Dental Journal
Majalah Kedokteran Gigi

Original article

Bone remodeling using a three-dimensional chitosan - hydroxyapatite scaffold seeded with hypoxic conditioned human amnion mesenchymal stem cells

Michael Josef Kridanto Kamadjaja
Department of Prosthodontics,
Faculty of Dental Medicine, Universitas Airlangga,
Surabaya – Indonesia

ABSTRACT

Background: *Bone regeneration studies involving the use of chitosan–hydroxyapatite (Ch-HA) scaffold seeded with human amnion mesenchymal stem cells (hAMSCs) have largely incorporated tissue engineering experiments. However, at the time of writing, the results of such investigations remain unclear.* **Purpose:** *The aim of this study was to determine the osteogenic differentiation of the scaffold Ch-HA that is seeded with hAMSCs in the regeneration of calvaria bone defect.* **Methods:** *Ch-HA scaffold of 5 mm diameter and 2 mm height was created by lyophilisation and desalination method. hAMSCs were cultured in hypoxia environment (5% oxygen, 10% carbon dioxide, 15% nitrogen) and seeded on the scaffold. Twenty male Wistar rat subjects (8 – 10 weeks, 200 - 250 grams) were randomly divided into two groups: control and hydroxyapatite scaffold (HAS). Defects (similar size to scaffold size) were created in the calvaria bone of the all-group subjects, but a scaffold was subsequently implanted only in the treatment group members. Control group left without treatment. After observation lasting 1 and 8 weeks, the subjects were examined histologically and immunohistochemically. Statistical analysis was done using ANOVA test.* **Results:** *Angiogenesis; expression of vascular endothelial growth factor; bone morphogenetic protein; RunX-2; alkaline phosphatase; type-1 collagen; osteocalcin and the area of new trabecular bone were all significantly greater in the HAS group compared to the control group.* **Conclusion:** *The three-dimensional Ch-HA scaffold seeded with hypoxic hAMSCs induced bone remodeling in calvaria defect according to the expression of the osteogenic and angiogenic marker.*

Keywords: *bone tissue engineering; chitosan-hydroxyapatite scaffold; human amniotic mesenchymal stem cells; hypoxia*

Correspondence: Michael Josef Kridanto Kamadjaja**,** Department of Prosthodontics, Faculty of Dental Medicine, Universitas Airlangga. Jl. Mayjen Prof. Dr. Moestopo No. 47 Surabaya, 60132 Indonesia. Email: michael-j-k-k@fkg.unair.ac.id

INTRODUCTION

Tissue engineering has been undertaken to remedy many medical conditions, for instance: complications arising from wound healing, bone defects, immune system responses, and donor-transmitted disease. Three dimensional scaffolds were created to provide adequate support forming an extracellular matrix that enables cells to proliferate and differentiate. Chitosan alone as a scaffold suffers from its mechanical strength. Chitosan could easily break and therefore not able to create a suitable matrix for cell delivery.[1] Carbonate apatite also faces the similar problem, its brittle nature has limited its application as a scaffold. Therefore, combining both materials are predicted to create stronger scaffold.[2] Scaffold made from Chitosan – Carbonate Apatite (Ch-CA) has been reported as producing a robust, interconnected three-dimensional (3D) porous structure which could support the proliferation and differentiation of osteoblast during osteogenic differentiation.[3,4]

Hydroxyapatite has chemical structure that similar to human bone, therefore it has good affinity towards the bone and subsequently form chemical bond directly to the hard tissue.[5,6] By combining chitosan and carbonate apatite into scaffold, this material was expected to increase its mechanical strength and reduce the degradation time. The biocompatibility of hydroxyapatite (HA) and the resemblance of its mineral composition to bone has rendered it an ideal material for bone tissue engineering

(BTE). The development of HA into a 3-dimensional (3D) scaffold or a support to mesenchymal stem cells (MSC) *in vitro* has also been extensively explored. HA scaffolds offer massive advantages within the field of BTE.[7,8]

Human amniotic mesenchymal stem cells (hAMSCs) derived from human placentas are known for their pluripotent properties; ability to differentiate into three forms of germ layer; and efficacy in reducing both potential inflammation and immune reaction.[9,10] Chitosan-Hydroxyapatite scaffold seeded with hAMSCs was expected to intensify osteogenesis. The aim of this study was to observe the effect of Ch-HA scaffold seeded with hAMSCs within tissue engineering techniques.

