Combination of Anadara Granosa Shell-Stichopus Hermanni Gel on Osteoblast-Osteoclast and Blood Vessels in Femur Healing

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Abstract

Bone graft is widely used as bone augmentation in dentistry. Anadara granosa shells contain calcium carbonate can be used as bone graft. Hyaluronic acid which is abundant in Stichopus hermanni can be used to increase osteoconduction and osteoinduction. The aimed of this research to determine the combination of Anadara granosa shells and Stichopus hermanni gel on osteoblast-osteoclast and blood vessels in bone healing process. 25 male wistar rats were divided into five groups, that were control, treated with bone graft derived from Anadara granosa shells (AGs), and another were treated with the combination of Anadara granosa shells and Stichopus hermanni with concentrations of 0.4%, 0.6%, 0.8% (AGSH1,AGSH2,AGSH3). All rats were intentionally defected on their right femur sized a half-diameter of a round bur. Those rats were sacrificed on day 14th, and their femur in the transversal side was cut continued with Haematoxylin eosin staining. The number of osteoblasts-osteoclasts and blood vessels were measured and statistically analyzed ($p<0.05$). Data analyzed showed significant differences between groups for osteoblast, control-treatment groups and AGs-AGSH3 for osteoclast and blood vessels.

Combination of Anadara granosa shells and 0,6% Stichopus hermanni gel most effectively increased the number of osteoblasts, and blood vessels, and decreased the number of osteoclasts on the bone healing process of femur.

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Introduction

Alveolar bone destruction can occur due to extensive pathological or traumatic conditions, that it has an impact on large bone defects resulting in bone resorption. The repair process in the bone defect consists of inflammatory phases (0-7 days), proliferation (weeks), and also remodeling (months). The process of vasoconstriction, hemostasis, and inflammatory cell infiltration occur in inflammatory phase. The peak of this inflammatory phase occurs on day 2 and disappears completely within 1 week. The proliferation phase where fibroblasts produce extracellular matrix, primary collagen, and fibronectin for cell migration and proliferation occurs on days 3-14. This phase is evidenced by angiogenesis, collagen tissue deposition, granulation tissue formation, and epithelial cell migration. The remodeling phase is characterized by tissue and collagen remodeling, epidermal maturation, and shrinkage.[1] Osteoblast activity appears on day 14, when Osteoprotegerin (OPG) gene expression increased relatively early during osteoblast differentiation. it is also associated with osteoclastogenesis where there is a decrease in the receptor activator of nuclear factor-B ligand (RANKL) - OPG ratio.[2]

The bone destruction could be corrected with the help of bone grafting in order to improve bone contour or new bone formation the bone. Bone graft is possible because bone tissue has the ability to regenerate well if provided space in which the bone will grow. Naturally, growing bones will transplant grafts in newly integrated areas. [3] On the 14th day, bone graft transplants showed a trash graft in the connective tissue and wind by the nucleated cells.[4]

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Bone graft can significantly fill the defect about 60-65%. With bone graft is expected to improve bone, it is better to use ordinary materials. The bone graft material must have three basic functions, namely osteogenesis, osteokonduksi, and osteoinduksi. Bone graft must be bioactive and biocompatible, acceptable to the body, well mechanized, and easily manipulated.[5]

Anadara granosa is a bivalve class, the Arcidae family of the genus Anadara, a type of shell commonly found in East Asia and in Southeast Asia. In this study used shells of Anadara granosa from the coastal region of Madura. The rounded fan, slightly oval, consists of two equal parts (symmetrical), having a palial line on a complete inner shell and a grooved outer palial outline. The inside is smooth with a shiny white color. Anadara granosa have red blood pigments (hemoglobin and myoglobin). This gives them better oxygen transfer allowing them to live in murky low oxygen environments in which most predators cannot thrive.[6] Anadara granosa is one of the most potent and economically valuable shells. Meat from Anadara granosa is used as a source of protein and minerals to meet the food needs of Indonesian society. Blood shell are a waste that is not widely utilized.[7] The structure of mussel shells is generally similar to the cancellous bone and the bone-like mechanical properties. Blood shells have a fairly high CaCO3 mineral composition (98.7%).[8] Based on Kamba and Zakaria studies[9], it is shown that the presence of calcium carbonate (CaCO3) crystals derived from shells has the potential to mimic the original composition, structure, and bone properties. The shells of Anadara granosa are a source of calcium in bone damage by making bone graft. Calcium carbonate that has biocompatible, osteoconductive and biodegrable properties, serves as a new bone deposition scaffold. Calcium carbonate having biocompatible, osteoconductive and biodegrable properties.

