Osteoblast on Porous HA-TCP Scaffold Derived from Blood Cockle Shells Synthesis: In Vivo Study

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Abstract

Porus scaffold has been desired as bone graft materials due to its important property to bone ingrowth. Hydroxyapatite (HA) has excellent biocompatibility and osteoconductivity. Moreover, TCP is a resorbable bone graft material. The blood cockle shell can be converted into HA and TCP by hydrothermal method. This study aimed to evaluate the effects of porous HA-TCP scaffold derived from blood cockle shells synthesis on the osteoblast in socket healing. HA-TCP was synthesized from blood cockle shells using hydrothermal method at 200oC for six and 12 hours (Group 1 and 2). The XRD was carried out to determine the HA-TCP composition. HA-TCP with gelatine combination (1:1) scaffolds were made using freeze dry method. The pore sizes were confirmed by SEM and a porosity test was carried out. The scaffolds were applied in the incisive socket of mandible rats for seven and 14 days. Thereafter, histological evaluations were perfomed. In the XRD results, Group 1 showed HA54,5%-TCP9,1%; Group 2 showed HA51,5%-TCP16,8%. The SEM image confirmed the pore sizes of Group 1 and 2 were in the range 41,76-64,8µm and 41,02-49,1µm. The scaffold porosity of Group 1 and 2 were 72,98% and 77,51%. The histological evaluation after seven and 14 days showed the osteoblast number of Group 2 were significantly greater than Group 1. This study suggested the osteoblasts in porous HA-TCP scaffold derived from blood cockle shell synthesis were dependent on the porosity of scaffold and HA-TCP composition. The osteoblast proliferation were prominent for the group with composition HA51,5%-TCP16,8% and 77,51% porosity.

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Introduction

With regard to the bone regeneration process, scaffold acts a temporary supporting structure for attaching and growing cells that forms a new bone ingrowth. The scaffold architecture plays a crucial role for bone regeneration process, such as the porosity, pore size and shape.[1] The pores characteristics are principal for the biological process in terms of new bone ingrowth process inside the scaffold.[2] The degree of bone infiltration into the porous block was depended on the porosity and pore size.[3] Furthermore, open porosity and sufficient pore size are substantial factors to cell nutrition transportation, tissue vascularity[4], protein absorption, cellular migration[5,6], osteoconduction.[7,8] The porous scaffold has a good osteoconductivity inside the pored scaffold and be able to be absorbed by body than dense scaffold. These cause the pored scaffold synthesize development are more demandable.

Hydroxyapatite (HA) is a main inorganic component to the bone and teeth which is commonly used as a bone graft.[9] Hydroxyapatite has a similar chemical, biological and crystallographycal characteristics with bone and teeth. Nevertheless, HA is a fragile and slightly resorbable.[10,11] Futhermore, tricalcium phosphate (TCP) is a bioresorbable material which makes it easier to absorb by bone.[12] The combination of HA and TCP could be an effective material by their properties such as osteoconduction, osteoinduction and resorbability.[13] HA and TCP

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combination is expected to assist the acceleration of socket healing process.

The socket after extraction would be going on reparative process after, which is called socket healing. It is consisted of four phases. The first phase is homeostasis that proceeded by inflammation phase.[14] Then, proliferation phase which is lasted from the third until fourteenth day. Proliferation phase is marked by fibroblast, angiogenesis, collagen forming and matrix synthesize.[15] Proliferation of osteoblast and osteoclast is happened on this phase. In addition, tissue regeneration is happened either on this phase.[16] The remodelling phase is characterized by tissue and collagen remodelling, epidermal maturation and shrinkage.[17]

Ridge preservation acts as a crucial management to maintain alveolar bone for retention, support and stabilization after extraction, particularly to implant placement, prosthetic restoration and stabilization. It can cause ridge volume loss, if there is no any immediate treatment. Physiologically, this condition is happened on sixth until twelfth months after extraction.[18] It also can cause ridge volume loss up to 40-60% in the first three years.[19] Bone graft is needed to retain alveolar bone height and thickness.

