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**EFFECT OF SNEAKHEAD FISH SCALE HYDROXYAPATITE AS AN
 ALTERNATIVE BONE GRAFT MATERIAL ON THE NUMBER OF WISTAR RAT
 FIBROBLAST**

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ABSTRACT

Background: Bone regeneration is a process to repair bone structure but has limited reparative capacity because the bone resorption process occurs in line with the regeneration process. Bone graft application is required to minimize resorption and guide bone regeneration, including in alveolar bone after tooth extraction. Snakehead fish scales are known to contain hydroxyapatite components as an alternative bone graft material to increase fibroblasts in tooth sockets. **Purpose:** To determine the effect of sneakhead fish scale hydroxyapatite as an alternative bone graft material on the number of Wistar rat alveolar bone fibroblasts after tooth extraction. **Methods:** This study was in vivo laboratory experimental study, single blind, with a post test only control group design. The samples used were Wistar rat which were divided into 4 groups after tooth extraction, the treatment group (HAp) was given hydroxyapatite from snakehead fish scales for 7 and 14 days and the control group was given distilled water for 7 and 14 days. Euthanasia of rat was carried out on days 7 and 14, then the jaw was cut off and histopatological preparations were made. The number of fibroblast was counted and analyzed statistically. **Results:** The Independent T-test showed that the number of fibroblasts was higher in the treatment group than control ($p\text{-value} > 0,05$). **Conclusion:** Hydroxyapatite from snakehead fish scales can significantly increase the number of fibroblasts in rat socket after tooth extraction.

Keywords: bone regeneration, fibroblast, snakehead fish scale, tooth extraction, wistar rat

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INTRODUCTION

Bone regeneration is a form of bone structure repair with a complex and dynamic process consisting of several phases, namely the inflammatory, reparative, and remodeling phase.^{1,2} Inflammatory phase is the first phase that occurs for 7 days from the beginning of bone damage. This phase is characterized by the migration of macrophage cells, monocytes, lymphocytes, PMN cells, and fibroblasts to the damaged area, the formation of new capillary blood vessels, and the release of cytokines and growth factors such as Platelet Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF), Transforming Growth Factor (TGF), and Angiopoietin 1 (Ang-1) which then stimulate angiogenesis.^{2,3} The reparative phase begins on the 3rd day to the 21st day dominated by platelets and granulation tissue, such as new blood vessels, fibroblasts, and macrophages. Fibroblasts begin to appear and form on the 3rd day after tooth extraction, their proliferation increases on the 7th day and will continue to increase until the peak of proliferation occurs on the 14th day to the 21st day.^{2,4} After fibroblast proliferation reaches its peak, extracellular matrix formation occurs in damaged area by fibroblasts until new immature bone (woven bone) is formed.² Remodeling phase begins with osteoclasts resorption of woven bone forming a cavity which is then filled by osteoblasts with osteoid. This new bone matrix forms lamellar bone which is stronger and mechanically stable replacing woven bone through the cooperation of osteoblasts and osteoclasts.⁵ Bone regeneration can occur as a normal reaction in an ability to heal damaged bones but has limited reparative ability because the bone resorption process occurs in line with the bone regeneration process.¹ Placement of bone graft on damaged bone is necessary to minimize resorption and guide bone regeneration.⁶

Bone graft is a material used as a bone tissue former that must be biocompatible, osteoconductive, osteoinductive, and trigger osteogenesis.^{4,6} Commonly used bone graft material is hydroxyapatite (HAp) with a material structure consisting of calcium phosphate complex minerals ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$).^{5,6} Various studies in Indonesia on the synthesis of HAp as an alternative bone graft material that utilizes natural materials from fish waste have been conducted, one of which is fish scales. Fish scales are known to accelerate the bone growth process with high bone density. In the study of Sulistiawati et al., it was found that HAp can be synthesized from snakehead fish scales (*Channa striata*).⁷

Fish scales are known containing several inorganic components with a percentage around 7%-25% which include calcium and phosphate. These components are the main components of HAp as an alternative bone graft material that can be used in bone regeneration.⁷ One example of bone graft application in bone regeneration is post-tooth extraction in the alveolar bone.⁴ Bone loss that occurs after tooth extraction requires bone graft to maintain the dimensions and contours of the bone.⁸ Bone graft can induce bone tissue formation by increasing macrophages and growth factors.⁴ Increased macrophages and growth factors can affect the proliferation of fibroblast cells to form an extracellular matrix in the formation of new bone.² Based on the background above, the purpose of this study was to determine the effect of HAp from snakehead fish scales as an alternative bone graft material on the number of alveolar bone fibroblasts in Wistar rats after tooth extraction.