Other materials that can be used to speed up the bone repair process are SH which is rich in glycosaminoglycans (GAGs).[10] GAGs play a role in increasing osteoblast activity. GAGs consist of sulfate (chondroitin sulfate, dermatan sulphate and heparin sulphate) and non sulfate (hyaluronic acid).[11] In the previous study, the characterization of GAGs content in golden cucumber with spectrophotometer showed that Hylauronic acid (HA) was the greatest content (75.7%), followed by 2% sulfuric heparan, chondroitin sulfate 1.72%, and dermatan sulphate 1.11%.

HA plays an important role in influencing the rate of cell migration in the process of wound aging, inflammation, angiogenesis, cell reproduction and proliferation.[12] HA research on bone repair processes with a concentration of 0.2% in chronic periodontitis had statistically insignificant results between clinical and histologic outcomes. In another study using HA with a concentration of 0.8% stated that HA accelerated bone regeneration by chemotaxis, proliferation and differentiation of mesenchymal cells.[13] The use of HA as a regenerative material, which was utilized in conjunction with Carbonate hydroxyapatite, could increased the number of osteoblasts, the expression of OPG and TGF-β1 in bone formation.[14]

Based on the above background, in this research will be made bone graft consisting of combination of Anadara granosa shell and Stichopus hermanni, by analyzing the number of osteoblast-osteoclast cells and blood vessels in the bone healing process in the experimental animals.

Materials and methods

Preparation of Anadara granosa shells

Anadara granosa shell preparation was done by collecting Anadara granosa shell taken from restaurant waste of sea food and cleaned from dirt. [15] Anadara granosa shells was boiled for 30 minutes. Then, the shells of Anadara granosa are then brushed on the outside and inside using water and soap without bleach, then dried at room temperature. After that, it is crushed using mortar and paste and done with 50 mesh sieving to get smaller particle yields. Furthermore Anadara granosa shell powder is calcined with oven furnace (Brand) temperature of 10000C. X-Ray Difraction (XRD) examination was performed to see the percentage of calcium carbonate content. Sterilization of Anadara granosa shell powder was performed using ultraviolet light (A10-UV-30, Cleatech®, USA)

Preparation of Stichopus hermanni

SH obtained from coastal areas of Madura with the criteria of weight and length that has reached 400-500 grams / tail. The body part

taken from the SH is only meat and the contents of the stomach discarded. After that the sea cucumbers are cleaned under running water. SH are washed with sterile aquades and cut into small pieces, blended (brand), with a ratio of 500 grams SH and 1 liter of sterile aquades, for 10 minutes. Furthermore, 2000 gram of SH mixture was carried out freeze dried to obtain dry rough formation from sea cucumber. Results of freeze drying that has been subsequently finely blended and sieved with a size of 50 mesh and obtained size 297 microns. To change the size of golden sea cucumber particle required high energy milling Elliptical 3D Motion (HEM-E3D) by Nanotech® Indonesia by inserting material and ball on vial with comparison of 2 g SH with 20 g of continuous spinning stainless steel balls. The vial is fitted into the HEM and is set working time and rest time. Working time is done where within the first 10 minutes of working time, the equipment is rested 10 minutes as well, and so on until 30 minutes of work and 30 minutes stop, then the tool is rested 30 minutes. At the time the appliance is rested the operator turns off the power and takes the vial and opens it for stirring. This is done up to 2 times (total time is 60 minutes of work and 60 minutes stop where there is a 30 minute interval from interval 1 and second). The results show the golden sea cucumber powder measuring ± 202 nm.

