Biocompatibility Testing of Hydroxyapatite-Chitosan-Chondroitin Sulfate Composite Scaffold as Bone Graft

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Abstract

The most commonly transplanted organ after blood is bone. One of bone implants developed today is bone scaffold. In this study, the biocompatibility test of the hydroxyapatite-chitosan-chondroitin sulfate composite scaffold was conducted based on the level of inflammation and the number of osteoblasts in the Javanese rabbits (Leprus nigricollis). This study is a true experi mental research with post-test only control design. The samples were divided into three groups, namely the negative control group, the positive control group and the treatment group. Each group has observation times of 7 days, 21 days and 56 days. The laboratory test results showed that the treatment group had the highest inflammatory level on day 7 (WBC 7.02 x 1012 sel/L) but it had the lowest inflammatory level (WBC 3.64 x 1012 sel/L) on day 56. Based on the histopathological results, the treatment group had the highest number of osteoblasts on the 7th, 21th, and 56th days which are 52.33 ± 10.73, 70.00 ± 26.99 and 61.67 ± 10.58 respectively. The amount of the woven bone, the lamellar bone, the havers system and the bone repair on the 7th, 21th, and 56th day was also the highest compared to the other two groups. It can be concluded that the hydroxyapatite-chitosan-chondroitin sulfate composite scaffold can be used as a bone graft.

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Introduction

A research done by the Indonesian Ministry of Health showed a comparison of the increase in injury prevalence nationally from 2007 to 2013, which were from 7.5% to 8.2%. The number of fractures due to traffic accidents has reached 24 million cases per year while the number of fractures due to osteoporosis has reached 350,000 cases per year[1]. In order to repair those fractures, bone graft is needed. Bone is the second most transplanted organ after blood with more than 2.2 million bone grafts have been carried out worldwide. In Indonesia (1997-2001), there has been a four times increase of demands for biomaterials and the needs for bone graft will continue to increase along with increasing cases of fractures due to trauma, tumors, congenital abnormalities, infections, and complications of joint prosthesis installation[2].

One of the most commonly used type of bone grafts is scaffold. The ideal scaffold should have a good biocompatibility, biodegradation that can be controlled, suitable pore size and a total porosity and it has to act as a bone matrix to form a new bone so that it does not require a second surgery[3]. Moreover, scaffold should also have mechanical properties that are suitable to the tissue in the implantation area[4]. In addition, the ideal scaffold should be able to support cell growth activity, also facilitate molecules and optimize tissue regeneration without causing local or systemic responses to the host[5]. Thus, bone scaffold biomaterial is the potential alternative for bone defect repair technique due to trauma, tumor resection and abnormal development[6]. Currently, the scaffold material that has been developed is in the form of composite, such as hydroxyapatite (HA) and chitosan composite. Hydroxyapatite is a bioceramic that is often used for bone implants because of the same chemical composition of bones and teeth and it is osteoconductive.

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Chitosan is a biopolymer that is often used as a bone scaffold because it is biocompatible, biodegradable and it can form pores making it suitable for cell growth and natural antibacterial. The scaffold from HA and chitosan composite has been studied more widely for applications as bone implants and other tissue engineering. In the study done by Venkatesan[3], chondroitin sulfate was added to HA-chitosan composite. The synthesis process was carried out by the freeze-drying method. The results obtained were total porosity > 89%, non-toxic, controlled biodegradation, and an increase of cell proliferation. In cartilage, chondroitin sulfate plays a role in maintaining elasticity integrity in tissue[7]. The study is developed with various comparisons of the composition of hydroxyapatite-chitosan-chondroitin sulfate. The result is an increase in the percentage of chondroitin sulfate so that the pore size increased and the porosity decreased while the degradation rate for 4 weeks allowed scaffold to remain during the bone growth period. In vitro biodegradation test results showed that the highest percentage of weight loss in each sample, which was observed in week four, is 23.8619%. Cell Viability showed that more than 92.1514%. The compressive strength obtained is around 2.6914 – 7.6233 MPa which has met the standard as cancellous bone substitute[8].