MATERIALS AND METHODS

This study is an in vivo laboratory experimental study, single blind, with a post-test only control group design. Subjects of this study were male white Wistar rats (*Rattus norvegicus*) with healthy physical conditions, age 3-4 months, and 200-250 grams in weight. A total of 24 rats were divided into 4 groups (n = 6 rats per group), namely treatment for 7 days, treatment for 14 days, negative control for 7 days, and negative control for 14 days. The object of this study was snakehead fish scales obtained from Seberang Ulu I District, South Sumatra, Indonesia. The snakehead fish went through an identification process first at the Biosystematics Laboratory, Department of Biology, Universitas Sriwijaya to ensure that the species used was *Channa striata*. This study has been approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Universitas Sriwijaya No. 067-2024.

HidroxyApatite Procedure

The research procedure began with cleaning the scales of the snakehead fish from other components by boiling at a temperature of 100°C for 10 minutes 4 times and rinsing with distilled water after each boiling until the water was clean. Then the scales were ground with a ball mill for 45 minutes, then put into a furnace at a temperature of 900°C for 16 hours. The scales of the snakehead fish were then sieved with a 200 mesh sieve. Furthermore, calcium nitrate ($\text{Ca}(\text{NO}_3)_2$) was made by dispersing 56 g of calcined CaO powder and 67 ml of distilled water, then added 81 ml of 68% nitric acid (HNO_3). After that, HAp synthesis of snakehead fish scales was carried out by reacting 100 ml of H_3PO_4 solution with 100 ml of ($\text{Ca}(\text{NO}_3)_2$) solution suspension, then ammonium hydroxide (NH_4OH) was added periodically to produce a pH of 10. The aging process was carried out by leaving it at room temperature for 24 hours, then the resulting precipitate was filtered with Whatman 42 filter paper and washed with aquabidest to remove by-products to produce a pH of 7. The precipitate was dried at 110 °C for 4 hours, then calcined with a protherm furnace at 900°C for 6 hours to increase its crystallinity and produced HAp powder measuring 200 mesh as shown in Figure 1. Evaluation was carried out on the snakehead fish synthesis powder using XRD to determine the characterization of HAp in the powder from the extraction of snakehead fish scales.



Figure 1. a) Snakehead fish scales, b) HAp powder from snakehead fish scales

Experiment Models Procedure

Before treatment, the rats were adapted for 1 week. Mandibular left incisor tooth extraction was performed in experiment models using 0.2 ml/head ketamine intramuscularly anesthesia. The socket was irrigated with sterile distilled water and it was ensured that the tooth was completely removed without any remaining tooth fragments. The HAp bone graft from snakehead fish scales was applied into the socket of the treatment group as high as the socket using a dental excavator, then compacted using a plugger. Socket suturing was carried out on the treatment group and the negative control group with 5-0 silk.

Histopathology Preparation

Histopathology sample preparation began with euthanasia of mice using chloroform after 7 and 14 days of treatment. The mandible bone of rat that had been incised was fixed, dehydrated with graded alcohol, cleared, then infiltrated into liquid paraffin and the embedding process was carried out. Paraffin blocks were made and paraffin block cutting was carried out with a 5 μm thick microtome. The pieces were placed in a water bath. Incubate with a hot plate and stain with hematoxylin eosin. Observation of histopathology results was carried out using a single blind technique using an Olympus Light Microscope with a magnification of 1000x to count the number of fibroblasts in 5 fields of view.

RESULT

The XRD diffractogram results show the highest peak at an angle of $2\theta = 31.62$ with other peaks at angles of $2\theta = 30.05$ and 34.98 (Figure 2). The peak is close to the JCPDS database no. 9-432 where the highest peak of pure HAp has an angle of $2\theta = 31.77$. Referring to the database, the compound formed in the snakehead fish scale extraction powder is HAp.

