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RESEARCH ARTICLE

The Characteristics and Potency of Limestone-based carbonate hydroxyapatite to Viability and Proliferation of Human Umbilical Cord Mesenchymal Stem Cell

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ABSTRACT:

Background: Limestone primarily consists of CaCO3 (calcium carbonate), which have a similarity to one of human bone component, hydroxyapatite (HA), an element of apatite group (Ca10(PO4)6(OH)2). There were several setbacks in the use of artificial hydroxyapatite in the bone repair process; one of them was its relatively higher crystallinity level compared to those of human bone apatite. The addition of carbonate element to hydroxyapatite could improve its characteristics, such as increasing the solubility, decreasing the crystallinity, and changing the morphology of the crystal. That caused carbonate hydroxyapatite is preferable to help in the bone repair process. **Aims:** This study aimed to find the effect of limestone-based CHA on viability and proliferation of hUMSCs, thus discovering the potential of CHA as a bone graft biomaterial candidate derived from limestone. **Methods:** This study used FTIR, EDX, and XRD assays to CHA powder sample derived from limestone found in Padalarang and Cirebon extracted by BBK. Two grams of the sample were placed in the sample holder and examined by computer software. EDX assay was conducted three times in three different points, and the means were recorded. In the XRD assay, a carbon tip was put to the sample holder to allow sample attachment. The recorded data was compared to JCPDS data. Toxicity and proliferation examination of CHA were conducted through MTT assay in human umbilical cord mesenchymal stem cell (hUCMSC) cell lines with four different doses: 50µg/ml, 25µg/ml, 12,5µg/ml, and 6,25µg/ml. **Results:** Limestone-based CA has hydroxyl (OH-), phosphate (PO_4^{2-}), and carbonate (CO_3^{2-}) functional groups. It has crystal particle formation and consists of O, Ca, and P elements. The result of the MTT assay showed limestone-based CHA is not toxic in all concentrations and has the proliferative ability. There were significant differences between the control and treatment groups. **Conclusion:** CHA has OH-, PO_4^{2-}, and CO_3^{2-} function group. It has crystal particle formation and O, Ca, and P elements as its composition, with a Ca/P ratio of 1,67. It shows no toxicity to hUCMSC in all doses and has the ability to stimulate hUCMSC proliferation.

KEYWORDS: Carbonate Hydroxyapatite, Limestone, Medicine, Cytotoxicity, Mesenchymal Stem Cells.

INTRODUCTION:

Bone grafting technic is often used for periodontal defect, apicoectomy, osteogenesis distraction, and sinus lifting in dentistry[1]. Periodontal disease prevalence in all ages in Indonesia is 96,58%[2,3]. Based on WHO data in 2018, severe periodontal diseases resulting in bone

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defect and tooth loss were found in 15-20% of middle age groups of 35-44 years old[2]. The demand for bone graft in the US amasses to more than 1.5 millions graft every year[4], even doubled in 2020[5,6]. Autograft is a gold standard of the bone graft, but its availability is limited, thus to meet the high demand of bone graft, many researchers try to discover alternative material sources for the bone graft. Regenerative medicine has become a great advancement in medicine technology, especially in tissue and organ failure treatment[7].

Several notable characteristics of bone graft are: having osteogenic, osteoinductive, and osteoconductive characteristics; able to stimulate neo-angiogenesis; showing no antigenic beam reaction, teratogenic and carcinogenic; having sufficient quantities for the supplies; showing good mechanical strength; having minimal morbidity risk; possessing hydrophilic characteristic; and easy to handle with a minimal budget[8,9]. It also needs to be non-toxic, stimulate cell attachment and proliferation[10], have good solubility, and appropriate degradation corresponds to bone remodeling timeline[10–12].

A recent study showed that hydroxyapatite (HA) could be extracted from limestone, which primarily consists of calcium carbonate ($CaCO_3$), as an alternative material for human bone defect replacement in medicine. $CaCO_3$ has a similarity to one of human bone component, hydroxyapatite (HA), an element of apatite group ($Ca_{10}(PO_4)_6(OH)_2$)[13].

