The Effectiveness of Deproteinized Golden Sea Cucumber (Stichopus Hermanii) Combination of Deproteinized Bovine Bone Xenograft in Stimulating the Formation of Bmp-2 and Opg in the Process of Bone Tissue Regeneration

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Abstract

Periodontitis is a disease that damages the periodontal tissue and can cause tooth loss. In recent years there has been an interest in carrying out regenerative efforts as periodontal therapy. Bonegraft derived from natural materials is the current choice because of the limitations of the available synthetic bonegrafts.

The aim of this study was to analyze the effect of golden sea cucumber on the increase of BMP2 and Osteoprotegrin on bone regeneration. This research is a laboratory experimental study with male guinea pigs as the subject. The number of samples is 30 guinea pigs. After 14 and 21 days sacrificed was performed for immunohistochemical examination. The expression of BMP2 and OPG had a significant difference between the sea cucumber group and the positive control group.

It can be concluded that bonegraft from golden sea cucumbers has a mechanism as bonegraft material so that it can be used as an alternative material in regenerative therapy.

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Introduction

The Global Burden of Disease Study in 2019 stated that periodontal disease is the 11th most common disease in the world, and shows that this disease still requires attention today. Periodontitis is a progressive disease that is the main pathogenic cause of tooth loss, involving the supporting tissues of the teeth such as alveolar bone, cementum, and the periodontal ligament. Pasarelli et al conducted a study on the reasons for tooth extraction in adults and concluded that the main cause of second tooth loss is periodontal disease after dental caries. (Caries 52.2% and periodontal disease 35.7%). [1,2]

The main goal of periodontal treatment is to stop the disease course and restore the function of the lost periodontal tissue. In recent years there has been a lot of interest in regenerating periodontal tissue, and bonegraft is the most widely used periodontal treatment option today. This material is used to fill or replace lost bone and can help the bone healing process, has the function of stimulating osteogenesis (bone formation) and providing mechanical support. This material also has bioactive, biocompatible, osteoconducting, and osteointegration properties. Bonegraft consists of several types, namely those derived from natural materials (autograft, allograft, xenograft) and synthetic (alloplast).3-6

Bovine Xenograft

Xenographs come from a variety of sources, including cattle, pig, horse, and corralling and are generally biocompatible with human bones. Bovine xenografts were the first patient-applied xenografts, are commercially available in a variety of products and are considered the most documented material of this category. They are characterized by osteoconductive properties, are deproteinized and lyophilized, do not cause an immune response. However, the granules of this material

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are thought to undergo poor or slow absorption, being surrounded by neoplastic bone tissue rather than entering the normal bone remodeling process. Processing at high temperatures to avoid immune reactions, allergies and infectious diseases such as spongiform encephalopathy is thought to be responsible for modifying the hydroxyapatite structure which further leads to reduced absorption potential.[6]

Periodontal Regeneration and Golden Sea Cucumber

Autograft is a graft that is transferred from one place to another but is still in the body itself, is the gold standard because it has osteoconductive, osteoinductive and osteogenesis properties, but the drawbacks are that the number of collections is limited, it is difficult to take the graft, has the risk of infection, the risk of blood loss, and causes inflammation. morbidity. Xenografts are grafts taken from different species usually from cattle or cattle for use in humans, the disadvantages of xenografts are that there is a risk of spreading infection between species and cannot produce live cells during the process of osteogenesis. Allograft is a graft that comes from one person to another both in the same or different species, the lack of which can evoke an adverse tissue response and host rejection response and immune system rejection.[3,4,6,7]

There are several shortcomings that are currently available with bonegrafts as well as enormous advances in the field of biomaterials science, encouraging experts to develop natural materials as bonegraft materials. One of the natural ingredients that attract attention today is sea cucumber. Sea cucumbers are marine invertebrates with cylindrical, flexible and rough skin. This animal contains insoluble collagen and is used as a dietary supplement and contains a large source of the polysaccharide chondroitin sulfate, has a complete nutritional content, low in fat, high in protein, and rich in essential amino acids such as arginine, lysine, and tryptophan and fatty acids (FAs). Several studies have reported that sea cucumbers have biological and pharmacological activities including anti-inflammatory, antimicrobial and antibacterial, antibiofilm, anti-angiogenic, anticancer, anticoagulant, anti-hypertension, antioxidant, antithrombotic, antitumor, wound healing with its flavonoid content.[8-11]

