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Characterization of Fabricated Bovine Hydroxyapatite Crystal as Socket Preservation Material: An SEM-EDX and X-ray Diffraction Study

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ABSTRACT

Aim: This research aimed to fabricate and characterize bovine hydroxyapatite (BHA) crystal by using scanning electron microscope-energy dispersive X-ray (SEM-EDX) and X-ray diffraction (XRD) test for the evaluation of its potential as socket preservation material.

Materials and methods: Bovine hydroxyapatite was fabricated from bovine cancellous bone that was washed and cut into small pieces. The bone was placed in an ultrasonic cleaner to remove the fat, heated in the oven at 1000°C for 1 hour and dried. The bone was then grounded into a powder and sieved through 150 µm sieves. Powdered BHA was then analyzed with SEM-EDX to analyze the structure and content of the HA element, and an XRD test was used to analyze the HA crystal.

Results: From the SEM test, BHA had a crystalline particle with hexagonal crystal with an average size of 197 µm. The EDX test indicated BHA has the elements of carbon (C) (50.71%), oxygen (O) (34.59%), sodium (Na) (0.35%), magnesium (Mg) (0.35%), phosphorus (P) (4.29%), calcium (Ca) (8.97%), and niobium (Nb) (0.74%). The XRD test showed that BHA contains hydroxyapatite (HA) crystal with 66% crystallinity degree.

Conclusion: The BHA was successfully fabricated through the process of calcination. Based on the characterization and analysis using SEM-EDX and XRD tests of BHA, it had the potential as socket preservation material to preserve alveolar bone after tooth extraction.

Clinical significance: The addition of BHA after tooth extraction has the potential to maintain alveolar bone quality and prevent dimension loss. This biomaterial helps and supports prosthesis treatment, such as implants, which need good alveolar bone quantity and quality.

Keywords: Biomaterial, Bovine hydroxyapatite, Socket preservation.

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INTRODUCTION

Tooth extraction is the second most frequently performed dental procedure in Indonesia, covering 7.9% of all dental procedures on research done by the Ministry of Health in 2018.¹ After tooth extraction, the dimensions of the alveolar bone will decrease by 30% due to resorption.^{2,3} Socket preservation was carried out to maintain the dimensions of the postextraction alveolar bone socket. Socket preservation material in the form of bone graft could come from natural or synthetic materials.⁴ Natural socket preservation materials are easier to obtain and do not require complex or expensive processing to be fabricated.⁵ Natural grafts can be in the form of autografts from the patient's body, allografts from the same species, and xenografts.

Human bone consists of 70% inorganic components, most of which are HA crystals and 30% organic components, most of which are type 1 collagen.⁵ Alveolar bone consists of 67% organic material and 33% inorganic material.⁶ HA [calcium hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$)] is the main inorganic compound that composes various hard tissues such as bones, teeth, dentin, and others. Currently, the demand for HA as a bone graft is very high. Due to the limited supply of HA autograft and allograft, HA from xenograft was developed.⁷ Xenograft is a graft taken from a different species from the recipient, such as an animal.⁸ There are two categories of HA xenografts that are often used, namely synthetic and natural. One of the natural sources of this material is bovine bone.⁹ In the field of dentistry, several commercial products use HA as a bone graft.⁵ The price of commercial HA is

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quite expensive when compared to the fabrication price of HA from bovine bone. One method of fabricating HA from bovine is the calcination method. The calcination method with thermal decomposition is easy to do and can produce HA in the form of nanorods.¹⁰

Bovine hydroxyapatite fabricated from bovine bone has shown potential as socket preservation material; still, it needs to be tested both elementally and structurally to prove its biological function. SEM is a tool that provides very accurate microscopic images of material. SEM could analyze organic and inorganic material on a nanometer to micrometer scale. EDX test could be done while doing SEM analysis. EDX test measures the X-ray pattern

emitted by the material to determine the chemical composition and quantity.¹¹ XRD is a test for analyzing crystal structure, size, component parameter, and degree of crystallinity by using an X-ray beam.¹² XRD works by measuring diffracted X-ray angles emitted from the material to the detector.¹³ Therefore, the present study aimed to fabricate HA derived from bovine bone and see the characterization of this material with an SEM-EDX and XRD to analyze the potential of this material as a substitute for alveolar bone for socket preservation.

