Dental Journal
Majalah Kedokteran Gigi

Dental Journal
(Majalah Kedokteran Gigi)
2017 December; 50(4): 194–198

Research Report

# The effects of *Anadara granosa* shell-*Stichopus hermanni* on bFGF expressions and blood vessel counts in the bone defect healing process of Wistar rats

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***ABSTRACT***

***Background:*** *Bone damage can be caused by various factors with treatment usually involving graft materials being applied to the defective area. Moreover, in the bone defect healing process, blood vessels are also considered to be an important energy source for cell proliferation. One of the angiogenic factors playing an important role in blood vessel formation is basic fibroblast growth factor (bFGF). Furthermore, synthesized hydroxyapatite derived from Anadara granosa (AG) shells constitutes one of the potential materials for use in bone graft. The gold sea cucumber genus Stichopus hermanni (SH) possesses the ability to stimulate endothelial progenitor cells inducing bFGF.* ***Purpose:*** *This study aims to investigate the effects of AG shells and SH on bFGF expressions and blood vessel counts within the bone healing process.* ***Methods:*** *Twenty four male Wistar rats were divided into three groups, namely: a control group (C), a treatment group was administered with blood cockle shell (AG), and a treatment group with blood cockle shell and golden sea cucumber (AG+SH). Defects were made on their femurs measuring half the diameter of a circular, no. 018. bur These rats were subsequently sacrificed on day 7 after surgery. The expressions of bFGF were measured by means of IHC technique, while the number of blood vessels was quantified using HE technique. The resulting data was subjected to statistical analysis using an Anova test followed by an LSD test ($p < 0.05$).* ***Results:*** *The one-way Anova test results combined with those of an LSD test showed there to be significant differences in bFGF expressions and blood vessel counts between the control group (K) and the treatment group (AG) as well as between the treatment group (AG) and the treatment group (AG+SH).* ***Conclusions:*** *A combination of Anadara granosa shell and Stichopus hermanni can increase the expression of bFGF and the number of blood vessels on day 7 during the bone healing process in Wistar rats.*

***Keywords:*** *Anadara granosa shell; Stichopus hermanni; basic fibroblast growth factor; blood vessel; bone healing*

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## INTRODUCTION

Bone damage or bone defects can be caused by congenital factors, trauma, infection, or jaw tumors.[1] When bone damage occurs, a healing process consisting of three stages, namely an inflammatory phase, a reparation phase and a maturation phase[2] will ensue. In the early stages, blood vessel vasoconstriction occurs followed by a hemostasis phase and one of inflammatory cell infiltration. A blood clot progressively filling the defective area will be replaced by granulated tissue. Thereafter, the fiber remains will diffuse into the bone leading to a temporary matrix derived from the blood clot being formed. Granulation tissue will also subsequently be formed. At the end of this

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 32a/E/KPT/2017.
 Available at http://e-journal.unair.ac.id/index.php/MKG
DOI: 10.20473/j.djmkg.v50.i4.p194–198

process, a remodeling phase characterized by tissue and collagen remodeling, epidermal maturation and wound shrinkage occurs.[3,4]

Another important factor accelerating the healing process of damaged bone significant in size and volume is new vascularization tissue formation on the bone.[5] The formation of bone vascularization is triggered by an angiogenesis process together with several other factors. One which plays an important role in the formation of bone vascularization is basic fibroblast growth factor (bFGF).[6] bFGF acts as an intermediary between the formation of vascularization in new bone and the occurrence of chondrocyte and osteoblast differentiations since it appears in the early phase of proliferation during the bone healing process.[6,7] bFGF is also considered to be one of the growth factors possessing the ability to induce all stages required for angiogenesis and is involved in both wound repair and tissue development.[8] In tissue healing, bFGF is regarded as a growth factor that plays a role in stimulating endothelial proliferation, migration and blood vessel formation. Together with platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), bFGF synergizes to neutralize tissue.[9]

As medical science develops, therapeutic modalities for the reconstruction of bone defects become increasingly available. Such modalities include local bone transport, bone elongation or reduction and bone graft.[5] Initially, the bone graft used was taken from the bones of the individual itself, but this can result in damage to other areas of the body.[10] This situation then prompted efforts to develop bone graft from other natural sources, such as *Anadara granosa* (AG) shells.

