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Research Report

Compressive strength and porosity tests on bovine hydroxyapatite-gelatin-chitosan scaffolds

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ABSTRACT

Background: *Degenerative diseases, aggressive periodontitis, trauma, jaw resection, and congenital abnormalities can cause defects in jaw bone. The surgical procedure for bone reconstruction currently performed is bone regeneration graft (BRG). Unfortunately, this procedure still has many disadvantages. Thus, tissue engineering approach is necessary to be conducted. The main component used in this tissue engineering is scaffolds. Scaffolds used in bone regeneration is expected to have appropriate characteristics with bone, such as high porosity and swelling ratio, low degradation rates, and good mechanical properties. For those reasons, this research used scaffolds made from bovine hydroxyapatite (BHA), gelatin (GEL), and chitosan (K)/BHA-GEL-K as one of biomaterial candidates for bone regeneration.* ***Purpose:*** *This study aimed to determine compressive strength value and porosity size of BHA-GEL-K scaffolds.* ***Method:*** *Compressive strength of BHA-GEL-K scaffolds was tested using autograph with speed 10 mm/ min with a load cell compress machine of 100 kN. Compressive strength was calculated by force divided to surface area. Porosity test was measured using SEM. Scaffold were coated with Pb and Au, then the porosity size is calculated with SEM at 100x magnification.* ***Result:*** *BHA-GEL-K scaffolds had a mean compressive strength value of 174.29 kPa and a porosity size of 31.62 + 147.06 lm.* ***Conclusion:*** *It can be concluded that BHA-GEL-K scaffolds has a good compressive strength, but not yet resemble real bone mass, while porosity of BHA-GEL-K scaffold is appropriate for bone tissue regeneration application.*

Keywords: *scaffolds; bovine hydroxyapatite; gelatin; chitosan; compressive strength; porosity*

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INTRODUCTION

Degenerative diseases, aggressive periodontitis, trauma, jaw resection, and congenital abnormalities can cause defects in jaw bone.[1,2] In a massive bone defect, gap can emerge. Massive bone defect is still a major challenge in the field of dentistry since bone healing process often cannot successfully restore the shape and size of the jaw bone as the same as the previous ones.[3]

A surgical procedure for bone reconstruction currently performed is bone regeneration graft (BRG). Unfortunately, this procedure still has many disadvantages. Tissue engineering approach is necessary to be conducted. Tissue engineering aims to regenerate damaged structures and tissues. The concept of tissue engineering is combining three basic components of a cell, scaffolds, and signal regulator, called as triad tissue engineering.[1]

Scaffolds are three-dimensional structure used as temporary replacement for damaged natural extra cellular matrixes (ECM). Consequently, the attachment, anchoring, proliferation, migration, and differentiation of cells as well as regeneration of tissue can occur. Scaffolds for bone regeneration have some criteria, such as good mechanical properties, high porosity and swelling ratio, and low degradation.[4,5]

The mechanical properties of scaffolds are expected equal with mechanical properties in normal bone. The mechanical properties can be measured by several parameters. The most widely used parameter is compressive strength. Compressive strength is an ability of a material to

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withstand the burden of pressure. Meanwhile, porosity of scaffolds is required for cell attachment. Porosity can also determine the mechanical properties of scaffolds. The size of the scaffolds porosity can be observed using a scanning electron microscope (SEM).[6]

In the last few years, biomimetic scaffolds made of hydroxyapatite, gelatin, and chitosan scaffolds began to be developed. Scaffolds with the composition of hydroxyapatite, gelatin, and chitosan is expected to resemble ECM bone, which consists of inorganic components (70%) and organic components (30%).[7] Hydroxyapatite is the biggest inorganic component. hydroxyapatite is osteoconductive and biocompatible, that is able to integrate well and strongly in the host bone.[8] Hydroxyapatite can also be obtained from synthesis and natural materials, such as bovine hydroxyapatite. Bovine hydroxyapatite has similarities with hydroxyapatite in humans, and is considered as non-toxic material.[3]

Gelatin is a type-I collagen considered as the major organic component in bone (90%). The main advantage of the gelatin is increasing attachment and growth of cells since gelatin has many arginine-glycine-aspartic acid (RGD) protein chains and sequences. Gelatin is also biodegradable and biocompatible, but has low biomechanical properties.[9]

