

Research Report

Endothelial cell cultured on HA/TCP/chitosan scaffold for bone tissue engineering

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ABSTRACT

Background: Angiogenesis is crucial for the success of bone reconstruction through tissue engineering. Currently, is still not known the activity of endothelial cells that is responsible for blood vessel formation, cultured in HA/TCP/chitosan scaffold. The ability of the scaffold to facilitate the proliferation and migration of endothelial cell to form blood vessel is essential for cell survival especially in the inner area of the scaffold that is susceptible for cell death if adequate vascularization is not occurred. **Purpose:** The purpose of this study was to evaluate the porosity of HA/TCP/chitosan scaffold and the biocompatibility of HA/TCP/chitosan scaffold to endothelial cells. **Methods:** Endothelial cells were isolated from umbilical vein (human umbilical vein endothelial cells/ HUVEC). HA/TCP/chitosan scaffold was made from two gelling agents and various basic washing solutions. The characteristic of scaffold was examined by scanning electron microscopy. The activity of HUVEC was evaluated by MTT assay. **Results:** Initial average scaffold porosity size range from 68 μm and increased up to 134 μm after 7 days incubation with 10 mg/L lysozyme. There was no significant difference in the viability of HUVEC incubated with the scaffold compared to control. **Conclusion:** HA/TCP/chitosan has a good biocompatibility for HUVEC. This condition supports the activity of HUVEC in the scaffold for angiogenesis process, to provide oxygen and nutrient necessary for osteoblast.

Key words: Endothelial cells, scaffold, hydroxyapatite, tri calcium phosphate, chitosan

ABSTRAK

Latar belakang: Angiogenesis merupakan proses yang penting untuk keberhasilan rekonstruksi tulang melalui rekayasa jaringan. Saat ini, aktifitas sel endotel pembentuk dinding pembuluh darah pada HA/TCP/chitosan scaffold belum diketahui. Kemampuan scaffold sebagai tempat proliferasi dan migrasi sel endotel untuk membentuk pembuluh darah penting untuk kelangsungan hidup sel osteoblast terutama di bagian dalam scaffold. **Tujuan:** Mengevaluasi porositas HA/TCP/chitosan scaffold serta sifat biokompatibilitas scaffold terhadap sel endotel. **Metode:** Sel endotel diisolasi dari vena tali pusat (human umbilical vein endothelial cells/HUVEC). HA/TCP/chitosan scaffold dibuat dengan variasi gelling agents dan dicuci dengan berbagai larutan basa. Karakter scaffold dievaluasi dengan scanning electron microscope. Aktifitas HUVEC dievaluasi dengan MTT assay. **Hasil:** Pada tahap awal, rata-rata ukuran porus 68 μm dan meningkat menjadi 134 μm setelah inkubasi dengan 10 mg/mL lysosyme selama 7 hari. Kultur HUVEC pada scaffold selama 24 jam tidak menunjukkan tingkat viabilitas yang berbeda dibandingkan dengan kontrol. **Kesimpulan:** HA/TCP/chitosan memiliki sifat biokompatibilitas yang baik terhadap sel HUVEC. Kondisi ini memberikan dukungan terhadap aktifitas sel HUVEC pada scaffold untuk proses angiogenesis yang akan memberikan oksigen dan nutrisi untuk osteoblas.

Kata kunci: Sel endotel, scaffold, hydroxyapatite, tri calcium phosphate, chitosan

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INTRODUCTION

Tissue engineering is a process of tissue regeneration and remodelling to repair, replace, maintain or increase organ function. This technique relies on the role of stem cells that have the potential to proliferate and differentiate in the scaffold with specific stimuli to form the desired tissue.¹ There are three important factors involved in tissue engineering technique: stem/progenitor cell; biomaterial (scaffold) to give structural support for organisation, growth and differentiation of cells in the process of tissue formation; and chemical and mechanical signals required to increase the proliferation and differentiation of cells. These three components can be developed *in vitro* and transplanted to the defect area *in vivo*.^{1,2} Tissue engineering technique has been developed as an alternative treatment for large-size bone defect reconstruction.

Recently, the development of a new biodegradable scaffold based on the biopolymer chitosan has been initiated by National Atomic Energy Agency of Indonesia in collaboration with the Laboratory of Oral Biology, Faculty of Dentistry, University of Indonesia. The new development to manufacture this material in Indonesia is important to build-up scientific and clinical expertise, to become independent from high cost-import of this material and to reduce the high treatment costs accordingly. The scaffold made from hydroxyapatite (HA), tri calcium phosphate (TCP) and chitosan.³⁻⁶ The rationale of this scaffold design is to have materials mixed in different proportions with different resorption rates. The polymer which can be degraded to a certain extent thus leaving behind macropores for colonization of osteoblasts. The calcium phosphate could then be resorbed by osteoclasts and replaced with new bone.

