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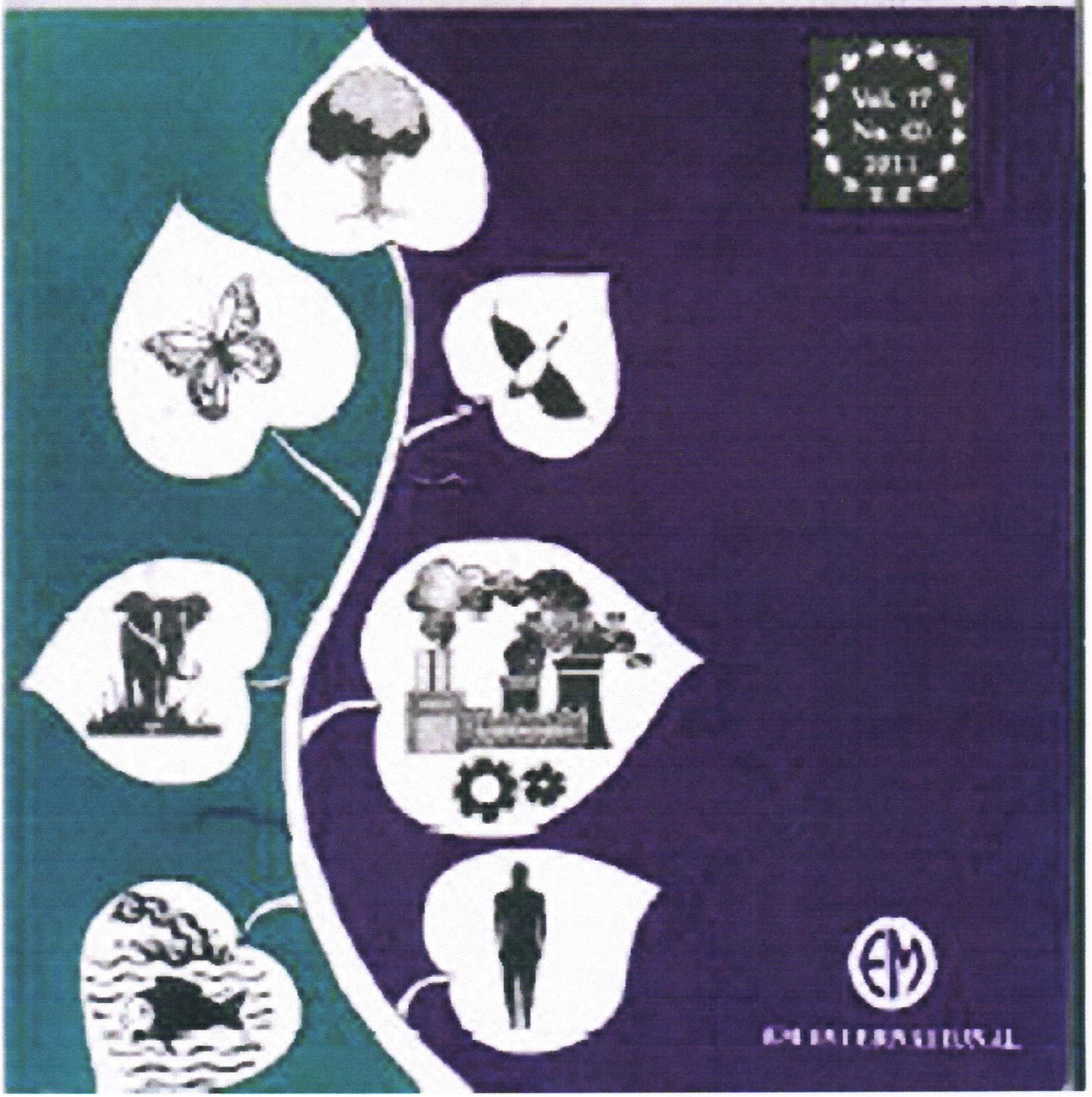

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Exploration of bovine bone waste as source of bovine hydroxyapatite synthesis and its composite with Gelatin-hydroxypropylmethyl cellulose as injectable bone substitute