MATERIALS AND METHODS

Universitas Airlangga, Faculty of Dental Medicine, Dr Soetomo Surabaya approved this research with ethical clearance No: 360/HRECC.FODM/VII/2021. This research fabricated HA at the Tissue Bank of Rumah Sakit Umum Daerah (RSUD), Dr Soetomo Surabaya. Bovine bones used in this study were derived from female cow bones from farms in Malang, East Java, Indonesia. At first, the bovine bones were cleaned and washed from any blood clots. Then, the part of the cancellous bone that contains a lot of sponges was cut into small pieces as needed (Fig. 1A). Washing was carried out in detail using hydrogen peroxide (H_2O_2) (Fig. 1B). The bone was cleaned with an ultrasonic cleaner at a temperature of 60°C so that the fat would melt (Fig. 1C). Ultrasonic cleaning is repeated until the bone is completely clean of fat and the color becomes white. Washing was carried out again with distilled water to remove H_2O_2 until it was clean (Fig. 1D). The bones were then dried at room temperature to minimize moisture content (Fig. 1E). Next, the powder was heated in a furnace with a temperature of 1000°C for 1 hour (Fig. 1F). After the heating process, the bone was rinsed with

distilled water 3–4 times to remove the toxic properties (Fig. 1G). Then dried again in an oven at a temperature of 50°–100°C until dry (Fig. 1H). After drying completely, the bone was ground with a bone miller until particles were formed (Fig. 1I). The fine and coarse particles were separated by a sieving machine with a size of 150 μm (Fig. 1J).

Specimen Characterization

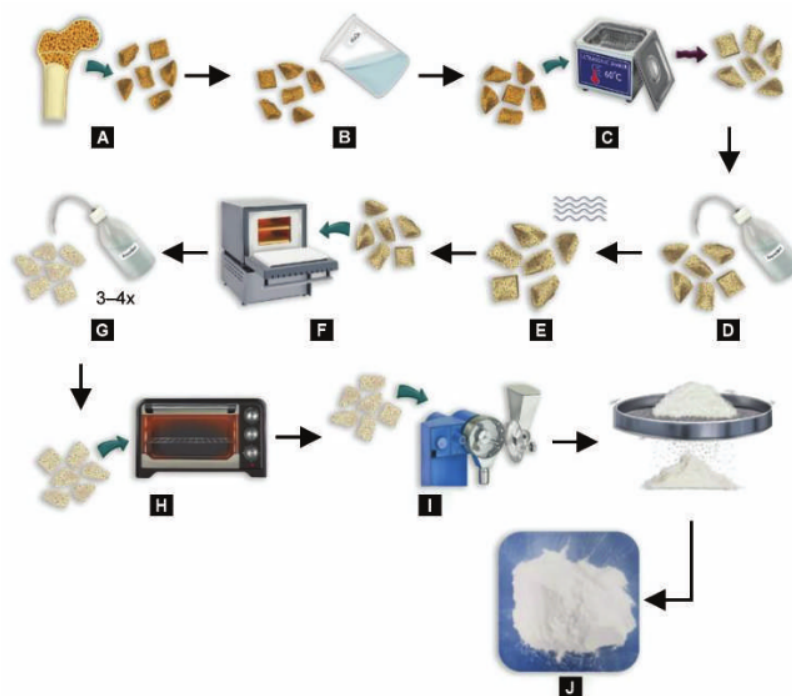
After the fabrication of HA powder from bovine bone, then it was characterized by SEM-EDX and XRD.

Scanning Electron Microscopy-Electron Dispersive X-ray Test

Scanning Electron Microscope test was carried out at Fakultas Matematika dan Ilmu Pengetahuan Alam, Institut Teknologi Bandung (JEOL, JSM 6510A). A total of 0.1 gm of BHA material was used in this analysis. The sample was then coated with gold (fine coater JFC-1600) before testing. The prepared sample was inserted in the chamber and vacuumed in high vacuum mode with a 15 kV secondary electron imaging detector. Magnification was set to 10,000 and 20,000x, after which the focus X stigma and Y stigma were adjusted to refine the image. Then the surface morphology of the pores, size of the pores, and the shape of the pores were analyzed.