Studies on the biocompatibility of biomaterials have become a very significant research recently. The important thing in evaluating the biocompatibility is to consider interactions between biomaterials with body tissues in the short or long term period, which can be seen from the tissue and cellular levels[9]. In addition, Hsieh conducted a study with scaffolds consisted of curdlan and poly (vinyl alcohol) which were implanted for 14 days in subcutaneous rats of Sprague-Dawley (SD). The results showed that there was no difference between the control group and the treatment group in terms of the whole blood results, namely red blood cells (RBC), white blood cells (WBC), platelets (PLT), as well as the comparison of neutrophils, lymphocytes, monocytes, eosinophils and basophils[10]. Based on the several pre-studies mentioned above, it is necessary to do a biocompatibility test of hydroxyapatite: chitosan: chondroitin sulfate scaffold with the composition of 50:35:15 wt%. The purpose of this study is to determine the biocompatibility level of hydroxyapatite-chitosan-chondroitin sulfate scaffold including WBC, RBC, PLT and WBC component ratio, osteoblast number, woven bone, lamellar bone, havers system and bone remodeling in an experimental study on Javanese rabbits (*Lepus nigricollis*).

Materials and methods

The materials used to make the composite scaffold samples include hydroxyapatite produced by BATAN, chitosan with 75% of degree acetylation (DA) and chondroitin sulfate from CV INTERLAB, 2% acetic acid, 10% NaOH and the Javanese rabbit (*Lepus nigricollis*) from the Faculty of Veterinary Medicine, Universitas Airlangga.

Synthesis of Hydroxyapatite-Chitosan-Chondroitin Sulfate Composite Scaffold

1.75 g of chitosan powder with acetylation degree of 75% was dissolved in 2% 200 mL acetic acid solution. Then the solution was stirred using a magnetic stirrer for ± 6 hours until the chitosan powder was completely dissolved in the acetic acid. After that, 2.5 g of hydroxyapatite was dissolved in 50 mL distilled water then mixed into chitosan solution using a magnetic stirrer. Following that, 0.75 g chondroitin sulfate was added gradually to the chitosan-hydroxyapatite solution while being stirred for 12 hours. The comparison of hydroxyapatite: chitosan: chondroitin sulfate used is 2.5g: 1.75g: 0.75g (50: 35: 15) wt%. The hydroxyapatite-chitosan-chondroitin sulfate solution which had already been prepared was moved into a pot bottle (3x3x3 mm^3) then it was frozen at -80^{0}C for 5 hours. This freeze-drying process was conducted for 30 hours. Furthermore, it was rinsed with 10% NaOH solution then washed with distilled water until the pH was neutral. After that, it was heated in the oven at 80^{0}C for 5 hours to remove the remaining water. Then the sample was ready to be used.

Implantation Procedures, Whole Blood and Osteoblast Examination

The rabbits were anesthetized with 50 mg/kg BB ketamine and 0.2 mg/kg BB diazepam. Then the lateral side of proximal femur area was disinfected with a betadine (povidone-iodine) solution and 70% alcohol. The rabbits were classified into three big groups: (1) the control group C1, C2, and C3, which consisted of rabbits without drilling nor scaffold composite implants;

(2) D1, D2, D3 groups which consisted of rabbits which were drilled without given any scaffold composite implant; (3) E1, E2, E3 groups, which consisted of rabbits which were done incisions on the lateral side of the proximal femur that were deepened until the femoral head bone appeared. The femoral head was drilled 4 mm deep and 4 mm in diameter. The composite scaffold was inserted into the femoral head bone that had been drilled. The incision was closed layer by layer. The rabbits were put into cages to be taken care of by giving them food and drink. After the operation, the rabbits were given cephalosporin antibiotics at 22 mg/kg bid for 2 weeks, pain killer Rimadyl at 2 mg/kg bid for 5 days to stop transamin bleeding, multivitamin 1x1 for 7 days, Levofloxacin at 6 x 1 drops for 7 then 4 x 1 drops for the next 7 days, and also given lubricant (Regefluidbid for 1 month)[11].

