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Suckermouth catfish bone extract as bone graft raw material for bone-healing promotes bone growth in bone loss

Extrato de osso de bagre como material de reparo promove o crescimento ósseo em perda óssea

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ABSTRACT

Objective: This study aimed to evaluate the properties of suckermouth catfish bone extract, which allows it to be adopted as a raw material for bone graft following its graft in an artificial defect of a rat model. **Material and Methods:** Hydroxyapatite (HA) from suckermouth catfish bone extract was characterized using Fourier-transform infrared spectroscopy (FTIR), and its toxicity was evaluated by Brine Shrimp Lethality Test (BSLT). This material was grafted on artificial defects in rats' femoral bones, which were observed immunologically by Enzyme-linked immunosorbent assay (ELISA) after one week and four weeks, and radiographically in the second week, and histologically in the second and fourth weeks. **Results:** FTIR shows that this material consists of phosphate, hydroxyl, and carbonate groups, while the BSLT results show that this material is not toxic. Observations by ELISA showed an increase in the expression of Tumor necrosis factor alpha (TNF-α) in defects with HA in the fourth week. Radiographically the defect did not show closure in the second week. In contrast, histological analysis showed a better bone healing process in the defect, which was applied with the HA of the suckermouth catfish bone. **Conclusion:** The HA extracted from the suckermouth catfish bone has beneficial properties as an alternative to bone graft raw material and, more investigated needed to support this biomaterial to be used in the treatment of bone loss.

KEYWORDS

Hydroxyapatite; Bone graft; Bone defect; Bone healing; Fourier Transform Infrared Spectroscopy.

RESUMO

Objetivo: Avaliar as propriedades do extrato de osso de bagre, que permitem sua adoção como material bruto para enxerto ósseo, em um defeito ósseo artificial em ratos. **Material e Métodos:** A hidroxiapatita (HA) do extrato de osso de bagre foi caracterizada usando espectroscopia infravermelha por transformada de Fourier (FTIR), e sua toxicidade foi avaliada pelo Teste de Letalidade do Camarão de Sal (BSLT). Esse material foi enxertado em defeitos artificiais nos ossos femorais de ratos. Análise imunológica por meio do ensaio imunoenzimático (ELISA) foi realizada uma e quatro semanas após a colocação dos enxertos. Análises radiográficas foram feitas na segunda semana e histológica na segunda e quarta semanas. **Resultados:** A FTIR mostrou que esse material é composto por grupos de fosfato, hidroxila e carbonato, enquanto os resultados do BSLT mostraram que esse material não é tóxico. As observações pelo ELISA mostraram um aumento na expressão do fator de necrose tumoral alfa (TNF-α) nos defeitos com HA na quarta semana. Radiograficamente, o defeito não apresentou fechamento na segunda semana.

Em contraste, a análise histológica mostrou um melhor processo de cicatrização óssea no defeito que foi aplicado com a HA do osso de bagre. **Conclusão:** A HA extraída do osso de bagre possui propriedades benéficas como alternativa ao material bruto para enxerto ósseo, sendo necessárias mais investigações para apoiar esse biomaterial a ser usado no tratamento da perda óssea.

PALAVRAS-CHAVE

Hidroxiapatita; Enxerto ósseo; Defeito ósseo; Cicatrização óssea; Espectroscopia no infravermelho por transformada de Fourier.

INTRODUCTION

Alveolar bone is a structure that supports teeth, protects nerves, blood vessels, and glands, and supports masticatory and facial muscles [1,2]. Alveolar bone loss can occur due to periodontal disease, cysts, tumors, post-extraction trauma, or other trauma [3-5]. The patient's quality of life can be significantly impacted by alveolar bone loss because it can lead to mobility issues and tooth loss if it progresses without treatment [3,6,7].