Blood vessel formation (angiogenesis) is prerequisite for bone formation (osteogenesis) therefore angiogenesis is crucial for the success of bone reconstruction through tissue engineering.⁷⁻¹⁰ Angiogenesis include migration, proliferation and differentiation of endothelial cells for new capillary from the existing vessels. Firstly, vascularization delivers oxygen for osteoblasts metabolic activity.¹¹ Previous study shown a decrease in alkaline phosphatase activity and collagen synthesis *in vitro* in low oxygen tension environment. This condition inhibits the differentiation of osteoblast cells possibly through the downregulation of Runx2.¹¹ Runx2 is the essential transcription factor for osteoblasts differentiation and for maintaining their differentiated state as Runx2 is needed for gene expression of collagen type I and most of bone non-collagenous proteins. Secondly, blood vessels carry variety of cells including progenitor cells from bone marrow and peripheral blood that are able to differentiate to osteoblasts under the appropriate signals. Evidences showed the possibility of pericytes, the cells on the blood vessel wall to differentiate to osteoblasts.¹² Third, blood vessel cells secrete paracrine factors that regulate bone cells metabolism. A number of angiogenic factors have also their osteogenic potential such as vascular

endothelial growth factor (VEGF).¹³ VEGF stimulate osteoblasts differentiation, showed by the increase in bone nodule formation and the increase in alkaline phosphatase activity in osteoblast cell line.¹³

Currently, it is not known how is the activity of endothelial cells that is responsible for blood vessel formation, culture in HA/TCP/chitosan scaffold is still not known. The ability of the scaffold to facilitate the proliferation and migration of endothelial cell to form blood vessel is essential for cell survival especially in the inner area of the scaffold that is susceptible for cell death if adequate vascularization is not occurred. The purpose of this study was to evaluate the porosity of HA/TCP/chitosan scaffold and the biocompatibility of HA/TCP/chitosan scaffold to endothelial cells. The urgency of this research that this HA/TCP/chitosan scaffold is expected to facilitate the growth of endothelial and osteoblasts cells to form new bone matrix for large bone defect reconstruction such as cleft palate and rehabilitation of post tumor resection, cases that are frequently found in Indonesia. The development of new scaffold material in Indonesia is important to reduce the dependency to imported materials so as to promote a more affordable treatment with local product.

MATERIALS AND METHODS

Chitosan is obtained by deacetylation of chitin, the structural element in the exoskeleton of shrimp with sodium hydroxide solution and precipitated by hydrochloric acid. HA and TCP are prepared by wet chemical method of calcium hydroxide and phosphoric acid sodium phosphate and calcium nitrate, respectively. The paste is exposed by radiation sterilization of 25 kGy radiation. A scaffold premix liquid is prepared by incorporating 6 gr chitosan in 10 ml 3% acetic acid with gelling agent of 1% hydroxypropylmethylcellulose (HPMC) or 1% carboxymethyl cellulose (CMC) and 0.2M phosphate solution for 2 hours under ambient conditions. To reduce the acid residue derived from chitosan dilution solution, the scaffolds were incubated with Na₂SO₃ or NaOH for 24 hours. The porosity of the scaffold was analyzed by scanning electron microscopy from three randomly regions of interest. Porosity was measured before and 1 week after enzymatic degradation with 10 mg/L lysozyme.¹⁴

Endothelial cells were isolated from umbilical cord human umbilical vein endothelial cells (HUVEC). Only breach deliveries with caesarean section were included in this study. Informed consents were signed by all patients. Umbilical cords of approximately 15 cm were stored in cord buffer transport medium containing 2 g/mL glucose (Sigma, St Louis, USA) in PBS with 1% penicillin-streptomycin and processed for culture within 4 hours. Isolation of HUVEC was performed according to Baudin *et al.*,¹⁵ with some modification. Umbilical vein were washed with sterile PBS with 2 cannulae fixed in both cord extremities to remove the remained blood clot. 0.2% collagenase I