Furthermore, the whole blood test was conducted on 7th, 21th and 56th days after implantation by drawing 5ml of the rabbits' blood then the whole blood was examined with hematology analyzer.

The examination of osteoblasts was done in the following steps. First, the rabbits were injected with ketamine and diazepam subcutaneously in the thigh and then sacrificed. After that, the tissue in the incision area was cleaned and disinfected with a 70% alcohol solution. The tissue was cut across with a thickness of 2 cm. Then the tissue was soaked in 10% buffer formalin solution with pH of 7.4 for about 18 to 24 hours. Lastly, the tissue was decalcified with EDTA then the histology test was performed.

The histology test was carried out by cutting the tissue near the implantation area with 3-6 µm deep. Next, the tissue was put carefully onto a glass object then it was stained with HE (Hematoxylin Eosin) dye for 1 minute. Furthermore, the number of osteoblasts can be calculated by observing through a light microscope with the field of view of 6. The histological test of bone regeneration was done by calculating the number of osteoblasts, woven bone, lamellar bone, havers system and bone repair on day 7, 21 and 56.

Results

Whole Blood Test

The whole blood observation consists of White Blood Cell (WBC), Red Blood Cell (RBC), platelet (PLT) and the WBC component ratios are shown in Table 1. As shown in Table 1, the observation on day 7 (C1, D1, E1) shows an increase number of WBC on samples D1 and E1 which indicates that an inflammatory process has occurred. The ratio of WBC components is still within normal range since there is no addition in the number of eosinophil and basophil. Moreover, the amount of RBC is also within normal range since there is no decrease in the number due to bleeding. The number of PLT (platelets) is also within the normal range which indicates that there was no excessive blood clotting process due to bleeding. The ratio of the WBC components shows that he number of eosinophil and basophil is <1 which means that there was no inflammation.

	Amount			
Samples	RBC (cells / L)	WBC (cells / L)	PLT (cells / L)	WBC component ratio Eosinophil / basophil / neutrophil / lymphocyte / monocyte
C1	3.83×10^{12}	2.40×10^{9}	69×10^{9}	0/0 / 51.7 / 41.5 / 6.8
D1	6.10×10^{12}	7.00×10^{9}	174×10^{9}	0/0 / 35.8 / 34.4 / 29.8
E1	7.02×10^{12}	$7,80 \times 10^{9}$	230×10^{9}	0/0 / 23.1 / 46.5 / 30.4
C2	5.67×10^{12}	3.70×10^{9}	116×10^{9}	0/0 / 59.3 / 20.1 / 10.6
D2	5.60×10^{12}	8.50×10^{9}	289×10^{9}	0/0 / 67.5 / 21.9 / 10.6
E2	6.77×10^{12}	2.40×10^{9}	33×10^{9}	0/0 / 35.2 / 40.1 / 24.7
C3	4.16×10^{12}	2.30×10^{9}	102×10^{9}	0/0 / 53.6 / 26.4 / 10.0
D3	6.24×10^{12}	$5,20 \times 10^{9}$	295×10^{9}	0/0 / 56.6 / 29.0 / 14.4
E3	3.64×10^{12}	1.70×10^{9}	38×10^{9}	0/0 / 34.5 / 34.6 / 30.9

Table 1. Results of the whole blood test for 7 days, 21 days and 56 days.

