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CHARACTERISTICS AND CYTOTOXICITY OF HYDROXYAPATITE FROM PADALARANG–CIREBON LIMESTONE AS BONE GRAFTING CANDIDATE

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ABSTRACT : Limestone, as a natural source has the main component of calcium carbonate ($CaCO_3$) and is abundantly existed in Indonesia. Calcium carbonate is one of the compounds that could be a precursor in the fabrication of hydroxyapatite (HA) that has been commonly used in clinical application. The center of ceramics in Indonesia (*BBK: Balai Besar Keramik*) has synthesize HA from limestone's calcium carbonate derived from mountains in Padalang and Cirebon, West Java, Indonesia. This study aims to evaluate the characteristics and cytotoxity of hydroxyapatite made from Padalarang-Cirebon limestone by BBK as a bone graft candidate. Characterizations of HA were Fourier Transform Infra-Red (FTIR), X–Ray Diffraction (XRD) and Energy Dispersive X-ray (EDX). Cytotoxicity of the samples were evaluated with MTT assay on umbilical cord mesenchymal stem cells (UC-MSCs) with the concentrations of 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.12µg/ml, 1.56µg/ml, 0.78µg/ml, 0.39µg/ml, and 0.19µg/ml. HA from limestone has hydroxyl (OH-) dan phosphate (PO_4^{2-}) functional groups, the particle was in the crystal form and has the composition of O, Ca, and P with the Ca/P ratio was 1.64. Toxicity was not found in all concentrations ($p > 5$). HA from Padalarang-Cirebon limestone by BBK has hydroxyl and phosphate groups, crystal particles, the composition of O, Ca, and P, Ca/P ratio of 1.64 and no toxicity, which satisfied the requirements of a bone graft candidate.

Key words : Hydroxyapatite, limestone, physical characterizations, cytotoxicity, regenerative medicine.

INTRODUCTION

Indonesia is a country that possesses abundant natural sources. One of the largest natural sources is limestone, which is widely spread on the whole country, reaching 28.68 million tons. West Java has approximately 2.96 million tons of limestone (Cifriadi, 2014). Main component of limestone is 95% calcium carbonate in calcite form which has not been optimally utilized (Apriliani *et al*, 2012). Calcium carbonate from limestone could be a precursor for HA fabrication.HA is the main component in bone formation that needed in the regeneration process in bone defect.HA products that commonly used in medical treatment currently provided by import products which is quite costly (Rumengan *et al*, 2017; Nugraha *et al*, 2019).

Hydroxyapatite [HA: $Ca_{10}(PO_4)_6(OH)_2$] is an anorganic component from bone which possess osteoconductive, osteoinductive, and osteointegration ability, and widely used in orthopedic and dental treatment (Turon *et al*, 2018; Szczeœ*et al*, 2017; Adi *et al*, 2014; Chandrasekar *et al*, 2013) particularly in bone grafting. Bone grafting is the treatment to replace bone loss caused by trauma or disease with a bone graft implantation to improve the bone regeneration ability (Sari *et al*, 2019). Bone grafts are classified into two main groups, natural bone graft (autograft, allograft, and xenograft) and synthetic bone graft or alloplastic graft (Kumar *et al*, 2013; Prahasanti *et al*, 2020).

Up to this day, autograft is still a golden standard among all of the bone graft materials due to its excellent osteoconduction, osteoinductions, and osteogenesis (Wang *et al*, 2017; Ebrahimi *et al*, 2017; Nugraha *et al*, 2020a). However, autograft has several drawbacks such as the extensive surgical procedures on the donor site that not

only cause additional pain, it also might potentially cause post-operative complication, extended stay in hospital, which eventually, would be expensive to afford. To overcome the problems, numerous kinds of materials have been developed to produce synthetic bone graft, such as bioceramics. One of the first bioceramics used as synthetic bone grafts is hydroxyapatite (Kumar *et al*, 2013; Hung, 2012; Fillingham *et al*, 2016; Ginebra *et al*, 2018).

