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In vivo histomorphological evaluation of geopolymer-carbonated apatite nanocomposites implanted on rabbit tibia at early bone healing

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ABSTRACT

Introduction: Dental implants have become more desirable treatment for replacing missing teeth. The mechanical properties and biocompatibility of titanium and zirconia are excellent but they are less bioactive. The chemical composition of the carbonate apatite is similar to enamel and dentin. Geopolymers are inorganic polymers and they are similar to ceramics, they have an excellent mechanical properties, bioactivity, biocompatibility.The purpose of this study is to assess the early bone healing in osseointegration at geopolymer-carbonated apatite (CHA) nanocomposites. **Methods:** Geopolymer-CHA nanocomposites with diameter of 3 mm and length of 6 mm is placed in tibia of eight male New Zealand White rabbit whose body weight is 3 to 3.5 kg and 6 month ages. Experimental subjects were randomly assigned to 2 groups for assessing the bone healing capability around samples to 14 and 28 days histomorphologically. Wilcoxon test was performed and $p < 0.05$ was considered significant, using Minitab software version 13. **Results:** Granulation tissue, woven, and lamellar bone were analyzed. In the 14th day revealed a reactive bone formation. Osteoblasts, osteoids, and osteocytes showed more mature and woven bone became denser on the 28th day. **Conclusion:** Geopolymer-CHA nanocomposites could be considered as a potential dental implant material from mechanical and biological properties point of view.

Keywords: Histomorphological, geopolimer-CHA nanocomposites, dental implant, early bone healing

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INTRODUCTION

Implant supported denture are able to restore the masticatory function which affects the quality of life for edentulous patients. Bone healing event after implant placement is preceded by several stages until osseointegration occurs, which characterized by close contact between the implant surface and the surrounding bone.[1,2,3] Currently, titanium and zirconia are the most widely used as dental implant materials due to their biocompatibility and mechanical properties. However, there is disadvantage the elastic modulus of titanium (2222.7 ± 277.6 MPa) and zirconia (90 GPa) values are greater than enamel (1338.2 ± 307.9 MPa) and dentin (1653.7±277.9 MPa), less bioactive to stimulate osseointegration even the surface has been modified.[4,5,6,7,8,9,10]

Recently a large amount of research has been undertaken to develop materials that are suitable and adaptable to the biological environment to stimulate bone healing using inorganic materials such as calcium phosphate to simulate the chemical properties of bones and teeth. As well as geopolymer according to Davidovit, geopolymers are inorganic materials that resemble ceramics, consisting of aluminosilicate precursors which are activated by a solution of alkaline activators such as sodium hydroxide and sodium silicate.[11]

Geopolymer have excellent mechanical properties including compressive strength, while from their biological properties they are bioactive, biocompatible, suitable to replace the hard tissue, and safe for the environment.[12,13,14,15,16] Combining geopolymers with other materials is a way of enhancing the excellent properties of geopolymers. According to Tippayasam et al.17 the bioactive properties and biocompatibility of geopolymers are shown by their ability to increase bone-cell activity for new bone formation. Supported by Cataura et al.18 that metakaolin-based geopolymer can be used as a hard tissue prosthesis.[12,17,18]

Carbonate and apatite are a mineral where apatite is calcium phosphate such as hydroxyapatite and fluorapatite. Carbobate apatite [Ca10(PO4) x(CO3)y(OH)z] is widely used as a biomaterial due to their similarity to bone and tooth composition, their ability to stimulate bone regeneration, allows bone growth onto and stimulates new bone formation. In vitro studies have demonstrated the role of calcium in the differentiation of preosteoblasts to osteoblasts.[12,19,20] According to Park et al.[21] calcium phosphate biomaterial is useful for bone repair because of their similarity to bone minerals and are biocompatible and osteoconductive.[21]

The process of healing bones after implant placement is repetitive process similar to bone development.[3,22,23] The first stage of the bone healing process is hematoma, followed by acute inflammation, granulation tissue, callus formation, and remodeling. The hematoma and inflammation stage lasted few days to a week after the fracture and is followed by the granulation tissue formation rich in mesenchymal cells which are the essential for bone formation and developing neovasculature which is the key factor for bone remodeling embedded in an unorganized extracellular collagen matrix in several days to a week after the injury, which as a starting point of bone healing.[24,25]