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ABSTRACT

Bovine bone waste is nowadays not really utilized to increase its economic value. The slaughtering center only used small amount of this waste just be used as food supplement or plant fertilizer. Bovine bone is rich with calcium with the similar structure with human natural bone. So, it could be used a source of biomaterial for bone application, which is hydroxyapaitite. This study was aimed to synthesis of Hydroxyapatite from bovine bone by high pressure-steamed, grinded and filtered process with a 80 mesh and characterized by Fourier transform infrared, Scanning Electron Microscopy and X-Ray Diffraction for determining the purity of the hydroxyapatite. Besides that, it would be combined with gelatin and hydroxyporpylmethylcelluose to form Injectable Bone Substitute (IBS). The IBS was synthesized by stirring 5% (w/v) Bovine Hydroxyapatite:Gelatin with a ratio of 45:55 and 2%(w/v) hydropropylmethylcellulose. The IBS was injected to Bovine Hydroxyapatite Substrate until it was set. The result was shown that the hydroxyapatite was 100% pure and stoichiometric ratio Ca/P was 1.718. The result of FTIR showed that the functional group of hydroxyls, amine, phosphate group, and Ca^{2+}—COO^{-} were present at wavelength of 3465.37 cm^{-}, 1059.52 cm^{-1}, 1642.53 cm^{-1}, and 1562.57 cm^{-1},respectively. The result of SEM showed that the surface of the substrate had dots which indicated the hydroxyapatite of the IBS. The XRD test showed that the degree of crystallinity increased from 99.15 to 99.50%. In conclusion, the bovine bone waste could be used as the source of hydroxyapatite and could be applied as IBS combined with gelatin and hydroxypropylmethylcellulose

Key words: *bone waste, Bovine Hydroxyapatite, Injectable Bone Substitute, HA substrate*

Introduction

The Slaughtering Center is a service unit for proving meat based on national standards, which are safe, healthy, intact and halal (ASUH). The high demand of cow meat in Indonesia which reached 490.420,77 tons and the imported meat which reached 550,000 cows for beef industry and its processed products encourages the development of the slaughtering center (RPH, 2019). This development required concerns in many aspects, especially the environmental aspects since the waste product from the slaughtering center has a potential to be the source of disease or pathogenic microbes. One of the root problems of slaughtering center in Indonesia is the minimal utilization of the waste products, such as bovine bone.

The bovine bone waste is rich of collagen and calcium structurally which is similar to protein and mineral of human bone in terms of chemical components, morphology, distribution, function and its pathology (Bano *et al.,* 2017; Odusote *et al.,* 2019). Almost 16.6% of cow weight is consisted of bovine bone. The data from Pegirian slaughtering center mentioned that each day they slaughter 200 cows with bone waste product of 99 ton each month. Nowadays, this bovine bone waste is already utilized as the source of food supplement product that is halal, livestock supplement product, and plant fertilizer to increase its economic value. Through the good sorting process, the bovine bone waste could be beneficial to be the source of biomaterial, which is hydroxyapatite because its calcium component reaches 1-5% and its phosphorus component reaches 0-1%, (Odusote *et al.,* 2019).

Hydroxyapatite could be used in the treatment of bone defect in a form of solid or semi-liquid, such as suspension or paste. Hydroxyapatite (HA) is calcium phosphate much used a substitute for material and repair of human bone tissue because it has in common composition with the chemical structure of natural bone. This material is non-toxic, bioactive and has high biocompatibility with surrounding tissue and can encourage the growth of new bone because of its porous structure (Hikmawati *et al.,* 2019; Noor, 2013). One of the aplication of hydroxyapatite is Injectable Bone Substitute (IBS). It is a material that could replace the bone in the form of suspension. IBS could be applied by injection to reach out to region of bone defect that is deeper and able to adjust the form of defect better mobility. IBS injection on a bone is expected to replace bone material (Putra *et al.,* 2019; Azami *et al.,* 2017; Putra *et al.,* 2018). Based on the composition, the IBS may be made from composite of hydroxyapatite and gelatin. Hydroxyapatite include ceramic material that is brittle (easily broken) so that it requires the other materials of the type polymer, such as gelatin. Gelatin is a polypeptide produced from the hydrolysis of bone collagen, skin and connective tissue (Mohammad *et al.,* 2014). Hydroxyapatite-gelatin composites are expected to be compatible. Therefore, it is needed for the addition of alendronate in IBS that serves to set the pace of degradation with completion remodeling bone by the osteoblast.