Analysis of glycosaminoglycan concentration by spectrophotometric method using sulfate GAGs assay

The analysis of glycosaminoglycan concentration was measured by spectrophotometric method using sulfate GAGs assay which refers to Jong et al.[16] modified with Zhou et al.[17]. A methylene blue stock solution was prepared by dissolving 25 mg methylene blue into 50 mL of bidestylates. A total of 5 mL of the stock solution was diluted with a bidestylate accent to a volume of 30 mL. The solution is then used as a reagent in the test of glycosaminoglycan concentration. The sample solution was prepared by mixing 0.25 mL of glycosaminoglycan solution into 0.25 mL of bidestylate and added 2.5 mL methylene blue. The sample solution was allowed to stand within 5 minutes to 2 hours and the absorbance was measured. The absorbance measurements were performed using a spectrophotometer at a wavelength of 540 nm for hyaluronic acid, chondroitin sulfate and sulfate dermatan, and 610 nm for heparin sulphate. The standard curves were obtained by measuring the standard absorbance values of hyaluronic acid (53747), chondroitin sulphate (C 4384), dermatan sulphate (D 3788) and heparin sulphate (H 7640) from Sigma Aldrich. Standard solution stock solutions are prepared by dissolving as much as 0.2 g Standard solution in aquades up to 100 mL volume. The standard test solution was obtained by diluting the standard solution stock solution to give a solution of concentrations of 200, 600, 1,000 and 1,400 ppm, respectively. This research was conducted at ULP, Pharmacy Laboratory of Airlangga University Surabaya.

Preparation of scaffold from Anadara granosa shells and Stichopus hermanni

The tools used in this study are animal cages, right femur bone storage tubes, scissors and scalpels, anatomical and resparatorium tweezers, animal scales, food and beverage venues, microtomes for cutting, light microscopes, object glasses and glass cups, bottles and shelves painting, 3cc syringe, round-brip (straight handpiece) Mcisinger® Germany size 18, micromotor 1200 rpm, razor shaver, dinner sonde, glass dappen, separating disc. In addition the materials used are calcined AG powder, dried SH with ± 202µm size, membrane, silk and sewing thread, needle, cotton or tissue, rat food, aquades for beverage rats are changed daily, 10% formalin buffer, ketamine hydrochloride, xylazine hydrochloride, povidine iodine 10%, 30% alcohol, 50%, 70%, 80%, 96%, novalgin, EDTA 10%.

Preparation of bone graft is done by mixing AG shell powder and SH gel. SH gel preparation is done by using a mixture of gelatin material. The gel preparation was prepared by mixing the 25 g of gel powder into 250 ml to obtain a viscosity of more than 800cps, so 10% gelatin was used by mixing 25 grams of gelatin into 250 ml of water. In this study using concentrations of 0.4%, 0.8% and 1.6%.

Cytotoxicity test

Cytotoxicity of hydrogels was determined by using a MTT assay, which is a standard for measuring the viability of cells via metabolic activity. Water-soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromid) is metabolically reduced in viable cells to a blue-violet insoluble

formazan. BHK-21 fibroblasts were cultured in DMEM medium. The cells were seeded onto 96 well plates at a density of 104 cells/ ml and incubated at 37^0C for 48h. After incubation, we aspirated the culture medium out of each well. Then, 100ml of culture medium containing the hydrogel extractions were added to each well. Cells cultured without the hydrogel extractions were used as the control. After another 24h of incubation, 20ml of MTT solution was added to each well and further incubated at 37^0C for 4h. After removing the culture medium from the plates, 100ml of isopropanol was added to each well and incubated for 20min at 37^0C in a shaker incubator. The absorbance of the resulting solution in each well was recorded immediately at 570nm using automated micro plate reader. The experimental data were plotted and the percentage of cell viability was obtained. Every sample was done at least in triplicate and the average was plotted. The following formula is to calculate the reduction of viability:

$$\text{Viab.}\% = \frac{100 \times 0D_e}{oD_b}$$

Where ODe is the mean value of the measured optical density of the 100% extracts of the test sample; ODb is the mean value of the measured optical density of the blanks. The higher the Viab. % value is, the lower the cytotoxic potential of the test item. If viability is reduced to<70% of the control, it has cytotoxic potential.[18]

