materials entailed washing the material with distilled aquadest before finely chopping it at a mixer ratio of 500 grams to 1 liter of distilled water until a smooth consistency was obtained. The next stage involved a drying process utilizing the freeze-drying method, the results of which were finely milled and sieved through a 50 micron mesh (297 μm). Producing powder of an even smaller micro size required high energy milling performed with an Ellipse 3D Motion (HEM-E3D) by Nanotech® Indonesia.[22]

Scaffold was produced by mixing 5% HA-TCP with 50ml of distilled water and 10 grams of gelatin before agitating the solution for four hours with a magnetic stirrer, placing it in a 96-well plate mold and conducting freeze-
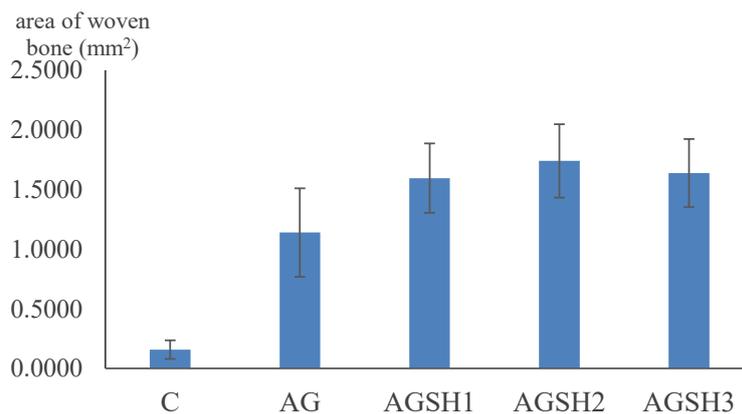
Figure 1. Extensive graph of woven bone showing bone graft application involving a combination of *Anadara granosa* and *Stichopus hermanni* shells on 2/3 apical post-extraction sockets.
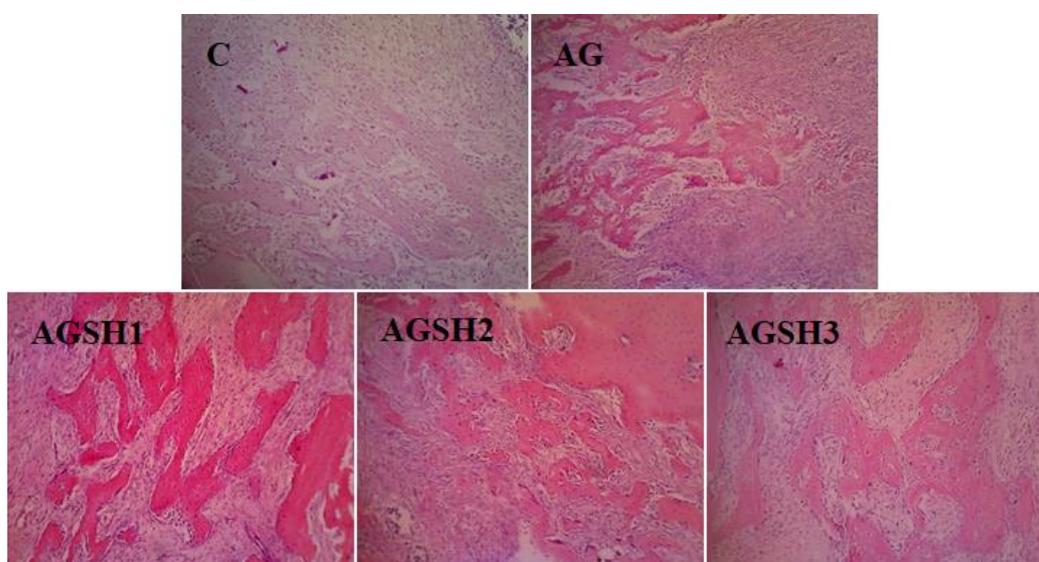

Figure 2. Wide histological picture of woven bone with bone graft application involving a combination of *Anadara granosa* shells and *Stichopus hermanni* on the apical 2/3 post-extraction socket on day 14. Observations were made by means of HE painting employing a light microscope at 100X magnification.

Table 1. *Post-hoc* test results (Tukey HSD)

Average		Mean difference (IJ)	*p*-value
Group (I)	Group (J)		
C	AG	-0.9817	0.000*
	AGSH1	-1.4381	0.000*
	AGSH2	-1.5823	0.000*
	AGSH3	-1.4804	0.000*
AG	AGSH1	-0.4564	0.122
	AGSH2	-0.6006	0.024*
	AGSH3	-0.4988	0.078
AGSH1	AGSH2	-0.1442	0.927
	AGSH3	-0.0424	0.999
AGSH2	AGSH3	0.1018	0.979

Note: * indicates a significant difference

Information:

This table shows a comparison of woven bone area between groups. The area of woven bone in the treatment group is greater than that of the control group. The negative difference means that the area of woven bone is smaller. The AGSH2 group has the largest woven bone area compared to the other three groups.

drying.[15] The final stage of producing this scaffold consisted of sterilization by gamma ray irradiation of 25 kGy supplied by the National Nuclear Energy Agency (BATAN).[23]

Research on the experiment subjects commenced with a seven-day period of acclimatization. Subjects were weighed and marked before being divided into groups and denied food until midnight when the anesthetic, namely; a dose of 10% ketamine, 0.1 cc/kgBB and 0.01 cc/100 g BW xylazine was injected intramuscularly into the right upper thigh[24]. Once cleaning of the aseptic extraction areas of the socket with water spray and antiseptic had been completed, extraction of the left mandibular incisor was effected using a needle holder and scaffold application. Application of the treatment involved dividing the 30 male rats into five groups. Gelatin alone was applied to the control group, scaffold from *Anadara granosa* shell was applied to the AG group, while the AGSH1-3 group received scaffold from the combination of *Anadara granosa* shell and *Stichopus hermanni* at concentrations of 0.4%; 0.8% and 1.6%. Suturing of the socket ensued with silk braid (USP. 3/0) supplied by DR. SELLA®. Novalgin® 0.09 cc/200gr BB analgesics and Interflox® 0.1cc/100gr BB antibiotics were applied to control any resulting swelling and pain.