Electron Dispersive X-ray Test

The samples in the SEM chamber were analyzed and mapped the chemical elements of the material using EDX spectroscopy (JEOL, JSM 6510-LA) at 10 μm magnification settings with 10 mm spacing. The results of the chemical element analysis are displayed in the form of graphs and color gradation maps.



Figs 1A to J: Process of fabrication HA from bovine bone: (A) Cutting of cancellous bovine bone; (B) Washing with H_2O_2 ; (C) Fat removal; (D) Washing with H_2O_2 ; (E) Drying at room temperature; (F) Burning in the oven; (G) Washing with distilled water; (H) Bone drying; (I) Bone grinding;

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X-ray Diffraction Test

X-ray diffraction test was carried out at the National Research and Innovation Agency (BRIN) for Physics Serpong. A total of 1 gm of BHA powder was used in this study. The Bruker D8 XRD machine (Germany) with cobalt cathode is used with a scan range of 10–90° with a speed of 0.1 seconds/step.

RESULTS

In this study, the calcination method with thermal decomposition was used to extract HA from bovine bone to remove organic material. The bovine bone powder is heated in a furnace to a temperature of 1000°C for 1 hour until the organic matter evaporates.

Scanning Electron Microscopy-Electron Dispersive Test Results

Figure 2 is an SEM image of BHA at (A) 10,000× and (B) 20,000× magnification. In this study, Image J software was used on SEM images with a magnification of 20,000× to measure the size of HA crystals. The picture shows an irregular hexagonal crystal structure with an average size of 1.97 μm, with the smallest size of 0.144 μm and the largest size of 4.494 μm.

Figures 3 and 4 are the results of BHA analysis with EDX. These results indicate the elements contained in BHA. In the picture, BHA shows the presence of the elements C (50.71%), O (34.59%), Na (0.35%), Mg (0.35%), P (4.29%), Ca (8.97%), and Nb (0.74%).

X-ray Diffraction Test Results

The analysis of HA peaks and measurements of the degree of crystallinity were carried out using the Origin 2019b program. In the results of the HA analysis, there is a sharp peak at 30.2°, 33.8°, 37.1°, 37.6°, 38.4°, 39.8°, 46.6°, 54.8°, 56.5°, 58.2°, 59.4°, 60.4°, 61.3°, and 62.7°2θ (Fig. 5). The measurement of crystallinity degree was carried out with $\frac{\text{crystalline area}}{\text{amorph area} + \text{crystalline area}} \times 100\%$. The degree of crystallinity in this HA sample is 66% which is HA crystal with a moderate degree of crystallinity. The X'Pert Highscore program was used to calculate the crystal size by Scherrer calculation. The crystal size of HA in the HA sample was 1087 nm.

From the test above, BHA contains HA crystals that has an average crystal size of 1.97 μm containing elements such as C, O, Na, Mg, P, Ca, and Nb with 66% degree of crystallinity. The result

of the test shows favors that BHA has the shape, elements, and crystal structure of a bone formation that has potential as a socket preservation material.

DISCUSSION

Tooth extraction will cause alveolar bone resorption, which could lead to dimension loss. Using graft material HA after extraction could help maintain alveolar bone height. Commercial natural HA is very expensive and hard to get for postextraction use. This study is aimed to create a cheaper and more readily available HA from bovine bone and analyze it using SEM-EDX and XRD. SEM-EDX test is to show the microscopic size and chemical component of HA crystal. XRD is used to characterize HA crystals.

Alveolar bone resorption can be divided into two stages. The first stage of this process is bone resorption which causes a decrease in bone dimensions. The second stage in this process is the remodeling of the buccal bone and woven bone, which will lead to more reduction in the dimensions of the alveolar bone. Alveolar bone disuse atrophy, decreases blood supply, and local inflammation also plays a role in bone resorption.¹⁴ Alveolar bone resorption complicates even the failure of placement of restorations such as implants. Implant placement requires ideal alveolar bone volume to achieve an adequate implant-to-bone ratio.¹⁵ Bone quality is also an important factor in the placement of implant restorations.¹⁶ Socket preservation material could come in many forms, such as HA powder from many sources. HA could come from allograft, xenograft, and alloplast. Allograft materials come from the same species but from a different individual. Allograft HA could come from frozen bone, freeze-dried bone, demineralized freeze-dried bone, and deproteinized bone. Xenograft material comes from a natural origin but from different species from the recipient. These include animal bones and coral. Alloplast is a graft that comes from a synthetic origin. Alloplast is made from calcium phosphates, glass ceramics, and polymers.^{17,18}