Hydroxyapatite is widely used in medicine for artificial bone and to fill the osteoporosis bone. Despite its biocompatible, non-toxic, and osteoconductive characteristics, HA shows a limitation in the bone repair process as it has higher crystallinity than human bone apatite[14,15]. According to previous studies, the addition of carbonate elements to hydroxyapatite could improve its characteristics, such as increasing the solubility, decreasing the crystallinity, and changing the morphology of the crystal[16].

Carbonate hydroxyapatite (CHA) is a nonstoichiometric version of HA[17]. Carbonate substitution on its crystal structure is known to weaken apatite structure and increase its solubility, resulting in CHA being more chemically similar to human bone.[18] Compared to HA, CHA has more distinct advantages in bone remodeling[16]. The chemical formula of CHA is $Ca_{10}(PO_4CO_3)_6(OH)_2$ with a Ca/P ratio of 1,6-2,0[16].

Limestone-based CHA from Cirebon, West Java, extracted by Balai Besar Keramik (BBK) Indonesia, has a potential application as bio-ceramic material in the medical field for a bone substitute in bone defect therapy. As a medical biomaterial candidate, it needs to pass several requirements, and one of the essential characteristics is the biocompatibility[19,20].

Biocompatibility examination in this study was conducted through cytotoxicity assay to quantify cell viability and cell proliferation, using MTT assay. MTT assay is a sensitive, quantitative, and reliable coulometric assay to quantify cell viability, cell proliferation, and cell activity. MTT assay relies on mitochondrial dehydrogenase in the living cell, reducing the yellow-colored MTT to purple-colored formazan crystal. The number of cell formation was accounted through spectrophotometry to show the number of the living cell[12].

The general procedure in tissue engineering uses the cell-based methodology approach, for example, by combining bioceramic scaffold with adult stem cell. Adult mesenchymal stem cells (MSC) can differentiate into various mesenchymal cell lineages, including cartilage and bone[21]. One of the sources of MSC is human umbilical cord mesenchymal stem cell (hUCMSC) originated from umbilical cord tissue[22]. The hUCMSC can be easily isolated and stored as it was a disposable tissue without any ethical issues[22]. It also can differentiate faster than BM-MSC[13], which made hUCMSC more preferable for this study.

As carbonate apatite is a bioceramic with bone substitute/bone graft material potential in dentistry[14], there is a necessity to identify its mechanical characteristics through FTIR, SEM, EDX, XRD, and biocompatibility characteristics, such as its cytotoxicity and proliferation ability in hUCMSC, before its clinical use in regenerative dentistry.

Thus, this study aimed to find the effect of limestone-based CHA on the viability and proliferation of hUMSCs, thus discovering the potential of CHA as a bone graft biomaterial candidate derived from limestone.

MATERIALS AND METHODS:

CHA synthesis:

The base material of HA was obtained from HA synthetization with the wet precipitation method. The carbonate source was obtained from $CaCO_3$ (Cirebon, Jawa Barat, Indonesia) and $MgCO_3$ (Brataco, Surabaya, Indonesia). Dry mechanosynthesis was done in single HA, HA-$CaCO_3$ combination with a ratio of 6.67:1, and HA-$MgCO_3$ combination with a ratio of 6.67:1. Each material was ground in a 5kg size of porcelain pot mill using 25-30mm size porcelain milling balls with the ratio of material: ball at 1:1. The rotation speed use for dry mechanosynthesis was 80rpm for 48 h.

Fourier Transform Infrared Spectroscopy (FTIR) assay procedure:
Limestone-based CHA was prepared. After switching on the FITR program (Shimadzu Corporation, Kyoto, Japan), FTIR 8400s option was chosen on the menu column. The scaffold sample was put on the sample holder and positioned in the interferometer of the FITR tool (Shimadzu Corporation, Kyoto, Japan). BKG start button was clicked to start the examination. CALC menus were optioned to see the peak number as the result of the spectra. The setting noise level was set at 0,1 and the threshold at 7,8. Those setting was to determine the appearances of the result data. The result of the FTIR was a graphic that needs to be consulted to the peak table (SOP from Shimadzu 2007).