Subsequently chondrocytes produce cartilage known as soft callus several weeks after the injury. At the same time, further bone formation occurs by intramembranous ossification especially in non-hypoxic areas. Mesenchymal cells differentiate into osteoblasts which in turn form woven bone. Blood vessel growth extending into the scaffold cartilage bridging the fracture gap, at the same time differentiating osteopregenitor cells into osteoblasts continues and followed by depositing woven bone on the cartilage scaffold, this stage lasts for several weeks or months and known as hard callus formation.

In the final stage, both immature woven bone and cartilage matrix are resorbed by osteoclasts, and the remodeling process begins and will last for several months to several years.[24] In this research, we performed in vivo histomorphological evaluation of geopolymer-carbonated apatite nanocomposites implanted on rabbit tibia at early bone healing. The purpose of this study is to assess the early bone healing in osseointegration at geopolymer-carbonated apatite (CHA) nanocomposites.

METHODS

Sample preparation

Sodium hydrogen carbonate, calcium nitrate tetrahydrate, di-ammonium hydrogen phosphate, sodium hydroxide were obtained from Merck and sodium silicate from Sigma-Aldrich. Kaolin was provided by the Center for Ceramics, Ministry of Industry in Indonesia. Metakaolin was obtained by heating kaolin at 800^0 C. Carbonated apatite was synthesized by precipitation method.as followed Ammonia solution was added drop-wise to 100 mL of calcium nitrate tetrahydrate 0.1 M and stirred until pH reached 9, followed by addition of 100 mL of diammonium hydrogen phosphate 0.06 M and 100 mL of sodium hydrogen carbonate 0.06 M. Ammonia solution was added to adjust pH to 9. Solution was stored at RT for 12 h.

The suspension was centrifuged at 8000 rpm. The precipitate was separated and dried in an oven at 80°C for 30 min. The sample was then calcined at 700°C for 2 h in air atmosphere. The final product was ground using a mortar, resulting in a fine white powder. The geopolymer sample was prepared by mixing metakaolin with alkali activator containing sodium silicate and 12 M NaOH with w/w ratio of 2:1. The resulting paste-like mixture was poured into an acrylic mold and stored at RT for 30 minute and then dried in the oven at 80° C for 20 h and samples were cooled to RT.

Biological characterisation

Material cytotoxicity testing was carried out using the trypan blue method to verify the morphology and viability of fibroblast cells. Sampels were tested in cylinder form with dimension of 3 mm and thickness of 6 mm and evaluated in duplicate. Samples were washed for 96 hours in demineralized water before used. Demineralized water is changed every 24 hours.

Fibroblasts are cultured in medium RPMI 1640 (Gibco, USA) before placed in 6 wells, each containing 100% cells, followed by an incubation stage for 24 hours, 48 hours, and 72 hours at 37 °C. After incubation, all culture media are aspirated into a centrifugal tube, each well is washed with 1 ml of saline phosphate buffer pH 7.4 (Gibco, USA) and collected into a centrifugal tube.

One ml of trypsin (Gibco, Denmark) is pipetted into each well, then incubated for 5 minutes. The incubated trypsin is aspirated and collected into each tube, cells are quantified with a hemocytometer (Neubauer Improved, Marienfeld, Germany) and cell morphology is analysed using a Motic Inverted Microscope (Olympus CK40) with a 10 MP resolution camera.

Physical characterisation

Material characterisation was carried out by Fourier-transform infrared spectroscopy (FTIR) measurements recorded with KBr pellets on Prestige 21 Shimadzu to detect the differences in functional groups. The spectra was measured at a resolution 4 cm^{-1} with the number of scans 40 and at wavelength 4500–400 cm^{-1}. X-ray diffraction (XRD) analysis measured on Rigaku using Cu anode with wavelength of 1.5406 Å to detect the crystalline structure. The XRD measurement was collected at 2θ range of 15°–60°.