HA could be chemically produced, or derived from natural sources. HA from natural source is non-stoichiometric and has trace elements such as Na and Mg, similar to the human bone. Natural HA usually extracted from mammalian bones such as bovine, aquatic sources like fish bone and fish scale, shells, and minerals such as limestone. Limestone formed due to the precipitation of animal shells or bones, or algae, which are rich in calcium carbonate ($CaCO_3$). This calcium carbonate is one of the compound that is very useful in the fabrication of HA (Mohd *et al*, 2019; Brzeziñska-Miecznik *et al*, 2016; Fernandez *et al*, 2018; Pham Minh *et al*, 2014).

Characteristics and toxicity of HA from Padalarang-Cirebon limestone produce by BBK needs to be evaluated before it can used. Cytotoxicity or biocompatibility test is an initial stage test for new materials that needed to be conducted before it can be applied, particularly in medical application. Therefore, this study aims to evaluate the characteristics and cytotoxity of HA from Padalarang-Cirebon limestone as a bone graft candidate.

MATERIALS AND METHODS

This study has undergone ethical clearance from Health Research Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga (205/HRECC.FODM/V/2019).

HA powder was obtained from BBK with the size of 100-150 µm. Limestone was synthesized through wet milling process inside a pot mill for 6 hours, then sieved through 150 mesh. Obtained suspension contains alkali calcium hydroxide [$Ca(OH)_2$]also known as milk lime (Eq. 1 and 2). Phosphoric acid [H_3PO_4] were added into milk lime and slowly mixed inside a reactor with controlled pH (Eq. 3). The obtainedsuspension were dried at 110°C, then calcined at 1200°C and finally dry milled to produce hydroxyapatite powder.

$$CaCO_3 \rightarrow CaO + CO_2 \quad (1)$$

$$CaO + H_2O \rightarrow Ca(OH)_2 \quad (2)$$

$$10Ca(OH)_2+6H_3PO_4 \rightarrow Ca_{10}(PO_2)(OH)_2+18H_2O \quad (3)$$

FT-IR analysis

Physical characterization of HA was to identify functional groups, the particles phase, and particle image and compositions. Identification of functional groups were evaluated using Fourier Transform Infra Red [FT-IR: Thermo Scientific;Nicolet iS10 FTIR Spectrometer; Thermo Fisher Scientific Inc., Massachusetts, USA] with 2 grams of powdered HA that put on the sample holder.

XRD analysis

Phase identification was evaluated using X – Ray Diffraction[XRD: X'Pert PRO PAN analytical; X'Pert³ MRD; Malvern Panalytical Ltd., Malvern, United Kingdom] and the result were compared with HA standard.

EDX analysis

Two grams of powdered HA were put on pin holder that has been sticked by carbon tip to attach the sample. Particles composition of sample were evaluated usingEnergy Dispersive X-ray [EDX: EDAX AMETEK; EDAX's Octane SDD Series for the TEM; EDAX Inc., New Jersey, USA].

MTT assay

Umbilical cord mesenchymal stem cells (UC-MSCs) were taken from culture stock in the form of cell line. Cells were washed with Phospate Buffer Saline (PBS), added with1 ml trypsin versene. Swarmed cells were homogenized with 10 ml Alpha MEM then put into 96 well microplate with the density of 2×10^5 in 50 µl and finally incubated for 24 hours. UC-MSCs were characterized with CD45 and CD105 on fluorescence microscope with the magnification of 100x (Prasetyo *et al*, 2020; Kuntjoro *et al*, 2020).

HA were dissolved in the Alpha MEM medium (Gibco, UK REF: 12000-014) then divided into 9 groups based on the HA concentrations: 50.00 µg/ml, 25.00 µg/ml, 12.50 µg/ml, 6.25 µg/ml, 3.12 µg/ml, 1.56 µg/ml, 0.78 µg/ml, 0.39 µg/ml, 0.19 µg/ml, then incubated for 24 hours. Samples (50 µl) were put into 96 well microplate with the density of $2x10^5$. After 24 hours incubation, Alpha MEM were removed from the microplate. MTT (Ultra Pure CAS:298-93-1) were added and dissolved in 10µl PBS, and incubated for 2 to 4 hours at 37°C. Microplate were added with 50 µl DMSO (Bioworld, CAS: 67-68-5) into each well until formazan crystals were formed (Nugraha *et al,* 2020; Kuntjoro *et al*, 2020). Optical density value of formazan was measured with ELISA reader (Glomax GM3000) with wavelength (ë) of 620 nm and viable cells percentage were calculated with below formula:

$$\% \text{ viable cells} = \frac{\text{Treatment group} + \text{Medium}}{\text{Cells} + \text{Medium}} \times 100$$