Scanning Electron Microscope Electron connected to Dispersive X-ray Spectroscopy (SEM EDX) assay procedure:
SEM EDX assay was done by put 2 grams of CHA sample on the pin holder with a carbon tip to allow the attachment of the sample. The prepared sample was then inserted into the EDX tool (EDAX AMATEK Inc., Pennsylvania, USA). In the computer, SEM software was opened, and beam on was started to begin the vacuum for 5 minutes. After vacuuming, the examination point was decided, and both SEM and EDX software were linked to collect the data from the sample.

X – Ray Diffraction (XRD) assay procedure:
The monitor of the XRD tool was circled the 2 gram of sample in the position of 2θ angles. The monitor was paralleled to allow its axis to go through with the appropriate angles to the rotation axis of the sample. The result of X-ray diffraction was printed to the paper with the radiation sources of Cu, KA, and filtered with nickel. The obtained data was compared to Joint Committee Powder Diffraction Standard (JCPDS). Diffraction value with diffraction intensity and angle was analyzed to determine the crystal structure by comparing to *Inorganic Crystal Structure Database (*ICSD) data for the tested sample[16,23].

hUCMSC isolation procedure:
The procedures of this study were ethically approved with IRB no. 205/HRECC.FODM/V/2019. The umbilical cord was obtained from *section cesaria*n deliveries. The cord was cut to 0.5mm^3 fragments after the vena and arteries were dissected. Enzymatic digestion through trypsinization using 0.25% trypsin was done for 40 minutes at 37°C, and after being centrifuged, the supernatant was discarded. That process was repeated twice. After trypsinization, the sample was put into PBS solution containing 0,75mg/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA) and 0,075 mg/ml DNAase I (Takara Bio, Shiga, Japan), incubated at 37°C for 60 minutes before being stirred in magnetic stirrer at 37°C. The mixture was filtered with 100μm filer to obtain pellet cell suspension.[17]

hUCMSC culture:
Cell culture was conducted in accordance to the protocol in the Stem Cell laboratories, Institue of Tropical Disease, Universitas Airlangga. Cells were planted with 1 x 10^6 cell/cm$_2$ density and were put into culture tubes. The growth medium contained α-modified essential medium (α-MEM), 20% *fetal bovine serum* (FBS) (Biowest), penicillin/streptomycin 100U (Sigma-Aldrich, St. Louis, MO, USA), and amphotericin (Sigma-Aldrich, St. Louis, MO, USA). The cultures were incubated in CO_2 5% incubator at 37°C. The growth medium was replaced every two days. After 80-90% confluence was achieved, the attached cell was transferred to other culture plates with 1 x 10^4 cell/cm^2 density with a growth medium for the expansion.[18]

hUCMSC characterization:
a) Immunocytochemistry:
The mesenchymal stem cell characterization was defined using immunohistochemistry examination. The monolayer cell was turned into single cell through the trypsinization process. After trypsin addition, centrifugation at 1600 rpm was done for 5 minutes. One ml of DMEM-LG growth medium was added to the cell pellet, and after resuspension, 20μl of the cell was planted to special object-glass. Object glass was put into a box with wet paper, and incubation was done for 1 hour at 37°C. After the incubation, the cell was fixated using formaldehyde 3% for 15minutes at room temperature. After that, it was washed in PBS four times, dried, blocked with PBS containing 1% serum for 15 minutes at room temperature, before being rewashed in PBS four times, then dried. Each sample was added with CD45 or CD105 antibody, labeled by FIT-C, and incubated at 37°C for 45 minutes. After being washed in PBS four times, water droplets surrounding the object-glass were dries using tissue. After dropping 50% glycerin on the object-glass, the cell was observed using a fluorescence microscope at 40x magnification. It was positive if fluorescence was observed in the cell and negative if there was nothing[19,22].

b) Viability and proliferation assay of hUCMSC:
A viability cell assay procedure was conducted using MTT assay. Before the assay, CHA was sterilized using ultraviolet (UV). On the microplate, each well was added with 200μl medium and 5 x 10^4 cells, following by incubation for 1x 24 hours at 37ºC with 5% CO_2 humidity.

