Golden Sea Cucumbers (Stichopus Hermanii) as Growth Factors of Stem Cells

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Abstract

Stem cells can give a new hope to accelerate wound healing process and can also be used for the treatment of various diseases, including diseases on periodontal tissues (periodontitis) since there is still not any materials known to be able to attach wobbly teeth perfectly. Adult stem cells derived from bone marrow have widely been used nowadays. However, the number of stem cells is limited, consequently, growth factors are necessary to increase the proliferation of stem cells. Unfortunately, growth factors that have been used are still expensive and difficult to obtain. Therefore, an alternative to the use of growth factors from natural materials that can potentially accelerate wound healing process is necessary to be developed. Purpose: Golden Sea Cucumbers (Stichopus hermanii) is rich in both growth factors that can repair damaged cells and protein that reaches up to 82% of all components of the golden sea cucumbers, 80% of which is collagen. Therefore, this research aims to prove how great the potential of the Golden Sea Cucumbers (Stichopus hermanii) as natural growth factors on stem cells is.

Method conducted aimed to analyze proliferation ability and osteogenic differentiation in Mesenchymal Stem Cells (MSC) with the provision of the Golden Sea Cucumbers (Stichopus hermanii). Results: Based on the results of MTT assay, the provision of the Golden Sea Cucumbers (Stichopus hermanii) in cultured MSCs could enhance the proliferation of stem cells. In the provision of osteogenic induction medium, mineralization emerged in MSC culture, stained with alizarin red. The provision of the Golden Sea Cucumbers on MSC can improve the proliferation ability of MSC and can also increase the ability of MSCs to differentiate into osteoblasts.

Keywords: Golden Sea Cucumbers (Stichopus hermanii), Growth factors, Mesenchymal Stem Cells.

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Introduction

Periodontitis is an infection of tissues supporting teeth that can trigger periodontal attachment loss of tooth tissue, resulting in wobbly tooth and premature tooth loss. Those cases are considered as big problems in the field of dentistry. Currently, the prevalence of periodontitis in Indonesia is quite high and still not declined. Many efforts to cope these cases have been carried out, either surgical or nonsurgical ones. However, a material that can attach wobbly teeth perfectly still has not been found. Therefore, this research on stem cells is expected to solve these problems.

Stem cells give a new hope to accelerate wound healing process and can be used for the treatment of various infectious diseases, including periodontitis. Stem cells are unspecialized cells that have two components, ie able to differentiate into various cell and capable of regenerating. Nevertheless, the use of embryonic stem cells is still prohibited in many countries. To avoid controversy, adult stem cells derived from bone marrow have been used recently.

However, the urgency / virtue of this research is the limited number of stem cells, so
growth factors are needed to increase proliferation of stem cells.1 Unfortunately, growth factors that have been used are still expensive and difficult to obtain. Thus, an alternative to the use of growth factors from natural materials that can potentially accelerate wound healing process is necessary to be developed.

Golden Sea Cucumbers (Stichopus hermanii) is rich in growth factors that can repair damaged cells and protein that reaches up to 82% of all components of the golden sea cucumbers, 80% of which is collagen. Therefore, this research aims to prove how great the potential of the Golden Sea Cucumbers (Stichopus hermanii) as natural growth factors on stem cells is.

Materials and methods

Adult Golden Sea Cucumbers (Stichopus hermanii) weighed 200-300 gr/head were obtained from the catch of fishermen in the area of Kalimantan Bontang. Next, those Golden Sea Cucumbers collected were washed with running water, and cut longitudinally to follow the body axis of the cucumbers. Their internal organs then were separated. Afterward, parts of the body left were drained on blotting paper before homogenized into a smooth texture1.

Next, Golden Sea Cucumber extract was prepared in several stages. First, 50 grams of the homogenized tissue was placed on 250 ml of the conical flask, and then added 100 ml of water (distilled water). Second, the mixture was shaken with water-bath shaker at a speed 80rev / min at room temperature for 4 hours. Third, the result of the mixture was centrifuged on 3.000rpm for 20 minutes. Fourth, the extract was frozen and dried, using a freeze-dryer (Heto models FD3, EN 87 164). Fifth, the results obtained were in the form of powder, which then was stored in sterile bottles at a temperature of 4°C2.