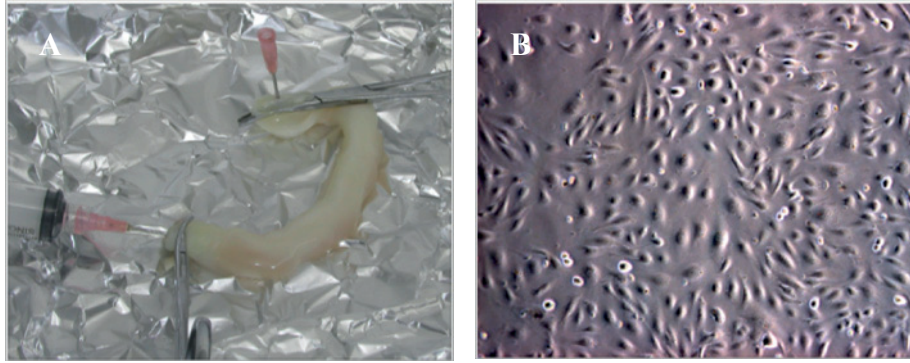


Figure 1. Isolation of human umbilical vein endothelial cells (HUVEC). A) Endothelial cells were isolated from the vein of an umbilical cord. Collagenase type I was injected into the vein, secured by surgical clamps and incubated for 7 minutes in 37° C incubator, B) HUVEC with 80% confluency after 5 days of culture.

(Gibco, California, USA) was injected into the umbilical vein and incubated briefly for 7 minutes in 37° C incubator to avoid any contamination of fibroblast or smooth muscle cells (Figure 1A). The cords were massaged gently to facilitate cell detachment and were washed with complete M200 supplement with LSGS kit containing fetal bovine serum, 2% v/v; hydrocortisone, 1 mg/ml; human epidermal growth factor, 10 ng/ml; basic fibroblast growth factor, 3 ng/ml; and heparin, 10 mg/ml (Gibco, California, USA). HUVEC were cultured 6 well-plate (Nunc, Roskilde, Denmark) for approximately 6 days until it reached 80% confluency (Figure 1B). HUVEC were then incubated with scaffold for 24 hour and tested for their viability by means of MTT assay.

HUVEC was cultured with various gelling agents (HPMC or CMC) and basic washing solutions (Na_2SO_3 or NaOH) of HA/TCP/chitosan after 24 days of incubation MTT assay was performed and as control HUVEC cultured in normal DMEM medium.

RESULTS

Evaluation of characteristic of HA/TCP/chitosan was performed by scanning electron microscopy (JEOL, JSM 6510, Japan) (Figure 2). Porous size was measured in three regions of interest of each sample with a magnification of 200–500 \times . Before enzymatic degradation, it showed the initial porous size of the scaffold range from 19 μm to 122 μm (Table 1). After enzymatic degradation with lysozyme the size of the porous increased range from 50 μm to 160 μm (Table 1). The highest increase was found the HPMC- Na_2SO_3 and HPMC-NaOH scaffold, where approximately 4.5-fold higher porous size was detected than the initial porous size.

HUVEC cells were cultured until 80% confluency (Figure 1B) and harvested using trypsin. HUVEC cells (5×10^3 cells) were plated in 96 well-plate with medium 200, LSGS kit and scaffold and incubated for 24 hours. Experiments were in duplo and repeated twice. MTT data

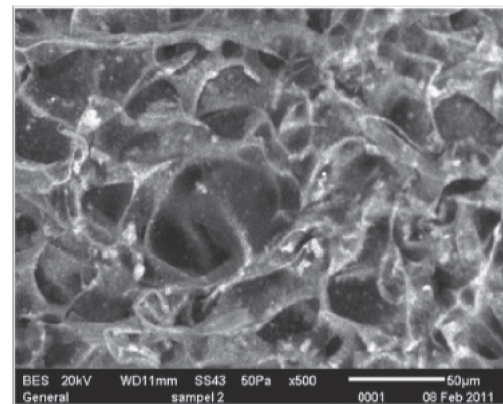


Figure 2. Porosity of the scaffold. Scanning electron microscope photograph of scaffold after 7 days of enzymatic degradation with 10 mg/L lysozyme.

showed no significant difference in the HUVEC incubated with scaffold compared to HUVEC incubated in normal medium. A comparable viability compared to the group where HUVEC was cultured with normal medium (Figure 3). The data showed the biocompatibility of the scaffold as HUVEC viability was unchanged with scaffold incubation compared to the control group.

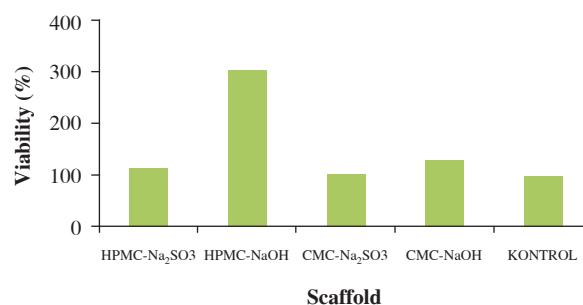


Figure 3. Viability of HUVEC after 24 hours incubation with various scaffold materials.