CHA sample was immersed in the medium for 1x24 hours and stored in the refrigerator at 4ºC before the assay. Cell viability was observed after the sample has

24 hours of contact with *hUCMSC, while proliferation was* observed after 72 hours of contact at 37ºC with 5% CO_2 humidity. MTT (Thermo Fisher Scientific, USA) was then administered for 25 µl on each well followed by incubation for 4 hours at 37ºC with 5% CO_2 humidity. The solution on each well was discarded, and 200 µl DMSO was added to dilute the crystals. Purple formazan was observed on each well using the inverted microscope. The microplate was then put into an ELISA reader (Bio-Rad, USA) with a 595 nm wavelength for the final result of *optical density* (OD).[20] To decide the viability and proliferation rate of hUCMSC (%), the following formula was used[24,25]:

$$\% \text{ cell viability} = \frac{\text{OD of treatment group+media}}{\text{OD of cell group+media}} \times 100$$

RESULTS:

FITR assay:

CHA has functional groups of hydroxyl (-OH), phosphate (PO_4^{3-}), and carbonate (CO_3^{2-}). The wavenumbers of the -OH function group was around 3700 – 3600 cm^{-1}, 3550 – 3500 cm^{-1}, and 300-1700 cm^{-1}. The PO_4^{3-} function group, has wavenumbers of 1100 – 950 cm^{-1}. Wavenumbers of CO_3^{2-} function group, was 1450 – 1410 cm^{-1} and 880 – 800 cm^{-1}.[26]

The result of FITR for CHA was in the form of the peaks on transmittance (%) to wavenumbers (cm^{-1}) graph. CHA from BBK showed -OH bonding at the peak of 3649.65 cm^{-1}. PO_4^{3-} was found at the peak of 961.62 cm^{-1}, 1026.48 cm^{-1}, and 1086.79 cm^{-1}. While CO_3^{2-} was shown by the peak at 1425.12 cm^{-1} as shown on Figure 1.

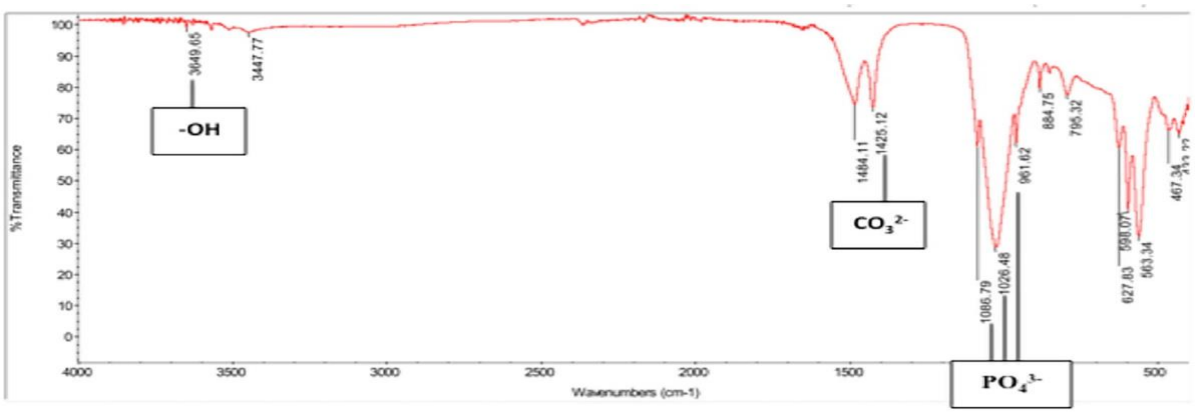
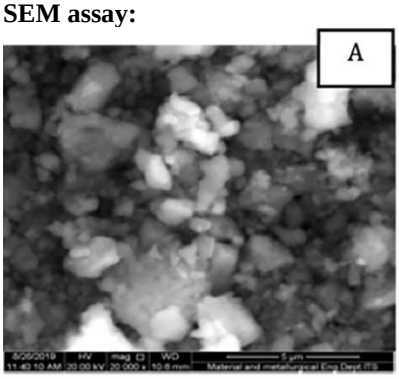
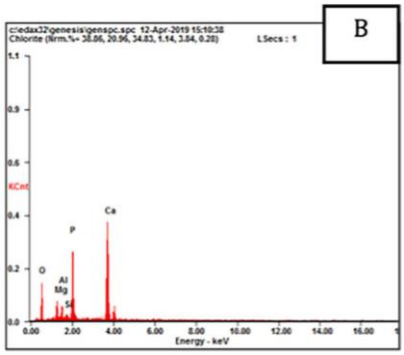


