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Experimental Research

Dentistry Section

Potency of Injectable Hydroxyapatite Chitosan Scaffold for Bone Regeneration

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ABSTRACT

Introduction: Several methods are used to enhance bone regeneration and new bone formation. Recently, there is significant clinical need to develop alternatives to autografts and allografts for bone grafting procedures. Biopolymer chitosan combined with hydroxyapatite have been investigated as bone grafts substitute in the form of injectable, porous and biodegradable structure.

Aim: To evaluate the effect of Injectable Hydroxyapatite Chitosan (IHAC) Scaffold for rabbit femoral condyle bone regeneration by assessing its histological view.

Matertials and Methods: The experiment was conducted in New Zealand White Rabbits. IHAC scaffold was transplanted into the femoral defect of treated rabbit, while the control rabbit's defect was left empty. Bone regeneration was analysed histologically using Modified Salkeld Histological Scoring four weeks postoperatively.

Results: Cortical bone fusion occurred better in the treated rabbit compared to control rabbit. Active osteoblasts were found in the periphery of mature bone. Cortical bone had undergone complete maturation. Control rabbit still showed the presence of haemorrhagic area and fibrocartilage that indicated early phase of bone regeneration.

Conclusion: IHAC scaffold is effective for treatment of bone defect by guiding the host response to regenerate the bone tissue.

Keywords: Bone formation, Biopolymer chitosan, Rabbit femoral condyle

INTRODUCTION

Annually, more than 2.2 million bone grafting procedures are performed worldwide in order to make sure an adequate bone healing in many skeletal defects [1]. Currently, autograft is the golden standard for treatment of bone defect [2]. However, there are several downsides associated with autograft. In the clinical application, a portion of bone is taken from separate donor site on patient's body. Hence, surgical procedure is conducted twice. This treatment is also related to new fracture, donor site pain, new nerve damage, infection and bleeding [3,4]. For these reasons, allograft has been investigated and approved but is also associated with a high potential risk of infection, disease transmission, and immunologic reaction. Therefore, tissue engineering can become treatment of choice with promising approach for bone regeneration particularly for large size bone defect regeneration [3,4]. There are three main factors involved: stem cell, scaffold and growth factor. Scaffold plays an important role as structural support. An ideal scaffold should be biocompatible, biodegradable, have high surface ratio that could facilitate the growth of cells to desired tissue [5] and allow sufficient transportation of gases, nutrition and any other important factor [6]. Recently, three-dimensional injectable scaffold made from Hydroxyapatite (HA) and chitosan named IHAC has been studied extensively by National Atomic Energy Agency of Indonesia in collaboration with Oral Science Research Center, Faculty of Dentistry, University of Indonesia [6]. Hydroxyapatite is a major inorganic component in human hard tissue including bone and teeth [6]. Now-a-days, researchers have extensively fabricated hydroxyapatite using biomimetic strategies and is used in many types of implant regeneration and bone regeneration [7,8]. Fabricated hydroxyapatite shows improved clinical outcome due to its similarity in chemical composition to natural bone including high osteoconductivity and biocompatibility [9]. Natural bone is a composite consisting of 60-70% hydroxyapatite as an inorganic component and other organic component. This organic component plays a vital role in maintenance of bone structure [10]. Absence of this organic component causes migration of hydroxyapatite particle from implanted site to healthy tissue [10]. Due to this condition, combining hydroxyapatite with biopolymer was investigated to find best material that can maintain good properties of hydroxyapatite. Chitosan is a chitin derivate that is obtained by chitin deacetylation [11]. It is the most abundant polysaccharide after cellulose that can be degraded by glucosaminidase and chitosanases. In addition, chitosan has been proven to have increased biocompatibility properties, minimal immune reaction and antimicrobial activity [11]. Another study reported that Deproteinized Bovine Bone Mineral (DBBM) also capable to regenerate new bone formation [12]. Additional Hydroxypropyl Methylcellulose (HPMC) is required in order to create injectable scaffold [13,14]. In the clinical practice, usage of injectable scaffold provides several advantages over the traditional (liquid-powder form) as it minimizes risk of infection, cost of treatment, scar formation and patient discomfort [3]. Also, it can reach the defect site having limited access. Previous study on IHAC conducted by Bachtiar EW et al., showed minimal toxicity and improved cell proliferation in vitro [11]. Therefore, evaluation on the potency of injectable hydroxyapatite chitosan scaffold in vivo is important as the development of this material is important to reduce the dependency to imported goods and promote more affordable medical treatment for local people in Indonesia. We have hypothesized that IHAC will accelerate bone regeneration in the area of defect.