Efforts to overcome challenges in recovering lost alveolar bone currently can be done with several types of treatment, one of which is the use of bone grafts which aim to replace damaged bone tissue using specific materials that can come from the patient's body, synthetic/chemical materials, or other materials [4,5,8]. This treatment shows a high success rate in rebuilding alveolar bone morphology [9-11]. Bone grafts must be biocompatible, osteoconductive, osteoinductive, and osteointegration to provide structural support and stimulate bone healing [4,5,12]. The primary raw material for bone grafts is hydroxyapatite (HA) with the formula $Ca_{10}(PO_4)_6(OH)_2$, which is an inorganic biomaterial compound that is a component of human bones, teeth, and dentine and can come from various natural sources, such as bovine bone, a mixture of pork bones with horse bones, fish scales, limestone, egg shells, cow teeth, and fish bones [13-17].

The need for HA for bone grafts has significantly increased in response to the rise in occurrences of alveolar bone loss [18-20]. This problem necessitates creating and investigating substitute HA materials for bone graft raw materials made from natural materials. Indonesia, a maritime nation rich in different marine biota that contains HA, can be exploited as a source of raw materials for bone transplants, such as fish bones [21,22]. In many lakes, the suckermouth catfish (*Pterygoplichtys pardalis*) is a species of fish whose bones can be utilized as a natural source of raw materials to synthesize HA, which is anticipated to help lessen environmental issues as fish populations rise. However, in Lake Tempe, South Sulawesi, Indonesia, suckermouth catfish are an invasive species that threaten the ecosystem's equilibrium and compete with native fish species, upsetting the food chain and resulting in the extinction of several endemic fish species [23].

Based on the problem of tooth loss due to alveolar bone loss and the potential content of HA from suckermouth catfish bones, this study innovates to seek HA alternatives by evaluating the properties of suckermouth catfish bones in their utilization as bone graft raw material for bone healing.

MATERIALS AND METHODS

Material preparation

The hydrothermal technique which refers to the procedure proposed by Alqap and Sopyan [24] which is modified by the procedure carried out by Chadijah [25], was adopted in this investigation to manufacture 100 suckermouth catfish bones as the HA raw material. The suckermouth catfish is obtained from Lake Tempe, which is located in the province of South Sulawesi, Indonesia. First, the flesh and bones of the suckermouth catfish are separated once it has been skinned. Next, the fish bones are cleaned, washed under running water, and dried in the air for 24 hours. Next, the fish bones were soaked in acetone solution (C_3H_6O) for 3×24 hours and exchanged daily to decontaminate protein in the fish bones. After that, the fish bones were placed in the oven for 30 minutes at 115°C. Next, fish bones were smashed in a mortar and sieved through a 200 mesh screen before being subjected to the calcination process. The following step is calcination, which takes place for five hours at a temperature of 700 to 800°C. The initial stage of HA synthesis was weighing 5.117 g of fish bone powder and then dissolving it in 100 mL of distilled water in a 250 mL Erlenmeyer.

Next, the solution was homogenized using a stirrer speed of 300 rpm at 90°C for 1 hour after the dissolved bone fish was combined with 100 mL of a 0.547 M solution of ammonium dihydrogen phosphate ($NH_4H_2PO_4$). Furthermore, the solution was processed hydrothermally using an autoclave for 1 hour at a temperature of 121°C and a pressure of 1 atm. Then, the sterilized solution was filtered using Whatman filter paper no. 42. The precipitate obtained was then washed three times using distilled water to remove the remains of ammonium dihydrogen phosphate, heated at 105°C for 30 minutes, and continued with the blasting stage, namely at 700°C for 5 hours and then at 900°C for 10 minutes. Finally, the obtained material was tested for toxicity and FTIR.

FTIR analysis

The fishbone powder was placed on an Attenuated Total Reflectance (ATR) plate at a controlled ambient temperature (25°C) and scanned using an FTIR spectrophotometer (ABB MB3000, Clairet Scientific, Northampton, UK) at a wavenumber of 4000 – 650 cm^{-1} equipped with a deuterated triglycine sulfate (DTGS) detector and potassium bromide (KBr) as the beam splitter, recorded for 32 scans at 8 cm^{-1} resolution. These spectra were recorded as absorbance values at each data point in triplicate.