Figure 1. FTIR assay result of CHA.

SEM assay:





C

EDX		
Unsur	Wt %	At %
O	36.47	56.24
Mg	04.46	04.41
Al	03.24	03.02
Si	00.92	00.73
P	17.05	13.29
Ca	37.02	22.30

Figure 2. (A) The result of SEM assay for CHA at 20,000x magnification, (B) EDX CHA, (C) the element contents of CHA.

Figure 2A shows the graphic result of the SEM assay for CHA samples extracted by BBK Indonesia. The graphic (see Figure 2B) is the energy comparison graphic using kilo electron volt (keV) as its unit, while the X-ray intensity used kilo count (kCnt) unit. The graphic shows CHA BBK contains oxygen (O), magnesium (Mg), aluminum (Al), silicate (Si), phosphor (P), and calcium (Ca). The percentage of each element contained by CA can be seen in Figure 2C, with the highest percentage is 38, 40 Wt % for Ca and the lowest is 00.92% for Si. The Ca/P ratio of molar is 1.67 (22.30 At% for Ca and 13.29 At % for P).

XRD assay:

The result of the XRD assay for limestone-based CHA can be seen in Figure 3. It shows a graphic pattern with a firm peak which can be interpreted that the sample has crystals formation.

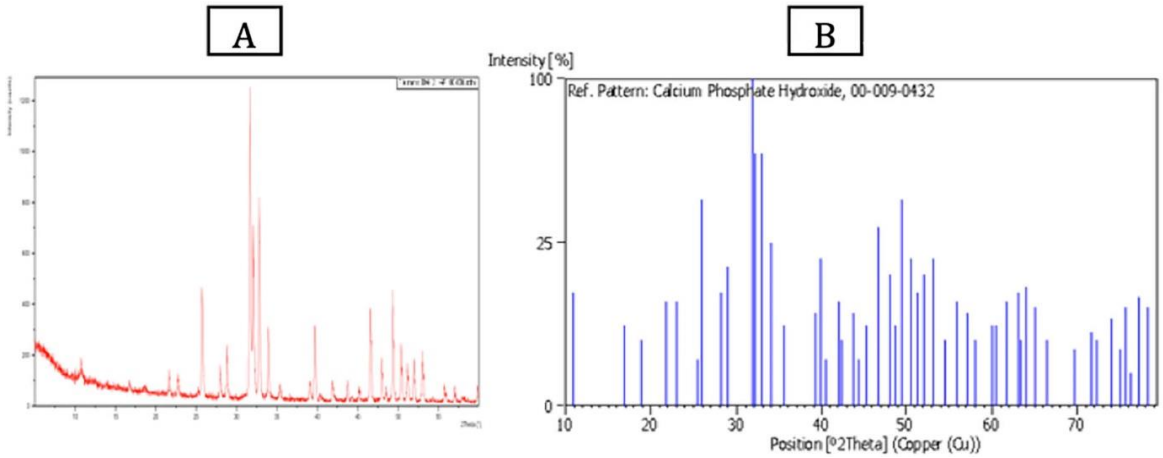


Figure 3. The XRD assay graphic result of limestone-based CHA (A), Joint Committee on Powder Diffraction Standards (JCPDS) with code 00-009-0432(B).