Materials and Methods

The materials used in this study was divided into two types that is material to the making of samples and materials for the characterization of samples. Materials for making of samples include hydroxyapatite from bovine, gelatin, alendronate, HPMC from Sigma Aldrich H7509 and distilled water. Materials for sample characterization include hydroxyapatite substrates from Tissue Bank Dr. Soetomo General Academic Hospital, and materials for making Simulated Body Fluid (SBF) such as aquabidest, NaCl, $NaHCO_3$, KCL, $K_2HPO_4.3H_2O$, $MgCl_2.6H_2O$, $CaCl_2.2H_2O$, Na_2SO_4, $(CH_2OH)_3CNH_2$ and HCL 1 M.

Bovine bone preparation from fresh cortical bone of mature bovine was used as raw material purchase from Slaughtering Center Surabaya, Indonesia. The bones were cut off and cleaned with water. Then the spongy parts were removed together with the bone marrow. The sample were boiled in water for 4 hours and each hour, the water was changed in order to avoid the saturation with fat and protein form the bones. The sample was then high pressure-steamed for 2 hours with each hour, the water was changed. The sample was dried in oven with temperature of 60! for 3 hours. The dried sample was immersed and shook in alcohol for three hours to get rid of the fat with water change in each hour. To obtain hydroxyapatite from calcination process, the sample was heated at temperature of 950! for five hours. The result was a clean white sample. The sample was then grinded and filtered with an 80 mesh. The characterization performed for the HA sample was Fourier Transform Infrared (FTIR) (Perkin Elmer). The morphology characterization was performed by using Scanning Electron Micro-

scope (SEM) with Energy Dispersive X-ray (EDX) (FEI Type INSPECT S-50). The crystallization and phase information were obtained by X–Ray Diffractometer (XRD) (PANalytical Type X'Pert Pro).

The synthesis of Injectable Bone Substitute (IBS) was started by dissolving gelatin powder into distilled water with a concentration of 5 % at temperature 40 ºC for 1 hour. The gelatin solution was added by hydroxyapatite powder with a composition ratio of HA-gelatin of 45:55 and stirred for 1 hour until homogeneous. HPMC 2 w/v% was dissolved with distilled water at temperature of 90 ºC and then allowed to stand until the temperature of 40 ºC. A solution of HPMC was mixed into the solution of HA-gelatin with the temperature of the mixture of 40 ºC and stirred for 6 hours until a viscous composite of hydroxyapatite-gelatin forms a suspension which is white in color. The sample was then injected to the hydroxyapatite substrate, the mass, volume, and density of the substrate was measured before and after the setting of IBS and then characterized by using Fourier Transform Infrared (FTIR) to observe the functional group of the release IBS in SBF, X-Ray Diffraction (XRD) test to observe the crystallinity of the release IBS, and the SEM of the substrate after the IBS released.

Results and Discussion

The hydroxyapatite was successful to be synthesized and it was characterized by using FTIR, XRD, and SEM. Figure 1 showed the spectra of the sample through FTIR test. There was hydroxyl group (OH^-) at wavenumber of 570, 602, and 3571 cm^{-1}, carbonate group ($-CO_3^{2-}$) at wavenumber of 1414 and 1455 cm^{-1} and phosphate group (PO_4^{3-}) at wavenumber of 472, 962, 1048, and 1089 cm^{-1}. All the functional groups to identify hydroxyapatite was found in the result with addition of carbonate functional group.

Figure 2 showed 100% purity of hydroxyapatite based on XRD test compared to the standard hydroxyapatite Ref Code 01-184-1998 in XRD instrument that was used in the sample characterization (Bano *et al.*, 2017). This result showed that the extraction method was successful in extracting the hydroxyapatite from bovine bone.