Toxicity test

In the present study, the toxicity test was carried out using the BSLA method. The toxicity of the fish bones was tested at concentrations of 62.5, 125, 250, 500, and 1000 ppm in 10 ml seawater solution and 0 ppm without the test substance as a control, which was added with 1% DMSO solvent (v/v). Then 30 Artemia salina Leach (nauplii) shrimp larvae aged 48 hours were used at each concentration tested. The toxic effect was obtained from observations by calculating the percentage of death of nauplii at each concentration within 24 hours, which was obtained by multiplying the ratio by 100%, namely the number of dead larvae divided by the number of initial larvae multiplied by 100% for each replication (three replications were used for each concentration). Then it was compared with the control, and the results were analyzed using probit analysis so that the LC_{50} value was obtained using the Software Package used for Statistical Analysis (Version 25.0; SPSS Inc., Chicago, IL, USA).

Grafting procedure

Twenty-four Rattus norvegicus rats aged 8-10 weeks with an average body weight of 188.365 g were used in this study. The animal study protocols were reviewed and approved by the Ethics Committee of the Dentistry Faculty of Hasanuddin University under approval no. 0065/PL09/KEPK FKG-RSGM UNHAS/2021 and conducted at Veterinary Captive Laboratory, Hasanuddin University (Makassar, Indonesia). Rattus norvegicus rats were divided into four groups based on the grafting material consisting of P0 (without grafting), P1 (100% HA fishbone), P2 (100% HA bovine), and P3 (50% HA fishbones and 50% HA bovine). The HA bovine that we used in this study were commercial HA (BATAN RESEARCH TISSUE BANK, FDBX Xenogrfat) produced by BATAN Jakarta, Indonesia. The grafting procedure was performed under general anesthesia using isoflurane inhalation. Approximately 0.1 g of HA powder P1 (n = 3), P2 (n = 3), and P3 (n = 3) was mixed with the blood of the test animals and then grafted onto the femoral bone of the rat after creating an artificial defect by drilling which resulted in a defect with a diameter of 2 mm and 2 mm depth. At the same time, control group P0 was left without graft material.

ELISA observation

In the first and fourth weeks after grafting, blood serum was taken from each group to test the effectiveness of the raw material using the Mouse Tumor Necrosis Factor Alpha ELISA kit (GenWay BioTech, San Diego, USA), which followed the manual instructions. Data from the test results were then analyzed using the dependent sample T-test with the Software Package used for Statistical Analysis (Version 25.0; SPSS Inc., Chicago, IL, USA) application.

Radiography analysis

The effectiveness of raw materials was also tested by radiographic examination, which was carried out randomly on experimental animals, one tail per group. In addition, radiographic examinations using x-rays (Toshiba MRAD-A32S, Toshiba Medical Manufacturing Co., Ltd, Tochigi, Japan) were carried out in the second weeks to determine the progress of bone growth.

Histopathological analysis

Testing the effectiveness of raw materials is also carried out by histopathological examination to determine the progress of osteoblast cell growth. Rattus norvegicus rats were euthanized in the second and fourth weeks after grafting, and the femur from each treatment group was taken. Samples were rinsed with distilled water, fixed with 10% formalin, and decalcified with 10% EDTA. Then the samples were washed in distilled water, dehydrated, embedded, trimmed, stained with Hematoxylin and Eosin (H & E), and examined via a digital image capture pathology scanner (Aperio CS model, Leica Biosystems, Buffalo Grove, IL, USA) under 400 × magnifications. Histopathological examination was only carried out in groups P1, P2 and P3.

RESULTS

FTIR analysis

Figure 1 shows the spectra of phosphate, carbonate, and hydroxyl groups in fishbone and bovine. The stretching vibrations of phosphate HA from fish bones and HA bovine are in the same wave number range, 1031-1093 cm^{-1}.

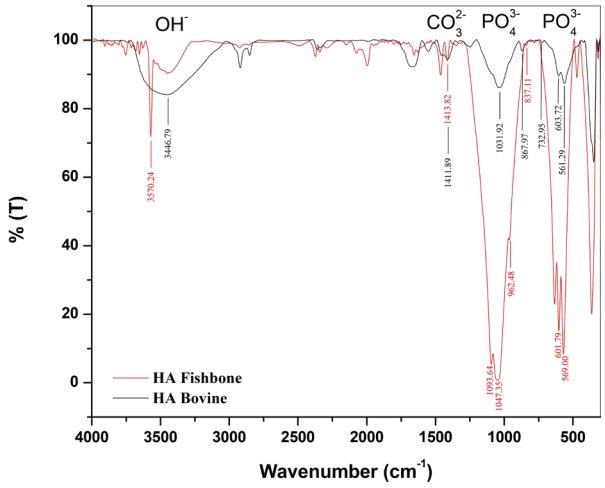

Figure 1 - Spectra of phosphate, carbonate, and hydroxyl groups in HA of fish bones and bovine.