Characterization of hUCMSC:

The characterization of hUCMSC was done using immunohistochemistry with the FIT-C label. The immunohistochemistry examination showed a negative result for CD45, a hematopoeitic stem cell marker. Both CD90 and CD 105 staining showed a strong expression of positive MSC under the fluorescence microscope. The hUCMSC characterization using CD 45, CD 90, and CD 105 markers under fluorescence microscope at 40X magnification.

Table 1. The viability and proliferation percentage of hUCMSC after direct exposure of limestone-based CHA

No	CHA concentration	N	hUCMSC viability 24 h			hUCMSC Proliferation 72 h		
			Mean OD	SD	% Viable cell	Mean OD	SD	% Viable cell
1	50 µg/ ml	5	0.59	3.89	101.70	0.77	2.55	113.61
2	25 µg/ ml	5	0.53	4.83	89.92	0.67	1.22	97.34
3	12.5 µg/ ml	5	0.52	4.82	87.76	0.65	1.58	95.22
4	6.25 µg/ ml	5	0.49	5.58	81.84	0.64	2.55	93.75

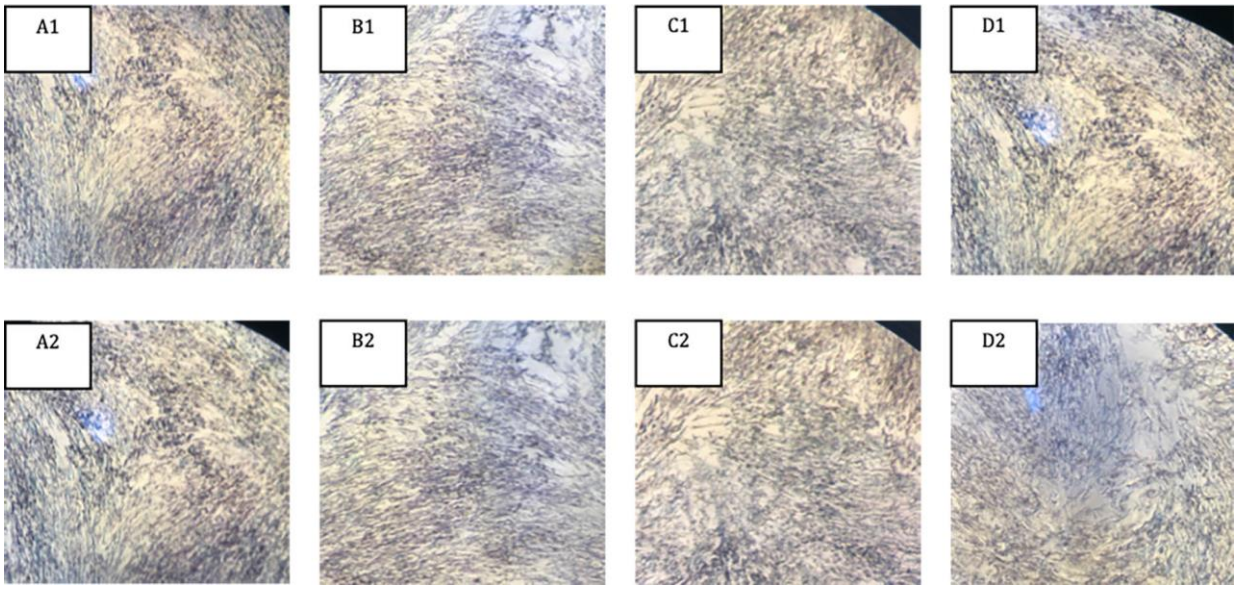


Figure 4. Microscopic photo of MTT assay for the treatment group after exposed with: CHA 50 µg/ ml for 24 h (A1) and for 72 h (A2); CHA 25 µg/ ml for 24 h (B1) and for 72 h (B2); CHA 12,5 µg/ ml for 24 h (C1) and for 72 h (C2); CHA 6,25 µg/ ml for 24 h (D1) and for 72 h (D2).