Pre-clinical test to the experimental animals

The experimental animals research was true experimental research with complete randomized design with the control group and the selection of experimental animals in the group is done randomly. The samples used were 25 Rattus novergicus Wistar types divided into five groups, where the selected criteria were male sex, age 2 months with body weight 150-357 gram. The study was conducted after obtaining approval from the animal ethics committee of the ethic team of Faculty of Dentistry, University of Hang Tuah, Surabaya.

The experimental procedure in this experimental animal was started with wistar rat acclimatization for 7 days, followed by Wistar rats divided into 5 groups, ie control group, treatment group given powdered shell powder (AGs), treated group application of combination of Anadara granosa shells and Stichopus hermanni gel with concentrations of 0.4% (AGSH1), 0.8% (AGSH2), 1.6% (AGSH3). After acclimation, surgery was performed on the os femur dextra.

At the beginning of the treatment the rat performed anesthesia by giving ketamine and xylazine at doses of 0.11 mL / 100 gr BB intramuscularly.[19] After the rat started unconscious , fur on the part to be done defects, shaved by using a Gillete shaver. Furthermore, 10% povidine iodine antiseptic was treated in the area for 5 min.[20] A 2 cm incision was performed using a One Med Indonesia surgical knife, and soft tissue (skin and muscle) was removed using a periosteal elevator brand Osung Defek on the femur os dextra lateral is done by using round bur brand Mcisinger® Germany size 18 using straight handpiece as deep as half diameter bur. After forming defects in the femur, an application is performed with the treatment according to the division of the group and closed by the administration of the membrane. Surgical procedure ended with suturing to cover skin and soft tissue.[21] Administration of novalgin novelgin 0.09cc/200g BB and antibiotic interflox 0.1cc/100g BB for 3 days. This procedure is needed to control the inflammatory process and pain. [22]

Intake of dextra animal femur was done on the 14th day after treatment, before rats performed euthanasia action by using cervical dislocation. [23] The existing femur preparation was skinned for post grafting bone, by cutting using separating disc, then inserted into in 10% formaldehyde buffer solution so that the tissue does not decompose, hardening the tissue and increasing the affinity of the tissue against the paint material. After the process of tissue fixation, the decalcification process is done using EDTA for 2 months. Femur specimens were prepared in the form of a sagittal piece preparation with HE painting. After that done counting the number of osteoblasts and osteoclasts in the defect area with a 400X magnification light microscope. The data obtained are analyzed to obtain a description of the distribution and data summary in order to clarify the results. Then tested the hypothesis by using parametric statistical test One-way ANOVA, followed by LSD test.

Results

In the spectrophotometric test, using standard solutions of hyaluronic acid (53747), chondroitin sulfate (C 4384), dermatan sulphate (D 3788) and heparin sulphate (H 7640) from Sigma Aldrich, showed glycosaminoglycan present in whole Stichopus hermanni. The complete can be seen in table 1.

Test Materials	Hyaluronic acid	Chondroitin sulphate	Dermatan sulphate	Heparan sulfat
Whole Stichopus hermanni	75,7%	1,72%	1,11 %	2 %

Table 1. Spectrophotometric test results of glycosaminoglycan content.

MTT assays were used to test the cytotoxicity of hydrogels. We can observe from the results that the group AGSH3 has the highest cell viability, and the others group (AGS, AGSH1, AGSH2) showed higher cell viability than did the control group. The percentage value of the cell viability of the treatment groups in the MTT assay showed above 50%. Thus, we can concluded thatthese hydrogels have no cytotoxicity.

In the experimental animal study on femur os performed for 14 days, clinically the defect in control had not closed, but in AGs group, the bone formation process was not optimal yet. Pathological conditions in control group showed that osteoblast has increased in grafting of AGs and AGSH (figure 1).

