Effect of Biphasic Calcium Phosphate Treated with Vascular Endothelial Growth Factor on Osteogenesis and Angiogenesis Gene Expression *In Vitro*

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Abstract — **Biodegradable biphasic calcium phosphate (BCP) scaffold holds tremendous potential for bone tissue engineering. It elicits response from cells such as endothelial cells (ECs) that are similar to those elicited by bone. ECs promote bone regeneration by stimulating both neovascularisation and osteogenesis. For an effective coupling of angiogenesis and osteogenesis, vascular endothelial growth factor (VEGF) is required. The aim of this work is to study the effectiveness of VEGF-added-BCP on the expression of osteogenesis and angiogenesis genes in ECs. Commercially obtained rat aortic ECs were cultured in the endothelial-cell growth medium. The cells were treated with three different modalities: BCP- only, VEGF- only, and VEGF-added-BCP. The optimal BCP and VEGF concentrations were determined. The cells were harvested at four different time intervals (day 3, day 7, day 10 and day 14) and were subjected to RNA isolation using RNA extraction kit (*analytikjena*, Germany). This was followed by performing reverse transcriptase-PCR (RT-PCR) (Qiagen, Germany) to amplify the osteogenesis and angiogenesis-regulated genes. The RT-PCR products were then electrophoresed. The gel image was captured using Image Analyser. Suitable concentration of BCP was 10mg/ml while optimal VEGF was 15ng/ml. Angiogenesis and osteogenesis genes were clearly expressed in ECs in response to treatments. Angiogenesis gene (VEGF) was highly expressed by VEGF-only treatment but showed some changes with added BCP. Osteogenesis genes (BMP-2, ALP, OC and OPN) were shown to be positively affected by both BCP and VEGF. Some genes were expressed at an earlier time interval compared to the other genes depending on the type of treatments. BCP-only treatment induced high expression of early regulating osteogenesis genes (BMP-2 and OPN). Mineralized gene markers (ALP and OC) were however, highly expressed with VEGF-added-BCP treatment. Combination of BCP and VEGF modality on ECs was suggested to initiate osteogenesis and angiogenesis related gene expressions earlier than the other modalities.**

Keywords—**Endothelial cells, biphasic calcium phosphate, VEGF, osteogenesis, angiogenesis.**

I. Introduction

Vascular endothelial growth factor (VEGF) is a potent, vital angiogenic cytokine and specific mitogen for vascular endothelial cells [1]. The vascularization of a scaffold is a complex process and is modulated by the presence of transplanted cells, exogenous and endogenous signaling proteins, and the host tissue reaction [2]. Bone repair cells, both osteogenic and angiogenic cells, represent a unique cell population which have been demonstrated to be closely associated with the process of bone regeneration [3]. ECs from peripheral blood circulation was the choice rather than from bone marrow because peripheral ECs are indeed superior to bone marrow-derived cells in their proliferative and vasculogenic potential, more committed, and less plastic than the one from bone marrow source [4,5]. For genetic and molecular information analysis, a rat model is widely considered as a powerful screening tool for gene and cell therapy *in vitro* and *in vivo* [6]. The use of ECs as a cell-based therapy for bone regeneration represents a significant departure from conventional thinking, that cell-based therapy should use mesenchymal stem cells (MSCs) because of the ability of these cells to act as osteoprogenitors [7]. New thinking suggests that ECs could change to MSCs in a process called epithelial-mesenchymal transition (EMT) [8,9]. EMT also exhibits multipotency by their ability to differentiate into osteoblasts, chondrocytes, adipocytes, smooth muscle cells or fibroblasts *in vitro* and *in vivo* [10, 9].

II. Materials and methods

Cell culture: Rat aortic ECs and all others reagents required for cell culture were purchased from Cell Applications, Inc. (San Diego, Calif, USA). ECs are cultured in the rat endothelial medium in accordance with the manufacturer's recommendations. VEGF was purchased from (GIBCO, USA). BCP powder was synthesized in Ceramic Laboratory, School of Materials and Mineral

J. Goh (ed.), *The 15th International Conference on Biomedical Engineering*, IFMBE Proceedings 43,
DOI: 10.1007/978-3-319-02913-9_61,

Resources, Engineering Campus, Universiti Sains Malaysia (USM). By wet precipitation with titration and heating process followed by calcination method. Different Ca/P ratio was prepared starting from HA with Ca/P ratio 1.67 to β-TCP with Ca/P ratio 1.50. For this study CaP ratio 1.52 has been selected in which HA/β-TCP range from 11/88 to 17/83. Average size of BCP powder is ranged from 12.23 to 150 µm. For cell viability test, PrestoBlue was purchased from Invitrogen Corp. (San Diego, USA).

