Stimulation of Osteoblast and Osteocalcin in the Bone Regeneration By Giving Bonegraft Golden Sea Cucumber

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Abstract
Regeneration therapy in periodontal defects and requires a material that is biocompatible, especially in the form of bone graft material, to reduce pocket depth, restore attachment, fill bone defects, and regenerate new bones, cementum, and periodontal ligaments. Golden sea cucumber (Stichopus hermanni) contains various bioactive components that are beneficial for human health.

Objectives is to analyze the process of bone regeneration after the administration of bone graft Golden Sea cucumber through an increase in the number of Osteoblasts and Osteocalcin. This study used raw material in Golden Sea cucumber. After going through the process of deproteinization and maceration, performed XRD and XRF test analysis. The study was an experimental laboratory with the subject being a male guinea pig. The number of samples in this study was thirty guinea pigs. After 14 and 21 days, guinea pig sacrificed for the histological examination and immuno-histochemistry.

Expression of Osteoblasts and Osteocalcin in the Golden Sea cucumber group significantly differ from the positive control group.

Bone graft material from golden sea cucumber has the bone regeneration mechanism as bone graft material, so golden sea cucumber can be used as an alternative material for bone regeneration.


Keywords: Bone regeneration, golden sea cucumber, stichopus hermanni.

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Introduction
Bones can regenerate their cells as part of the process of responding to trauma or infection. Similarly, in the alveolar bone, the presence of an infection that occurs in the alveolar bone will cause severe bone loss if not treated.¹

Periodontitis is one of the most common diseases of the oral cavity in humans, characterized by damage to supporting tissues of teeth such as alveolar bone, ligament periodontal (PDL) and cementum. Its pathogenesis involves complex interactions between host immune responses to localized microbial colonies in the periodontal section and modifying host factors, including smoking, genes, and systemic disorders such as diabetes, cardiovascular disease, and rheumatoid arthritis.² ³ ⁴ There are 57.6% of Indonesia's population experiencing oral health problems, one of which is an infection of periodontal tissue, according to the health ministry's RISKESDAS in 2018.⁵

Regeneration therapy in periodontal defects requires biocompatible material, especially in the form of Bone Grafting (BG), to lower pocket depth, restore attachment, fill bone defects, and regenerate new bone, cementum, and periodontal ligaments. In periodontal surgery, wound healing patterns consist of primary healing and secondary healing. Successful healing of periodontal wounds after treatment can support optimal periodontal tissue regeneration.⁶ ⁷ ⁸

Indonesia is a country that has high
potential marine resources because most of its territory is in the form of waters. One of them is The Golden Sea cucumber (Stichopus Armani), better known as gamat, a group of echinoderms that have long been used as nutritional ingredients and traditional medicine in Asian countries. Sea cucumber contains active ingredients that have therapeutic properties, antioxidants and can accelerate wound healing.9,10 Research in Golden Sea cucumber as a drug in dentistry has only developed in the last decade. It is expected that its bioactive compounds can provide new hope for treating various diseases, including as an adjunct therapy in dentistry.11,12,13,14

Regenerative Treatment of Periodontal Tissue

Creating a regeneration process from tissue defects is challenging because it requires enough power to maintain its mechanical strength and architectural properties. Using cost-effective and straightforward techniques requires optimal tissue and surfaces for cell attachment, migration, proliferation, and differentiation. Bone regeneration is the process involving many cellular and molecular events similar to bone formation during embryogenesis. Many growth factors released include Transforming Growth Factor-β (TGF-β), Bone Morphogenetic Protein (BMPs), Vascular Endothelial Growth Factor (VEGF), Basic Fibroblast Growth Factor (bFGF), and Platelet-Derived Growth Factor (PDGF).15,16,17,18

Regenerative treatment of periodontal tissue should reduce probing depth, clinical attachment strengthening, and radiographic replenishment of bones and defect closure through periodontal regeneration (i.e., formation of root cementum, periodontal ligament, and new alveolar bone). Bone graft materials used in regenerative treatments can come from the same species (Allograft), other species (autografts), synthesis materials, hydroxyapatite (Alloplastic), and from the other species, not human (Xenograft).19,20,21

Regeneration in periodontal effect can occur when the remaining bone walls are still preserved and can provide blood supply and mechanical support for the placement of bone fillers at resort sites. Treatment of periodontal tissue bone defects can be an autologous bone graft, allogenic bone graft, and substitute regeneration technique.22,23,24

Alveolar bone regeneration follows a series of events, among others:
- Hemostasis and the formation of blood coagulum
- Inflammatory phase
- Angiogenesis: the growth of new cell cells and capillaries

Mesenchymal cell activity, temporary non-mineralization matrix deposition followed by interactive processes involving mineralization, differentiation of bone-forming cells and eventually the formation of new bone o the role of growth and differentiation factors o the process of woven and lamellar bone formation.