RESULTS

Fourier Transform Infra-Red (FT-IR)

FT-IR result showed peaks in the graph with transmittance (%) as Y-axis to wavelength (cm^{-1}) as X-axis. HA showed OH bond at the peak of 3569.28 cm^{-1} which includes in the range between 300 – 3700 cm^{-1}. Phosphate group (PO_4^{3-}) has wavelength range between 1100–950 cm^{-1} (Stuart, 2008 p.96). Phosphate ions indicated by the peaks at 961.30 cm^{-1}, 1024.23 cm^{-1}, dan 1086.51cm^{-1}.

X – Ray Diffraction (XRD)

XRD pattern of HA from Padalarang and Cirebon limestone has the sharp peaks that indicates the crystal form of the samples.Peaks of the samples were compared with the ones of HA standard (Sigma-Aldrich) to confirm whether HA obtained in this study correspond to pure HA, and not contain other phases. Analysis of the samples was taken by observing and comparing the three highest peaks as representatives between the samples and standard. First highest peak position of HA samples were at 31.6329Å, second highest at 32.7728Å and third highest at 32.0332Å.

Energy Dispersive X-ray (EDX) analysis

EDX analysis on the HA from Padalarang and Cirebon limestone has shown that the sample contained 59.48% of CaO and 36.19 of P_2O_5. Thus, it was calculated that the Ca/P ratio of the sample was 1.64.

UC-MSCs characterization

UC-MSCs were characterized using CD45 and CD105 and analyzed using fluorescence microscope with 100x magnification.

MTT assay

Quantity of formazan crystals measured using spectrophotometry 620 nm to obtain the optical density value of formazan.

The highest percentage of viable cells was at the HA concentration of 50 µg/ml with the amount of 90.88%. The lowest percentage was at the concentration of 0.19 µg/ml with the amount of 81.40% (Table 1). Viable cells percentage in all concentrations were more than 50% (CD_{50}) and viable cells increase with respect to higher concentrations (Fig. 4). Post Hoc analysis and Tukey

Table 1 : Optical density and viable cells percentage of UC-MSCs after treated with hydroxyapatite derived from Padalarang-Cirebon limestone(OD).

No.	HA concentration	N	Average±SD	% Viable cell
1.	Cell Control	8	0.583±0.015	100
2.	50 µg/ml	8	0.535±0.049	90.88
3.	25 µg/ml	8	0.534±0.046	90.60
4.	12.5 µg/ml	8	0.526±0.086	89.10
5.	6.25 µg/ml	8	0.514±0.054	86.69
6.	3.12 µg/ml	8	0.512±0.033	86.30
7.	1.56 µg/ml	8	0.508±0.009	85.70
8.	0.78 µg/ml	8	0.505±0.000	85.10
9.	0.39 µg/ml	8	0.504±0.023	84.80
10.	0.19 µg/ml	8	0.486±0.028	81.40