The sharp uptake of phosphate HA phosphate from fish bones was detected at wave numbers 1047-1093 cm^{-1} and HA bovine phosphate groups at wave numbers 1031.92 cm^{-1}. The wave numbers 569 cm^{-1} and 601 cm^{-1} indicate a symmetrical stretching vibration of the HA phosphate of fish bone compared to the bovine HA phosphate group at waves 603.72 cm^{-1} and 561.29 cm^{-1}, both of which are at the same wave number range, namely, 561.29-603.72 cm^{-1}. The stretching vibration of the HA hydroxyl group in the fishbone was detected at a wave number of 3570.24 cm^{-1}. The wave numbers of 1413.82 cm^{-1} and 837.11 cm^{-1} indicated carbonate groups' presence in the fishbone's HA. While, carbonate group in the HA bovine detected at waves 1411.89 cm^{-1}, 867.97 cm^{-1}, and 732.95 cm^{-1}.

Toxicity test

Nauplii that were exposed to the extract of the fish bones at the highest concentration, namely 1000 ppm, showed the highest mortality of 20%. However, probit analysis of the HA of the fish bones as shown in Figure 2 depicted that the LC_{50} value was 49137.4644 ppm (> 1000 ppm) which showed that the HA of the fish bones was not toxic.

ELISA observation

Table I shows the average expression of TNF-α from experimental animals after four weeks of treatment. All treatment groups except P0 experienced an increase in TNF-α expression in the fourth week, and the test results of the four types of treatment in the first and fourth weeks showed a significant difference (p=0.037).

Radiography analysis

In Figure 3 it can be seen that until the second week for each defect made on the femur and given HA according to the type of treatment P0, P1, P2, and P3 showed no significant healing process which was marked by all defects still

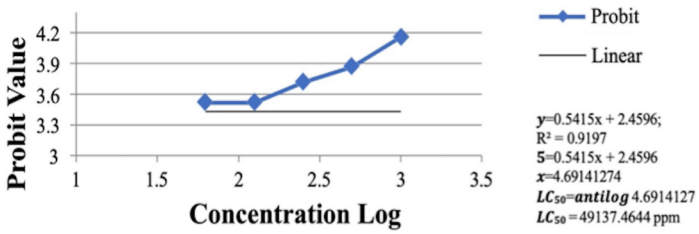

Figure 2 - Correlation between mortality percentage (probit value) of nauplii and HA concentration of fish bones.

visible radiolucent (red circle) which indicates that complete closure has not occurred.

Histologic evaluation

Figure 4 shows the results of H&E staining of the sample defects treated with HA at weeks 2 and 4. At week 2, groups P1 and P2 showed a more pronounced formation of granulation tissue in the defect area treated with HA compared to group P3. Furthermore, in the fourth week, all treatment groups showed granulation tissue formation. However, the P1 group showed denser granulation tissue formation than the P2 and P3 groups, which indicated better bone renewal in the P1 groups.

Table I. The average TNF-α expression value of the test animals in the first and fourth weeks

Treatment Groups	1st Week	4th Week	*p*-value
P0	192.804[a]	120.082[b]	0.037*
P1	73.691[a]	154.842[b]	
P2	203.846[a]	253.560[b]	
P3	150.570[a]	280.274[b]	

*Dependent sample T test; $p < 0.05$: significant. The value with different superscript letters in a column are significantly different.