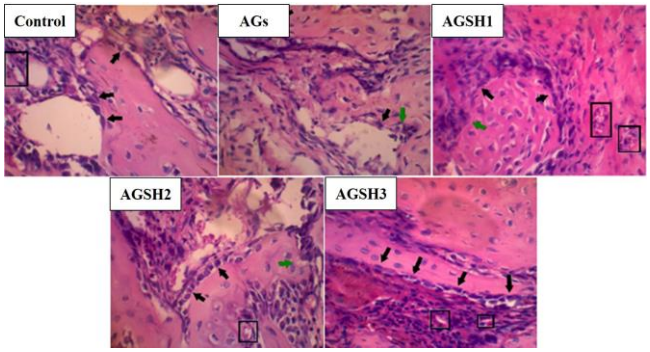

Figure 1. Histological section with application of grafting combination from Anadara granosa shell and Stichopus hermanni in bone healing process of femur at 14th day.

Histologic features show proliferation of osteoblast in the defect areas of femur in all study groups (figures 2).The result of osteoblast count shows the mean of osteoblast in control=9.671.63; AGs=20.831.72; AGSH1=32.17±2.04; AGSH2=39.83±2.04; AGSH3=46.832.48, mean number of osteoclast in control=7.832.32; AGs=5.00±1.27; AGSH1=3.50±1.05; AGSH2=2.83±1.33; AGSH3=2.00±0.89 and mean number of blood vessels in control=3.17±1.33; AGs=4.83±1.17; AGSH1=6.83±1.17; AGSH2=7.50±1.52; AGSH3=7.67±2.34.

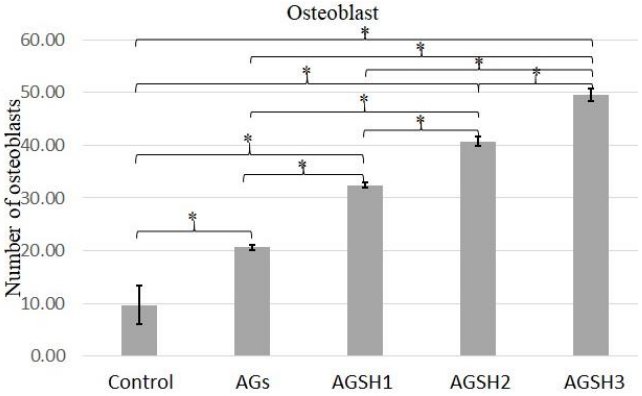

Figure 2. Effect of grafting combination of Anadara granosa shell and Stichopus hermanni to osteoblast.
Control= group not given anything ; AGs = group given only Anadara granous shell; AGSH1 = group given bone graft from Anadara granosa shell and 0.4% Stichopus hermanni gel; AGSH2 = group given bone graft from Anadara granosa shell and 0.8% Stichopus hermanni gel, AGSH3 = group given bone graft from Anadara granosa shell and 1.6% Stichopus hermanni gel.

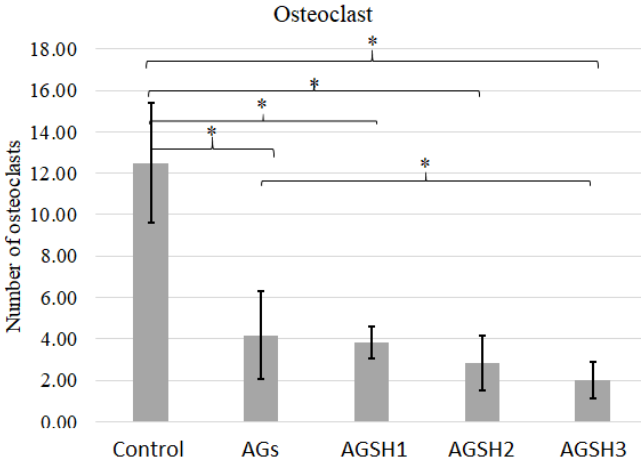

Figure 3. Effect of grafting combination of Anadara granosa shell and Stichopus hermanni to osteoclast.