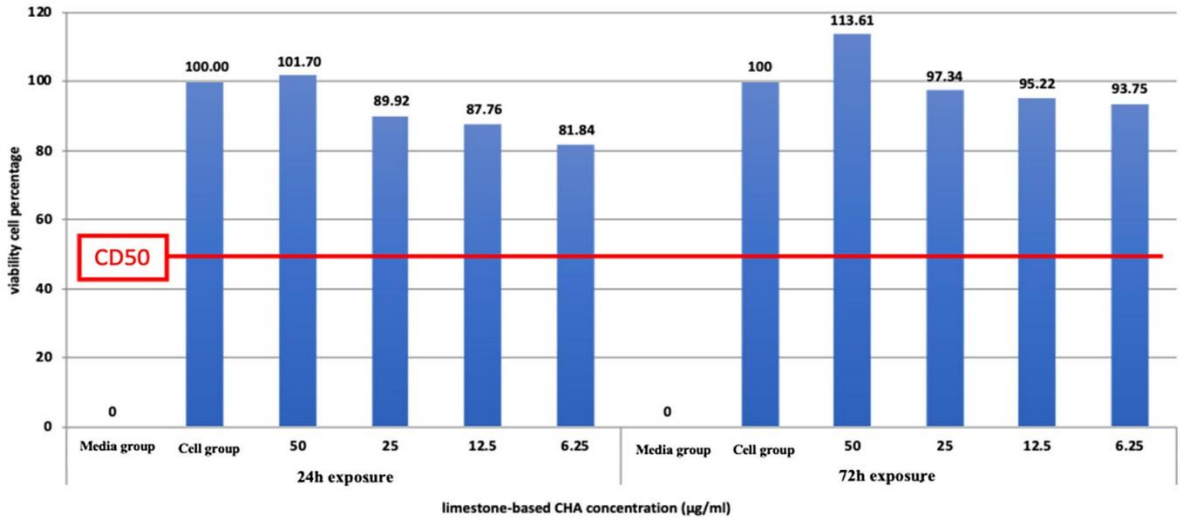


Figure 5. Correlation graphic between limestone-based CHA concentration and the mean of viability cell percentage after exposure for 24 h and 72 h.

Based on Table 1, the highest mean of viability cell percentage was found in cells exposed with CHA 50 µg/ml for 24 h (101,70%) and for 72 h (113.61%). In contrast, the lowest was found in the cell exposed with CHA 6.25µg/ml for 24 h (81,84%) and 72h (93,75%).

The graphic in Figure 7 showed the viability cell percentage of all treatment groups higher than 50% (CD_{50}), and the number went higher with higher concentration and longer contact duration. The analysis of one-way ANOVA continued with post hoc Tukey HSD assays demonstrated significant differences between each treatment

DISCUSSION:

The characteristic assays of CHA in this study aimed to analyze the functional group, particle formation, contents, and cytotoxicity of limestone-based CHA. This study also observed the proliferation of hUCMSC after exposure to limestone-based CHA. FTIR assay meant to identify the presence of hydroxyl (-OH), phosphate (PO_4^{3-}), and carbonate (CO_3^{2-}) functional groups[23]. CHA in FTIR assay showed the presence of OH bonds for the peak in 3649.65 cm^{-1}. Phosphate was observed by the appearance of peaks in 961.62 cm^{-1}, 1026.48 cm^{-1}, and 1086.79 cm^{-1}. Carbonate was identified with the peak in 1425.12 cm^{-1}. The hydroxyl formation group was recorded by the typical peal in the region of 3800-3200 cm^{-1}, originated from the hydrated inorganic compounds. Hydrated inorganic compounds were the result of the production process of hydroxyapatite with water addition, which lead to the water molecule on the crystal structure of those materials producing a peak due to the stretching and bending of O–H bonds. Phosphate groups (PO_4^{3-}) in FTIR assay observed from the presence of peaks in the phosphate bond range, which was 1100 – 950 cm^{-1} [26].