MATERIALS AND METHODS

The isolation and culture procedure were performed following the securing of approval from the faculty's research ethics committee (No. 378/Panke. KKE/VII/2015). Material from a newly-formed amnion was peeled from the chorion and rinsed using phosphate-buffered saline (PBS). The amnion was then soaked in Ringer's lactate (RL) containing 2.5 µg/mL gentamycin and 1000 U/mL amphotericin which had been obtained from Gibco ^{TM} Amphotericin B, New York, USA.

The isolation and culture of hAMSCs using a modified Soncini's protocol. Small, fine pieces of amniotic membrane were treated with 0.25% trypsin in order to remove the epithelial cells. Centrifugation of five minutes duration at 2,000 rpm was carried with the supernatant subsequently being removed. This procedure was then repeated. The supernatant was washed using PBS containing 0.075 mg/ml DNase 1 (Takara Bio, Shiga, Japan) and 0.75 mb/ml type IV collagenase (Sigma-Aldrich, St. Louis, MO, USA). Incubation of the amnion was performed at 37°C for 60 minutes. Filtration and centrifugation lasting five minutes were performed to obtain cells. Single cells culture was created then using collagen–coated discs. The medium for the cells consisted of Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) at a ratio of 1:1, added to fetal bovine serum and 10 ng/mL human leukemia inhibitory factor (Gibco BRL, Gaithersburg, MD, USA). This medium was replaced every three days. Once the cell growth had reached a confluent stage (80%), the cell was split using trypsin. The laboratory stem cell protocol was implemented as the isolation procedure. hAMSCs were cultured in hypoxia chamber (1% oxygen, 5% carbon dioxide, and 94% nitrogen).

Chitosan-hydroxy apatite scaffold was prepared by dissolving 200mg of medium-molecular weight Ch powder (Sigma-Aldrich, St. Louis, MO, USA) into 5ml of ethanoic acid at room temperature and mixing them for 15 minutes. 15ml of sodium hydroxide solution was used for neutralising purposes in obtaining chitosan gel. Furthermore, samples of the chitosan gel were mixed homogenously with 200mg of HA prior to centrifuging at 1,500 rpm for ten minutes. After extraction of excess water, the solution gel was placed into the specific mold to produce scaffolds (5 mm diameter and 2 mm height). Before being transferred to a drying machine, the gel was frozen for two hours at -80°C.[3,4]

Human amniotic mesenchymal stem cells were deposited onto a 96-well cell culture plate (M96) at a density of 5 x 10^4 cell/well and incubated at 37°C for 24 hours with CO_2 5% concentration. Once the cell proliferation population had reached 80%, Ch-HA scaffold was added together with 100 µL of growth medium. The cells underwent a second incubation at 37°C for 20 hours with 5% CO_2. After the addition of 5 mg/mL MTT reagent (25 µL/well), the cells were incubated a second time for four hours before being observed under an inverted microscope. The scaffold and medium were removed and added to 200 µL/well DMSO. A 595 nm wave length ELISA reader was employed to read the absorbance, while the living cells were counted by means of a Cell Counting Kit.[3,4]

2% glutaraldehyde was used to fixate hAMSCs-seeded on Ch-HA scaffold at 40°C for 2-3 hours. The subsequent stage in the procedure consisted of washing with PBS solution three times every five minutes. After exposure to osmic acid 1% for 1-2 hours, the cells were washed again with PBS. A 15-minute dehydration procedure using alcohol at varying concentrations (30-100%) was also completed for each concentration. The scaffold was dehydrated using a critical point drying (CPD) device, attached to a stud pad with specific adhesive, and coated with pure gold. The scaffold was examined under a scanning microscope and photographed by means of a scanning electron microscope (JEOL JSM-T100, Japan).[3,4]

20 male Wistar rats were used as the animal subjects of the experiment. The inclusion criteria applied were as follows: aged 8-12 weeks old and weighing 100-150 grams. The subjects were randomly divided into a control group and a treatment group of equal size which were observed during weeks 1 and 8.