Hardness values of all samples were measured by HMV-G21ST series Shimadzu Micro Vickers Hardness Tester. Nanocomposites specimens were prepared in cylinder of 5 mm diameter and 6 mm thickness, with indentation load of 100 gf on three different points for each sample. Specimens for diametral tensile strength were prepared in a cylinder of 6 mm diameter and 3 mm thickness and for compressive strength were prepared in a cylinder of 4 mm diameter and 6 mm thickness. The measurement of the specimens was conducted with load cell F 1 kN, crosshead speed of 1 mm/s. Specimens for three points bending were prepared with bar of 25 mm × 5 mm × 2.0 mm with load cell 1 kN, 1 mm/s, and span of 10 mm.[26,27]

Animal

Eight male New Zealand rabbits, aged 6 month of age (weight 3.0-3.5 kg) were used in this research. During the experiment, each rabbit was kept in their own cage and fed once per day with standard laboratory diet and given tap water chow *ad libitum*. Animal selection, management, and surgery protocol had already gaining approval from IPB University Ethical Committee numbered 151/KEH/SKE/VIII/2019. The study began after the animal had been adapted for 2 weeks.

Study design

The research was conducted on eight healthy male breeding New Zealand white rabbits, 6 month of age and weight of 3.0-3.5 kg. Experimental subjects were randomly assigned to 2 groups for evaluating the early stage of bone healing capability around samples, one group of 4 rabbits were evaluated to 14 days, and the other ones were to 28 days.

Surgical procedure

Rabbit fur is shaved and the surface cleaned with iodine solution in tibia metaphysis before surgery. Rabbits were anesthetized with combination of ketamin hydrochloride® (Pharmamadix Corp, Peru) 10 mg/kg and xylazine hydrochloride® (Interchemie werken "De Adelaar" BV, Venray, Holland) 3 mg/kg intramuscular. Additionally, a local anesthetic of lidocaine® 0.5 ml/adrenaline (PT.Bernofarm Pharmaceutical company, Jakarta) was applied subcutaneously. During surgery all rabbits were infused with lactated Ringer's solution. After the skin and subcutaneous tissues incision, muscles and periosteum were dissected to expose the bone surface of tibia metaphysis. Bone defects of 3 mm wide and 6 mm deep were performed by low speed drill with continuous irrigation.[28]

Samples with 3 mm in diameter and 6 mm in length were thoroughly rinsed with sterile saline before insertion and positioned in tibia metaphysis as showed in Fig. 1A. The wound was closed with resorbable 3.0 polyglygolic acid coated white sutures (Surgifit®, Busan, Korea), and the samples were allowed to heal under the skin as showed in Fig.1B. Analgesic Fortis® (Dong Bang Co, Ltd, GYeonggi-do, Korea) 1,1 mg/kg and Genta-100® (Interchemie werken "De Adelaar" BV, Venray, Holland) 10 mg/kg were administered via intramuscular injection after surgery and following 3 days after surgery, topical application of nebacetine ointment® (Pharos, Jakarta) in the wound area until healed.

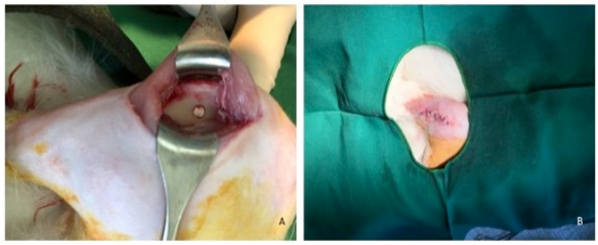

Figure 1. (A). Sample with 3 mm in diameter and 6 mm of length was positioned in tibia metaphisis; (B). Primary wound closure and sample was left to heal

Animal sacrification and retrieval of specimen

The rabbits were euthanized used overdose of penthobarbital sodium phenytoin 0.5 cc/kg body weight intravenous after 14 days and 28 days postoperative. Tibia was dissected and a segment of metaphysis about 2.0 cm in length comprising the sample was obtained for histological study. All dissected bone segments were fixed in 10% neutral-buffered formalin solution for 24 hours.