One type of sea cucumber that has great benefits is the golden sea cucumber (Stichopus hermanii). Pringginies, et al in 2017 conducted a study and stated that one of the sea cucumber species that has antibacterial compounds is Stichopus hermanii. Tamara, et al in 2015 conducted a study and concluded that Stichopus hermanii was able to inhibit gram-positive and gram-negative bacteria, compared to other types of sea cucumbers. Adam M, et al 2022 Golden sea cucumber (Sticopus Hermanii) is a marine microorganism that is very usefull in human life.[12-14]

Golden sea cucumbers are very useful in the process of tissue regeneration, especially periodontal tissue. Damage to bone tissue will go through a remodeling process in its healing. Bone remodeling occurs with the aim of maintaining bone strength and mineral homeostasis. Various cell biomarkers that play a role in the bone remodeling process, including BMP2 and Osteoprotegerin (OPG). Bone Morphogenetic Protein 2 (BMP2) is a superfamily of TGF which plays a role in a number of processes of bone development and formation. OPG means bone protector because it protects bone from excessive resorption by limiting bone resorption by osteoclasts. OPG binds to RANKL and inhibits the interaction of RANKL with RANK thereby affecting osteoclast formation and function.[15-17] Until now, research on the effect of golden sea cucumber on bone regeneration is still limited, especially looking at its effect on changes in bone remodeling marker cells. Therefore, researchers are interested in conducting research on the effectiveness of the use of golden sea cucumbers (Stichopus hermanii) on BMP2 and OPG levels in bone tissue regeneration, so it is hoped that the results of this study will be taken into consideration in the selection of sea cucumbers as natural ingredients to be used as bone graft materials in bone grafting. tissue regeneration therapy, especially periodontal tissue.

Materials and methods

This research was conducted from July to October 2022, and has been approved by the ethics commission No.0060/PL.09/KEPK FKG-RSGM UNHAS/2022. This research is a true experimental laboratory research with a post-

test only control group design. The preparation of sea cucumber extract and deproteinized was carried out in the biology laboratory of Makassar State University. The preparation is made in two ways, namely deproteinization and maceration and then mixed together to become bone graft material. After that, XRF analysis was carried out to see the compound content in the bonegraft, and XRD analysis to see crystallization. The maintenance and treatment of experimental animals was carried out at the Doc Pet clinic in Makassar, then continued with making slides in the Anatomical Pathology Laboratory, Faculty of Medicine, Hasanuddin University, and reading of BMP2 and OPG immunohistochemical analysis was carried out at the Faculty of Medicine, Brawijaya University, Malang.

The material used in this study was a type of golden sea cucumber (Stichopus hermanii). The picture of the golden sea cucumber can be seen in picture 3. The sample used a male guinea pig (Cavia porcella) with inclusion criteria of 3-4 months of age, body weight 200-500 grm, healthy condition (enough hair, not shedding and active movement and good feed consumption). This study uses 6 groups and 5 samples per group so that the total number of samples was 6x5 equal to 30 guinea pigs.

Preparation of gold sea cucumber bonegraft material:

a. Deproteination method: The sea cucumbers are cleaned of remaining dirt and then dried manually in direct sunlight to dry and then dried in an oven. The sea cucumbers were cut into small pieces and blended until smooth with a size of ± 60 Mesh. The sea cucumbers were added with 3.5% sodium hydroxide (NaOH) in a ratio of 1:10 (w/vol) to the mashed golden sea cucumber flour. After that, it was heated with an electric stove at a temperature of 65°C while stirring with a magnetic stirrer for 2 hours. The mixture is cooled at room temperature to facilitate the filtration process and the precipitate is obtained and the drying process is in an oven at a temperature of 65°C.[18]

b. Maceration Method: Sample preparation was carried out by washing the golden sea cucumbers, then drying manually in direct sunlight to dry, it is recommended to dry in the oven. The golden sea cucumbers were cut into small pieces and mashed to a size of ± 60 Mesh. Approximately 700 g of sea cucumbers were homogenized and extracted using the maceration method with a volume ratio of 1:3 methanol. The sample was left for 72 hours with solvent changes every 24 hours and stirred with an orbital shaker. The resulting maserate is then filtered and concentrated with a rotary evaporator at a temperature of 40°C until the extract is obtained

Animals model:

a. Defect manufacture and application of sea cucumber extract and deproteinazed sea cucumber extract. The guinea pigs were shaved on the femur and then disinfected using betadine and the guinea pigs were anesthetized with ketamine (0.4 – 0.6 ml/kg or 0.1 – 0.15 ml/head). One of the guinea pig's femurs was incised vertically and then a muscle evaluation was performed to gain access to the femur. A cavity with a diameter of 3 mm and a depth of 2 mm was made with a round bur, irrigated with 0.9% NaCl. 10 guinea pigs were given a combination of deproteinazed sea cucumber extract and 10 guinea pigs were given BATAN bonegraft as a positive control. Then sutured with absorbable vicryl 5.0.