Bovine bone has a morphology and structure that is similar to human bone. HA from bovine bone also has the same chemical composition as human bone.¹⁹ Bovine bone also has the main mineral of human bone, namely HA.²⁰ HA extracted from bovine bone by thermal decomposition has good biocompatibility so that it can decompose naturally, can be absorbed naturally, and can be integrated with the bone. BHA is also more available, not limited to religious beliefs, and more economic.⁵ HA also has mechanical

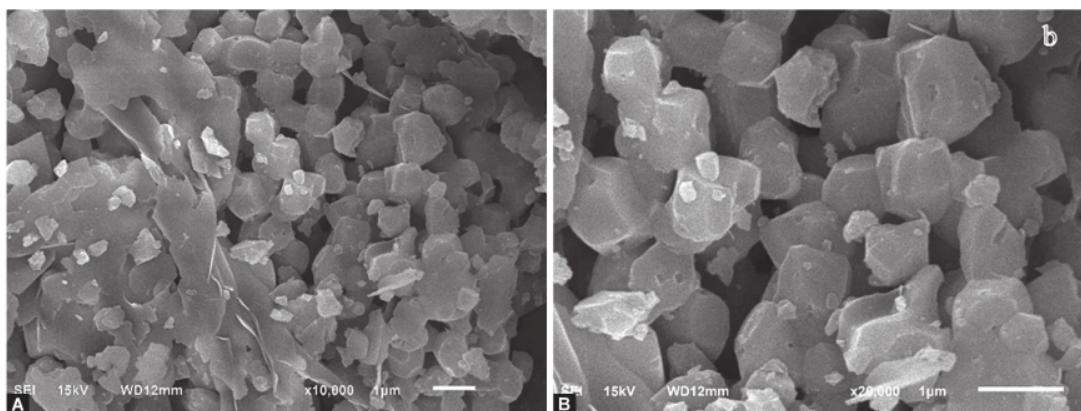


Fig 2A and B: Scanning electron microscope images (A) BHA at 10,000x magnification and (B) BHA at 20,000x magnification

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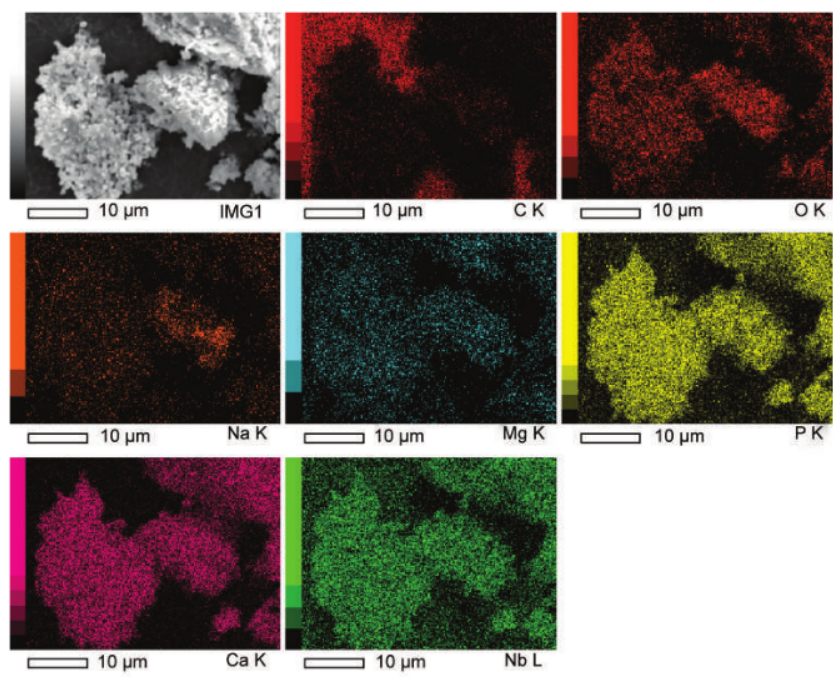


Fig. 3: Energy dispersive X-ray images of C, O, Na, Mg, P, Ca, and Nb element mapping from BHA