In this study, we evolved HA-TCP derived from blood cockle shells synthesis using hydrothermal method with variation of sintering times and then produced porous HA-TCP scaffold by freeze drying method. In order to evaluate which HA-TCP scaffold porosity is most suitable for bone regeneration, different HA-TCP compositions were produced and tested in vivo using a socket after extraction of insisive of Rattus Novergicus Wistar type.

Materials and methods

Preparation of HA-TCP scaffold

HA-TCP powder was synthesized from blood cockle shells which had been cleaned and ground to form a smooth texture. Then calcined at 100° for three hours in an oven furnace (Naberterm, Germany). As part of the hydrothermal process, the hydroxyapatite powder was mixed with Ammonium Dihydrogen Phosphate and heated at 200°C in an oven furnace (Naberterm, Germany) with a sintering time of 6 and 12 hours. After completion of this process, the powder was rinsed with distilled water and methanol PA. The powder was then heated to a temperature of 50° for four hours and at 900°C for a further three hours. HA-TCP powder had been filtered with a 200mesh filter to produce a powder size of <74 µm. Futhermore, HA-TCP scaffold was subsequently produced by mixing 25% HA-TCP with 10% gelatin (wt%) (1:1), frozen at a temperature of -80° C for five hours and freeze dried for 30 hours. HA-TCP scaffolds were sterilized by gamma radiation 25 KGy (Gray) dose.

Evaluation of composition HA-TCP powder

An XRD test was conducted using an Xpert-Pro PANalytical at an angle of 2θ= 5°- 60° to identify the crystallization phase and content of the material using X-ray electromagnetic radiation. An identification phase was then performed by comparing the hydroxyapatite diffraction pattern with data from International Center for Diffraction Data (ICDD).

Evaluation of pore size and porosity

Pore were confirmed by scanning electron microscopy (SEM) with a 1000x magnification. The porosity of the HA-TCP scaffold was evaluated using a liquid displacement method determined by the percentage of liquid absorbed by scaffold after being immersed in ethanol. A porosity test measured the volume of the samples, weighing their dry weight before soaking them in 96% absolute ethanol for 48 hours. The samples were weighed to determine the wet mass of the specimens. The porosity of the samples was then calculated using the following equation below.

$$\text{Porosity (\%)} = \frac{m_b - m_k}{\rho_{\text{liquid}} \times V_b} \times 100$$

mb = wet mass of specimen (gram)
mk = dry mass of specimen (gram)
Vb = volume of specimen (cm3)
ρ liquid = density of water (1 gr/cm3)

In vivo procedure

Twenty four males Rattus Novergicus Wistar type (weight: 150 – 350 g) were used for the study, following the Ethics Committee for Animal Research at the Faculty of Dentistry, Universitas Hang Tuah. All surgical procedures were performed under general anesthetic using

ketamine and xylazine at doses of 0.11 mL / 100 gr BB intramuscularly. After shaving and aseptic treatment of the surgical sites with 10% povidine iodine antiseptic, incisive teeth was extracted and then HA-TCP scaffold was applied in the socket randomly. Thereafter, the gingiva around the socket was sutured with a silk suture. The animal pain and discomfort were minimized via analgesic and antibiotik therapy for three days.

Histological preparation

After seven and 14 days of healing, the animals were euthanized via an anesthesia overdose. The specimens were removed en bloc and subsequently perfused in 10% formaldehyde buffer solution for 48 h. After fixation, the decalcification process is done using nitric acid. Then, dehydration process was conducted in in a series of ethanol (70%–100%) and infiltrated. After complete infiltration, the samples were embedded in a paraffin. The embedded paraffin blocks were grind sectioning to a final thickness of 45 µm and were then stained with Hematoxylin Eosin (HE). The histological analyses were performed using a light microscope with a 400X magnification.

Statistical analysis

The statistical analysis was performed using a parametric statistical test One-way ANOVA, followed by post hoc LSD test using the computer software SPSS (SPSS Inc., Chicago, IL, USA). Statistical significance was set at 95%.

Results

Characteristics of HA-TCP scaffold

Blood cockle shells were converted into HA-TCP using hydrothermal method successfully. The XRD result was showed in table 1. HA-TCP was synthesized from blood cockle shells using hydrothermal method at 200°C with a sintering time of 6 hours (Group 1) showed HA 54,5% and TCP 9,1%. Moreover, HA-TCP was synthesized from blood cockle shells using hydrothermal method at 200°C with a sintering time of 12 hours (Group 2) showed showed HA 51,5% and TCP 16,8%.