Remodeling newly formed bones: The incorporation of osteoclasts and osteoblasts that continues throughout life.25,26

The bone graft material is widely used in periodontal surgery for periodontal bone regeneration and is the standard gold for periodontal treatment. Compared to open-flap debridement procedures, bone-replacement grafts generally increase bone levels and clinical attachment levels and reduce probing depth. Bone grafts play a role in the correction of periodontal bone defects through the process of osteoinduction and osteoconduction. Osteoinductive materials can induce bone formation by activating undifferentiated mesenchymal cells to form osteoblast cells, while osteoinductive materials act as material, new bone formation scaffolding.27,28,29,30

Biomarkers of bone remodeling can consist of two types, markers of bone resorption or osteoclasts and markers of bone formation, i.e. osteocalcin produced by osteoblasts and are non-collagen proteins. The process of resorption and bone formation is used as a marker of bone regeneration.31,32,33,34

Golden Sea cucumber (Stichopus hermanni)

Golden sea cucumber (Stichopus hermanni) is one type of marine biota that benefits from regenerating cells, which is often used to heal various diseases. In addition, sea cucumber contains 86.8% protein (functional as a building block that plays a role in the process of growth and maintenance of body tissues), minerals (one of the minerals essential for bone and tooth formation is calcium Ca), mucopolysaccharides, glycosaminoglycans (GAGs), antiseptics, flavonoids, droitin, saponins, hyaluronic acid, omega 3, 6, and 9 as well as
Amino acids 21. Phosphorus and calcium contained in Golden Sea cucumber can increase osteoblast and osteoclasts resorption. In addition, the content in sea cucumbers that calcium ions will be used as bone raw materials in osteocytes and ultimately play a role in forming new bone. Calcium metabolism has a dominant role in the process of bone formation. 

The purpose of this study was to analyze the process of bone regeneration after the bone graft of golden sea cucumber through increasing the number of Osteoblasts and Osteocalcin, as well as produce a natural material that had bone regeneration effect that can accelerate healing so, it expected to be used as a periodontal tissue regeneration material that can be widely used in the Dentistry.

**Materials and methods**

This research was a true experimental design with post-test design – only control group design. The research was conducted at the Biology Laboratory of Makassar State University to make bone graft of Golden Sea cucumber on experimental animals: Deproteinized and maceration and mixed into one until a bone graft preparation is formed from Golden Sea cucumber. After that, XRF analysis tested to see the compounds in the bone graft content of Golden Sea cucumber through X-rays and XRD analysis to see the crystallization structure phase through the peak of crystals of diffraction. Pet Shop Lacoste for animal maintenance trials, Laboratory of Anatomical Pathology Faculty of Medicine Hasanuddin University to manufacture osteocalcin and Osteoblast slides, and Laboratories biochemist - Biomolecular Faculty of Medicine, University of Brawijaya Malang for slide reading Osteocalcin and Osteoblast.

After adaption process during seven days, thirty-male guinea pigs were randomly selected and divided into three groups (ten each group), namely group A received (golden sea cucumber’s bone graft), group B received (bovine bone xenograft (BATAN)) and group C, without any treatment. On the eighth day, weight loss was carried out on each group of guinea pigs. Then the manufacture of bone defects in the femur. After 14 days and 21 days, five guinea pig from each group were sacrificing for the next test. This research was approved by the ethical committee of the Faculty of Dentistry, Hasanuddin University.

**Making bone-graft Golden Sea cucumber:**

*De-proteinezed Method:*

Preparation of the sample is done by washing sea cucumber from the remaining dirt left behind, then manually drying with the sun directly until dry. After that, it is recommended to dry using an oven.

Sea cucumber is cut into small shapes, and she has it blended to smooth with a size of ± 60 Mesh.

Make bone-graft preparations through the deproteinized method by adding Sodium Hydroxide (NaOH) 3.5% with a ratio of 1:10 (b/vol) to the mashed powder Golden Sea cucumber. After that, heated with an electric stove at a temperature of 65°C while stirring with a magnetic stirrer for 2 hours. (Amri, Ilza, & Sumarto, 2018)

Lastly, it should be cooled at room temperature for easy filtration process to obtain the precipitate and drying process in the oven with a temperature of 65°C.