Histological preparation

After 24 hours of fixation and additional 96 hours of decalcification with a commercial EDTA-hydrochloric acid mixture (Surgipath Decalcifier II, Leica Biosystem, USA). The bone segment was cut longitudinal with a plane of the sample, dehydrated used ascending grades of 70%, 80%, 90%, and 96% alcohol, followed by absolute ethanol 1 and absolute ethanol 2 for two hours each concentration. Dipped in xylol 1, xylol 2, and xylol 3 and followed by paraffin infiltration (Thermo Scientific Histoplast, Cheshire, WA7 1TA, UK). The sample was cut to a thickness of 5 micrometers using microtome, and placed on a slide that had been coated with poly-L-lysine (Sigma– Aldrich, Gillingham, UK).

Put the slide on a hotplate with a temperature of 56-60^0 C for 10 minutes, then stored in an incubator at 38^0-40^0 C for 1 night. After incubated, slides were deparafinized with xylol, followed by rehydration with xylene for 10 minutes and under running water for 5 minutes. Samples were stained with Haematoxylin and Eosin (H&E) to identify areas of new bone formation.

Statistical analysis

Analysis was performed on the percentage of granulation tissue, woven bone, and lamellar bone using Wilcoxon test and $p<0.05$ was stated as statistically significant. Statistical tests were conducted with Minitab version 13 software.

RESULTS

Mechanical properties and characterization of geopolymer-carbonated apatite anocomposites

Enamel is mostly calcified, the hardest tissue, and has a role in resisting pressure during mastication and protect dentin because of it wears resistance due to the hardness. While dentin forms the major part of the tooth and functions in the absorb bite forces because of it is higher force resistance due to the modulus of elasticity.Considering the implant as a substitute for an artificial root that will be in close contact with the surrounding bone so it can distributed into the mastication load that not damage the bone tissue, this synthetic implant material is compared with standard mechanical values of enamel and dentin in the order to simulate dental tissue.

Study showed hardness value (99.207 ± 19.352 VHN) was higher than the hardness value of the dentin (53–63 VHN), compressive strength value (102.849 ± 7.648 MPa) exceeded the enamel value (38.4–86 MPa), but less than dentin (163.1–224.3 MPa). Tensile strength value (12.892±1.651 MPa) is greater than that of enamel 8–35 MPa. Modulus elasticity of sample (13316.650 ± 1576.606 MPa) was closed to dentin (15.000 MPa).[29] Fourier-transform infrared spectroscopy spectrum of sample shown by a profile at 3566 cm^{-1} associated with strong and sharp peak attributed to O-H streaching vibration of carbonate apatite.[30] The other peaks are related to the P-O vibrations, namely 1014-1052 cm^{-1} and 659 cm^{-1}.

Characteristic of carbonate bands also observed at 1415,1462 and 875 cm^{-1} and sample confirm the formation of carbonated apatite type A and B as shown by the peak at 1417 cm^{-1}, 1412 cm^{-1} and type B carbonates at the peak 1415 cm^{-1}[30] as showed in Fig,2 XRD pattern showed the sample consist of pure hydroxyapatite phase according to PDF 2.841998 The hydroxyl apatite in CHA is shown by three main peak of the hydroxyapatite phase angles 2θ of 31.80°, 32.23°, and 32.96°, corresponding to the (121), (112), and (300) crystal plane of the hexagonal structures of hydroxyl apatite as showed in Fig.3