Fourteen days after application to the socket, the experiment subjects were sacrificed and their mandibular preparations subsequently removed and placed in a 10% buffered formalin solution. After tissue fixation, decalcification was carried out by application of ethylenediaminetetraacetic acid (EDTA) for one month. Mandibular specimens were made in the form of sagittal fragments with hematoxillin eosin staining. The area of woven bone was measured in the socket area with an Image Raster® at 100X magnification with resulting data from each group being tabulated. The statistical analysis employed was a one-way ANOVA parametric test followed by a Tukey-HSD test.

RESULTS

The effects of the granule scaffold combination *Anadara granosa* shell and *Stichopus hermanni* administered were evaluated on day 14 after application to the socket. Formation of woven bone in the control group (C) occurred on day 14. Bone graft application from AG shells indicated an increase in woven bone formation. The combined administering of AG SH shell bone graft (0.4; 0.8; 1.6) increased the formation of wider woven bone (Figure 1).

The application of bone graft from AG shells did not sufficiently expand the area of woven bone (AG). Broad woven bone was increased through the application of bone graft shell in groups AGSH1; AGSH2; AGSH3, while the largest expansion in woven bone area was found in the AGSH3 (combination *Anadara granosa* shell and *Stichopus hermanni* 0.8%) group (Figure 2).

Statistical analysis carried out using SPSS version 23 IBM® 2015 indicated normal and homogeneous distribution

of data (0.05), confirmed by the results of a Shapiro-Wilk test and a Levene's test. In the one-way ANOVA test results (p>0.05) significant differences existed in the woven bone variables across all treatment groups. However, significant differences between one group with another did not occur. This can be seen in the multiple comparison test using Tukey-HSD (Table 1).

DISCUSSION

The process of socket healing commenced with the occurrence of vascular damage in the socket resulting in platelet aggregation and blood clot formation.[25] The inflammatory response, characterized by infiltration of inflammatory cells and macrophages, released proinflammatory cytokines and growth factor. This growth factor played a role in the process of angiogenesis and stimulated the formation of fat. One growth factor actively stimulating an increase in the number of fibroblasts and preosteoblasts and greater differentiation into mature osteoblasts is FGF.[26] Mature osteoblasts secrete osteoid, type I collagen, growth factors, alkaline phosphatase. Calcified irregular collagen tissue will form woven bone.[27]

Formation of new woven bone was initiated on the seventh day and became visible along the lateral alveolar wall and the base of the socket.[28,29] Woven bone formation increased during the second week of the socket healing process. The newly formed woven bone was surrounded by numerous newly differentiated osteoblasts. These produced a bone matrix with a high proportion of osteocytes in the lateral wall of the socket and extending to its center, thereby reaching the old trabecular bones.[30]

The application of blood shell granules (*Anadara granosa*) containing HA-TCP serves as a framework for the growth and development of mesenchymal cells into osteoprogenitor cells.[31] The hydroxyapatite structure has a stoichiometry similar to that of bone mineral, while *TCP* is a biodegradable compound that can release calcium ions.[32,33] In the study conducted by Zhang *et al.* (2015),[34] providing scaffold of gelatin/β-TCP composites induced osteogenic differentiation of bone marrow stem cells (BMSC) in vitro through activation of Ca^{2+}-sensing receptor signaling *(CaSR)* which is proven to increase the expression of RUNX2, BMP2, COL-1 and OCN.

Hyaluronic acid contained in the *Stichopus hermanni* interacts with CD44 to initiate signal transduction through MAPK activation which induces ERK1/2 phosphorylation and AP-1 activation resulting in cell migration due to the release of various growth factors.[35,36] This activation, in turn, triggers the proliferation and differentiation of osteoprogenitor cells into osteoblast cells which play an important role in the formation of bone matrix. This process was demonstrated by this study in which groups which had been administered with a combination of *Anadara granosa* and *Stichopus hermanni* shells experienced a greater

Sari, *et al.*/Dent. J. (Majalah Kedokteran Gigi) 2019 December; 52(4):177–182

181

increase in their area of woven bone on the fourteenth day compared to that of the C and AG groups.

The largest area of woven bone was found in the group to which *bone graft* from a combination of *Anadara granosa* shell and *Stichopus hermanni* 0.8% had been administered. The different concentrations of whole *Stichopus hermanni* also affected the formation of woven bone due to the initial process of interaction with *CD44*. This was indicated by the insignificant difference between C and AG with the combination group following the addition of whole *Stichopus hermanni* 0.4% and 1.6%. However, a significant difference was shown between the C and AG groups following the addition of whole *Stichopus hermanni* 0.8 %. This is because *Stichopus hermanni* contains other glycosaminoglycans, such as chondroitin sulfate and keratin sulfate, which can affect the ability of *CD44* to bind AH. Modification of bonds due to N and O chains in chondroitin sulfate produces a negative effect on the ability of *CD44* to bind AH.[37] It can be concluded, therefore, that the administration of scaffold granules from a combination of *Anadara granosa* shells and *Stichopus hermanni* can accelerate the formation of woven bone on the fourteenth day after tooth extraction in order to prevent alveolar bone resorption (socket preservation). The concentration of *Stichopus hermanni* 0.8% is the most effective in terms of accelerating the formation of woven bone on the fourteenth day after tooth extraction

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