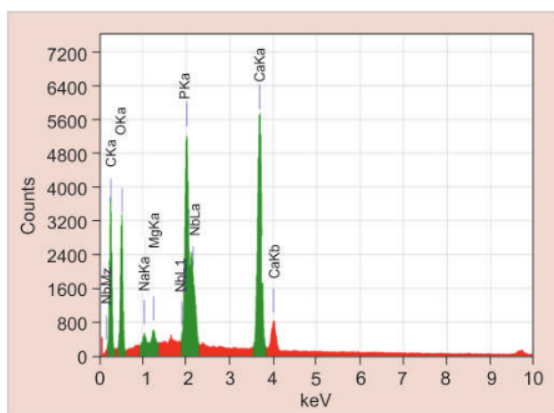


Fig. 4: Energy dispersive X-ray graph test results from BHA

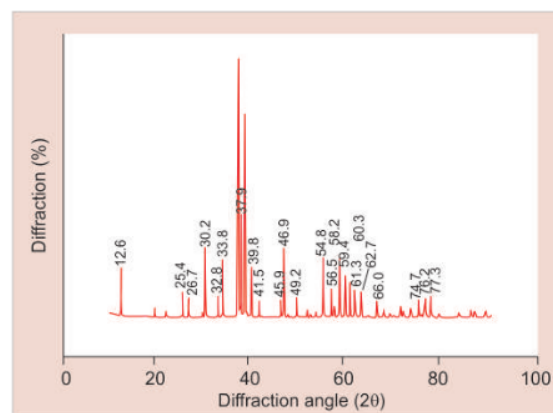


Fig. 5: X-ray diffraction graph from BHA

properties that are almost the same as human bone. This material has an important role in socket preservation.^{21,22}

Beef consumption in Indonesia is quite high. In 2020, 1,276,473 beef cattle were slaughtered and produced around 168,267 tons of waste beef bones.²³⁻²⁵ So, bovine bone waste produced in Indonesia is quite a lot, but the processing is not optimal, which makes bovine bones have a low selling value. To increase the selling value of bovine bones, processing can be done, one of which is extracting HA from the bones.

Bovine bones contain 70% HA which is similar to HA in human bones. The content of HA in bovine bones can make it a good candidate for socket preservation material.²⁴ HA from bovine bone has long been developed so that it has an affordable price and better quality. Bovine bone is considered as organic waste. Bovine

bones can be recycled and reused to increase their resale value. One way to increase the market value of bovine bone is to make it a source of HA for graft biomaterials in dental practice.²¹ This research tried to process bovine cancellous bone and fabricate biomaterials that are useful as bone replacement materials or to maintain bone tissue after tooth extraction.

In this study, HA biomaterial derived from bovine bone was made by calcination process with thermal decomposition. Ramirez-Gutierrez et al. successfully created pure HA without any organic compound from the bovine bone using calcination through thermal degradation at 900°C.²⁶ The bovine bone powder in this research is heated at 1000°C for 1 hour.²⁷ This was done to remove other organic matter that will evaporate at a temperature of 350°C.²⁸ HA also has stability up to a temperature of 1200°C.²⁹ The



advantage of the calcination method is that it can remove all organic components and all disease genomes. This provides safety when used as a graft material.²⁷

The bone used in this study was taken from the cancellous bone. Cancellous bone is a porous bone that is easily powdered and calcined. The porosity of the graft material is required for bone regeneration. The size and amount of porosity of the graft material can affect the osteoconductive properties, biodegradation, connection, migration, adhesion, proliferation, and differentiation of cells.^{30,31} The higher the porosity of the graft material, the easier it is for growth factors to penetrate the material and increase vascularization and angiogenesis.³⁰

Scanning electron microscope is a type of electron microscope that images the surface of a sample by scanning it with an electron beam. The electrons that interact with the atoms that make up the sample produce a signal that contains information about the sample from the surface topography, morphology, and composition of a material. SEM can detect and analyze surface fractures, provide microstructural information, view surface contamination, perform qualitative chemical analysis, and identification of crystalline structures.³²