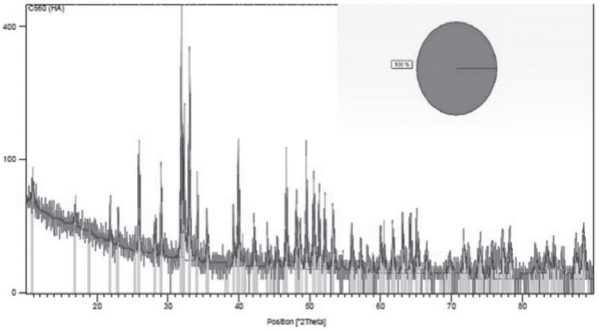

Fig. 2. XRD spectra of pure hydroxyapatite originating from bovine bone

The morphology and content of the hydroxyapatite was tested by using SEM test. The result was shown in Figure 3 and 4. The atom ratio between calcium and phosphate based on stoichiometric ratio Ca/P was 1.67 (Bano *et al.*, 2017; (Maulida *et al.*, 2015), while the extraction result from the bovine bone analyzed by EDX (Figure 4) was 1.718. This number showed that the calcium content was higher than the phosphate content. This may be

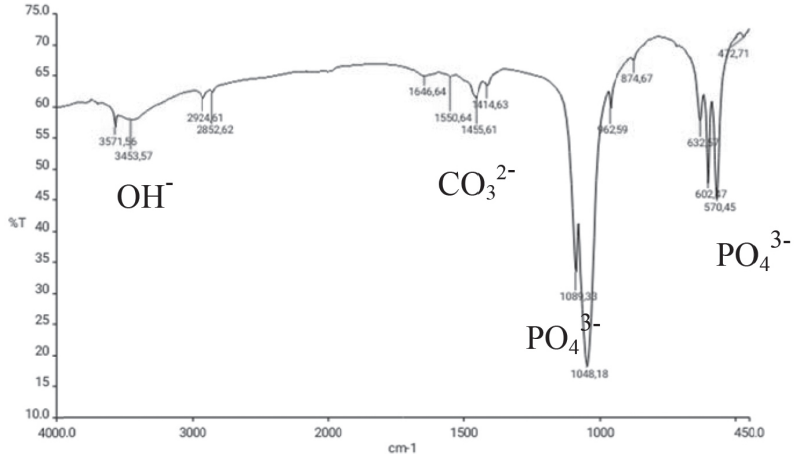

Fig. 1. FTIR Spectra of Bovine Hydroxyapatite (BHA) $\{Ca_{10}(PO_4)_6(OH)_2\}$

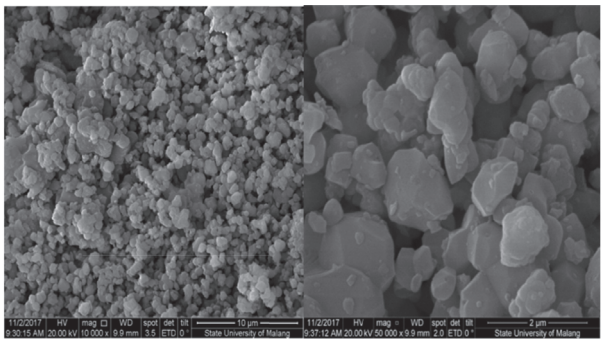

Fig. 3. The SEM image of Bovine Hydroxyapatite particles particles, with magnification of 10.000 and 50.000.

caused by calcium carbonate that is one of the bone components.

All the result of hydroxyapatite characterization originating from bovine bone waste indicated a good quality of hydroxyapatite from the functional group by FTIR, component and crystallinity by XRD test and morphology and content by SEM-EDX test. In term of result, the hydroxyapatite from bovine bone waste has slight difference compared to commercial hydroxyapatite, such as the presence of carbonate functional group in the FTIR result and Ca/P number in the XRD result. This condition is not crucial for its application as biomaterials, since in natural bone also has carbonate ions. The carbonated-hydroxyapatite was mentioned that it exhibited more bone formation in rabbit that has bone defect. It was also mentioned that it did not show rejection, necrosis, or infection clinically or histologically (Jebahi *et al.*, 2012).

The synthesized hydroxyapatite was then used as composite with gelatin and Hydroxypropylmethyl cellulose (HPMC) called Injectable Bone Substitute (IBS). The IBS was suitable with the previous result performed by Putra et al (2019) and Maulida *et al.* (2019) who also used hydroxyapatite for IBS application for osteoporosis and spinal tuberculosis case, respectively (Hikmawati *et al.* (2019); Putra *et al* (2019). The IBS was almost 100% injectable and had normal pH. The result was also characterized in term of functional group, morphology in substrate, and crystallinity.