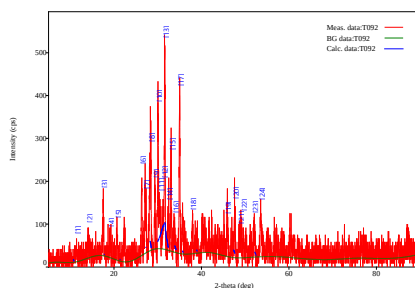


Figure 2. XRD diffractogram of HAp powder from snakehead fish scales.

Histopathological examination showed fibroblast in irregular purple cytoplasmic with oval-shaped nucleus in dark purple (Figure 3). The shape of the fibroblasts in the preparation appeared to have irregular purple cytoplasmic branches and there was an oval-shaped nucleus in dark purple as seen in Figure 3.

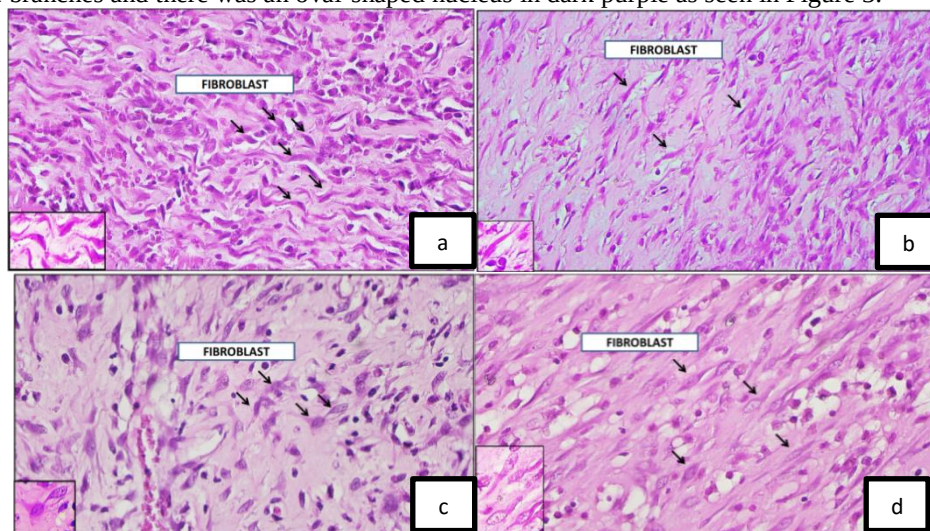


Figure 3. Histopathology of fibroblasts in four research groups: a) HAp 7 days; b) HAp 14 days; c) Control 7 days; d) Control 14 days.

The average results of the number of fibroblasts showed in Table 1.

Table 1. Average number of fibroblasts in the research groups

Research Day	Research Group	N	Average	SD
7 days	HAp group	6	170.13	13,908
	Control group	6	98.03	14,825
14 days	HAp group	6	149.41	21,220
	Control group	6	96.96	4,924

Statistical analysis

The results of the normality test using the Nonparametric Kolmogorov-Smirnov test showed a significant value of more than 0.05 ($p > 0.05$), meaning that the research data in each group were normally distributed. The results of the homogeneity test with Levene showed that the significant value was more than 0.05 ($p > 0.05$), meaning that the variation data was homogeneous. The homogeneous data were then continued with the Independent T-test statistical test. The results of the Independent T-test statistical test showed a significant difference in the average number of fibroblasts in the treatment group (HAp) on day 7 compared to the average number of fibroblasts in the negative control group on day 7 with a p -value < 0.05 . A significant difference was also shown in the group on day 14.