Table 1. Porosity of the scaffold before and 7 days after enzymatic degradation with 10 mg/L lysozyme at 37° C

Scaffold	Porosity (μm)*		Increased of porosity (%)
	Before enzymatic degradation (Mean \pm SD)	After 7 days of enzymatic degradation (Mean \pm SD)	
HPMC-Na ₂ SO ₃	19 \pm 12	160 \pm 60	534
HPMC-NaOH	33 \pm 28	134 \pm 55	402
HPMC-NaTPP	68 \pm 39	50 \pm 48	73
CMC- Na ₂ SO ₃	95 \pm 69	59 \pm 29	62
CMC-NaOH	122 \pm 40	93 \pm 45	77
CMC-NaTPP	72 \pm 33	89 \pm 47	125

DISCUSSION

For tissue engineering, the ideal scaffold should have the characteristic: biocompatible, biodegradable, high surface area/and volume ratio that could support the attachment, proliferation and differentiation of cells.¹⁶ The design of our scaffold is materials mixed in different resorption rates. Natural polymer of chitosan which can be degraded to a certain extent thus leaving behind macropores for colonization of osteoblasts. The calcium phosphate could then be resorbed by osteoclasts and replaced with new bone. Data presented in the literature demonstrated the ability of chitosan to be degraded by enzyme to become oligosaccharide that is easy to degrade.¹⁴ It can also form a complex with connective tissue such as collagen and glycosaminoglycan to develop interconnected three dimensions structure.¹⁴ Research works on enzymatic degradation of chitosan for human use have been carried out mostly in lysozyme¹⁴ since it is found in various human body fluids, including serum (concentration 4–13 mg/l) and tears. We studied porosity of the scaffold in its initial form and after *in vitro* enzymatic degradation with lysozyme. The data of the present study showed the immersion of scaffold in lysozyme in PBS solution for 7 days increased the porous size of the scaffold as high as 5-fold. The increase in porosity size gives a positive effect as it allows the infiltration of osteoblast and endothelial cells to the inner core of the scaffold. The migration of endothelial cells inside the scaffold to form the vascular beds has a crucial role for the survival of cell and scaffold complex. Initially, cell and scaffold complex relies on diffusion of nutrition supply from the surrounding existing vascular until the vascularized bone is developed. This phase is a critical period, particularly in the large defect reconstruction where the diffusion of nutrition effectively ranged from 150-200 μm from the existing vessels. The deficiency of nutrition for cells in the inner core of the scaffold might prevent the formation of new bone and result in tissue necrosis.¹⁷

The data of the present study showed the potential of HA/TCP/chitosan as the ideal scaffold for bone tissue engineering. It revealed that the locally made scaffold composed of HA/TCP/chitosan do not have the negative effect on the viability of endothelial cells. Metabolic

activity of HUVEC incubated with HA/TCP/chitosan was comparable to the activity of HUVEC in normal medium. In contrast, metabolic activity of dental pulp cells incubated with HA/TCP/chitosan scaffold was significantly increased (data not shown). The differences could be explained by the osteoconductive property of the scaffold that could induce the proliferation of dental pulp cells and their differentiation to the osteoblastic lineage. The results indicated the good biocompatibility property of scaffold toward endothelial cells.

As the blood vessel formation is prerequisite for bone formation, angiogenesis is therefore crucial for the success bone reconstruction through tissue engineering technique. In light of the critical role of endothelial cells (ECs) in the angiogenic process, a necessary step to evaluate and properly predict the vascularization potential of biomaterials is to assess the interaction of ECs with the respective substrate. HUVEC represent a model for any research on general properties of human ECs as HUVEC is the most simple and available human EC type, accurate for the preparation of large quantities of cells. The present study indicated a good biocompatibility property of scaffold material to HUVEC.

The new development to manufacture this material in Indonesia is important to build-up scientific and clinical expertise, to become independent from high cost-import of this material and to reduce the high treatment costs accordingly. The scaffold made from hydroxyapatite, tri calcium phosphate and chitosan for bone tissue engineering purpose. The current study evaluated the biocompatibility of HA/TCP/chitosan scaffold to HUVEC. Proliferation of mammalian cells on a particular matrix takes place in three stages: first the cells on the matrix, then the spread and finally they divide in the presence of nutrients. Attachment of HUVEC to the scaffold, migration of the scaffold to facilitate the blood vessel formation in the inner core of the scaffold will be studied in the near future.

The conclusion of this study is HA/TCP/chitosan has a good biocompatibility for HUVEC. This condition supports the activity of HUVEC in the scaffold for angiogenesis process for oxygen and nutrient supply necessary for osteoblasts, successful bone tissue engineering could be achieved accordingly.

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