The result of FTIR of the IBS was shown in Figure 5. The hydroxyl group was found at wavenumber of 3465.37 cm^{-1}. This functional group became deeper compared to the hydroxyl group in the bovine hydroxyapatite since in the synthesize process. We used distilled water as solvent to form the suspension of IBS. The phosphate and the carbonate functional group were found at wavenumber of 1059.52 cm^{-1}, 602.59 cm^{-1}, and 1453.54 cm^{-1}, respectively as the functional group of the synthesized hydroxyapatite. The presence of amina group indicated the existence of gelatin in the mixture at wavelength of 1642.53 cm^{-1}. The hydroxyapatite and gelatin also made an interaction of Ca^{2+} and COO^{-} which was shown at wavenumber of 1562.57 cm^{-1}. This result also suitable with the previous study which showed the same behavior (Hikmawati *et al.* (2019); Putra *et al.* (2019); Putra *et al.* (2018); Maulida *et al.* (2015); Rachmawaty *et al.* (2019). The use of hydroxyapatite forms the bovine bone waste did not show a different behavior when it is applied as Injectable Bone Substitute (IBS).

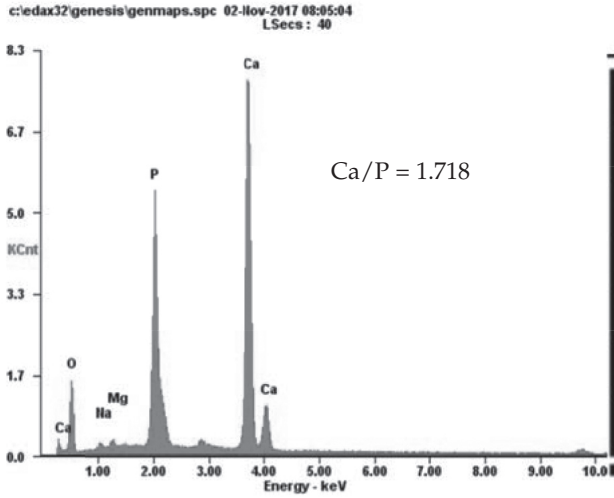

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Fig. 4. EDX result of Bovine Bone Originated- Hydroxyapatite

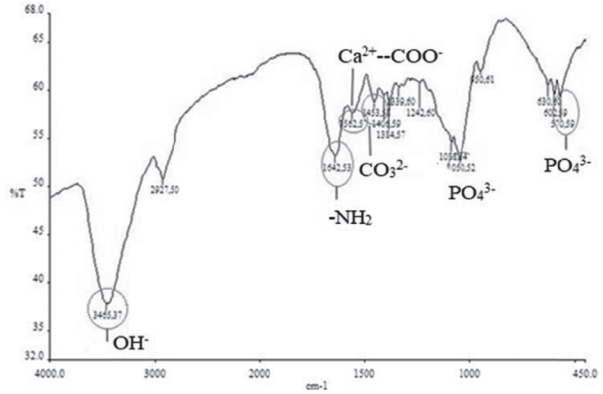


Fig. 5. FTIR Spectra of IBS based on Bovine Hydroxyapatite-Gelatin-HPMC

The result of XRD test of HA substrate before and after IBS injection showed that the hydroxyapatite content in the substrate increased from 99.15% to 99.50%. The content of the substrate was almost 100% hydroxyapatite. The increase HA content was caused by the application of the IBS which also contained HA in its mixture. This result also indicated that the IBS could increase the calcium content of the substrate which represented the natural bone.