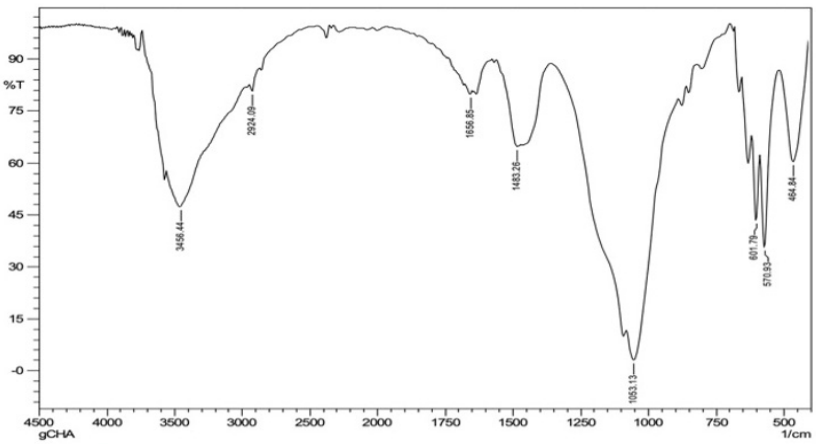

Figure 2. FTIR spectra of geopolymer-carbonated apatite nanocomposites

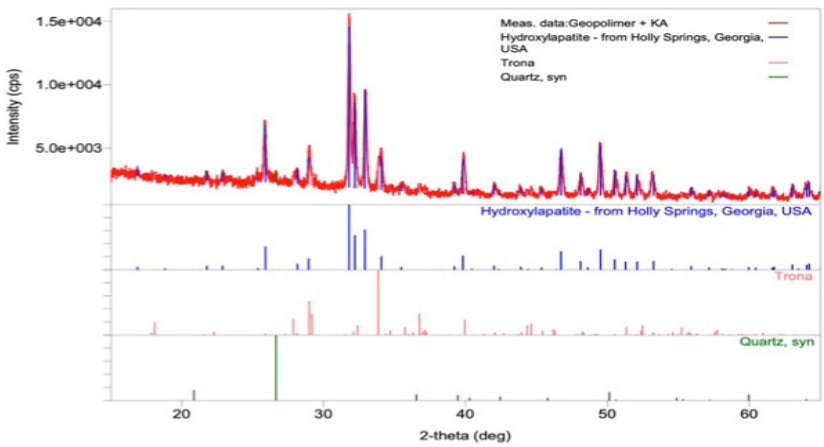

Figure 3. XRD pattern of geopolymer- carbonated apatite nanocomposites

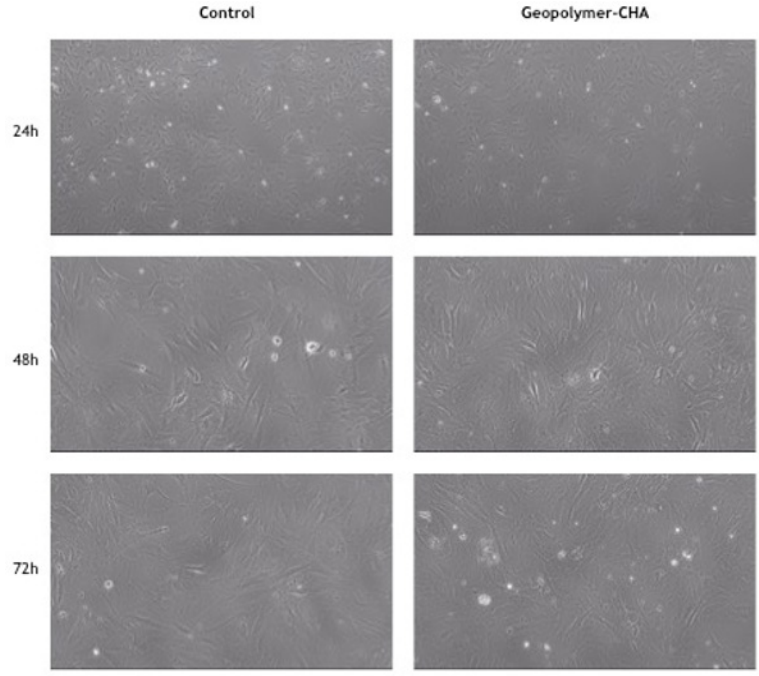

Figure 4. Microscope images of mouse embryonic fibroblasts after 24, 48 and 72h incubation on (A). Control group; (B) Geopolymer-CHA nanocomposites. The bar denotes 50 µm