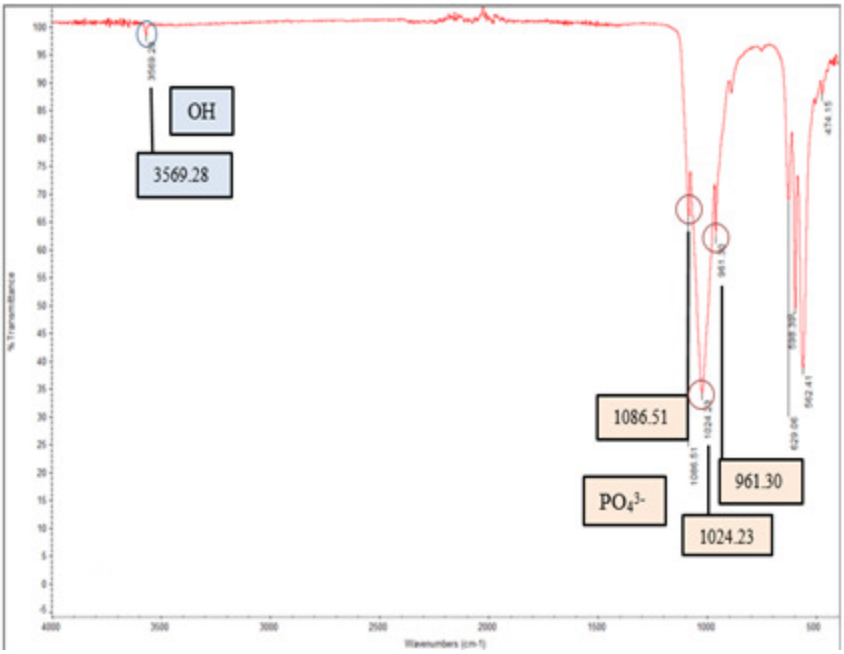

Fig. 1 : Fourier Transform Infra Red (FTIR) of HA from limestone derived from Padalarang dan Cirebon.

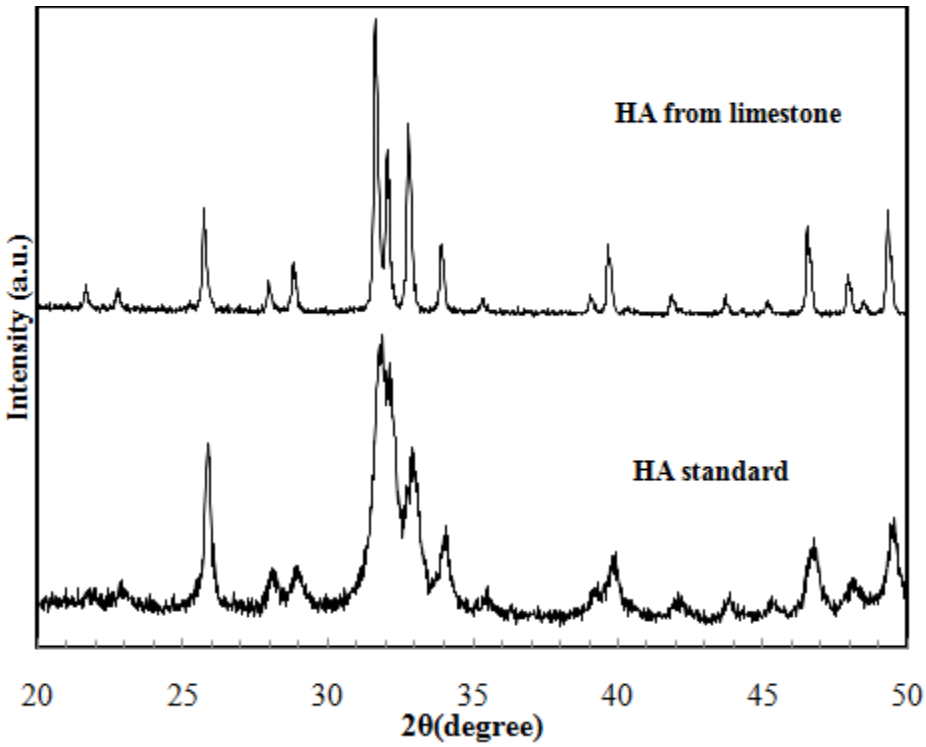

Fig. 2 : Comparison of XRD patterns between hydroxyapatite from Padalarang and Cirebon limestone(top) and hydroxyapatite standard (bottom).