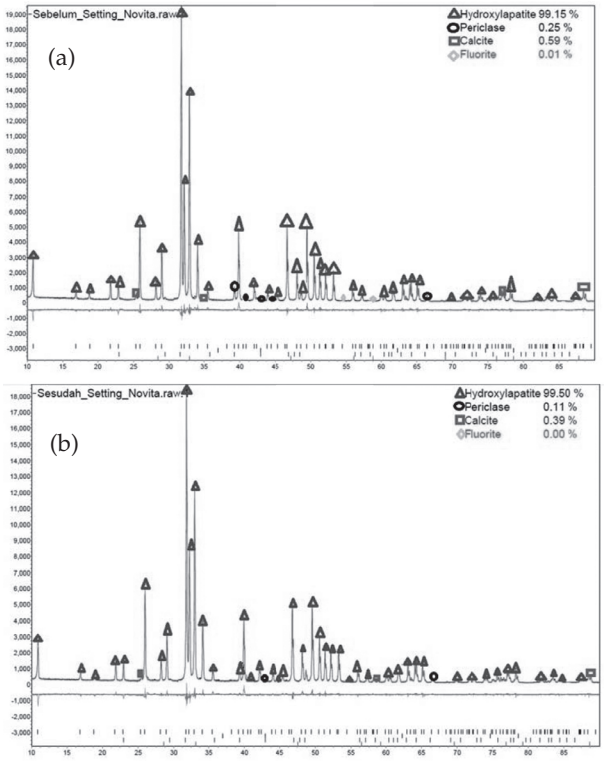
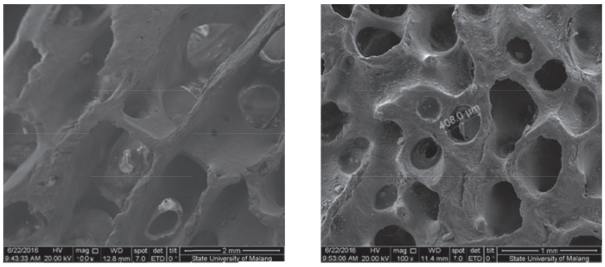


Fig. 6. XRD Spectra of HA Substrate (a) Before Injection and (b) After IBS Injection

The last characterization was morphology test by using SEM. The SEM image result was shown in Figure 7. The application of IBS in the substrate made the surface of the substrate had dots which represented the hydroxyapatite crystal from the IBS (Hikmawati *et al*. (2019); Putra *et al.* (2019). Before the application of IBS, the surface of the substrate was smooth and only form bigger pores compared to the one in the substrate after the injection of IBS. The presence of IBS could fill the pores of the substrate. In term of application, this behavior was beneficial since the bone substitute could fill the hollow part of the substrate which showed the capacity of bone defect.



(a) **(b)**

Fig. 7. SEM Image of HA Substrate Before and After IBS Injection

Conclusion

In conclusion, based on the results of this study, it can be concluded that the bovine bone waste could be used as the source of biomaterial, which was hydroxyapatite. The characteristics of bovine hydroxyapatite was also similar to the commercial hydroxyapatite which the addition of carbonate functional group based on FTIR result and the Ca/P number of 1.718. The synthesis of Injectable Bone Substitute (IBS) was successful in combination with gelatin and hydroxypropylmethylcellulose. The result of FTIR, XRD, and SEM test showed that the application of IBS in the HA substrate is beneficial as it would be used as bone filler in the bone defect. For future studies, the use of bovine bone waste should be optimized by calculating the amount of hydroxyapatite could be synthesized from the amount of bovine bone waste.

Acknowledgments

The authors would like to deliver gratitude to Halal Center Universitas Airlangga for the support in this study.

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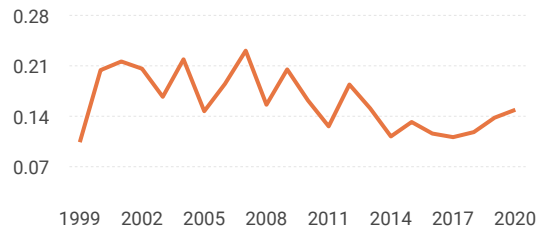

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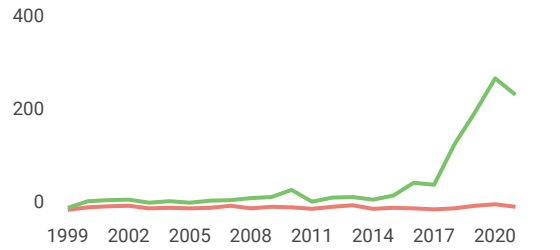

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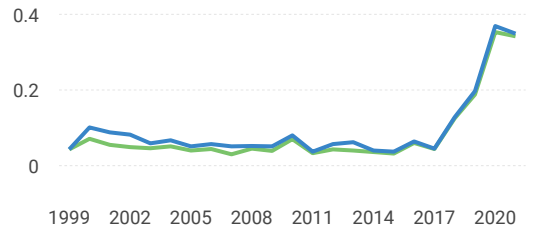