b. Thread Removal of Bone Tissue

10 guinea pigs were sacrified on day 14 and 10 guinea pigs were sacrified on day 21. The specimens were taken using sterile minor surgery and then the bone tissue was stored in a sterile pot containing 10% formalin. The bone specimens were brought to the Anatomical Pathology Laboratory, Hasanuddin University for making immunohistochemical slides, then the slides were read in the bio-biomolecular laboratory, Faculty of Medicine, Universitas Brawijaya.

Results

Data normality test using Shapiro-Wills test obtained p value> 0.05 indicating that the data used in this study is normally distributed. Homogeneity test used Levene's test with p>0.05. This indicates that the data in this study have the same variance (homogeneous). The next test is the ANOVA test to compare the levels of BMP2 and OPG between the three groups both on day 14 and 21.

Table 1 and figure 1 shows the mean value of the positive control, negative control and test BMP2 groups with respect to the time of observation. Where seen on day 14, the highest value in the test group (9.80), then the positive control group (9.00) and the lowest in the negative control group (4.80). The same result was also seen on day 21 where the highest value was in the test group (13.00), then the positive control (12.40) and the lowest was in the negative control group (6.60). The value ($p<0.05$) indicated a significant difference in the mean BMP-2 expression in the treatment group on days 14 and 2.

GROUP	SAMPLE (n)	DAY	MEAN	SD	Value P
P (Sea Cucumber)	5	14	9,80	1,48	
K+	5		9,00	1,58	0.000*
K-	5		4,80	1,30	
P (Sea Cucumber)	5	21	13,00	2,12	
K+	5		12,40	2,30	0.000*
K-	5		6,60	1,14	

Table 1. Comparison of BMP2 levels between groups.

*One Way ANOVA Test (Significant, *p<0.05,CI 95%).

GROUP	SAMPLE (n)	DAY	MEAN	SD	Value P
P (Sea Cucumber)	5	14	8,80	1,48	
K+	5		7,40	1,67	0.002*
K-	5		4,60	1,14	
P (Sea Cucumber)	5	21	13,20	1,48	
K+	5		11,00	0,71	0.000*
K-	5		7,20	1,30	

Table 2. Comparison of OPG levels between groups.

*One Way ANOVA Test (Significant, *p<0.05,CI 95%).

Table 2 and figure 2 shows the comparison of the average number of OPGs for each observation group on the 14th and 21st days. On the 14th day of observation, the highest average OPG was in the P group (sea cucumbers) of 8.80 and the lowest average was in the K- group of 4.60. from the ANOVA test results obtained p value (0.000) < 0.05, which means there is a significant difference in OPG between treatment groups on day 14. Meanwhile, on day 21, the highest average OPG was in group P (sea cucumbers) of 13.20 and the lowest average was on group K- of 7.20, from the ANOVA test results obtained p value (0.000) <0.05 which means there is a significant difference in OPG between treatment groups on day 21.

Immunohistochemical staining was performed using BMP-2 and anti-OPG antibodies against 30 guinea pig bone tissue preparations. Observations using an obtilab microscope with 1000 magnifications showed BMP-2 expression as a brown area. The following is an immunohistochemical description of negative control, positive control group and test group on days 14 and 21 (figure 3 and 4).

In immunohistochemical observations, the expression BMP-2 in positive control and test group showed a larger brown area than the expression BMP-2 in negative control. This indicates that the expression of BMP-2 in the test and positive control groups is higher than the negative control.

Meanwhile, for day 14 and 21, the expression of BMP-2 can be seen in Figures 3. The brown area indicating BMP-2 expression was larger in the positive control group and the test group, compared to the negative control group. When compared with the picture on day 14, the brown area on day 21 (positive control group and test group) looks bigger, indicating greater BMP-2 expression.