Mesenchymal Stem Cell (MSC), moreover, was isolated from the femoral bone marrows of Wistar rats. First, adult male Wistar rats aged about 3 months with a weight of approximately 200 grams were sacrificed after anesthesia with ether. Second, their femur bone was taken, and then both ends of the bones were cropped. Third, the bone marrows were taken by aspirating the femurs by spraying with a syringe containing medium Dulbecco's modified Eagle's (DME, Sigma, St. Louis, MO) supplemented with 200 units / ml heparin. Fourth, after the aspiration, the bone marrows were immediately mixed with 3 ml of DME medium, which had been added heparin. Fifth, 500 grams of them were centrifuged for 5 minutes. After the centrifugation, supernatant portion (the part of the medium that is translucent) was removed and then added DME medium without heparin. Sixth, the bone marrows were cultured on six well plates (Corning Inc., Corning, NY) using DME medium added 10% Fetal Bovine Serum (Hyclone, Logan, Utah) and antibiotics (100 units / ml of penicillin G and 100 ug / ml of streptomycin)3.

After 3 days, the medium disposal was performed to remove cells not attached to the dish, and a new medium was given. This medium replacement was performed for three days. After the cells in confluent state, passage was conducted using 0.05% trypsin-EDTA. Next, the cells were washed and cultured again in 60- or 100-mm tissue culture dishes (Corning). After the cell passage was confluent, passage was conducted again, so the cells could be used for further research. When the cells are not used immediately, the cells should be stored in liquid N22.

In addition, the proliferation ability of mesenchymal stem cells were observed by dividing them into three groups, namely mesenchymal stem cells added the Golden Sea Cucumbers (Stichopus hermanii) as growth factors, a group that was not given the Golden Sea Cucumbers (Stichopus hermanii), and a group given FGF as a control. Cells were cultured in 96 / well plates in DME medium. 100 mL of the Golden Sea Cucumbers (Stichopus hermanii) with a dose of 320 ug / ml was added into the group given the Golden Sea Cucumbers (Stichopus hermanii). Meanwhile, in the group without the Golden Sea Cucumbers (Stichopus hermanii), the provision was not performed given is not done adding gold Cucumber (Stichopus hermanii), and the cells were cultured for four days. Next, MTT assay was conducted using MTT assay kit. The absorbance of the supernatant then was performed using colorimetric (Elisa reader) with a wavelength of 550 nm3.

Afterwards, the osteogenic differentiation ability of mesenchymal stem cells was observed by dividing them into three groups, namely the group with the combination of mesenchymal stem cells and the Golden Sea Cucumbers.
(Stichopus hermanii), the group without the combination of the Golden Sea Cucumbers (Stichopus hermanii), and the control group with the cells cultured for 21 to 28 days in DME medium added 10 mM beta-glycerophosphate, 100 nM dexamethasone, and 50 ug / ml of ascorbic acid 2-phosphate (medium A). The control group was not given medium A, but only cultured in DME medium. Alizarin red staining then was performed³.

Results

In Figures 1, there were 15 grams of dried sea cucumber extracts derived from 800 grams of fresh golden sea cucumbers.

In Figure 2, the MSC, looked like fibroblastic, attached to the bottom of the dish, and after the culture media was changed every three days, the number of MSC was getting higher.

Figure 1. Golden Sea Cucumbers.

Figure 2. MSC (Control).

Figure 3. MSC combined with the Golden Sea Cucumbers.

As shown in Figure 3, the proliferation of mesenchymal stem cells combined with the provision of the Golden Sea Cucumbers was comparable to the proliferation of mesenchymal stem cells combined with the provision of FGF in Figure 4.

Figure 4. MSC combined with FGF.
Based on Table 1, the results of MTT assay showed that the provision of the Golden Sea Cucumbers was comparable to the provision of FGF on mesenchymal stem cells.

<table>
<thead>
<tr>
<th>No</th>
<th>Media Control</th>
<th>Cell Control</th>
<th>FGF</th>
<th>Sea Cucumbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.07</td>
<td>0.356</td>
<td>0.448</td>
<td>0.410</td>
</tr>
<tr>
<td>2</td>
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<td>0.352</td>
<td>0.444</td>
<td>0.478</td>
</tr>
<tr>
<td>3</td>
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<td>0.376</td>
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<td>0.438</td>
</tr>
<tr>
<td>Total</td>
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<td>1.084</td>
<td>1.338</td>
<td>1.326</td>
</tr>
<tr>
<td>Mean</td>
<td>0.073</td>
<td>0.381</td>
<td>0.448</td>
<td>0.442</td>
</tr>
</tbody>
</table>

Based on Table 2, data obtained were homogeneous and normal. Thus, Anova test was conducted to analyze significant difference between the provision of golden sea cucumber, the provision of FGF, cell control, and media control. Next, HSD test was performed. The results of HSD test showed that there was no significant difference between the provision of golden sea cucumber and the provision of FGF.

After the stem cell and the Golden Sea Cucumbers were cultured for 28 days and then added osteogenic induction medium, mineralization emerged in the cultures stained with alizarin red as shown in Figure 5.

Table 2. Results of Anava Test, MTT assay (OD).