In the graphs (figures 2 and 4), the average number of osteoblasts and blood vessels was found to be lowest in the control group, while the highest in the AGSH3 group. However, in the average number of osteoclasts (figure 3) the results are almost identical. On the

contrary, the number of osteoclasts in the control was highest, while the lowest was in the AGSH3 group.

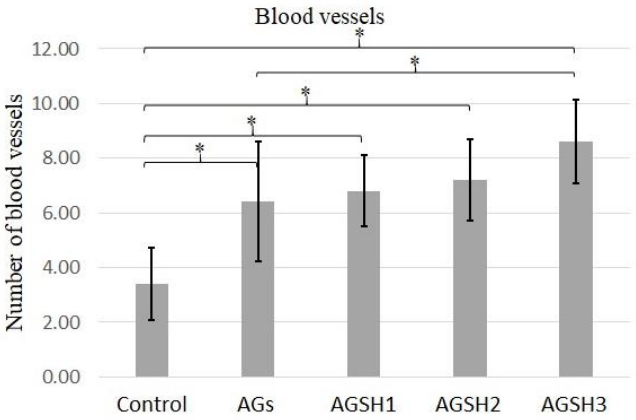

Figure 4. Effect of grafting combination of Anadara granosa shell and Stichopus hermanni to blood vessels.

Anova test results showed a significant difference in osteoblast variables, osteoclasts and blood vessels ($p < 0.05$). However, in the tukey-HSD post-hoc test, the osteoblast variables showed significant differences between the groups with each other. In the osteoclast variable, significant differences were shown in control group with all treatment groups, and group AGs with AGSH3. In the blood vessel variable significant differences were shown in control group with AGSH1-AGSH2-AGSH3 groups, and group AGs with AGSH2-AGSH3.

Discussion

Bone defects can cause damage to cells, blood vessels and bone matrix. The body responds to the damage by beginning the inflammatory process of hematoma formations, blood clots through accumulation of polimorphonucleates (PMNs), lymphocytes, platelets, blood monocytes, macrophages, neutrophils, and osteoclasts. In hemostasis, the cells that play a role are platelets or platelets that will undergo adhesion and aggregation.[24] Furthermore, the fibrinogen will be activated into fibrin fibrils which in turn will polymer into a stable blood clot.[25] Macrophages are phagocytic cells that are produced in bone marrow that play an important role in inflammation. Macrophages play a role in removing cytokines consisting of proinflammatory, anti-inflammatory and Growth factor. These macrophages will trigger the secretion of pro-inflammatory cytokines such as Tumor Necrosis Factor (TNF), interleukin-1 1 (IL-1) and interleukin-6 (IL-6) as inflammatory mediators to strengthen the immune response and increase metabolic processes. Cytokine , IL-1, TNF-β, IL-6, and IL-11 affect RANK receptor action to bind to RANKL present in osteoblasts so that osteoclast activity increases and leads to bone resorption.[26] Osteoclasts and osteoblasts regulate a dynamic balance in the bone remodeling process. [27]

Macrophages also secrete growth factor (PDGF, VEGF, FGF, TGF, and ANG-1), which in turn stimulate angiogenesis.[1] The tissue that is undergoing the healing process, the supply of oxygen and nutrients is very important as a source of energy for cells to proliferate. The process of providing nutrients and oxygen is mediated by the presence of angiogenesis in the early phase of proliferation. The increase in the number of blood vessels will provide the necessary nutrients the tissue to immediately begin the proliferative phase.[28] The proliferative or reparative phase begins when the inflammatory phase releases the cytokine and growth factor resulting in fibroblasts proliferation to form the extracellular matrix and the formation of calcium salts through an attachment, thus forming a woven bone.[29] This can be seen in Group K which is a group with normal bone healing.