In addition, chitosan has the same structure as glycosaminoglycans (GAG), a non- collagen organic component of bone. Chitosan is biocompatible, biodegradable, bioactivity, and osteoconductive. Chitosan also has anti-microbial activity. Chitosan can help cell attachment, differentiation, and migration.[6] Integration of hydroxyapatite (HA), gelatin (GEL), and chitosan (K) in scaffolds is expected to improve the mechanical and biological properties so that it can be considered as ideal scaffolds used for bone regeneration. gelatin hydroxyapatite, and chitosan scaffolds with a ratio of 70:15:15 (w/ w/ w) is a good biomaterial candidate for tissue engineering in bone.[10]

Unfortunately, there are still no recent researches on gelatin hydroxyapatite, and chitosan scaffolds explaining the origin of the hydroxyapatite used. Therefore, this research focused on the use of hydroxyapatite derived from bovine bones developed by Bank of Tissue in Dr. Soetomo Hospital, Surabaya. This study aimed to determine the value of compressive strength and the size of porosity derived from BHA-GEL-K scaffolds with ratio 70:15:15 (w/ w/ w). BHA-GEL-K scaffolds are expected to be biomaterials for regenerative therapy development in bone defects in the field of dentistry.

MATERIALS AND METHODS

Materials used were BHA (a particle size of <150 µm made from bovine bones produced by Bank of Tissue in Dr. Soetomo Hospital, Surabaya), gelatin (Rousselot 150 LB 8, Ghuangdong, China), chitosan (Sigma 448877, St. Louis, USA), a deacetylation degree of >81%), 10% NaOH (Brataco Chemica PT., Surabaya, East Java, Indonesia), 2% acetic acid, and distilled aqua (PT. Duta Farma).

BHA-GEL-K scaffolds conducted in this research were based on a modification of procedures for producing scaffolds from previous research. 9 ml of 2% acetic acid (Brataco Chemica PT., Surabaya, East Java, Indonesia) was mixed with 0.375 grams of gelatin using magnetic stirrer (DragonLab, MS-Pro-H280) (DragonLab, MS-Pro-H280, Beijing, China). 1.75 grams of BHA was mixed with 5 ml of distilled aqua, and allowed to settle. The sedimented BHA that had already been wet was soaked into the mixture, and then added 0.375 gram of chitosan and 2 ml of 10% NaOH. The mixture was put into scaffolds molds. Scaffolds were made with two different sizes diameter of 8 mm and a height of 10 cm, and diameter of 5 mm and a height of 5 mm. Scaffolds in molds then was frozen -80° C for 24 hours and then dried using freeze dryer (VirTis Bech Top "K" Series, SP Scintific Pennyslvania, USA) for 2 x 24 hours.[10,11]

Compressive strength test was performed on scaffolds with a diameter of 8 mm and a height of 10 cm. The sizes of scaffolds used were adjusted with the specification of the tools used. The sizes of the surface area of BHA-GEL-K scaffolds were measured. Autograph table (Shimadzu Ag-10 TE) was covered with paper. Scaffolds were placed in the middle of the table with the vertical axis position, perpendicular to the plane of the samples. Autograft tool was switched on, and the samples were pressed with a speed of 10 mm/ min with a load cell compress machine of 100 kN until scaffolds were distorted. The tool then automatically stopped, and the number figured out was noted.[12] Compressive strength value then was calculated using the following formula:[13]

$$\text{Compressive strength } (N/mm^2) = \frac{\text{Force (Newton)}}{\text{Surface area } (mm^2)}$$

In this research, the number generated by the autograft tool is in a unit of kgf (kilogram force). The compressive strength value is in a unit of N/ mm^2, so this value has to be converted to Newton first before compressive strength value is calculated. Compressive strength value commonly used is in a standard unit of Pascal (Pa), so the final compressive strength value then has to be converted into kPa.[12,13]

Scaffolds used for porosity test were scaffolds with a diameter of 5 mm and a height of 5 mm. Porosity size measurement procedure was performed by using SEM (FEI, Inspect-S50, Hillsboro, Oregon, USA). In the initial preparation, SEM holders were taken and coated with carbon tape. Carbon tape used was double-sided carbon tape. One side was attached to the SEM holders, and the other side was attached to the scaffolds. Samples in the form of scaffolds are non-conductor materials that has to be coated before using them. Coating was conducted using a sputter coater (SC7620, Qourum Technologies Ltd., East Sussex, England) by inserting BHA-GEL-K scaffolds and

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their holders in the sputter coater, and vacuum then was performed for 30 minutes. After the vacuum was done, plasma coating was conducted for 3 seconds using Au and Pb. The samples having coating were put into the SEM holders, and vacuum was carried out for 5 minutes. After that, the program for the SEM was started, and then SEM images appeared on the monitor. Pictures were taken at magnifications of 75x, 100x, and 250x. Scaffolds porosity measurement was performed on pictures with a magnification of 100x. In one field of view, the largest porosity size was selected corresponded to the number of samples required. Porosity size was measured by drawing a line on the selected porosity.