The packaging on a tightly sealed sterile bottle.

*Method of Maceration:*

Preparation of the sample is done by washing golden sea cucumber from the remaining dirt left behind, then manually drying with the sun directly until dry. After that, it is recommended to dry using an oven.

Golden sea cucumber is cut into small shapes, and she has it blended to smooth with a size of ± 60 Mesh.

The method of maceration by soaking Golden Sea cucumber powder in NaOH solution for 3 x 24 hours. After that, it is filtered with filter paper. Then evaporated in a water bath to get deposits of extract Golden Sea cucumber.

**Treatment of experimental animals:**

Guinea pigs were adapted for seven days, on the eighth day, weight scales were carried out on each group of guinea pigs. Guinea pigs are anesthetized using ketamine (20mg/kg).

Furthermore, the surgical stage was performed on one guinea pig femur, done inside horizontal with scalpel no. 15.

Made a 3 mm profound defect on the femur bone using round bur.

The application of bone graft Golden Sea cucumber was in the treatment group.
Application of bovine bone graft in the positive control group, and saline solution was in the negative control group.

Before the wound was closed, antibiotics were given first, then suture using absorbable vycril 5.0.

**Results**

The normality and homogeneity of the result data was tested using the *Shapiro-Wills* test. The ANOVA one way test is carried out to determine the influence of each test group on the expression of Osteocalcin and Osteoblast cells in the formation of male guinea pigs. The results of ANOVA’s one-way analysis showed significant differences between groups (p<0.001).

Table 1 shows the lowest values on group K- on days 14 and 21, namely 5.20 and 8.20, while the highest values are in group K + on day 14, which is 8.40 and group P on day 21, which is 12.60, in addition, the second highest is in group P on day 14, which is 8.20 and group K + on day 21, that is 12.00.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day</th>
<th>OCN Sample</th>
<th>Mean</th>
<th>SD</th>
<th>Value P</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>14</td>
<td>5</td>
<td>8.20</td>
<td>1.30</td>
<td></td>
</tr>
<tr>
<td>K+</td>
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<td>8.40</td>
<td>1.82</td>
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<tr>
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<tr>
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<td>21</td>
<td>5</td>
<td>12.00</td>
<td>2.35</td>
<td>0.013*</td>
</tr>
<tr>
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<td>5</td>
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<td>1.92</td>
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</tr>
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Table 1. The lowest values on group.

<table>
<thead>
<tr>
<th>Group</th>
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<th>Osteoblast Sample</th>
<th>Mean</th>
<th>SD</th>
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<td>1.58</td>
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</tr>
<tr>
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<td>0.016*</td>
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<td>21</td>
<td>5</td>
<td>9.00</td>
<td>1.58</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Demonstrate the lowest value is in group K- on.

In table 2 demonstrate the lowest value is in group K- on days 14 and 21, namely 6.40 and 9.00, while the highest value is in group K + on day 14, which is 10.20 and group P on day 21, which is 13.00, in addition to the second highest in group P on the 14th day, that is 10.00 and group K+ on the 21st day, which is 12.80 (Fig1,2).
Osteocalcin in Osteoblast cells and histological examination of Osteoblast cells. The expression Osteocalcin indicates a positive reaction to the brown coloring of the cytoplasm, which indicates the reaction of osteocalcin and anti-osteocalcin. Observations using a light microscope at a magnification of 1000x shown in figures 3 & 4.

Discussion

To help the regeneration process in a defect in the bone, the one of strategy is to provide a substitution material in the form of bone graft that has osteogenic, osteoinductive and/or osteoconductive properties. This bone graft material should ideally be biocompatible, easy to shape, well-integrated with the original bone, have good mechanical properties with an ideal replacement rate, and be predictable with a reasonable patient acceptance rate. In this study, we used materials derived from Golden Sea cucumbers processed into bone grafts. Researchers chose to use maceration and deproteinization techniques based on previous studies. Isolation and characterization of cellular and extracellular components of a material play a role in the development of molecular markers thought to reflect bone formation or bone resorption, thus aiding in diagnostic and therapeutic assessment. Research aims to analyze the process of bone regeneration after bone-graft Golden sea cucumber through improving the number of osteoblasts and the expression of Osteocalcin. The result of this study is expected to produce a natural ingredient that has a bone regeneration effect that can accelerate healing so that it can be used as a regeneration material—periodontal tissue.