AG shells have a calcium carbonate ($CaCO_3$) content of more than 95%.[11] In reality, $CaCO_3$ also has the potential to act as bone substitute material. However, bone structure and composition consist of hydroxyapatite (HAp). Therefore, $CaCO_3$ material must be converted into HAp structure through a calcification process in the body. As a result, this research utilised HAp synthesized from AG shells as bone graft material through a precipitation process to restore damaged bone.[12]

HAp, an inorganic salt contained in bone, has a chemical synthesis form similar to that of bone, non-immunogenic properties and a stable crystal form.[13] In addition, HAp is non-degradable, a quality required by graft materials. During the healing process in bone, hydroxyapatite releases calcium phosphate, thus increasing the saturation of body fluids and precipitating the biological apatite of the body in the area of the defect. The biological apatite contains endogenous proteins and acts as a matrix for the attachment and growth of osteogenic cells.[14]

Consequently, when a bone graft is carried out, the bone will undergo bone-repair phases involving hemostasis, inflammation, proliferation, revascularization, soft callus and hard callus formation and, finally, a remodeling phase.[15] The use of HAp in bone graft can also stimulate macrophages in the area of defects enabling the production of cytokines that stimulate growth factors, including FGF. In order to promote more rapid bone repair significantly greater in both size and volume, new vascularization must occur in the bone.[5] The new vascularization takes place through a process of angiogenesis triggered by several factors, one of which is bFGF.[15] bFGF acts as an intermediate between new bone vascularization and chondrocyte and osteoblast differentiations since it emerges in the early phase of proliferation during the bone healing process.[6,7]

Therefore, the healing process of bone damage will be accelerated through the addition of osteoinductive materials, one of which can be derived from marine biota, such as sea cucumbers whose osteoinductive properties can even convert cells in the graft into osteoblast cells composing the bone.[16] Sea cucumbers (*Stichopus hermanni)* are animals with high economic and nutritional value which also offer bioactive contents, such as glycosaminoglycans (GAGs), protein and polyunsaturated fatty acid (PUFA). The GAG content of sea cucumbers has positive effects on the healing of wounds. Meanwhile, sulfate chondroitin acts as a bFGF mediator in order to bind the bFGF. Thus, the more optimal the sulfate chondroitin, the more it may be assumed to increase the expressions of bFGF involved in the regulation of the angiogenesis process.[17] In addition, another content of *Stichopus hermanni* (SH), PUFA, consists of arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are useful in the bone healing process.[18] The research reported here aims to determine the effects of *Anadara granosa* shells and *Stichopus hermanni* on bFGF expressions and blood vessel counts in the repair of damaged bone.

## MATERIALS AND METHODS

This research was fully experimental incorporating a Completely Randomized Design whose samples consisted of twenty-four 5-month old male rats (*Rattus novergicus*) weighing 200–250 grams. The samples were then divided into three groups (n = 8), namely: a negative control group (C) which received no treatment, a treatment group with the administration of AG shells (AG group) and a treatment group administered with AG shells and 10% SH (AG + SH group).

Preparation of this research was initiated with the manufacture of graft derived from AG shells synthesized into HAp powder through calcination, precipitation and sintering processes. Sterilization was achieved using an ultraviolet ray unit (A10-UV-30, Cleatech, USA). On the other hand, graft material used with another treatment group was made by mixing AG powder and SH powder through a freeze-dried procedure. A milling process incorporating the use of high energy milling Elips 3D Motion (HEM-E3D) by Nanotech® Indonesia was subsequently carried out in order to obtain micro-sized results.[19]

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 32a/E/KPT/2017.

DOI: 10.20473/j.djmkg.v50.i4.p194–198

The Wistar rats were acclimatized for seven days, before their femurs were defected/were made defect. They were anesthetized by the intramuscular administering of an 0.11 mL/100 gr BW dose of ketamine and xylazine.[20] Once the rats had lost consciousness, the fur in the dextral area of the femur to be defaced was shaved off. Thereafter, the exposed skin was smeared with antiseptic (10% povidine iodine) for five minutes before a 2 cm-long incision was made with a scalpel in the soft tissues (skin and muscle) and lifted using a periosteal elevator. A defect was made on the dextral and lateral areas of the femoral bone (5 mm from the third trochanter)[15] with a straight hand piece, equivalent in depth to half the diameter of a circular bur (Mcisinger® Germany size 018).