RESULTS

Scaffolds were made by mixing powdered BHA, GEL, and K as seen in Figure 1. The results of compressive strength and porosity tests on BHA-gel-K scaffolds can be seen in Table 1. The mean value of compressive strength was 174.29 + 31.62 kPa, while the mean porosity size was 147.06 µm + 27 02 (n = 7).

Porosity size was measured based on the pore diameter of the SEM image. Eleven porosity sizes were measured and used as samples. SEM images with a magnification of 75x resulted can be seen in Figure 2. With the magnification of 75x, almost the entire porosity surface of BHA-GEL-K scaffolds could be seen. With a magnification of 100x and 250x, porosity sizes seemed much larger. The blue arrow indicates porosity on the surface of the scaffolds.

Figure 3 shows SEM images at 100x magnification used in this research. In the picture, how porosity measurements conducted can also be seen. Porosity measurements were performed by selecting the largest porosity to represent the scaffolds porosity. Green lines were drawn from one point to another. The length of the green lines was the size of pores in the scaffolds. The length of the green line then was calculated using a unit of µm. The total number of the measured porosity size was 11.

Figure 1. BHA-GEL-K scaffolds.

DISCUSSION

Tissue engineering is one of therapeutic methods that have been widely used and developed recently. Tissue engineering is regarded as a promising procedure for a biological component of a bone substitute that can be used in all kinds of bone damage.[6] Scaffold is also considered as an important component in tissue engineering. Scaffolds act as substitute for ECM so that bone regeneration can occur. Every tissue actually needs scaffolds that has different biomechanical and biological properties from others.[5]

Table 1. Results of compressive strength and porosity tests on BHA-Gel-K scaffolds

Characteristics	Minimal value	Maximal value	Mean ± SD*
Compressive strength values (kPa)	120.00	200.00	174.29 ± 31.62
Porosity size (µm)	107.20	208.60	147.06 ± 27.02

* SD: Standard Deviation

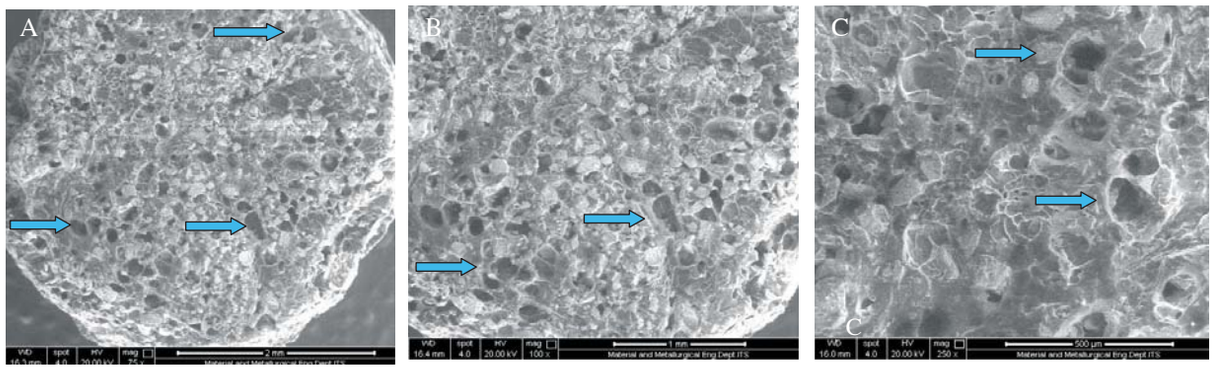

Figure 2. (a) Results of SEM test and porosity measurement test at the magnification of 75x; (b)100x; and (c) 250x.

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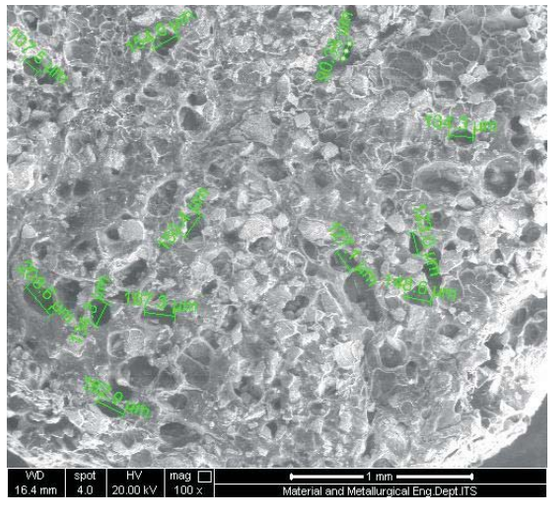

Figure 3. Porosity measurement process of BHA-GEL-K scaffolds.