An anaesthetic procedure was performed 4-6 hours after the subjects were denied further food and water. 20mg of Ketamin HCL (Ketalar, Ireland) per kg of body weight and 3mg of Xylazine (Xyla,Ireland) per kg of body weight were injected intramuscularly. A mid-longitudinal skin incision was then made on the cranium dorsal surface after an aseptic procedure had been completed. The periosteum of the cranium was separated from the surface in order to produce a flap. A 2 mm diameter, circular, low speed bur (NSK, Japan) was used to create the bone defect 5 mm in diameter. The scaffold was implanted and sutured in order to re-attach the wound area but only in the treatment group.[3,4] The defect was subsequently sutured with blue nylon 5-0 mono suture (Ailee Co. Ltd, Busan, Korea).

The subjects were sacrificed during weeks 1 and 8 in order to obtain the required specimens. The implantation region was decalcified and embedded in paraffin to produce microscopic specimens. In order to highlight the angiogenesis and trabecular bone area, the specimens were stained with Hematoxylin and Eosin, while post-scaffold implantation
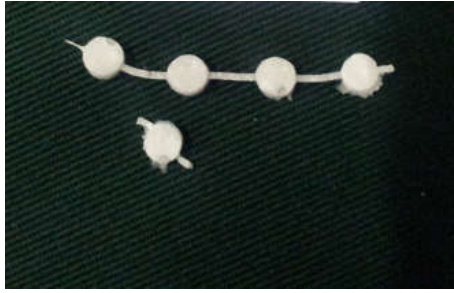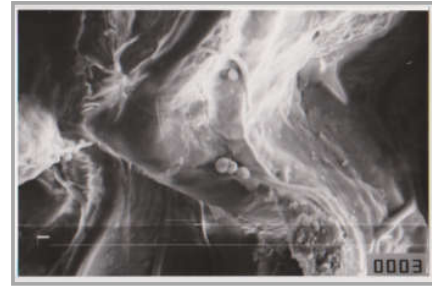
Figure 1. Ch-HA scaffold.