C1 : Negative control group day 7
D1 : Positive control group day 7
E1 : Implants group *scaffold* day 7
C2 : Negative control group day 21
D2 : Positive control group day 21
E2 : Implants group *scaffold* day 21
C3 : Negative control group day 56
D3 : Positive control group day 56
E3 : Implants group *scaffold* day 56
WBC : white blood cell
RBC : red blood cell
PLT : platelet

The observation on day 21 (C2, D2, E2) shows an increase in the number of WBC ion samples C2 and D2 which indicates that an inflammatory process has occurred. However, there was a decrease in the number of WBC on sample E2 due to the absence of inflammatory process. The ratio of the WBC components is still within normal range because there is no increase in the number of eosinophil and basophil. The

amount of RBC is also within normal range since there is no decrease due to bleeding. Furthermore, the number of PLT (platelets) is also within normal range which indicates that there was no excessive blood clotting process due to bleeding. The ratio of the WBC components shows that the number of eosinophil and basophil is <1 which means there was no inflammation.

The observation on day 56 (C3, D3, E3) shows that there is no increase in the number of White Blood Cells (WBC) which indicates that there was no inflammatory process any longer. It also shows that the ratio of WBC components is still within a normal range because there is no increase in the number of eosinophil and basophil. The Red Blood Cell (RBC) is also within normal range since there is no any decrease due to bleeding. The number of PLT (platelets) is still within the normal range which indicates that there was no excessive blood clotting process due to bleeding. The ratio of the WBC component shows that the number of eosinophil and basophil is <1 which means that there was no inflammation.

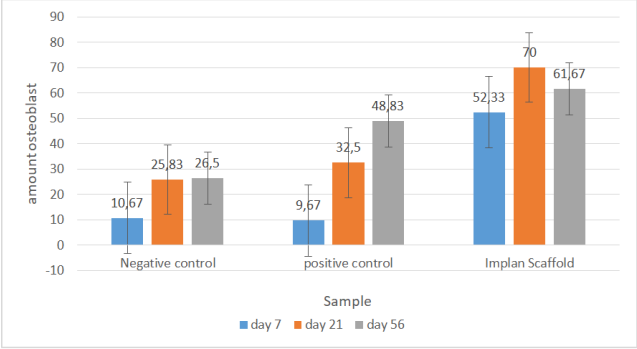


Figure 1. The percentage number of osteoblasts in the negative control group (C), the positive control group (D) and treatment group (E) of scaffold on day 7, day 21 and 56 (Blue, red and green depicts day 7, day 21 and day 56 respectively).

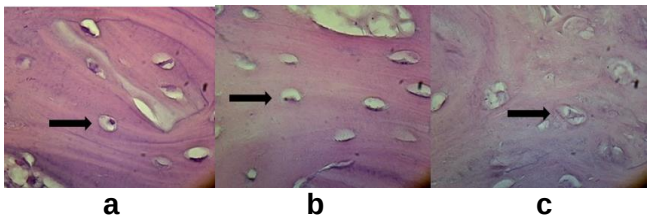
The Calculation Results of the Number Osteoblast

Osteoblast cells can be seen by using HE staining for the negative control group (C), the positive control group (D) and the treatment group with scaffold (E). The number of osteoblast cells for each group can is depicted in Figure 1. It can be seen that the number of osteoblast cells varies for each group, which was obtained by adding up all the cells found in the field of view of 6 at 1000x magnification.

Based on data on the number of osteoblasts for each study group, it can be seen that samples E1, E2 and E3 have the highest number of osteoblast cells compared to the negative control group (C) and the positive control group (D), which are E1 (52.33 ± 10.73), E2 (70 ± 26.99) and E3 (61), 67 ± 10.58). This difference occurs because of the implantation of scaffold to the rabbits' bones which results in osteoblast cells growing rapidly. Furthermore, it will accelerate bone growth. However, the number of osteoblasts on sample E3 decreased (61.67 ± 10.58) which was due to osteoblast cells that had turned into bone. The increase in osteoblast growth and a decrease in the number of red blood cells show that the hydroxyapatite-chitosan-chondroitin sulfate scaffold is good for bone growth.