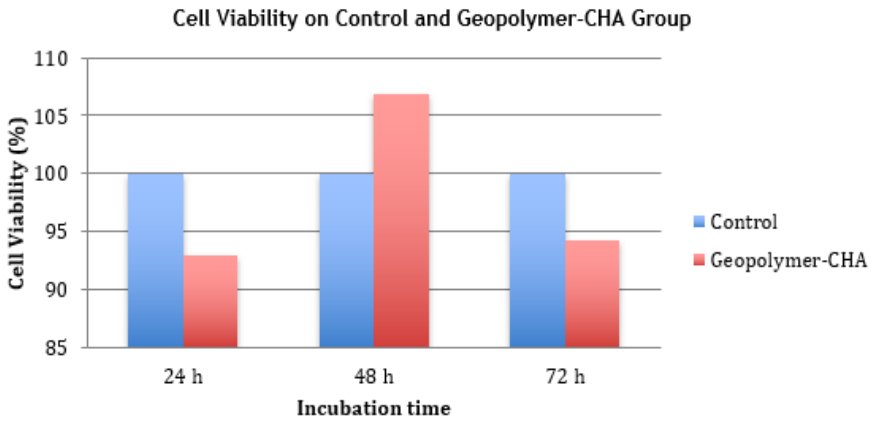

Figure.5 Cell viability of embryonic fibroblasts on control and geopolymer-CHA groups after 24, 48 and 72 h incubation

In vitro fibroblast cell viability test showed that the number of living cells above 80% and no morphological changes. It is noteworthy to mention here that apparently samples were biocompatible. After incubation for 72 h, the percentage of living cells was 94.2% as showed in Fig. 4 and Fig.5

Histological examination 14 days postoperative

In 14 days postoperative, there are no inflammatory infiltrate, bone resorption, likewise allergic reactions, abcesses, and infections during the observation period. Our histological results showed a gap between the sample and the surrounding bone has been filled with granulation tissue containing mesenchymal cells, development of new blood vessels, and fibroblasts in an organized extracellular collagen matrix as showed in Fig.6.

The results of these observations are consistent with Florence et al.[24] statement that the hematoma and inflammation stages will end within a few days to 1 week after the bone fracture and will be replaced by granulation tissue formation. The proliferation of blood vessels will result a better blood supply which allows the recruitment of mesenchymal cells which then differentiate into osteoprogenitor cells, osteoblasts, and eventually lay out woven bone contained of immature osteoids, which indicated

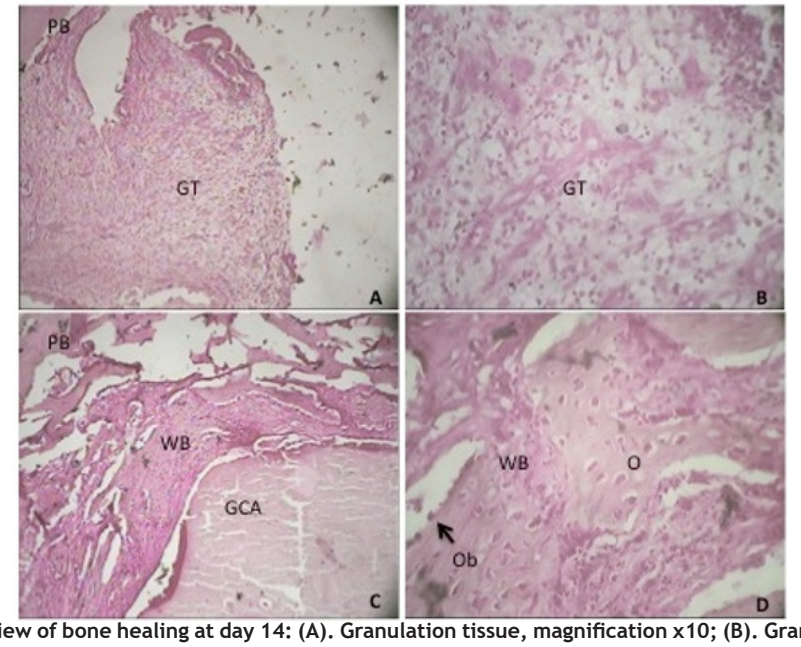

Figure 6.Histological view of bone healing at day 14: (A). Granulation tissue, magnification x10; (B). Granulation tissue consist of vascular proliferation, fibroblasts, and inflammation cells, magnification x40; (C). Parent bone (PB), woven bone (WB) - reactive bone formation, geopolymer-carbonated apatite (GCA), magnification x10; (D). Woven bone (WB), osteoids (O), Osteoblasts (Ob), original magnification x40