Based on the XRD assay, there was a clear graphic for each peak, and it has the same peak pattern compared to JCPDS, which means the particle formation of those materials was crystal formation. The comparison result between the XRD assay of CHA and JCPDS data has similar positioning. Based on those similarities, it can be concluded that limestone-based CHA was in accordance with standard data of JCPDS for diffraction groups of apatite mineral (CaOH) from hydroxy calcium phosphate compound with reference code of 00-009-0432. The result of the XRD assay on CHA was supported by the study of CHA using hydrothermal treatment, which resulted in the carbonation reaction resulted in more peaks. This finding demonstrated that the crystal of CHA from the carbonation reaction was largely produced but in a smaller size. HA was fully transformed into CHA after 72 h of carbonation. The result of carbonation was expected to show a good tissue reaction for biomedical application[9].

The composition of limestone-based CHA containing oxygen (O), calcium (Ca), and phosphor (P) was proved by EDX assay. Based on the result of EDX, CHA BBK Indonesia contains oxygen (O), magnesium (Mg), aluminum (Al), silicate (Si), phosphor (P), and calcium. The percentage of each element in CHA can be observed in figure 2(C). Calcium has the highest concentration with 38,40 Wt %, while the lowest was Si with 00,92%. The comparison of Ca/P molar were 22,30 At % and 13,29 At %, thus the Ca/P ratio was 1,67 (Figure 2).

Cell viability is an important marker in the tissue engineering concept, as this assay was conducted to observe the toxicity of the tested material. This study conducted viability and proliferation assays on hUCMSC after exposed to limestone-based CHA at four different concentrations: 50µg/ml, 25µg/ml, 12,50µg/ml, and

6,25µg/ml. The viable cell showed an active metabolism of mitochondria by turning MTT into a purple-colored formazan product (Figure 6 and 7). The dead cell lost its capability to turn MTT into formazan; thus, the color intensity formed by formazan could become the living cell marker[27].

Limestone-based CHA extracted by BKK in the concentration of 50 – 6,25µg/ml has a viability cell percentage higher than 50%, which according to CD_{50}, all concentrations showed no toxicity. It was supported by a previous study that claimed that through cytology, the biocompatibility standard of biomaterial was 60%, which means a biomaterial considered biocompatible is the percentage of the viable cell after exposure and contact is more than 60%[28]. Other research used IC_{50} standard as biocompatibility standard of biomaterial. The IC_{50} standard stated that a biomaterial is compatible if the viable cell percentage after exposure and contact was 50%[24,25,29–31]. The high viability cell percentage in this study can be resulted by the high ratio of Ca/P in CHA. Based on the results of this study, higher concentration of calcium resulted in higher proliferation. Limestone-based CHA from Padalarang and Cirebon extracted by BKK has a Ca/P ratio of 1.67, similar to the Ca/P ratio of human bone, in the range of 1,33-1,67[9,32]. A high concentration of Ca will increase the expression of the Ca channel, increased cell proliferation. Calcium sensing receptor (CaSR) can detect the changing of external Ca^{2+} concentration and activate the Ca channel to increase e Ca^{2+} influx. A higher concentration of Ca^{2+} will result in a higher influx, which stimulates cell proliferation as the cellular response[33,34].

Cell viability increased possibly due to cellular activity with similar conduct as the cellular activity happened in the human body when extracellular matrix (ECM) interacted with cell[35]. The living cell could not directly interact with foreign material. Those interactions need adhesive molecule proteins such as fibronectin (FN), vitronectin (VN), fibrinogen (FG), and laminin (LN). Adhesive molecule protein *in vivo* was obtained from physiological fluid adsorption, while *in vitro, it* was obtained from protein adsorption from media culture. The adsorbed adhesive molecule protein produced a protein layer on the CHA surface which played an essential role in cell and biomaterial bone graft interaction[18].

CONCLUSION:

CHA from BBK Indonesia has -OH, PO_4^{3-}, CO_3^{2-} functional groups. It has crystal particle formation containing O, Ca, and P elements with a CA/P ratio of 1,67. All concentrations tested showed no toxicity to hUCMSC and had the ability to stimulate the proliferation of hUCMSC.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation.

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