Figure 2. SEM image of cells attached and proliferated into the scaffold pores (SEM, 1000x magnification).
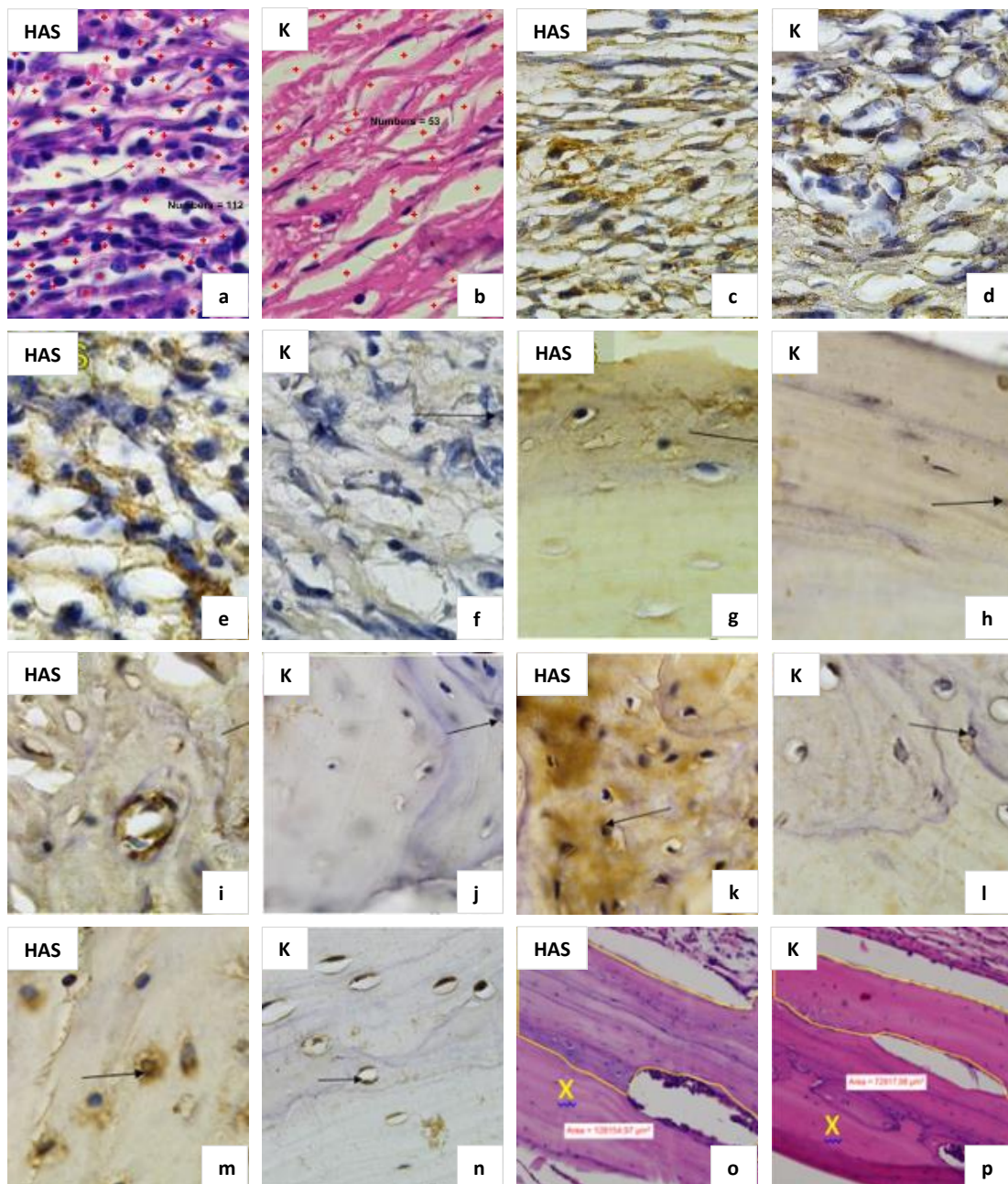

Figure 3. Angiogenesis (a and b), VEGF (c and d), BMP2 (e and f), RunX-2 (g and h), akaline phosphatase (i and j), type-1 collagen (k and l), osteocalcin (m and n) and trabecular bone area (o and p) with 1000x magnification. HAS: Hydroxyapatite-chitosan scaffold group seeded with hAMSCs. K: control group.

Kamadjaja/Dent. J. (Majalah Kedokteran Gigi) 2021 June; 54(2): 68–73

71

immunohistochemical staining (using mouse anti-human monoclonal (Novus Biological, USA) and polyclonal (Thermo Scientific, USA) antibody: BMP2, RunX-2, Alkaline phosphatase, Type-1 collagen, Osteocalcin, and VEGF of the specimens of cranium calvaria preparations was carried out. The Remmele Scale Index was used to measure the raw data. A Nikon H600L (Tokyo, Japan) light microscope with 1000x magnification and a DS Fi2 300-megapixel digital camera with image processing software (Nikon Image System) were respectively employed to examine the specimens and observe the tissue.[4] The data were presented as mean values, and standard deviation. Statistical Package for Social Sciences (SPSS) software version 15.0 (SPSS Inc., Chicago, IL, USA) was used to analyse the data by means of an ANOVA test and $p < 0.05$ was considered statistically significant.