After the defect had been created in each rat, the application of the treatment material was conducted according to the division of the group. A pericardium membrane was applied (from the Tissue Bank at Dr. Sutomo Hospital Surabaya). Sutures were then used to close the skin and soft tissues by means of cat gut (USP. 3/0) and silk braid (USP. 3/0) both manufactured by DR. SELLA®. After surgery, the animals received a 0.09 cc/200 gr BW dose of novalgin and a 0.1 cc/100 gr BW dose of interflox in order to control infection and swelling. Seven days later, the rats were sacrificed, and their os femur taken to make preparations using separating discs. The discs were then soaked in a 10% formaldehyde buffer solution to prevent the tissues from decomposing, to harden them, to increase the refractive index of various tissue components and to improve the affinity of the tissues against the staining materials used. After completion of tissue fixation, a decalcification process of two months' duration was performed using ethylenediaminetetraacetic acid (EDTA).

The os femur specimens were subsequently prepared in the form of sagittable piece preparations with HE and IHC staining techniques using polyclonal antibodies (anti-bFGF (basic fibroblast growth factor), bs-0217R, by BIOSS®, Massachusetts-USA). The number of blood vessels in the defective areas was then observed by means of a light microscope (Olympus® CX21, Japan) at 400× magnification. Data relating to the osteoblast counts obtained in each group was tabulated. The data was then analyzed statistically by means of a one-way Anova parametric test followed by an LSD test.

## RESULTS

The observation findings relating to bFGF expressions and the number of blood vessels were evaluated on the seventh day. Based on the Anova test results, there was a significant difference in the mean of bFGF expressions and blood vessel counts ($p < 0.05$). The mean of bFGF expressions indicating the positive reaction of fibroblast cells in the negative control group (C) stood at 11.33 ± 2.42, 13 ± 2.37 for the treatment group following the administration of AG, and 32.50 ± 4.14 for the treatment group following that of AG + SH. On the other hand, the mean of blood vessel counts in the negative control group (C) was 6.68 ± 0.82, 10.25 ± 0.38 for the treatment group with the administration AG, and 20.37 ± 0.55 for the treatment group with the administration of AG + SH. Blood vessels observed in the course of the research reported here constituted the formation of a lumen surrounded by a layer of endothelial cells seen in the area of the femur os that was defective (Figure 1 and 2).

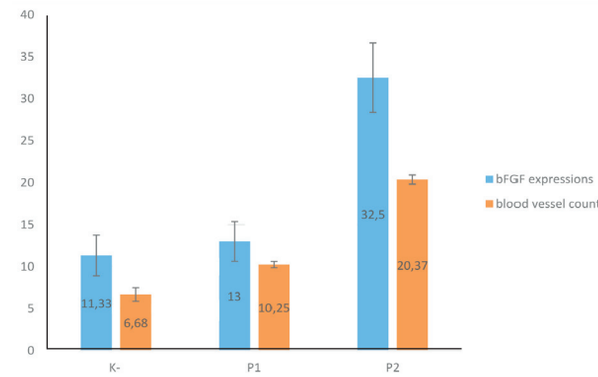


**Figure 1.** The mean of bFGF expressions and blood vessel counts in each group.

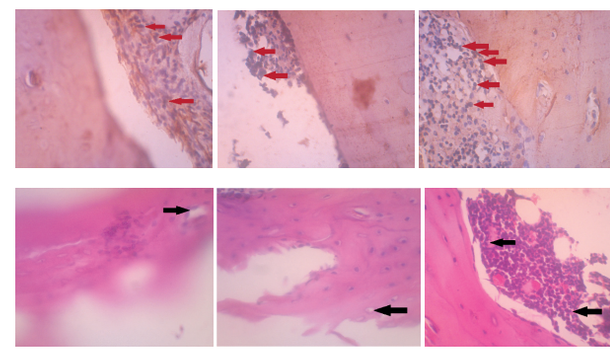

**Figure 2.** HPA images of bFGF expressions (red arrows) and blood vessel counts (black arrows) in each group.

Clinically, the defects in the AG group were slightly closed, and the process of bone formation appeared to have initiated, but not optimally. The LSD test results indicated a significant difference between the AG group and the C group. On the other hand, the defects in the AG+SH group, from a clinical perspective, demonstrated a greater degree of closure, indicating a more optimal healing process. This was supported by the results of the LSD test revealing that there was a significant difference between the AG+SH group as well as both the C group and the AG group (Table 1).