Compound	Group 1 (%)	Group 2 (%)
Tri-Calcium Phosphate (TCP)	9,1	16,8
Hydroxyapatite (HA)	54,5	51,5
Aragonite (CaCO3)	22,2	20,8
Calcium Hydroxide	3,0	3,0
Calcium Oxide	5,1	7,9
Calcite	6,1	-

Tabel 1. The composition of HA and TCP.

Group	Pore size (µm)	Porosity (%)
Group 1	41,76 - 64,87	72,98
Group 2	41,02 - 49,14	77,51

Tabel 2. The pore size and porosity of porous HA-TCP scaffold.

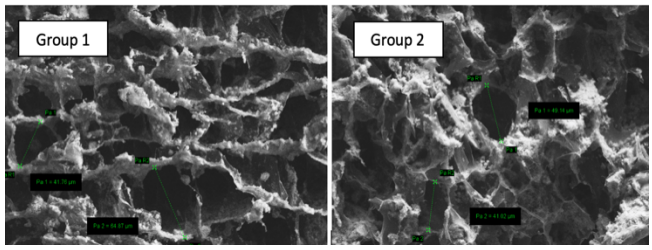

Figure 1. SEM images of the different porosity and pore sizes of porous HA-TCP scaffold.

The SEM image of the tested specimen is presented in figure 1. The pores of scaffold were confirmed in all groups. The SEM image confirmed that the pore size of Group 1 was in the range 41,76-64,87 µm and Group 2 was in the range 41,02-49,14 µm. As shown in table 2, the porosities in the range 72,98 – 77,51% were successfully obtained. The porosity of the porous HA-TCP scaffold was based on the percentage volume of void space contained in the scaffolds. Group 2 showed the highest porosity compared to Group 1.

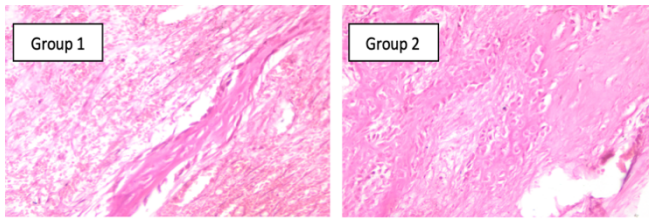

Figure 2. Histological images of osteoblast at seven days.

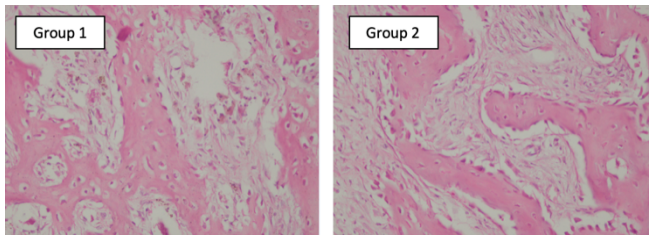

Figure 3. Histological images of osteoblast at 14 days.

Histological analysis

After seven and 14 days in vivo, the osteoblasts were observed in all of the groups

(figure 2 and 3). The histological evaluation is presented in figure 4. There was significant differences between the groups after seven days of healing, Group 2 (25,83 ± 3,68) presented a significantly higher osteoblast number than Groups 1 (19,83 ± 2,23). Moreover, Group 2 (33,28 ± 2,88) also presented a significantly higher osteoblast number than groups 1 (24,56 ± 2,05) after 14 days of healing. Group 1 showed there was increasing number of osteoblast after seven and 14 days. Futhermore, Group 2 also showed the increasing number of osteoblast after seven and 14 days.