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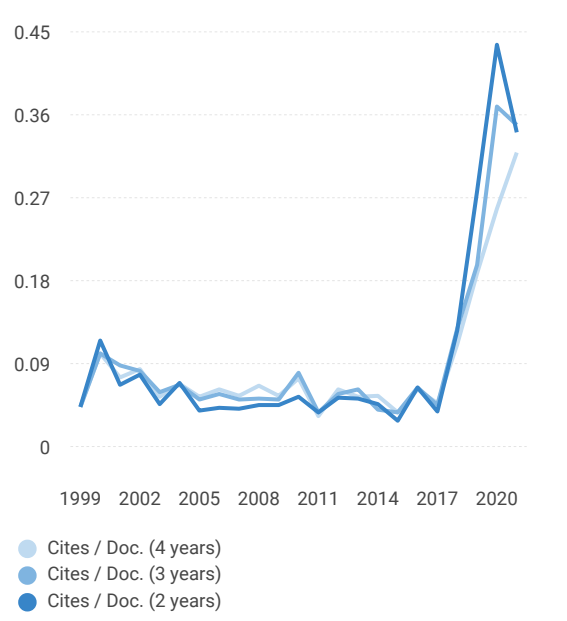

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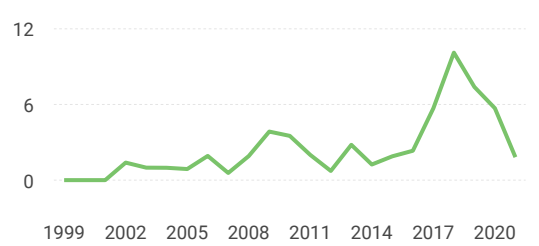

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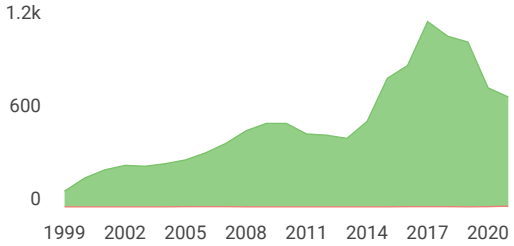

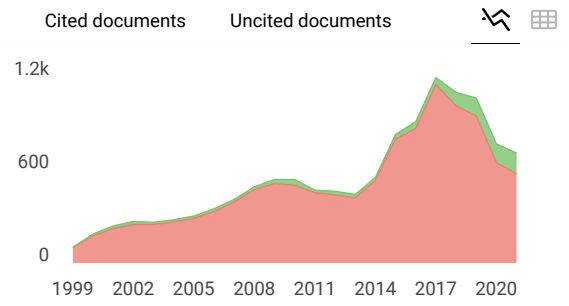

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Y **yayan** 4 years ago

Dear Scimago team

Dear sir/ma'am 1 need information about when EEC journal will be indexed in 2018 by Schimago since my article is published at EEC on September 2018. I am wait for your respond

best regards

yayan

SCImago Team

Elena Corera 4 years ago

Dear Yayan,

articles published in 2018 are not over yet. 2018 indicators will not be available until June 2019. We cannot see what will happen in the future with this journal. SCImago receives the data from Scopus / Elsevier annually and does not have the authority to include, exclude or modify the data provided by Scopus.

Best Regards,
SCImago Team

SCImago Team

Elena Corera 5 years ago

Dear Zainal Abidin,

thank you very much for your comment. Unfortunately, we cannot help you with your request, we suggest you contact journal's editorial staff so they could inform you more deeply. You can find contact information in SJR website https://www.scimagojr.com

Best Regards,
SCImago Team

T ARULKUMAR 5 years ago

Dear sir,
What is the impact factor of this Journal for citing in my article which was published in July 2017?

reply

SCImago Team

Elena Corera 5 years ago

Dear user, SCImago Journal and Country Rank uses Scopus data, our impact indicator is the SJR. Check our page to locate the journal. We suggest you consult the Journal Citation Report for other indicators (like Impact Factor) with a Web of Science data source. Best Regards, SCImago Team

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Horatio (Satire 1,1,106)