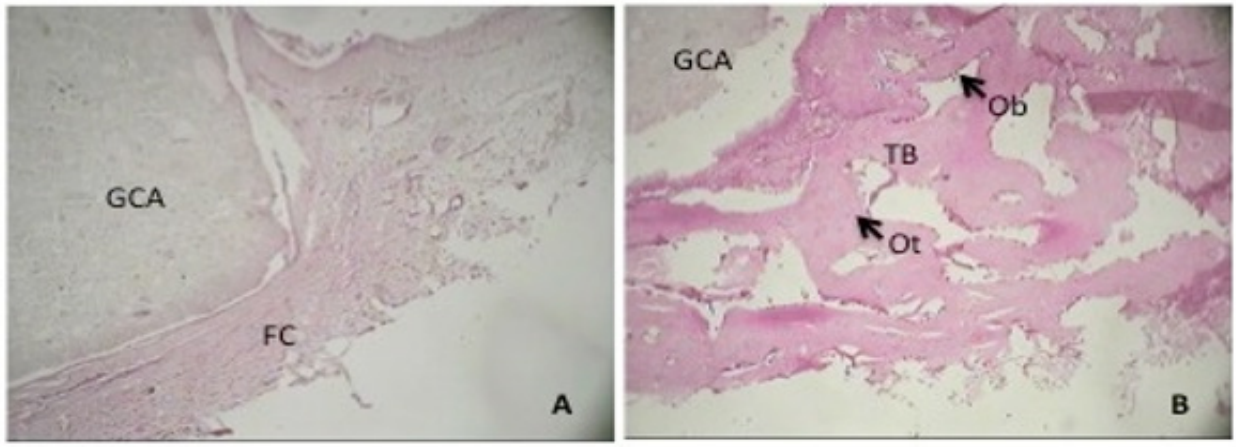

Figure 7. Histological view of bone healing at day 28: (A). Dense fibro collagen adjacent geopolymer-carbonated apatite (GCA), magnification x10; (B). Parent trabecular bone (TB) moved toward geopolymer-carbonated apatite (GCA), Osteoblasts (Ob), Osteocytes (Ot), magnification x40

there was reactive bone formation as showed in Fig.6. This situation was consistent with Chug et al.[31] statement that the formation of osteoid-rich woven bone signifies the beginning of new bone formation.

28 days postoperative

In 28 days postoperative, dense fibro collagen connective tissue that will be cartilage was observed between the samples surface and adjacent bone as showed in Fig 7. These results are consistent with the statement of Florence at.al[24] that chondrocytes produce cartilage and will contact both ends of the fracture within a few weeks of injury. This tissue of cartilage is known as soft callus.[24] Fibroblasts are not as much as 14 days postoperative. The amount of osteoblasts, osteoids, and osteocytes almost the same at 14 days postoperative but immature osteoblasts, osteoids, and osteocytes at 14 days postoperative showed more mature, while the woven bone became denser.

These results are consistent with Chug et al.[31] that at the end of four weeks osteoblasts form a thick layer of tissue around the implant, collagen fibers orient themselves parallel to the implant surface.[31] As stated Christina et al.[2] the progression of wound healing was marked by

Table 1. Granulation tissue, woven bone, lamellar bone at 14 and 28 days

Variable	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
Granulation	0.875	0.125	0.354	0.000	1.000	1.000	1.000	1.000
Woven bone	0.875	0.125	0.354	0.000	1.000	1.000	1.000	1.000
Lamellar bone	0.875	0.125	0.354	0.000	0.000	0.000	0.000	1.000

Table 2. Wilcoxon signed rank test of granulation tissue , woven bone, lamellar bone at 14 and 28 days

	N	N for Test	Wilcoxon Statistic	P	Estimated median
Granulation	8	8	31.5	0.069	1
Woven bone	8	8	31.5	0.069	1
Lamellar bone	8	8	4.5	0.069	0

the formation of previously absent woven bone and maturation of osteoids after 4 weeks of the healing phase.[32] By Haematoxylin and Eosin (H&E) staining, different cell locations and shapes could be identified. Osteocytes, osteoblasts are present in hard tissue components while fibroblasts are in connective tissue. Wilcoxon rank test showed no significant different in percentage between granulation tissue, woven bone, and lamellar bone on days 14 and 28 with p value 0.069 as in Table 1 and 2.