MATERIALS AND METHODS

This experimental research was conducted from January 2016 to August 2016 in Oral Biology laboratory, Faculty of Dentistry, and Histology laboratory and animal house, Faculty of Medicine, University of Indonesia, Indonesia. Prior to the research, ethical clearance was obtained from Animal Ethic Commission of Bogor Agricultural Institute, Indonesia (No. 05-2015IPB). In this experiment, we used two New Zealand adult Rabbits (weight 3 kg). The rabbits were randomly assigned as control rabbit and treated rabbit. Rabbits

were quarantined for two weeks preoperatively and received proper food and water throughout the project.

Surgical Procedure

General anesthesia containing a mixture of ketamine (1.5 mL) and xylazine (0.5 mL) was injected by intramuscular injection. The femoral condyle area was shaved and rinsed with Povidone-Iodine [4]. Longitudinal incision was made in femoral condyle area followed by the elevation of soft tissue. A defect of 1 cm depth and 5 mm in diameter was made in the exposed bone, 2 cm length from the tip of femoral condyle using bone bur. Defect of control rabbit was left empty, while defect of treated rabbit received IHAC scaffold [Table/Fig-1].

[Table/Fig-1]: Surgical procedure. (a) Defect was made using bone bur; (b) Defect of of 1cm depth and 5mm in diameter was made in rabbit's femoral condyle; (c) IHAC Scaffold placed in defect area.

Histologic Processing

After four weeks, the rabbits were euthanized and defect site was removed. The time period of four weeks was taken because formation of hard callus (which marked the mature stage of bone) occur after four weeks.

Then, the adjacent tissue was removed from the sectioned bone. The Bone was immersed in 10% formalin for 24 hour, followed by alcohol with concentration 70%, 80%, 95%, and 100% each for 24 hours. After dehydration and decalcification process, bone was embedded in paraffin block. Serial 5-µµm- thick paraffin block were cut through the center femoral condyle defect, subsequently staining was performed with Haematoxylin and Eosin (H&E) for microscopic evaluation.

Histologic Analyses

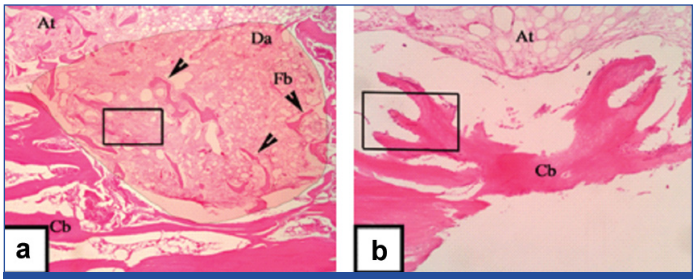
The histological slides were examined using binocular microscope (Olympus, China) at 40X and 100X magnification. The examined was carried out in Histology Department, Faculty of Medicine, University of Indonesia, by an experts in Histology on the histological slides. The experts performed descriptive histological evaluation based on modified Salkeld Histological Scoring so that the expert can obtain the histological healing score for each rabbit [12]. The higher score shows better bone formation [Table/Fig-2].

Histological Scoring	Description
0	No healing
1	Healing with fibrous union
2	Healing with fibro-cartilaginous or cartilage union
3	Bone healing with mineralized cartilage union
4	Mature bone healing

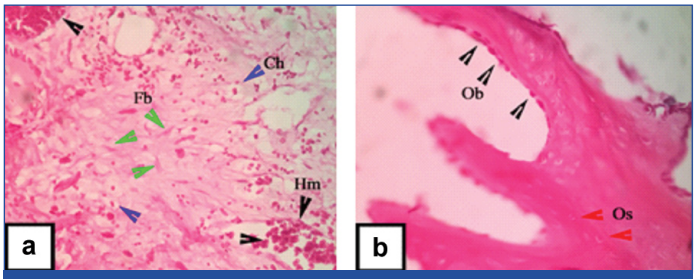
[Table/Fig-2]: Level of bone healing.
Modified Salkeld Histological Scoring of bone healing [12].

RESULTS

Bone regeneration was evaluated histologically after four weeks post scaffold transplantation into femoral condyle defect [Table/Fig-3,4]. In control rabbit histological view, The concave area is the central of defect area. Dark-red zone indicates mineralized cortical bone (hard tissue), while the light-red zone indicates un mineralized bone and soft tissue. Histological evaluation in defect area showed poor bone formation, indicated by the minimal formation of mineralized bone fragment in some area [Table/Fig-3a]. Presence of haemorrhagic



[Table/Fig-3]: Histological observation at four weeks postoperatively on control rabbit: (a) and treated rabbit; (b) in 40X magnification. Adipose tissue (At), Defect Area (Da), Bone fragment (Fb), Cortical bone (Cb).