RESULTS

The chitosan–hydroxyapatite was a solid 3D scaffold 5mm in diameter and 2mm thick (Figure 1). Toxicity tests incorporating the use of MTT Assay indicated that Ch-HA scaffold was not harmful to the hAMSCs culture. The percentage of viable cells found in the Ch-HA scaffold was 79.42 %. SEM imaging showed that cells were able to attach themselves to the Ch-HA scaffold's porous surface that was embedded in the calvaria bone defect (Figure 2). Histological image of seeded cells in the scaffold are shown on Figure 3. All groups were examined for expression of RunX2, BMP2, VEGF, alkaline phosphatase (ALP), type 1 collagen, osteocalcin, angiogenesis and trabecular bone area after 8 weeks. All treatment groups possessed a higher mean value than the control group (Figure 4).
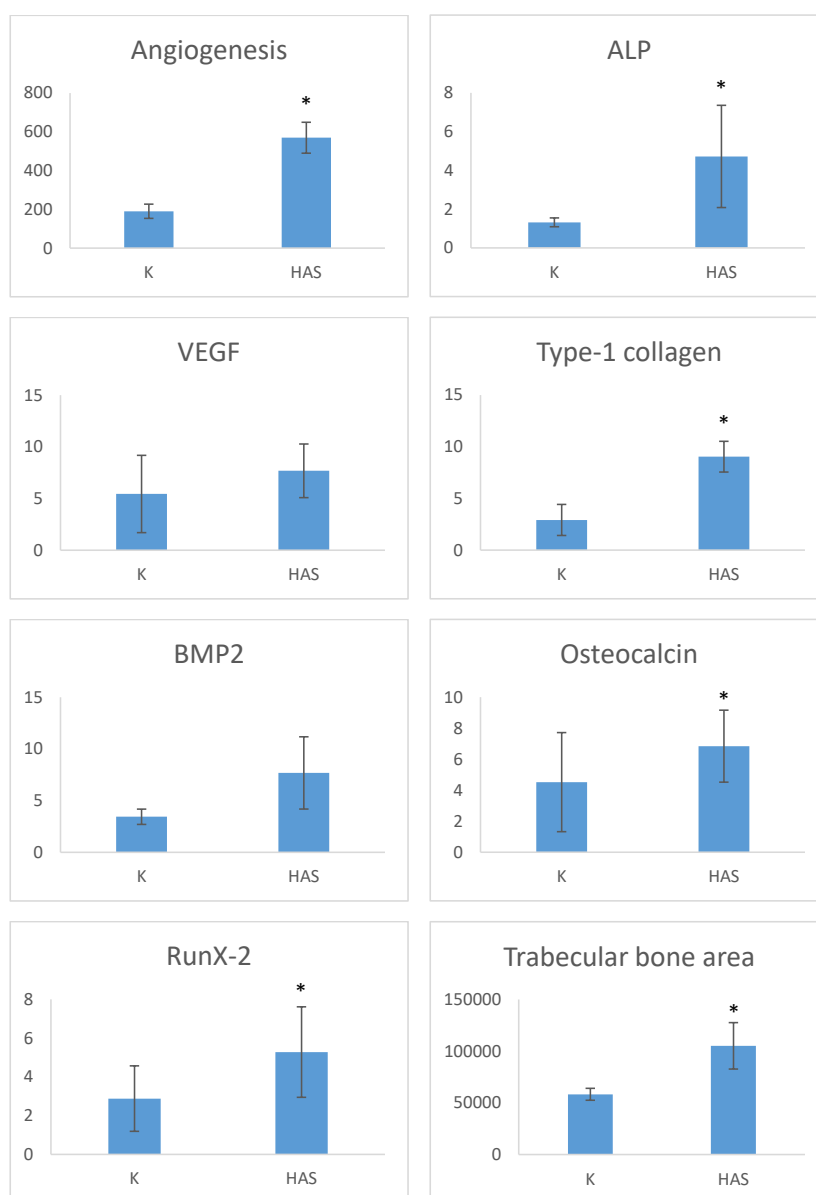

Figure 4. The mean value and standard deviation for several parameters observed after 8 weeks. K : control group; HAS: Hidroxyapatite – chitosan scaffold. *: $p < 0.05$ showed statistically significant.