<table>
<thead>
<tr>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
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<tbody>
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<td>.280</td>
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<tr>
<td>.003</td>
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<td>.000</td>
<td></td>
<td></td>
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<tr>
<td>.282</td>
<td>11</td>
<td></td>
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</table>

Figure 5. Osteogenic Differentiation.

Discussion

Stem cells are immature cells or undifferentiated cells that have an ability to produce cells similar to the cells themselves as well as an ability to differentiate into at least one cell type. Stem cells differ from progenitor cells, cells that can differentiate into only another cell type. Stem cells can divide and have self-renewing properties for long term. Stem cells are unspecialized, which also does not have a specific function and an ability to transform into specialized cells. Stem cells can also be found in dental pulp tissue that serves to produce odontoblasts, cells play a role in the formation of reparative dentin.

Mesenchymal stem cell (MSC), moreover, is defined as adult stem cells that are multipotential and have an ability to differentiate into various kinds of cells, such as osteoblasts, chondrocytes, adipocytes, fibroblasts, muscle
cells, and neural cells. MSC can be found in various kinds of adult body tissues, eg: adipose tissue, periosteum, synovial membrane, muscle, dermis, pericytes, blood, trabecular bone, and bone marrow. Bone marrow is the source of the majority of MSC as well as the most accessible ones.

In addition, the number of MSC contained in bone marrow is just 0.01 to 0.0001% of the total nucleated cellsbone marrow. Growth factors play an important role in expanding the number of MSC. MSC is a stem cell reacting positively to Stro-1 monoclonal antibody, but reacting negatively to CD34 antigens. Thus, besides an effort to increase the number of cells, an effort to regulate the differentiation process of MSC is also a very important factor. In vitro, MSCs can be directed to differentiate according to our will. For example, to be able to differentiate into osteoblasts, MSC should be cultured in osteogenic medium containing beta-glycerophosphate, ascorbic acid, and dexamethasone. For the differentiation process of MSC, some of the growth factors play an important role. The combination of bFGF and BMP2 in vivo can improve MSC's ability to form bone tissue. BMP and BMP receptors may influence the ability of MSCs to differentiate into bone tissue or fat tissue.

In the healing process, stem cell / progenitor cell is an important factor. These cells will proliferate and immigrate to the area of wounds, and then differentiate into the specific cells that will replace cells that die because of their injury. In patients with old age, the number of stem cells will be reduced so that the provision of exogenous stem cells is very useful. To get a sufficient number of cells, the stem cells can be propagated outside the body, and after that these cells can be applied to the cavity by using appropriate scaffold. It should be noted that the effort to add the number of stem cells should not eliminate the ability of these cells to differentiate. Growth factor is a material that can assist in the proliferation and differentiation of stem cells. Growth factors are often used in the following processes, namely transforming growth factors-beta (TGF-beta), bone morphogenetic proteins (BMP), platelet derived growth factor (PDGF), and fibroblast growth factors (FGF).

MSC derived from bone marrow, furthermore, will be attached to the base cell culture dish, while the other cells will not stick to the surface and will be lost during the change of the culture medium. Morphologically, MSCs are cells similar to fibroblasts. MSC began to attach to the surface of the dish on the 7th day, and the cells then will quickly form colonies and multiply. MSCs are cells that have an ability to duplicate themselves into the same cells with them (self-renewal capacity), but the ability of MSC to proliferate will decrease as the number of passage performs. Besides that, the age factor of the hosts will also affect the proliferation ability of MSC.

In this research, FGF-2 with a concentration of 5 ng / ml can increase the proliferation of MSC. The results of this research show that FGF-2 can improve the growth rate and life span of MSC derived from ilium and tibia bones of rabbits and humans. However, the mechanism of FGF-2's ability to increase the proliferation of MSC has still not known with certainty. FGF-2 is a growth factor that interacts with a receptor on the cell surface.

Receptors of FGF-2, moreover, can be classified into type I receptor since polypeptide receptor of FGF-2 can only penetrate transmembrane cells one time only. Signaling pathway of FGF-2 that can increase cell proliferation may be based on the Ras pathway. Receptors of FGF-2 can be detected on the surface of bone marrow cells, which means the stem cells from bone marrow can respond if there are exogenous FGF-2. With the stimulation of FGF-2, the receptors of FGF-2 will dymmerize and undergo autophosphorylation. Tyrosine kinase derived from the receptors that have been activated will bind to Grb2 via SH2 domain, while SH3 domain derived from Grb2 will bind to SOS. SOS will change GDP from Ras proteins into GTP causing activation of Ras. The active Ras then will activate Raf. Next, the activated Raf will bind to MAP2K, and then activate it. Afterward, MAP2K will activate MAPK, which will cause the phosphorylation of transcription factors. As a result, it will activate genes that play a role in the proliferation and differentiation of cells, such as c-myc.