Discussion

Osteoprotegerin (OPG) was first discovered in 1997. It acts as a receptor by blocking the binding of RANKL to its cellular receptor, RANK. OPG is a member of the TNF receptor super family and is produced by osteoblasts. OPG is an osteoclastogenesis inhibitory factor that prevents the function of RANKL. The RANKL/OPG balance system regulates bone metabolism. Therefore, the OPG level can be used as an indicator of bone regeneration. The results of this study are expected to produce a natural material that has a bone regeneration effect that can accelerate healing so that it can be used as a periodontal tissue regeneration material. To assist the regeneration process in a defect in bone, one of the strategies is to provide a substitution material in the form of a bonegraft that has osteogenic, osteoinductive and/or osteoconductive properties.[19-22]

Observation of OPG level can be done through immunohistochemical examination. Research using marine biota was also carried out by Hasanuddin Thahir, et al. in 2020 carried out the extraction of hydroxyapatite of snakehead

fish using the sol gel method and observed its effect on osteocalcin expression. And research on Yanti, et al in 2013 where sea sponge extract can inhibit the growth of Staphylococcus aureus bacteria found in the oral cavity. As for several other studies using golden sea cucumbers, including Dian Mulawarmanti in 2019 resulted that the active content of sea cucumbers is useful for wound healing. The use of combinations with other biota/compounds enhances the therapeutic effect of using gold sea cucumbers as adjuvant therapy in the oral cavity. another study was conducted by Mega Safithri in 2018, where the extraction of collagen from golden sea cucumber was carried out to see the tyrosinase enzyme inhibitory activity.[23-24]

The results of data analysis show that there are significant differences in BMP-2 and OPG between groups on days 14 and 21 (table 1 and 2). The highest OPG was in group P (sea cucumber) compared to the positive control group and negative control group. This shows the ability of sea cucumber extract combined with deprotinized sea cucumber in bone regeneration. In line with research conducted by I Ketut et al in 2017, the combination of Stichopus hermanii gel and Hyperbaric Oxygen Therapy (HBOT) to accelerate orthodontic treatment resulted in an increase in OPG that binds to RANK-L so that osteoclasts are inactive and osteoblasts form new bone thereby increasing tooth movement in orthodontic treatment. this is also in line with research conducted by Dian Widya in 2015 showing that gold sea cucumber extract has a lot of active ingredients involved in the wound healing process. Research on Putri Pirda in 2019 concluded that gold sea cucumber gel can accelerate wound healing in the proliferative phase in diabetes mellitus wounds.[25-27]

This study was conducted to examine the effectiveness of gold sea cucumber extract combined with protein deproteinase as a bone graft material in bone regeneration, which was characterized by an increase in BMP-2 expression. The presence of BMP-2 will accelerate bone healing, increase mineralization, remodeling, and biomechanical stiffness.[28-29]

In this study, the positive control used was BATAN bonegraft, which contained hydroxiapatite. The combination of golden sea cucumber extract with protein deproteinase (test group) showed a higher and significant mean value than the control group indicating that the content of gold sea cucumber extract was able to increase the bone regeneration process.

The content of hyaluronic acid in golden sea cucumbers can interact with CD44 to initiate signal transduction and activate AP-1 (alkaline phosphatase) which results in cell migration due to the release of various growth factors. Activation will trigger the proliferation and differentiation of osteoprogenitors into osteoblast cells which play an important role in the formation of the bone matrix. This is in line with Setiawati's research which found that the application of hyaluronic acid carbonate hydroxiapatite in the post-extraction socket of wistar rats could increase osteoprotegerin (OPG) and TGF-β. 5 times compared to control. An increase in TGF-β indicates an increase in BMP-2.[30-32]

Mardiana also stated that chondroitin sulfate has antiosteoclastogenic and flavonoid effects that increase OPG expression, increase osteoblast differentiation, and decrease RANKL expression. Harun also revealed that another ingredient of golden sea cucumbers that can increase the production of TGF-β is proteoglycans. Proteoglycans can modulate growth factors such as vascular endothelial growth factor and FGF and can regulate TGF-β activity and the production of collagen and fibrin types I and III. The release of TGF-β will increase collagen synthesis, and the wound healing process will occur faster. [33-37]

Golden sea cucumbers contain flavonoids that can inhibit osteoclast differentiation thereby inhibiting bone resorption and stimulating osteoblast differentiation. Flavonoids can increase the activity of alkaline phosphatase (ALP), collagen and bone morphogenetic protein genes (BMP-2, BMP-4, and BMP-7).[15,38-43]

BMP2 will be produced in large quantities starting at the end of the inflammatory phase to the initiation phase after 2 weeks and will continue until the completion of the masturbation phase of new bone tissue. BMP2 expression will continue to increase until it reaches day 21, so this is the reason in this study taking observation days 14 and 21.[44-47]

Conclusions

Deproteinized golden sea cucumber (Stichopus hermanii) and deproteinazed bovine bone xenograft as bonegraft preparations can increase the expression of BMP2 and OPG in

bone regeneration.

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Declaration of Interest

The authors report no conflict of interest.

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