DISCUSSION

In this study, an attempt was made to combine chitosan with hidroxy-apatite in order to create Ch-HA scaffold. Chitosan was combined with hydroxyapatite to increase the mechanical strength of the scaffold and decrease the degradation time of the material. Similar previous study shown that chitosan addition on a scaffold immersed in synthetic body fluid yielded a stronger mechanical strengh, greater strain, and more stable characteristic.[11] Another similar study also shown thatchitosan-hydroxyapatite scaffold had good biocompatibility and provided enhanced strength.[12] hAMSCs could attach to and proliferate effectively within the scaffold's porosity. The interconnected interstices of the scaffold were highly retentive and expected to be an excellent niche for osteoblast proliferation and differentiation. The ideal pore structure for tissue engineering scaffold ranged from 40-300 μm since this enabled vascular tissue migration and tissue growth.[4,13]

The potential of hAMSCs to act as a form of xenogenic MSCs during bone tissue engineering procedures has been thoroughly investigated. Several studies utilising xenogenic hAMSCs transplantation in various organs of rats confirmed a less intense immune reaction that could affect the tissue healing process.[14–16]

The first three days post scaffold-implantation consists the inflammatory phase, that is, the initial bone healing stage. During this phase, the hypoxic condition of the Ch-HA scaffold and the degranulation of platelets resulting from hematoma trigger increased VEGF expression that, in turn, induces angiogenesis which is essential in early healing processes. Functional capillary tissues provide nutritional intake, essential bioactive molecules, and adequate oxygen tension.[17] Angiogenesis plays an important role in the healing process in bone defects because it ensures cell survival in the scaffold.[18] Mesenchymal stem cells placed in hypoxic conditions enhance the expression of angiogenic factors, mainly VEGF.[19]

During the early stages of the regeneration process, the proliferation of MSC was followed by the differentiation of osteoblast. External signals produced by MSC and osteoblasts, particularly BMP2 protein, influence this regeneration process. In later stages, activation of transcription factor RunX-2 led by BMP2 helped induce MSC differentiation of preosteoblast and osteoprogenitors, which, in turn, continued to form a collagen and non-collagen bone matrix.[20]

The bone matrix maturation level was shown by the expression of type 1 collagen fibers. Mineralisation within the bone matrix maturation process will be influenced by type 1 collagen in previous stages. If the maturation level of bone matrix increases, type 1 collagen fibers will also be thicker.[21]

In this experiment, matured osteoblast marker was identified by osteocalcin. Osteoblast specifically expressed osteocalcin that is a non-collagen protein present in bone matrix.[22,23] In the treatment group the area of trabecular bone at the end of eight weeks was significantly higher compared to that of the control group, leading to the conclusion that new bone formation in the treatment group rate was higher than that in the control group.

The process of osteogenesis indicated by the expressions of ALP, type-1 collagen, and osteocalcin produced a better result in the treatment group compared to the control group. Therefore, the maturation level of bone matrix in the treatment group at the end of eight weeks was higher when compared to that of the control group. Mesenchymal stem cells could undergo differentiation to become osteoblasts, thereby producing the apropriate environment or stimulus. During osteogenic differentiation, several markers such as ALP, type 1 collagen, and osteocalcin were expressed by MSCs. At the time, when osteoblasts turn into osteocytes, ALP activity decreases. The latest marker of mature osteoblasts expressed by osteocytes was osteocalcin.

In this study, certain limitations occurred, including lack of systematic complication. The purpose of this research was to focus on regeneration of calvaria bone defects using hAMSCs and chitosan–hidroxyapatite scaffold. The study reported here should be continued to include research on their clinical application for bone augmentation. In conclusion, combining Ch-HA scaffold and hAMSCs could be used as an alternative bone tissue engineering method in order to escalate the clinical use of bone formation.

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