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**Table 1.** Results of the Post-Hoc LSD test for osteoblast

| Dependent variables | Group (n = 8) | AG (Group administered Anadara granosa paste only) | AG+SH (Group administered a combination Anadara granosa and Stichopus hermanni paste) |
|---|---|---|---|
| bFGF | C (Control) | 0.411 | 0.000* |
| | AG (Anadara granosa paste only) | – | 0.000* |
| Blood vessels | C (Control) | 0.234 | 0.000* |
| | AG (Anadara granosa paste only) | – | 0.001* |

Note: * significant difference if $p < 0.05$

## DISCUSSION

Angiogenesis is an important stage of the proliferative phase in the bone healing process. During this phase, the endothelial cells migrate to new tissues and undergo proliferation. The new endothelial cells will then attach to each other to form new tubular vessel structures. The interaction between the endothelial cells and the extracellular matrix components plays an important role in regulating the formation of new blood vessels.[21]

During the normal healing process, angiogenesis initiation usually begins with the local release of both pro- and anti-angiogenic growth factors by endothelial cells. This release occurs in response to inflammation caused by injury-inducing inflammation and accumulation of hypoxia-inducible factor-1a (HIF-1a) to hypoxia.[22] Under these conditions of hypoxia, the endothelial cells will trigger bFGF, resulting in micro-vascular growth. bFGF will subsequently begin to produce mature endothelial cells in order to synthesize further new blood vessels. Fibroblast growth factor (FGF) and VEGF then bind to receptors on the cell surface complemented by tyrosine kinase activity. The activation of the kinase receptor enables the incorporation of signal transduction pathways regulating the proliferation, migration and differentiation of endothelial cells.[23] This suggests that during the normal bone healing processes, bFGF expressions remain visible because of the hypoxic conditions of tissue damage (bone). Similarly, in the C group, the number of bFGF expressions remained visible without any external treatments (Figure 1).

Moreover, the HAp applications studied through this research aimed to serve as a framework or defect filler matrix. HAp has insufficient connecting mineral cavities leading to imperfect degradation results. These large, thin cavities then allow bFGF to form new blood vessels (scaffolds). HAp and bFGF are influential in the migration process of mesenchyme cell progenitors, including endothelial cell progenitors and also in the stimulation of macrophage cells in the defective areas.[24,25] Similarly, the expressions of bFGF and the number of blood vessels in the AG group differed significantly from those in the C group.

Furthermore, optimal osteoconduction and osteoinduction processes are also essential to the healing of damaged bone. Considerable current research on bone substitute materials has, therefore, tried to combine both properties of osteogenesis with a polymer material.[26-29] One of the various polymeric materials used in performing bone grafts is GAG. GAG consists of sulfate and non-sulfate compounds. Sulfate and heparin sulfate are categorized into GAG sulfate compounds that can have positive effects on the wound healing process since chondroitin sulfate and heparin sulfate can bind bFGF.[17] GAG content is found in the flesh of SH.[30] On the other hand, hyaluronic acid categorized into non-sulfate compounds of GAG plays a complex role in cell adhesion, cell proliferation and cell movement. Variations in cell responses induced by hyaluronic acid are proliferation, migration and cytokine synthesis mediated by the determinant molecule-44 cluster (CD44) presented on the cell surface. Hyaluronic acid also interacts with a receptor for hyaluronan-mediated motility (RHAMM) located on the cell surface to initiate endothelial cell migration.[31] The RHAMM ligand interaction mainly occurs in endothelial cell motility and interaction with CD44 ligand triggering endothelial cell proliferation. These two receptors then work together to facilitate the formation of new blood vessels.[32] The CD44 involvement of depolymerized hyaluronic acid subsequently leads to endogenous bFGF release which, in turn, stimulates the proliferation and growth of new blood vessels.[33]

In addition to GAG, SH also contains high levels of protein and amino acids which play an important role in the inflammatory phase by increasing the immune response and modulating the inflammation to immediately enter the process of bone healing (reparative).[34] Moreover, sea cucumbers also contain PUFA which is useful for mediating control inflammation and regulating cell proliferation, including endothelial cells in the formation of new blood vessels.[35] Similarly, in the AG+SH group, bFGF expressions and blood vessel counts were significantly different from those in both the C group and the AG group. Finally, it can be concluded that the combination of *Anadara granosa* shells and *Stichopus hermanni* used as bone substitute can effectively increase the expressions

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DOI: 10.20473/j.djmkg.v50.i4.p194–198

of bFGF and the number of blood vessels in the healing of bone damage in Wistar rats.

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