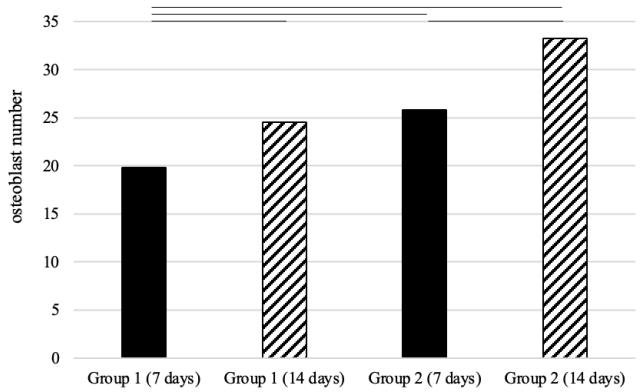

Figure 4. The osteoblast number after seven and 14 days. *p < 0.05. Values are shown as the mean.

Discussions

In this study, we have evaluated the number of osteoblast of porous HA-TCP scaffolds with various HA-TCP compositions derived from blood cockle shells synthesis after seven and 14 days of healing. HA and TCP are bone graft material that commonly used in the medical field. Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) (HA) is a main inorganic component to bone and teeth that generally used as bone graft. Due to its biocompatibility, osteoconductivity, chemical and biological properties which are similar to the bone.[10,20] Whereas, the tricalcium phosphate (TCP) has a similar physical and chemical compound to the bone mineral structure and human teeth.[12,21] Tricalcium phosphate has an easy absorbable properties, bioactive, biocompatible and osteoconductive. The combination of HA and TCP, that is composed by an appropriate composition, would be enabled effective as bone graft composition to their osteoconduction, osteoinduction and resorbability.

This study observed the osteoblast after seven and 14 days due to the duration of the proliferation phase of socket healing process. With the regards to the influence of HA-TCP composition, it has been indicated that the optimal balance of HA and TCP composition may be an essential factor for proliferation of osteoblast. HA-TCP scaffold with composition of HA 51,5% and TCP 16,8% which applied for seven and 14 days presented significantly more osteoblast number compared to the other group. It can be explained that the number of osteoblast dependent on HA and TCP composition. Moreover, Hydroxyapatite (HA) is a biocompatible and osteoconductive materials that it would stimulate Vascular Endothelial Growth Factor (VEGF) which plays an important role to proliferate microvascularitation for bone regeneration process. Furthermore, it can also stimulates the osteoblast proliferation increasing.[22] The osteoinductive properties of hydroxyapatite could promotes the differentiation and osteoblast proliferation.[9] Prahasanti et al reported that osteoblast were presented in hydroxyapatite group and osteopontin as an early osteoblast marker was detected.[23] TCP could increase osteoblast proliferation than other calcium compounds. Futhermore, TCP also promotes osteopontin, up-regulation osteopontin and influences the proliferation, differentiation and matrix deposition by osteoblast activation.[24] Osteopontin initiates the osteoblast differentiation and maturation.[25] Osteopontin has an important roles to the attaching osteoblast in matrix bone, the controlling bone remodelling process, and the differentiation and activation signals of osteoclast.[26]

The regardless of the pore size and porosity of HA-TCP scaffolds, the histological analysis showed the proliferation of osteoblast. A porosity 77,51 % with a pore size of 41,02 - 49,14 µm presented the highest porosity and showed significantly more osteoblast number than the other group. It could be explained that the increasing of osteoblast number was dependent on the pore size and porosity of HA-TCP scaffolds. It was also suggested in the other study that the osteoblast respond differently according to the porosity and pore size. Aminatun et al reported that a combination Chitosan-Chrondroitin Sulphate/Hiroxyapatite scaffold with 79,4% porosity showed the highest cell viability. It also suggested that the higher

porosity allows more osteoblast cells to grow.[27] The porosity provides a threedimensional space for bone tissue formation and a substrate for cell adhesion.[23] This result is in agreement with that Cheng et al, where osteoblasts exhibited cell-type dependent responses to construct porosity.[28]

Conclusions

The results of this study suggest the osteoblast in porous HA-TCP scaffold derived from blood cockle shell synthesis were dependent on porosity of scaffold and HA-TCP composition. The osteoblast was most prominent for the group with HA-TCP with composition HA 51,5%, TCP 16,8% and 77,51% porosity after seven and 14 days in vivo. The optimal balance of HA and TCP composition, pore size and porosity may be an essential factor for proliferation of osteoblast.

Declaration of Interest

The authors report no conflict of interest.

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