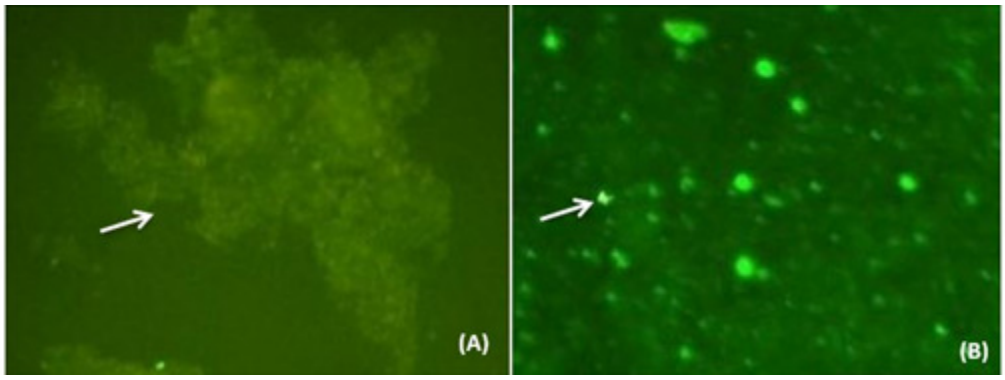

Fig. 3 : UC-MSCs characterizations with immunocytochemistry staining with the label of FIT-C. Staining resulted in (A) negative expression of CD45 (white arrow) dan (B) strong expression of CD105 (blue arrow).

HSD showed no significant difference between each groups of HA concentration.

DISCUSSION

Hydroxyapatite or HA is one of the bioceramics that possess good osteoconductivity and osteinduction which eventually would improve osteogenesis in bone regeneration (Mehta *et al*, 2013). Precipitation method was used for the synthesis of HA which derived from Padalarang-Cirebon limestone (Habibie *et al*, 2017). This hydroxyapatite need to physically characterized to identify functional groups, particles form and phase, and content of the materials. The obtained characteristic results would be used to establish the potential the materials as a bone graft candidate.

Functional groups were analysed with FT-IR to identify hydroxyl and phosphate functional groups. Hydroxy group marked by typical peak at 3800–3200 cm^{-1} that originated from hydrated inorganic compounds, which possibly came from water content that infiltrate during the production of HA. The water combined with crystal structure of the materials causing stretching and bending of O-H bond and generated peak at that position. Phosphate group on FT-IR was shown at the range of phosphate bond of 1100–950 cm^{-1} (Stuart *et al*, 2005).

The presence of hydroxyl and phosphate group showed the typical groups in common hydroxyapatite. The results of this FT-IR also corresponded to some of the previous studies, one of them was the study of HA fabrication from egg shells, which exhibit peaks at 3570.35