Histology

Histological analysis of the rabbits' femoral head bone tissue was done by observing the bone tissue of the femoral head directly under a light microscope (Nikon H600L) which was equipped with a 300 megapixel DS Fi2 camera. The histological results of the negative control group, the positive control group and the scaffold treatment group are shown in Figure 2–7.



a **b** **c**

Figure 2. Histology images of the negative control group (a) day 7, (b) day 21 and (c) day 56. The black arrows show the osteoblast cells (HE staining, 1000x magnification).

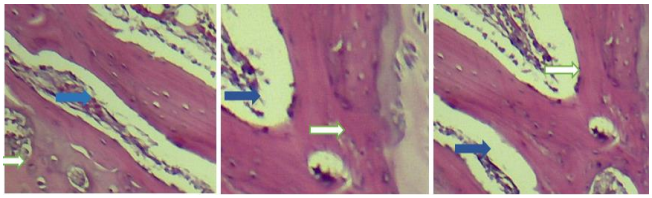
The observation consists of the number of osteoblast cells, the woven bone, the lamellar bone, and the havers system until bone repair occurred. Observations of the woven bone, the lamellar bone and them havers system can be seen at 400x magnification. Observation of the woven bone leads to the lamellar bone with differences in shape and structure. In the woven bone, the resulting matrix is irregular, while the lamellar bone forms a regular matrix. The havers system was formed from a set of lamellar bones

that forms a compact circle into a new bone (bone repair).

Histology Observation of the Negative Control Group

In Figure 2, the arrow shows osteoblasts which are characterized by the presence of a round nucleus in the basal part of the cell. Osteoblasts are formed is still small and immature and it was mixed with blood cells, either a negative control group day 7, 21 and 56.

The observation result of the woven bone, the lamellar bone, the havers system and the bone repair in the negative control group is shown in Figure 3.



a b c

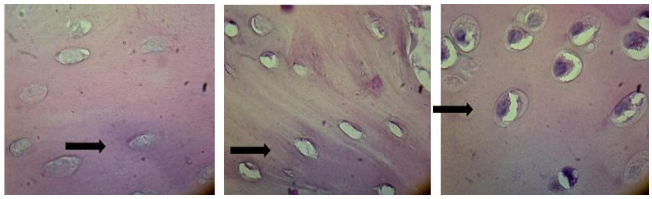
Figure 3. Histology images of the negative control group (a) day 7, (b) day 21 and (c) day 56. White arrows indicate woven bone. The blue arrow shows the lamellar bone that forms the havers system (HE staining, 400x magnification)

The presence of the woven bone and the lamellar bone is characterized by differences in shape and structure. In the woven bone, the resulting matrix shape is irregular, whereas in the lamellar bone the matrix formed is regular. The havers system is formed by a set of lamellar bones which forms a circular compact bone into a new bone (bone repair). There was no any noticeable differences between the woven bone, the lamellar bone and the havers system in the negative control group on days 7, 21 and 56.

Histology Observation of the positive control group

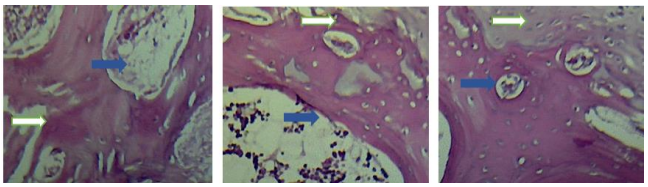
On the 21th day and 56th day of observation, osteoblast cells increased more than the on the 7th day. The number of osteoblasts increases while the blood cells decrease, which indicates that the process of bone formation has already started while the inflammatory process decreased.

The observation results of the woven bone, the lamellar bone, the havers system and the bone repair in the positive control group can be seen in Figure 5.



a b c

Figure 4. Histology images of positive control group (a) day 7, (b) day 21 and (c) day 56. The black arrow shows the osteoblast cells (HE staining, 1000x magnification).