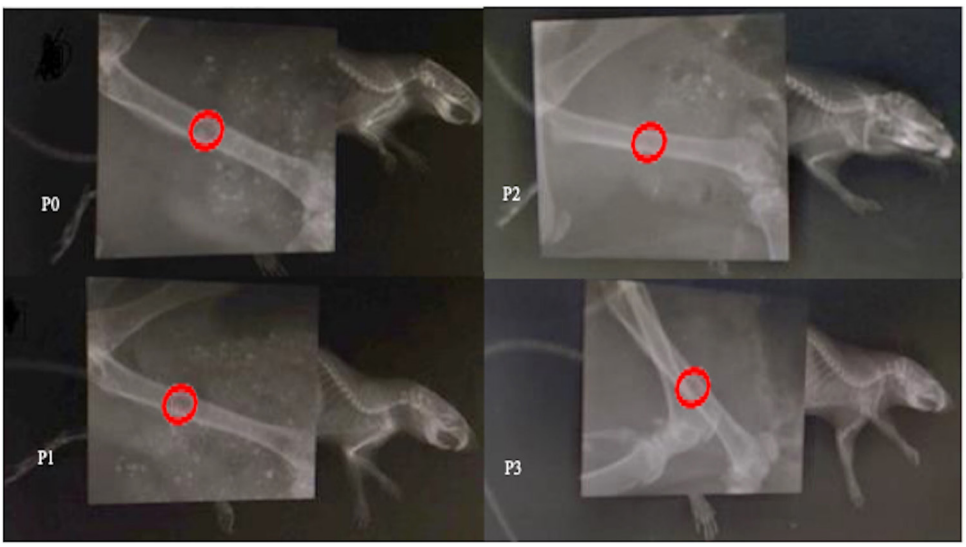

Figure 3 - Radiographic picture showing a radiolucency in the defect of the femur in the second week of the test animal.

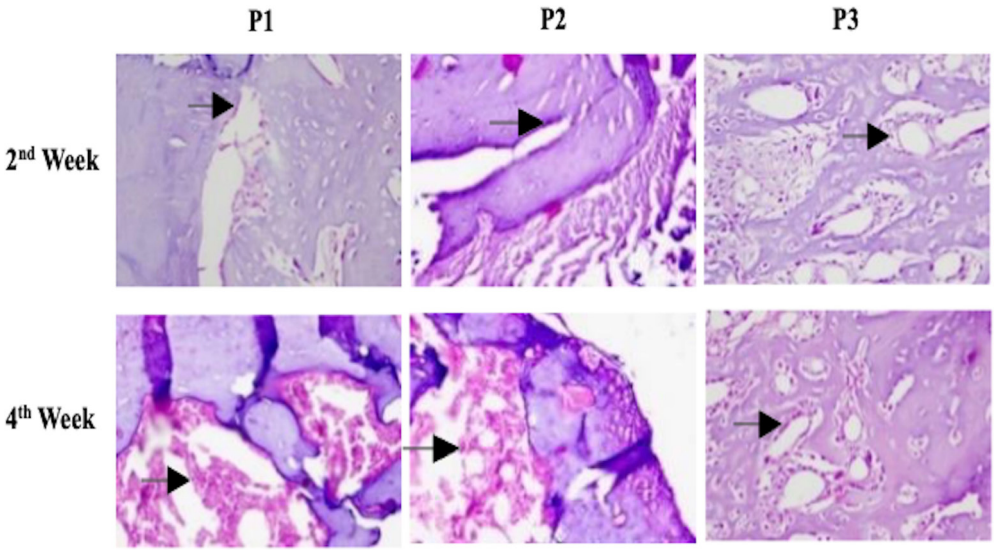

Figure 4 - H&E staining result of the P1, P2, and P3 treatment groups at 2 and 4 weeks after grafting with various HA. (Black arrow: defect with HA).

DISCUSSION

The use of hydroxyapatite as a bone substitute has been widely developed because the structure of hydroxyapatite is component of human bone. In addition, the bioactive bio-ceramic hydroxyapatite is also biocompatible, biodegradable, non-toxic, osteoconductive, and osteoinductive, making it an auspicious material for use as a replacement and bone regeneration in cases of bone damage and defects [26-29]. In this study, FTIR spectroscopy was used to characterize HA. It detected the presence of phosphate, hydroxyl, and carbonate groups in both the HA of fish bones and HA bovine. In the present study, the FTIR spectroscopy analysis showed there is no significant different between HA of fish bones and HA bovine. Compared to normal bone, bone and dentin have the most carbonates, which contain up to 8% of the weight [30]. While in some synthetic HA, carbonate groups are absent [31]. Thermal stability during the synthesis process can affect the loss of carbonate groups, so this must be controlled to ensure the biomaterial exhibits sufficient biological behaviour [32]. In this study, HA preparation was carried out using the hydrothermal method, which has the advantage of minimizing material loss [33].