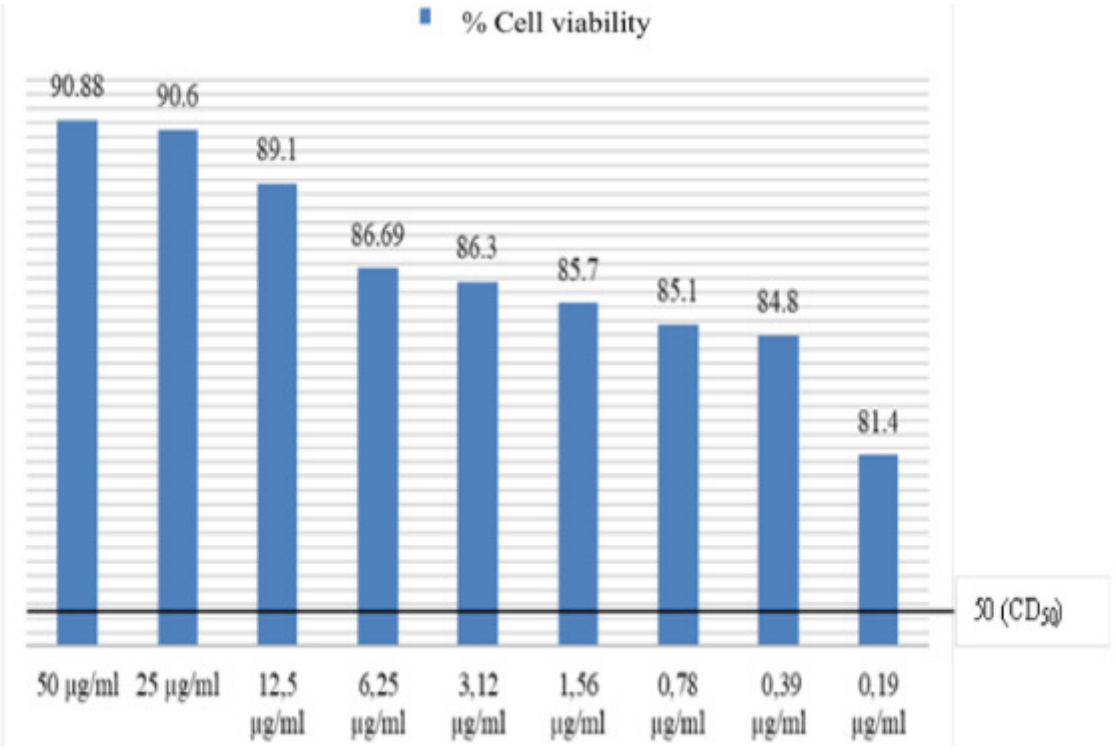

Fig. 4 : Graph of the relation of HA samples with the viable cell percentage.

cm^{-1}, which was typical mark of hydroxyl group and also at 926.81 cm^{-1} dan 1024.95 cm^{-1}, which was typical phosphate groups (Khandelwal *et al*, 2016). Other research that studied HA fabricated from shells and bovine also showed dominant presence of hydroxyl (–OH) and phosphate(PO_4^{3-}) groups (Adi *et al*, 2014; Khoiruddin *et al*, 2015). Hydroxyapatite synthesis from limestone by BBK resulted in the samples that possess hydroxyl and phosphate groups, which are the common functional groups which are present in commercial hydroxyapatite.

XRD result of the samples showed sharp peaks and also showed similar pattern with the standard which represent that the HA samples obtained in this study has crystal forms, which also a typical pattern of common HA. The XRD result also supported by previous similar studies that are using precipitation method to synthesis hydroxyapatite. XRD pattern of HA samples displayed various intensity, three highest peaks were taken to be observed, and the intensities were 100%, 63.32% dan 54.41%. Standard data also used three highest peaks with the intensities of 100%, 76.14% dan 64.27%. The three peaks of each were also compared in terms of peak positions. The comparison resulted in similar position between HA samples and standard. Based on this consistency, HA from Padalarang and Cirebon limestone are compatible to the standard.

EDX analysis has confirmed that the HA sample has a dominant composition of oxygen (O), calcium (Ca), and phosphor (P), with the highest peak was calcium, followed by phosphor and finally oxygen. Other studies regarding the synthesis of HA, such as the one using the wet chemical methods (Chandrasekar *et al*, 2013) or HA derived from egg shells (Khandelwal *et al*, 2016) have produced similar composition. Calcium phosphate ratio was calculated from the composition in EDX result and it was 1.64, which in the range of Ca/P ratio in the human bone (1,33-1,67).

Cytotoxicity of HA from Padalarang-Cirebon limestone which given in various concentration of 1.57 to 50 µg/ml showed more than 50% of viable cells, which are conform with CD_{50} and exhibited that all concentrations of HA caused no toxic response (Telli *et al*, 1999). High viability in this study could be affected by the high Ca/P ratio on HA. High amount of calcium would increase the proliferation, due to the increase of calcium channel expression. Calcium sensing receptor (CaSR) could detect any external Ca^{2+} concentration change and increase Ca^{2+} influx. Higher influx in higher calcium ions concentration would induce cellular response such as cell proliferation. However, too high extracellular Ca^{2+}ions concentration could object to overexpression of calcium channel. When Ca^{2+} ions enter the cytoplasm, calcium phosphate concentrations would increase. This increase would stimulate endoplasmic reticulum to secrete intracellular Ca^{2+} ions that would disrupt intracellular

Ca^{2+}homeostasis, as a secondary messenger in maintaining cell function, and would resulted in mitochondrial mediated apoptosis (Saunders *et al*, 2007; Motskin *et al*, 2009; Yuan *et al*, 2010).

CONCLUSION

Hydroxyapatite extracted from limestone possessed hydroxyl and phosphate groups, has crystal form, has the highest elemental compositions which were O, Ca and P and Ca/P ratio was 1.64, which were typical characteristics of hydroxyapatite. Toxicity were absence which satisfied the requirement of bone grafting candidate.

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