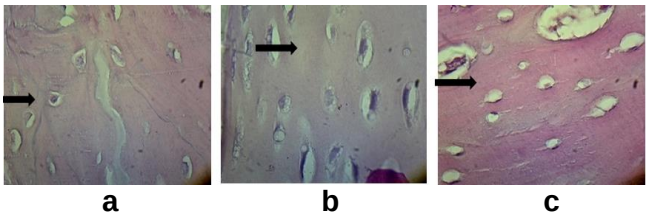


a b c

Figure 5. Histology images of positive control group (a) day 7, (b) day 21 and (c) day 56. The white arrow shows the woven bone. The blue arrow shows the *lamellar bone* that forms havers system (HE staining, 400x magnification).

The presence of the woven bone, the lamellar bone, the havers system and the bone repair on the 56th day were almost the similar to on the 21th day. The difference is that the number of forming cells were more mature and a perfect new bone regeneration has already occurred which is indicated by the increasing number and the regularity of the havers system.

Histology Observation of the scaffold treatment group



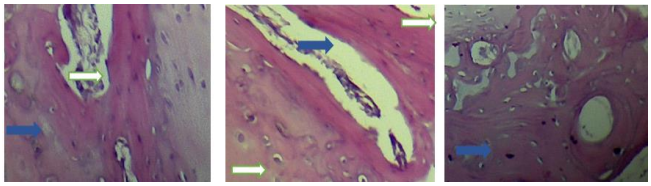
a b c

Figure 6. Histology images of treatment of scaffold implantation (a) day 7, (b) day 21 and (c) day 56. The black arrow shows the osteoblast cells (HE staining, 1000x magnification).

At the time of treatment of 56 days osteoblasts increasing treatment time than 21 days. On the treatment group scaffold 21 day and 56 days looked osteoblasts already mature and experienced remodeling process. The scaffold treatment group 56 days the process of

remodeling more than 21 days. This indicates the scaffold of hydroxyapatite-chitosan-chondroitin sulfate can form a good remodeling process that could form a perfect bone regeneration (Figure 6).

The observation result of the woven bone, the lamellar bone, the havers system and the bone repair in the scaffold implantation treatment is shown in Figure 7.



a b c

Figure 7. Histology images of treatment group of scaffold implatation (a) day 7, (b) day 21 and (c) day 56. The white arrow shows the *woven bone*. The blue arrow shows the *lamellar bone* that forms *havers system* (HE staining, 400x magnification).

Samples	*woven bone*	*lamellar bone*	Havers system	*Bone repair*
C1	+	+	+	-
D1	+	+	+	-
E1	+	+	+	-
C2	+	+	+	-
D2	+	++	+	+
E2	++	++	++	+
C3	+	+	+	-
D3	+	+	++	++
E3	+	++	++	++

Table 2. Histology observation data of woven bone, lamellar bone, Havers system.

- = not formed
+ = slightly formed
++ = formed more

The woven bone, lamellar bone, havers system and bone repair at day 7 began to form, and more were formed after 21 days and 56 days. The difference occurs in the number of cells that are formed which were more mature and also the new bone regeneration was perfect. Those differences were characterized by the increasing number and regularity of havers system. The perfect new bones usually takes longer time to form. The remodeling process at the bone regeneration stage takes months to years to get a perfect regeneration process.

The result of histology observations of woven bone, lamellar bone, havers system and bone repair for 7, 21 and 56 days of the three groups are presented in Table 2.

There were no significant differences in the parameters of woven bone and havers system in the three groups on day 7, day 21 and day 56. However, the woven bone and the havers system in the implant treatment group on day 21 were more than in the positive control group (Table 2).

Bone remodeling is the last stage in the bone regeneration process. Osteoblasts will replace the trabecular bone into compact bones. The content in the hydroxyapatite-chitosan-chondroitin sulfate composite scaffold helps to form, induce and produce new bone. The treatment of implant for each group and the recuperation times have influences on the bone regeneration process. The time difference also affects the recuperation process of bone regeneration. The longer the recuperation time, the more perfect bone regeneration.