[Table/Fig-4]: Histological observation at four weeks postoperatively on control rabbit: (a) and treated rabbit; (b) in 100X magnification. Haemorrhagic area (Hm, black arrowhead), Fibroblast (Fb, green arrowhead), Chondrocyte (Ch, blue arrowhead), Osteocyte (Os, red arrowhead), Osteoblast (Ob, black arrowhead).

area, chondrocyte and fibroblast can be seen in defect area of the control rabbit [Table/Fig-4]. These three components indicate that the bone formation is still in early stage of bone healing. Based on Modified Salkeld Histological Scoring, control rabbit score is 2 as the fibrocartilage is the dominant component found in this histological view [15]. Meanwhile, histological view of treated rabbit showed increased compact bone formation compared to control rabbit. This is indicated by union of bone fragment into well formed cortical bone [Table/Fig-3b]. Increased number of osteocytes can be found in this newly formed bone. Many active osteoblasts can be found embedded along the peripheral of cortical bone [Table/Fig-4b]. Osteoblast is in flat shape which means osteoblast is actively differentiate into osteocyte [16]. The changes of treated rabbit were significant indicated by the presence of abundant osteoblasts and osteocytes and also better union of cortical bone. Based on Modified Salkeld Histological Scoring, treated rabbit score is 4 as the osteogenesis already reached the mature stage.

The resume of histological evaluation in control and treated rabbit is showed in [Table/Fig-5]. Positive sign indicates the presence of bone-healing feature. The negative sign indicate the absence of them. Control rabbit has four positive and already reached the un-mineralized bone formation, but haemorrhagic area and inflammatory cells still can be found. It means there was impairment in the bone healing process. On the other hand, treated rabbit already reached the highest stage of bone healing indicated by the presence of osteocyte, osteoblast and mineralized cortical bone.

Better bone formation	Feature in bone healing	Control group	Treated group
	Hematoma	+	–
	Inflammatory cells	+	–
	Fibroacrtilage callus	+	–
	Unmineralized bone	+	+
	Mineralized bone	–	+

[Table/Fig-5]: Comparison of control rabbit and treated rabbit in bone healing and regeneration progress.

DISCUSSION

Ideal scaffold should possess the characteristic: biocompatible, biodegradable, have high surface ratio that could facilitate the growth of cells to desired tissue and allow sufficient transport

of gases, nutrition and any other important factor. Composite scaffold made from natural polymer and bone-like hydroxyapatite have been investigated intensively and show promising clinical outcome [11]. Hydroxyapatite and chitosan are good materials for bone regeneration due to biocompatibility, osteo-conductivity and biodegradability [11]. In this paper, we have described the evaluation of IHAC scaffold transplantation in femoral bone of New Zealand Rabbit using Modified Salkeld Histological Scoring. By using this scoring system, the quality of bone regeneration can be evaluated. From the histological analysis, we observed discrepancy in bone-healing progress between control and treated rabbit. The treated rabbit showed progressive increase in bone regeneration indicated by the union of mineralized cortical bone with the remodeling phase is still in process. On the other hand, the defect area in the control rabbit consists of bone fragment and dominated by callus. This finding is similar with research on Hydroxyapatite-Chitosan conducted by Chen Y et al., that showed complete healing of bone defect in rabbit received nano-hydroxyapatite/chitosan composite, while defect in control rabbit was still visible [3]. HA and chitosan is reported to enhance the process of osteoblast proliferation and differentiation [4]. Scanning Electron Microscope (SEM) evaluation of composite scaffold conducted by Kong L et al., showed many osteoblast cells adhered into the pores walls and distributed well [13]. The results showed that the combination of HA and chitosan increased the biocompatibility of scaffold [13]. Chitosan cationic feature plays a vital role in interaction with some anion element such as Glycosaminoglycan (GAG) and proteoglycan. Glycosaminoglycan is known to be responsible in retaining and accumulating the cytokines and growth factor so it will enhance the process of bone regeneration [13]. Research conducted by Liu H et al., showed other possible mechanisms of bone regeneration in HA-CS (Hydroxyapatite-Chitosan) transplanted defect. Hydroxyapatite/chitosan scaffold promotes bone regeneration by enhancing the adhesion, proliferation and activation of integrin-Bone Morphogenetic Protein (BMP) signaling pathway [14]. Further study needs to be conducted with larger sample size and analysing other biomarkers quantitatively. In the future, IHAC can be projected to be the solution for many skeletal problems as it is easy to use, affordable and accessible.

CONCLUSION

IHAC scaffold are capable in supporting the adhesion and differentiation of osteoblast. This condition supported the bone regeneration in treated rabbit, indicated by complete union of cortical bone and fast progression of bone regeneration. Therefore, IHAC is potential in promoting and guiding bone regeneration in bone defect. Further research on the development of IHAC scaffold is required to create more promising hard tissue graft for bone tissue engineering.

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