Selection of materials used for composing scaffolds is an important factor in the success of using scaffolds since the materials selected will affect the final and functional characteristics of the tissue formed.[14]

In this research, scaffold was made of mixed materials derived from BHA, GEL, and K. hydroxyapatite is an osteoconductive material with the main composition of calcium (Ca) and phosphate (P). Calcium and phosphate in hydroxyapatite can act as regulators of signal in bone tissue engineering, as well as basic ingredients in formation of new bone tissue.[15] In this research, a natural hydroxyapatite used was derived from bovines. BHA used was manufactured by Bank of Tissue in Dr. Soetomo Hospital, Surabaya with product validation in accordance with the standards set by the Ministry of Health and APASTB (Asian Pacific Association of Surgical Tissue Bank).[16] BHA has no toxic properties, but similar to human HA, it has a porosity size of 150-360 µm and high osteoconductivity, as well as can easily integrate with the surrounding bones.[17] BHA, consequently, has been widely studied and applied for bone tissue regeneration. A previous research even indicates that the use of BHA scaffolds planted with MSC on rabbit defected bone can regenerate the bone well. It is characterized by an increase in type I collagen and osteocalcin.[2]

Other main components in this research were gelatin and chitosan. The objectives of adding gelatin and chitosan as adhesive materials for binding BHA is to improve the effectiveness of BHA in binding the active ingredients and to reduce the fragility of BHA.[12] Gelatin is a denatured collagen product that plays an important role in cell adhesion and proliferation. Application of gelatin for bone regeneration is generally combined with other materials since gelatin has a low mechanical property.[9] Scaffolds made of HA-GEL is considered as a suitable environment for the growth of periodontal ligament fibroblasts, human mesenchymal stem cells (HMSC), and primary cells from human pelvic bone.[18] Chitosan, on the other hand, is a deacetylation product of chitin which has similar structure to GAG, a non-organic component in bone collagen. GAG modulates bone precursor cells to the defect area, and helps cell differentiation to regulate protein that is essential for the bone regeneration.[19]

Consequently, the combination of BHA, GEL, and chitosan is expected to form scaffolds similar to ECM in the bones. Those components of scaffolds interact with each other. Organic signals of chitosan and gelatin then will cover hydroxyapatite particles. This is consistent with interactions that occur in normal bone components, in which organic components will cover inorganic components.[11]

Mechanical properties of scaffolds are factors that also must be taken into account in the process of making scaffolds. Scaffolds must be strong enough to withstand mechanical stresses derived from the surrounding tissue. Therefore, the low mechanical properties on scaffolds can cause dimensional changes in scaffolds.[12] The value of compressive strength in this research was lower than the value of compressive strength in canselous bone, 2-12 MPa.[20] This can happen because in this research there was no crosslink agent added. The addition of crosslink agent will trigger crosslinking between molecules of the materials forming scaffolds.[12] In addition, the materials can also make crosslink bonds between the molecules stronger and prevent molecular bonds between the molecules shifting. The stronger bonds between the molecules then will improve the mechanical and biological properties of scaffolds.[21]

Another important property that should be owned by scaffolds is an appropriate size of porosity. Porosity size is related to cell adhesion and migration as well as diffusion of nutrients and removal of metabolic waste.[4] In this research, all samples had a pore size of more than 100 µm, and the mean porosity size of BHA-GEL-K scaffolds was 147.06 µm. This is consistent with a previous research stating that the minimal pore size of scaffolds is 100-150 µm.[6] The appropriate porosity size is used for the attachment of MSC-sized 17.9-30.4 µm.[22] The porosity size that is too small will lead to limited cell migration as well as will disrupt nutrient and metabolic waste diffusion. When this occurs, it will cause necrosis of scaffolds. Meanwhile, the porosity size of scaffolds that is too big will cause the cells to be easily separated from the scaffolds.[4,6]

It can be concluded that the BHA-GEL-K scaffolds has a good value of compressive strength, but not yet resemble bone mass. Therefore, further researches use BHA-GEL-K scaffolds with addition of cross linking agent should be conducted to increase the value of compressive strength. However, the porosity of BHA-GEL-K scaffolds is suitable for bone tissue regeneration application.

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