The results of the SEM test show that the HA crystal structure is hexagonal, which is in accordance with the research by Ruksudjarit et al., which examined HA from bovine extracted using a thermal calcination process.³³ The results indicate an average HA crystal size of 1.97 μm . The crystal size of these HA sample crystals is within the micro size (2–3 μm). Micro-sized crystals can help stem cell differentiation and matrix synthesis, stimulate cell proliferation, chondrogenic gene expression, and matrix production.³⁴ HA with micro size can also increase the osteogenic and cementogenic ability because it can help the attachment, spread, and proliferation of cells.³⁵ The process of making HA by thermal decomposition produces HA in the form of rods.¹⁰

Energy dispersive X-ray is one of the most frequently used tests to analyze materials. This test is very easy to do because this test kit is an addition to the SEM machine and can be done in a matter of minutes.³⁶ X-rays have a specific energy response for each element. This can happen because the higher the atomic number, the stronger the emission of energy from the X-rays.³⁷ In this study, EDX was used to determine the elemental content in HA powder.

The EDX test results from human alveolar bone showed that human bone contains elements of calcium, phosphorus, and oxygen which are the main constituents of HA. There are also trace elements of iron, copper, zinc, bromium, strontium, Mg, lead, and Na which are found in human bones in small amounts. The results of the EDX test of BHA indicate that BHA contains elements of Ca, P, and O which are the major components. It also found, elements of Mg and Na as minor components. This is in accordance with the research of Maidaniuc et al. also found elements of Ca, P, O, Mg, and Na in BHA.³⁸ The similarity of the elemental content of alveolar bone and BHA makes this material can be used as a bone replacement material.

In this study, HA was produced with a Ca/P ratio of 2.09. According to research by Jaber et al., the lower the thermal temperature used, the higher the Ca/P ratio.³⁹ The Ca/P ratio in human bone is 1.67, if the Ca/P ratio of an HA is below 1.5, it produces a biphasic polarized HA material, namely β -tricalcium phosphate. The higher the Ca/P ratio, the purer the resulting HA. The presence of Na content in BHA can increase biocompatibility and osteointegration.⁴⁰

X-ray diffraction analysis is an analysis that uses the degree of XRD to see the characteristics of a crystal.⁴¹ XRD analysis can also be used to identify the structure, crystal size, elements, lattice parameters, and degree of crystallization through the diffraction peaks that appear.¹² Therefore, XRD is a technique that can analyze the crystalline phase of various materials such as HA.⁴¹

After analyzing the XRD peaks on HA which was carried out with the X'Pert highscore program, HA bovine according to the mineral reference $\text{HA Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ from The International Centre for Diffraction Data, Powder Diffraction FileTM.⁴² Calculation of the degree of crystallinity showed that the resulting HA had a moderate degree of crystallinity, which was 66% which indicated natural HA.⁴¹ HA with a moderate degree could increase the proliferation of osteogenic cells and can release particles from HA crystals in the surrounding tissue.^{44,45} BHA has a moderate degree of crystallinity and micro size that can help bone healing because HA is not easily absorbed.^{43,46}

Hydroxyapatite in powder form is easier and more useful for dentistry. Also provides a repeatable quality in clinical use.⁴⁷ HA powder can be used for a variety of applications. HA powder can be applied as bone filler in the repair of bone defects, fixation of implants, and bone grafts.⁴⁸ BHA is a bioactive material that has osseointegration, osteoinduction, and osteogenesis properties.⁴⁹ The bioactive properties of BHA can stimulate osteoblast cells to form osteoid, which will accelerate osteocyte formation.²² However, HA has limitations, namely its slow resorption so it cannot be absorbed properly. The osteoconductive nature of HA makes it difficult for resorption. The remaining unresorbed HA can have a negative effect on the mechanical properties of the regenerated bone.⁵ Currently, there's no literature about contraindications for BHA in clinical usage.⁵⁰

This study on BHA is currently limited to SEM-EDX and XRD characterization tests. In the future, it's necessary to run other supportive tests, such as cytotoxicity tests and analyze them to observe BHA biocompatibility as socket preservation material. After the cytotoxicity test, clinical tests on BHA will be done on the animal subject and then on human subjects.

CONCLUSION

Hydroxyapatite can be produced naturally from bovine bone. The results of the characterization analysis using SEM-EDX and XRD showed that this material has a crystalline structure and elements that can replace human alveolar bone. Therefore, this material has the potential to replace the lost bone structure or as socket preservation after tooth extraction.

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