Group AGs is a group given bone graft of Anadara granosa shells containing calcium carbonate. This addition was expected to accelerate bone healing because calcium carbonate serves as a skeleton for allowing bone formation and improves bone healing and acts as a mineral reservoir that helps in the formation of new bone.[30] The shell powder of the blood shell serves as a scaffold that acts as a place, the environment for growth, development, and differentiation of mesenchymal cells into osteoprogenitor cells. With the help of Growth factor, osteoprogenitor cells migrate to the defect area and differentiate into osteoblasts. Differentiated osteoblasts will activate alkaline phosphate, type-1 collagen and osteocalcin. The mature osteoblasts will congregate and form the osteoid until the bone cavity is filled, causing an increase in bone matrix, which will affect the bone healing process.[31] In the histological section, bone formation observe well organized

in the treatment groups on the 7th day compared to the control groups and increase on the 14th day that indicated by the presence of maximum bone formation after extraction in rats.[32] Macrophages and bone related growth factors such as VEGF assist in the formation and improvement of bone by enhancing the recruitment of mesenchymal stem cells that can differentiate into buffer tissues such as blood cells, and vascular endothelium depending on where mesenchymal stem cells are attached.[33,34] This was shown in the AGs group having significantly different osteoblast-osteoclast and blood vessel counts compared to the control group (see table 1, $p > 0.05$).

In group AGSH1-3 were added preparations of gel having better adhesion than powder. Good material attachment to the defect can help speed up the bone healing process This gel contains the active content of whole Stichopus hermanni of 0.4%, 0.8% and 1.6%. Stichopus hermanni has an active ingredient rich in glycosaminoglycans. Among the existing glycosaminoglycan, hyaluronic acid (75.7%) is the highest content in Stichopus hermanni which is treated by the freezed-drying method.

Hyaluronic acid can interact strongly with leukocytes through CD44 receptors that have contributed to the retention of these cells at the site of inflammation.[35] This association with the CD44 receptor in inflammatory cells may modulate cell behavior for cell activity including proliferation and migration in inflammation. Hyaluronan induces a receptor-mediated signal by interaction with an HA binding protein on the macrophage cell surface. The interaction of HA with CD44 and Receptor for Hyaluronan-Mediated Motility (RHAMM) induces the grouping of CD44 receptors and activation of mitogen-activated protein kinase (MAPK) regulated by intracellular RHAMM, resulting in phosphorylation of extracellular signal-regulated protein kinases 1/2 (ERK1 / 2) and akt which will convert macrophage 1 (M1) to macrophage 2 (M2), so M2 modulation is larger than M1, so the network repair response is more prominent. This M2 modulation activates activator plasminogen (Ap-1) and increases the secretion of an anti-inflammatory cytokine. The presence of AP-1 activation enhances the regeneration process that is stimulated by various growth factors. This process will increase the number of blood vessels due to angiogenesis and increased osteoblast proliferation. It is evident in this study that the proliferation of osteoblasts and endothelial cells in the blood vessels in the group who were supplemented with Stichopus hermani were more numerous than control group and the treatment groups (AG alone).

HA bonds with TNFα-stimulated gene-6 (TSG6)-convolved CD44 receptors may inhibit the association of Toll-like receptors-4 (TLR4) with Myeloid differentiation primary response 88 (MyD88), thus suppressing nuclear factor kappa B (NF-KB) activation. This will prevent the expression of proinflammatory proteins (IL-6, TNFα, and IL-1β) and increase the expression of anti-inflammatory proteins in macrophages such interleukin-4 and 10 (IL-4 and IL-10).[36,37] This causes the number of preosteoclasts present in the inflammatory process of bone destruction to decrease resulting in a decrease in the number of osteoclasts.

In this study AGSH3 group is a group that has significant differences both with control group and AGs, compared to group AGSH1 and AGSH2. Although there was almost no significant difference between the groups of AGSH3 and AGSH1-2.

Conclusions

Stichopus hermanni is one of the marine biotas that have a high content of hyaluronic acid (75.7%). Aplication of Stichopus hermanni gel and Calcium carbonato of Anadara granosa shells increased the number of osteoblasts, blood vessels and decreased the number of osteclasts in the bone healing process. The most effective concentration addition of Stichopus hermanni gel was 1.6%.

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Declaration of Interest

The authors report no conflict of interest.

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