Biocompatibility test: Extracts for indirect tests obtained from BCP powder under standardized conditions were based on references from International standards (ISO 10993 part 5, 1999). BCP was sterilized using autoclave. The ECs were exposed to varying concentrations, serial dilutions of 10, 5 and 2.5mg/ml. Treated medium was changed daily. For each experiment, cells were lysed and transferred into a 96-well plate with standardized cells number, incubation in a humid atmosphere of 5% CO2 at 37°C, untreated media for control group. Viability was assesed at different time points (1, 3 and 7 days), where absorbance was read at 570 nm with 600 nm as reference wavelength using a Tecan Elisa reader. The experiment was repeated 3 times.

Optimizing the VEGF concentration, by RT-PCR: ECs of passage 5 were collected and cultured in 25 cm^2 flasks with ECs media treated with different concentration of 5, 10 and 15 ng/ml of VEGF. Cells cultured with untreated media were used as a control. The cells were collected on day: 3, 7, 10 and 14. Different initial numbers of cells were used for different days. Cells harvested and cell pellets were preserved at -80°C. The experiment was repeated 3 times.

RNA Extraction: Cell pellets were retrieved from the -80°C. RNA were extracted by following the manufacturer's protocol. RT-PCR was performed to amplify the selected genes. PCR products were then electrophoresed on agarose gel. The gel image was captured using Image Analyser.

Osteogenesis and angiogenesis-regulated genes test: After determining the optimal concentration of BCP and VEGF, the cells were treated with three different modalities: BCP-only, VEGF-only, and VEGF-added-BCP. The cells were harvested at four different time intervals (day 3, day 7, day 10 and day 14) and were subjected to RNA isolation using RNA extraction kit. This was followed by performing RT-PCR to amplify the osteogenesis and angiogenesis-regulated genes. The RT-PCR products were then electrophoresed. The gel image was captured using Image Analyser. RT-PCR was performed with specific primers as follows: rat GAPDH (F) 5'-TGAAGGTCGGTGTCAACGGATTTGGC-3' (R) :5'-CATGTAGGCCATGAGGTCCACCAC-3' ; [11], rat VEGF (F):5'-ACGAATGGTGAAGTTCATGGATGT-3' ; (R): :5'- AAGCTCATCTCTCCTATGTGCTGG-3' ; [12], rat OC(F):5′-AGGACCCTCTCTCTGCTCAC-3′,(R):5′-AACGGTGGTGCCATAGATGC-3′; [13], rat OPN (F): 5'-ATGAGACTTGCAGTGATTTGCTTTTGC-3';(R):5'CTCATCTGT GGCATGGGGATACTG-3'; [14], rat ALP (F): 5'-CAGGATTGACCACGGGCACC-3' ; (R): 5'-GCCTGGTAGTTGTTGTGAGC-3' ; [15] and rat BMP-2 (F): 5'-GCTGTCTTCTAGTGTTGCTGCTT-3';(R):5' TTCGGTGCTGGAAACTACTAT-3' ; [16].

III. Results

Viability test with concentration 10mg/ml showed higher proliferation activity of ECs compared with the other concentrations as well as control (Fig. 1). VEGF gene expression level of 15 ng/ml on day 3 was significantly different compared to 0, 5 and 10 ng/ml concentrations (Fig. 2). However, there were many fluctuation of VEGF gene expression level when treated with 5 and 10ng/ ml VEGF protein. On the other hand, ECs treated with 15ng/ml consistently expressed a higher level of VEGF-regulated gene regardless the day of treatment (Fig. 3).

Angiogenesis and osteogenesis genes were clearly expressed in ECs in response to treatments. Angiogenesis gene (VEGF) was highly expressed by VEGF- only treatment but showed some changes when added with BCP. Osteogenesis genes (BMP-2, ALP, OC and OPN) were shown to be positively affected by both BCP and VEGF. Some genes were expressed at an earlier time interval compared to the other genes depending on the type of treatments. BCP-only treatment induced high expression of initial-regulated osteogenesis genes (BMP-2 and OPN). Mineralized gene markers (ALP and OC) were however, highly expressed with VEGF-added-BCP treatment (Fig. 4).

Our results showed that BMP-2 gene expression was higher in days 3 and 7 and decrease afterwards in both BCP group and VEGF+BCP group. VEGF gene expressed in early phase of bone healing and up regulated from days 3 to days 7 (peak levels), decrease significantly between days 7 and 14, whereas expression of ALP increased until day 14. OC start to express slightly in day 3 and 7, increased significantly in day 10 for VEGF+BCP group. No expression of both OC and ALP was seen in control group.