DISCUSSION

Bone healing after implantation is a fracture healing process that recapitulates bone development. The first cascade of fracture healing is hematoma formation followed by acute inflammation, granulation tissue, callus formation, and remodeling. Several days to week after implantation, the inflammatory stage is replaced by granulation tissue which is the key factor for bone formation.[24] On the tissue level of bone formation is characterized by the initial deposition of collagen matrix in a irregular oriented pattern which referred as woven bone.

In this study histomorphological evaluation showed loosen woven bone with immature osteoids in its matrix formed adjacent the sample area which showed there is a reactive bone formation as demonstrated in Fig.6, Futhermore, immature osteoblasts are observed to be arranged around the sample area, and osteocytes are embedded with the newly formed bone on the 14th day. On the 28th day, woven bone shows more denser with mature osteoids in the matrix, osteoblasts and osteocytes are more mature than on the 14th day and scattered around the sample area irregularly. This result shows that geopolymer-carbonated apatite nanocomposites have a potential of osteoconductivity and bioactivity properties by initiating and supporting osteogenesis in the initial healing cascade. Osteoconductivity refers to the ability of a material to allow unimpeded bone growth onto or throughout.[33] While bioactivity refers to the ability of a material to develop a direct, adherent, and strong bonding with the bone tissue.[34] This result was in the a agreement according to Tippayasam et al.[17] reported that the bioactive properties and biocompatibility of geopolymers were shown by their ability to increase bone-cell activity for new bone formation.[17] Cataura et al.[18] stated that metakaolin-based geopolymer could be used as a hard tissue.[18]

Ramazanoglu et al.[35] classified ossification into 3 groups, namely biotolerant, where new bone formation occurs around the bone and migrates towards the implant surface (distance osteogenesis), bioinert that was characterized by the formation of new bone directly on the implant surface (contact osteogenesis), bioreactive which was the implant allowed new bone formation actively on the implant itself.[35] Our result indicated that geopolymer-carbonated apatite nanocomposites had the potential of biotolerant and bioreactive properties characterized by distance osteogenesis and samples allowed new bone formation as showed in Fig.7 In this study, analysis was carried out on granulation tissue, woven bone, and lamellar bone with consideration of granulation tissue contains many mesenchymal cells and neovascular.[24] Mesenchymal cells will differentiate into osteoprogenitor and osteoblast

cells which are supported by angiogenesis. The presence of granulation tissue indicates an early stage of new bone formation.[25] Osteoprogenitor cells would differentiate into osteoblasts that directly laid out woven bone, indicated the development of cortical bone, followed by collagen matrix deposition in regular and parallel orientation known as lamellar bone.[32]

Although statistically showed the presence of no significant different in the percentage between granulation tissue, woven bone, and lamellar bone on the 14th and 28th day, however histological observations showed the different woven bone densities between days 14 and 28, as well as the level of maturity of bone cells which showed more mature on the 28th day. Considering implant is an artificial root substitute that will be close contact with the surrounding bone, the mechanical properties of dental implant material should not exceed the mechanical properties of tooth structure to preserve the parent bone bed.

In this study, it was found that geopolymer-CHA nanocomposites reached the range of dentin hardness value, tensile strength and compressive strength reached the range of enamel, while modulus elasticity reached the range of enamel and almost reached dentin. In vitro biocompatibility test showed sample was not toxic, in vivo histomorphological evaluation demonstrated good response of bone cell on the sample surface, indicated by the initial bone healing which characterized by formation of granulation tissue, woven bone rich in osteoid and surrounded by osteoblast around samples. From the mechanical and biological properties point of view, our result showed that geopolymer-carbonated apatite nanocomposites was potential candidates as dental implant materials.

Limitations of this study include the small number of rabbits in each group and the short time limit of observation from operation and euthanized.

CONCLUSION

Geopolymer-CHA nanocomposites could be considered as a potential dental implant material from mechanical and biological properties point of view.

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