Discussion

Bone regeneration begins with the inflammatory stage. This stage occurs from the first day of injury to the 5th day, which causes the formation of hematoma (precipitation of blood) in which the ends of its fragment are invaded by macrophages. On the 5th day the fibrin, new blood vessels and osteoblasts are formed. Osteoblasts play a role in synthesizing collagen and glycoprotein components[12]. In addition, osteoblasts also synthesize bone matrix elements that are mostly composed by type 1 collagen and glycoprotein. The osteoconductive and bioactive properties of the hydroxyapatite-chitosan-chondroitin sulfate composite scaffold will accelerate the mineralization process which helps forming a new bone. Moreover, osteoblasts precipitate new matrix organic elements that form osteoid[13]. Mineralization calcium phosphate is extracellularly stimulated by osteoblasts which produce a collagen-rich matrix called osteoid[14].

The osteoblast membrane contains several enzymes, one of which is alkaline phosphatase that plays a role in the process of bone calcification. Bone cells derived from calcified osteoblasts are called osteocytes. Schiecker stated that the alkaline phosphatase enzyme causes an increase in phosphate concentration so the calcium phosphate bonds

are formed in the form of hydroxyapatite crystals that will settle in the bone. When osteocytes detect damages in the bone, the osteoblasts begin to activate to do bone resorption. The bone resorption results in osteoblast and osteoclast activity to increase again. This continual activity of osteoblasts and osteoclasts will form the woven bone[15].

The 7-days observation is the final phase of the inflammatory stage, which can be seen from the increase number of WBC (White Blood Cell) in the positive control group and the implant treatment group. In this phase, the number of osteoblasts has begun to increase slightly while the woven bone, the lamellar bone and the havers system formed were still few.

The 21-days observation is the final phase of the proliferation stage, which was can be seen from a decrease in the WBC number both in the positive control group and the implant treatment group. The number of osteoblasts increased significantly, especially in the implant treatment group. The woven bone, the lamelar bone and the havers system have been formed which are clearly shown in the histopathology slides for the implant treatment group. The increase shows that the type of material used influences the bone formation process.

The 56-days observation is a remodeling phase, which can be seen from the number of WBC that were normal in all treatment groups. The number of osteoblasts has decreased due to reduced bone growth. The resorption of the woven bone is marked by a decrease in the number of woven bone in the histopathology slides. Meanwhile, the lamellar bone was perfectly formed which is characterized by the presence of collagen fibers arranged in parallel and circular in the middle part that causes the formation of the havers system. An increase in the number of the lamellar bone and the haver system shows a complete bone regeneration[16].

Parameters for the bone regeneration can be seen from the presence of the woven bone, the lamellar bone, the havers system and the bone repair. There were significant differences in the lamellar bone and the bone repair in the negative control group, the positive control group and implant treatment group on day 7, day 21 and day 56. This showed that the calcium addition causes mineralized callus to be absorbed and replaced with the lamellar bone.

The difference was not significant in the parameters of the woven bone and the havers system in the negative control group, the positive control and also the implant treatment group on days 7, 21 and 56. The number woven bone and the havers system in the implant treatment group on day 21 were higher than the positive control group.

Bone repair is the last stage of the bone regeneration process. In this stage, osteoblasts replace the trabecular bone into compact bones. The content of the hydroxyapatite-chitosan-chondroitin sulfate composite scaffold helps to form, induce and produce a new bone. The implant treatment group and the recuperation time affect the bone regeneration process. The difference in the recuperation time also affects the bone regeneration. The longer the recuperation time, the more complete bone regeneration is formed.

Conclusions

Hydroxyapatite – chitosan - chondroitin sulfate scaffold with a composition of 50: 35: 15% wt which does not affect the occurrence of inflammation in the bone growth process, and it can improve the process of bone growth and bone repair.

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Declaration of Interest

The authors declare no conflict of interest

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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