The biocompatibility of a biomaterial is very important so that failure does not occur when using it. When in contact with tissues, biomaterials in the form of graft materials will induce foreign body reactions, which trigger the aggregation of large numbers of immune cells and inflammatory cytokines, inducing acute and chronic inflammatory responses and fibrous reactions [34]. So that if the biomaterial has a high enough toxicity, it will result in tissue damage and even affect some of the functions of the body's organs [34,35]. In this research, the toxicity test showed that the HA of the fish bones is not toxic. Several studies have shown that nano-hydroxyapatite has a toxic potential that can cause cell death due to exposure of macrophages to high levels of nano-hydroxyapatite due to the release of high levels of intracellular calcium, which disrupts the homeostasis of intracellular calcium [36]. However, nano-hydroxyapatite has physical properties that resemble natural bone minerals and is easily absorbed to stimulate bone-tissue regeneration [36-38]. This study produced HA from a 200-mesh sieve equivalent to 74 microns. Even on a micro scale, hydroxyapatite can still encourage bone-tissue regeneration through extracellular pathways and does not interfere with calcium homeostasis [39].

The bone healing procedure consists of three phases: inflammation, bone repair/production, and bone remodeling [40,41]. Inflammatory cytokines such as TNF-α are essential factors in the process of bone healing, and this study showed that HA application would increase TNF-α secretion in the fourth week. This indicates that defects receiving HA treatment can stimulate the release of cytokines resulting in increased expression of TNF-α from endothelial cells through an inflammatory reaction, the initial phase of the bone healing process. Therefore, in the radiographic appearance, there was no wound closure in all treatments in the second week. In the biological process of bone repair, the final phase, namely the remodeling phase, begins with the formation of granulation tissue [42]. Histological analysis in this study showed that the bone healing process had been seen in the second week. However, the defects that received treatment with HA suckermouth catfish bones expressed more granulation tissue in the fourth week than the other treatment groups, indicating better bone repair. As discussed above, suckermouth catfish bone can be used as a raw material for making bone grafts because of its hydroxyapatite content which can promote better bone healing. However, the limitations of this study were the limited characterization evaluation of the material, the use of a small sample size, the histological examination, which did not involve samples with defects without administration of HA material, and the brief evaluation period. Therefore, further studies with a complete evaluation of material characteristics, histological examination for all treatment groups, larger sample sizes, and long-term evaluation periods are needed to support the current study's findings.

CONCLUSION

FTIR analysis showed the presence of carbonate, phosphate, and hydroxyl groups, which are components of HA and also a component of bone formation. A toxicity test using the BSLT method showed that the HA of the fish bones is not toxic. TNF-α quantification from the results of the ELISA showed an increase in TNF-α expression in the fourth week in defects receiving HA treatment. Radiographs showed that the defect had not completely closed after the HA application in the second week. At the same time, the histological findings showed that the defects applied with the HA of the fishbone had a better bone healing process.

Therefore, the HA extracted from the bone of the suckermouth catfish has beneficial properties as an alternative to bone graft raw material and, more investigated needed to support this biomaterial to be used in the treatment of bone loss.

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Author's Contributions

ND: Conceptualization, Validation, Supervision. NH: Conceptualization, Validation, Writing (Review and Editing). NN: Methodology, Data Curation. NA: Methodology, Data Curation. AAMAL: Investigation. RSS: Investigation. Shaffati Shaffa: Investigation. SS: Data Curation, Writing Original Draft Preparation

Conflicts of Interest

The authors declare no conflict of interest.

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Regulatory Statement

This study protocols were reviewed and approved by the Ethics Committee of the Dentistry Faculty of Hasanuddin University under approval no. 0065/PL09/KEPK FKG-RSGM UNHAS/2021.

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