DISCUSSION

Bone graft from HAp snakehead fish scales can increase the number of fibroblasts in rat sockets after tooth extraction in the reparative process which is indicated by an increase in the number of fibroblasts in the group treated with bone graft from HAp snakehead fish scales compared to the negative control group with distilled water. These results are in line with preliminary result by Rianti et al (9). HAp from Indonesian limestone enhanced the bone defect healing through the enhancement of osteoblasts, fibroblasts and reduction of osteoclasts number.⁹

HAp has osteoconductive properties in the form of an ideal scaffold in providing a suitable environment for fibroblasts to attach, grow and differentiate. Ca^{2+} and PO_4^{3-} in HAp also play a role in fibroblast cell division.⁹ This study is also in line with research conducted by Naini et al (10) reported that HAp as a scaffold can increase the number of fibroblasts in Wistar rat post-tooth extraction sockets.¹⁰ Another statement in line with this is known in the research of Ramadhani et al (2). that HAp administration can stimulate macrophages in damaged areas.² This can affect the increase in growth factors that initiate the formation of fibroblasts.

HAp increase the activity of macrophages to produces growth factors such as PDGF, TGF- β , FGF, and VEGF to stimulate the angiogenesis process.² PDGF will stimulate fibroblasts to migrate to the wound area and undergo mitosis. TGF- β will increase fibroblast proliferation and form collagen to repair damaged tissue structures.^{8,11} FGF will stimulate the migration and proliferation of fibroblasts.¹² VEGF plays a role in activating endothelial cells and is guided by PDGF and TGF- β to proliferate and differentiate to form new capillaries.¹ The formation of new capillaries will increase the recruitment of cells, one of which is fibroblasts to produce proteins and Extra Cellular Matrix (ECM) needed to support the formation of new capillaries. This process will accelerate the regeneration and remodeling of fibroblast cells so that it can increase the number of fibroblasts.^{13,14}

Fibroblasts in the bone regeneration process will form after 3rd days after tooth extraction, increase on the 7th day until reaching maximum proliferation on the 14th to 20th day, and slowly decrease until the next phase, namely the remodeling phase.⁴ This study showed a higher number of fibroblasts on the 7th day compared to the number of fibroblasts on the 14th day in both the treatment and control groups. Referring to the period of alveolar bone regeneration in rats after tooth extraction when converted to humans, namely bone formation in rats after tooth extraction is completed on the 28th day and is equivalent to 64 days in humans, this study related with the theory. The number of fibroblasts in rat sockets after tooth extraction which is higher on the 7th day is related that the number of fibroblasts will be maximum on the 14th to 20th day in humans.¹⁵

Another study conducted by Naini et al (10). showed that in the control group and the HAp treatment group in Wistar rat sockets after tooth extraction, there was an increase in the number of fibroblasts from day 3 to the highest number on day 14.¹⁰ This shows a difference in results with this study. This study is in line with the study conducted by Vieira et al (16). that the number of fibroblasts in post-tooth extraction sockets reached a peak value on day 7, decreased in number on day 14, and disappeared until the next phase.¹⁶ This difference can occur because growth factors that initiate fibroblast formation such as PDGF, TGF- β , and VEGF have the highest number on day 7.^{16,17} The high number of these growth factors can cause a high number of fibroblasts.¹⁸ The increase in the number of fibroblasts was accompanied by a high average deviation value in this study. The high value is likely influenced by the difference in the magnitude of trauma during rat tooth extraction so that there is a difference in cell initiation response in the bone regeneration process.¹⁹

This study showed that fibroblast decrease in 14 days, it namely 149.41 from 170.13. This decrease in number indicates that the process that occurred has entered the last phase, namely the remodeling phase.¹⁸ Remodelling phase synthesize the collagen by fibroblasts that has been mediated and some fibroblasts have differentiated into fibrocytes.³ Granulation tissue formed from collagen synthesis and extracellular matrix by fibroblasts will be replaced by immature bone so that the number of fibroblasts decreases and then the remodeling phase occurs.¹¹ In addition, in the study of Yontasya et al. it is known that HAp can increase the activity of FGF in the wound area.⁴ The amount of FGF is at its peak on the 14th day.¹² FGF also functions as a signaling control in bone formation, namely the differentiation of fibroblasts into osteoblasts.² Due to this differentiation, the number of fibroblasts on the 14th day will decrease.

Based on the results and discussion of the study, it can be concluded that the administration of bone graft from hydroxyapatite from snakehead fish scales has a significant effect on increasing the number of fibroblasts in the sockets of Wistar rats after tooth extraction.

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