Several studies on natural ingredients, whether combined with other ingredients or not having good results, when used as bone grafts for bone regeneration, include the research conducted by Ashrin MN (2021) which combined Anadara granosa and gelatin with various concentrations for bone regeneration. The result of this research is that there is a significant difference in the compressive and tensile values of Anadara granosa's scaffold, with the highest value found in the gelatin concentration of 20%. Meanwhile, for the pore size and hydrophobicity of the scaffold which matched bone regeneration biomaterials, the scaffold with the lowest ratio was obtained (ari et al, 2018). The combination of the use of bone grafts and non-absorbable membranes has also been shown to accelerate bone regeneration, where the function of the membrane here is to prevent bacteria from entering the socket or wound.

Bargowo L (2020) in his research extracted collagen from gouramy fish scales to see its effectiveness in bone regeneration. The result of this research is that there is an increase in osteogenesis and angiogenesis in the process of bone regeneration. In line with the research of Pratiwi et al (2017) that chicken shank collagen scaffold can increase VEGF expression at week 2 during bone regeneration. Another study by Utari kresnadi (2020) which combined propolis extract and bovine bonegraft in tooth extraction sockets, found that TGF-ß expression increased in osteoblast cell formation and reduced IL-1ß expression and decreased the number of osteoclasts in alveolar bone regeneration. The same thing happened to the addition of Platelet Rich Plasma on implant placement can increase bone regeneration on the surface of the implant after 2 weeks of implantation. Nguyen et al (2021) also demonstrated a marked increase in contrast density was observed after 2, 4, 6, and 8 weeks, in which the radiolucent areas were gradually replaced with increased contrast density in the extracted rabbit socket.

This study showed that by giving bone graft material Golden Sea cucumber to defects in the bone femur guinea pigs, the number of Osteoblast and expression of Osteocalcin increased significantly. Significant compared to defects that are not treated. Gold is rich in growth factors so that it can repair damaged cells. Protein contained up to 82%, 80% of which is collagen. High collagen content in Golden Sea cucumber plays a role in cell regeneration. Collagen stability will improve inflammation recovery and increase the number of fibroblasts and osteoblasts. It has a beneficial effect on osteogenesis. Osteocalcin, a bone calcium-binding protein, a large part synthesized by osteoblasts, odontoblasts, and condrosits Hypertrophic, plays a role in the resorption and mineralization of bones, and is a specific marker of the bone formation process. Bone biomarkers play an essential role in the diagnosis, monitoring, and discovery of drugs. The ability to monitor health status, disease onset & development and treatment outcomes through noninvasive means is a desirable goal in the promotion and delivery.
of health care. Our results show the excellent effectiveness of bone graft materials because, in addition to organic materials, Golden Sea cucumber also contains an-organic material that plays an anti-organic material. Inflammation plays a role in bone mineralization that can increase bone synthesis for the formation of new bone. In line with research from Safina et al. (2016) stating that the material Golden Sea cucumber emphasis effectively increases the number of Osteoblasts. Golden sea cucumber also contains cell growth factor (GCF), the one of its components is VEGF which plays a role in inducing neovascularization (angiogenesis) and osteoblast differentiation for bone formation in vivo. This is also in line with research conducted by Arundina Ira et al. (2016) which stated that the administration of Gold Sea Cucumbers on MSCs can increase the proliferative ability of MSCs and can also increase the ability of MSCs to differentiate into osteoblasts. In line with previous research by Rima Parwati et al. (2019) stating that the collagen content of golden sea cucumber can help increase the amount of Osteoblast in the bone regeneration process. The study showed that hyaluronic acid contained in golden sea cucumbers can increase TGF-β resulting in cell migration due to the release of various growth factors. This activation, in turn, triggers the proliferation and differentiation of osteoprogenitor cells into osteoblast cells that play an essential role in the formation of the bone matrix. Based on the results of observations in this study, it is known that the expression of Osteocalcin and the number of Osteoblasts increased in the entire research group. Significant improvement results are seen as the healing process occurs. The osteogenic effect exerted by the bone-graft material of golden sea cucumber is very effective compared with the positive control group and negative control. Lining with Wozney (2002), saying various bone graft materials are used to improve bone ability and bone healing, its relative success and depending on the many factors. More research is needed to augment the osteoinduction effect of golden sea cucumber material in stimulating the bone healing.

Conclusions

The organic and an-organic ingredients of Golden Sea cucumber (Stichopus hermanni) extracted and put together into bone grafts can regenerate bone tissue. This can be seen from the accretion of the Osteoblasts and the expression of Osteocalcin in the defect of bone femur guinea pig.

Acknowledgments

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Declarations of Interest

The authors report no conflict of interest.

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