Golden sea cucumber (Stichopus hermanii) commonly used in powdered form, moreover, is usually made into a lotion or a topical ointment. Sometimes, it is mixed into clay and applied as a facial mask, or put into a tea and consumed for stomach complaints.
people believe that the solution of sea cucumbers can heal wounds, blisters, and skin infections, as well as has a beneficial effect on the immune system, so sea cucumbers can be considered to have a lot of growth factors\textsuperscript{1-12}.

Therefore, sea cucumbers that are rich in growth factors can be expected to be able to repair damaged cells. Protein contained is up to 82\%, 80\% of which is collagen. The high content of collagen in the sea cucumber is capable of causing cell regeneration briefly. According to previous researches, gamat is soluble in water, so it is so readily absorbed in liver without undergoing detoxification\textsuperscript{1}. Extract gamat dynapharm products even shows that fibroblast proliferation can be increased since the golden sea cucumber extract can stimulate PDGF and TGF -β to interact and stimulate FGF to increase fibroblast proliferation, so wound healing process can occur rapidly\textsuperscript{12}.

In other words, Golden Sea Cucumber can also accelerate wound healing process since it contains antibacterial extract, antioxidant components, and fatty acids, which are able to relieve pain due to irritation \textsuperscript{2}. In general, golden sea cucumber contains glycosaminoglycans (heparan sulfate, hyaluronan), proteoglycans, saponin, omega 3, calcium, and zinc. Glycosaminoglycans contained in sea cucumber has a long monomer chain, consequently, extraction selected should use water in order to make the elements contained in the sea cucumbers not damaged during the extraction\textsuperscript{13}.

Based on some literatures, the water extract of the Golden Sea Cucumbers contains glycosaminoglycans that are essential for cell response to extracellular growth factors, such as FGF. FGF binds to the part of glycosaminoglycans, namely heparan sulfate, then brings FGF receptors on the plasma membrane and trigger cell division. The combination of glycosaminoglycans binding to proteins will form proteoglycans. Proteoglycans can act as inflammation modulator, immune response, as well as cell growth and differentiation, so proteoglycans can stimulate macrophage formation\textsuperscript{14}.

Increased FGF, VEGF, and PDGF by macrophages, furthermore, can lead to increased fibroblast proliferation and angiogenesis. This condition is regulated by the presence of CD44 / HSPG (Heparan Sulfate Proteoglycans) and syndecan 2, receptors of GAGs on activated macrophages. In addition, proteoglycans derived from one of GAGs, hyaluronan, can participate in the recruitment of leukocytes via interaction with CD44, and also can activate inflammatory cells, such as macrophages. Migration and proliferation of cells are primarily driven by transforming growth factor-β (TGF-β), and FGF is produced by macrophages\textsuperscript{15-16}.

The contents of the sea cucumber influencing the wound healing process are calcium and zinc. Influx of calcium acts as a second messenger of growth factor signals. Thus, proliferation of cells requires increased level of free calcium in the cell. Consequently, it needs improvement of the influx of extracellular calcium. On the other hand, zinc affects cellular metabolism of calcium. Another effect of zinc is to protect thiol protein on calcium channels from oxidation. Therefore, adequate zinc level is essential for platelet aggregation and uptaking of calcium. Allegedly, the primary function of zinc is associated with signal transduction in the cell proliferation process\textsuperscript{17}.

Based on several literatures, glycosaminoglycan contained in the sea cucumber can play a role as a modulator of inflammation, as a result, inflammatory products, such as macrophages become more active and greater\textsuperscript{15}. Proteoglycans consisted of several glycosaminoglycans can modulate the ability of heparin-growth factor binding, such as vascular endothelial growth factor (VEGF) and FGF. Proteoglycans, which have the same core protein, can also regulate the activity of TGF-β and the preparation of fibrils in the collagen type I and III. The release of TGF-β then can cause an increase in collagen synthesis. The first collagen synthesized is the collagen type III, then transformed into collagen type I, which has greater tensile strength and leads to healing process\textsuperscript{18}.

The content of sea cucumbers that can affect the formation of collagen, furthermore, is omega 3. In researches on wound care, omega 3 therapy is known to have a positive correlation to the increased production of collagen. Omega 3 can increase collagen production associated with prostaglandins. In inflammation, PGE2 is produced, as a result, collagenase is increased, while collagen formation is decreased, resulting in the decreasing of procollagen mRNA transcription. Meanwhile, omega-3 can reduce the level of PGE2 in inflammation\textsuperscript{19}.
Conclusions

Finally, based on these results, it can be concluded that the provision of golden sea cucumber on MSC proliferation may increase the ability of MSCs, comparable to giving FGF. Besides, the provision of the Golden Sea Cucumbers can improve the ability of MSCs to differentiate into osteoblasts.

Declaration of Interest

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References