

Original

Enhanced Osteogenic and Angiogenic-Related Gene Expression of Human Dental Stem Cells on Biphasic Calcium Phosphate Scaffold Treated with Vascular Endothelial Growth Factor: Part I

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Abstract: To study the effectiveness of vascular endothelial growth factor (VEGF) added with biphasic calcium phosphate (BCP) on the expression of osteogenesis and angiogenesis-related gene in dental stem cells (DSCs). The cells were treated with three different modalities; BCP group, VEGF group, 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. Osteogenesis and angiogenesis-regulated genes was amplified using reverse transcriptase-PCR (RT-PCR). The RT-PCR products were then electrophoresed. The gel images were captured using Image Analyser AlphaEaseFC™. Angiogenesis and osteogenesis genes were clearly expressed in DSCs in response to treatments with 75mg/ml BCP and 5ng/ml VEGF. Angiogenesis gene VEGF was highly expressed by VEGF treatment group but showed some changes when added with BCP. Osteogenesis genes (BMP-2 and OPN) were 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 treatment induced high expression of initial-regulated osteogenesis genes (BMP-2 and OPN). Combination of BCP and VEGF modality on DSCs was suggested to initiate osteogenesis and angiogenesis-related gene expressions earlier than the other modalities. In our design, uncontrolled release of VEGF protein showed inhibition of BMP-2 at mRNA level in VEGF-BCP combined group.

Key words: Dental stem cells, BCP, VEGF, Osteogenesis, Angiogenesis

Introduction

Dental stem cells (DSCs) in particular, hold great promise to bone tissue strategies due to their advantages with high proliferation rate ability makes it a good tool that goes far beyond regenerative medicine and considered excellent candidate stem cells for hard and soft tissues regeneration and organ transplantation in bone tissue engineering. DSCs treated with appropriate cues could differentiate into osteoblasts, endothelial cells (ECs), chondroblasts, neural and smooth muscle cells similar to that of other types of mesenchymal stem cells (MSCs)¹. Recently, studies indicate that the human dental tissue contains precursor cells, named dental stem cells DSCs that offer advantages because of lower morbidity during the harvesting procedure and show self-renewal and multilineage differentiation capacity^{2,3}. DSCs have the ability to generate not only dental tissues but also bone tissues.

The success of tissue engineering depends on oxygen and nutrient transport to the implanted cells^{2,4}. If blood vessel formation at the transplanted tissue cannot be established rapidly, necrosis of the transplant will occur⁵. Therefore, in order to evaluate human DSCs are

good candidates for tissue engineering, it is of utmost importance to investigate the effectiveness of these stem cells to induce angiogenesis and osteogenesis². In addition, under appropriate conditions, DSCs have a superior immunoregulatory capacity that abolishes T-cell alloreactivity than bone marrow-MSCs, may not elicit humoral immune responses. Thus it is immune privileged^{6,7}. This finding plus their high proliferative ability makes DSCs a good candidate for tissue engineering and organ transplantation⁸.

Biodegradable BCP is considered as a good scaffold for bone substitution for tissue regeneration. VEGF is a key regulatory molecule that promotes the growth of new blood vessels by acting in ECs with a powerful mitogenic activity⁵. Due to their high proliferation rate and efficiency in producing bone chips, DSCs seem to be the best attractive candidates to study bone formation with respect to bone marrow-MSCs⁹. In tissue regeneration, it is important to consider the possible impact of the combination of the scaffold and the stimulator on processes linked to cell proliferation and function. Angiogenesis, the formation of capillaries from pre-existing blood vessels, is a key process in tissue engineering. If blood supply cannot be established rapidly, there is insufficient oxygen and nutrient transport and necrosis of the implanted tissue will occur. This type of blood vessel

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development is indispensable for physiological processes such as wound healing and reproduction²). VEGF exerts its effects through induction of vascularization which in turn plays an important role in bone growth and development. It also supports the survival and activity of bone-forming cells as well as migration and differentiation of primary human osteoblasts¹⁰). Therefore this study aimed to evaluate the effectiveness of VEGF added BCP on the expression of osteogenesis and angiogenesis related gene in DSCs.

Materials and Methods

Cell culture: Commercially obtained DSCs from (AllCells, LLC Company, Emeryville, CA, USA) were cultured in the Dulbecco's Modified Eagle Medium (DMEM) high glucose, (4.5g/l), Fetal bovine serum (FBS), Dulbecco's PBS (without Ca⁺² and Mg⁺²), Penicillin/streptomycin, liquid, Triple™ Express Stable Trypsin Replacement Enzyme without Phenol Red and all others reagents required for cell culture and VEGF protein were purchased from Invitrogen, (GIBCO), Faraday Ave, Carlsbad, CA, USA in accordance to the manufacturer's recommendations. Preparation of BCP powder was synthesized in ceramic laboratory, School of Materials and Mineral Resources, Engineering Campus, Universiti Sains Malaysia (USM). 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 chosen. In this study, HA/β-TCP powders were blended to get a powder mixture with a HA/β-TCP weight ratio 11/88 to 17/83%. For cell viability test PrestoBlue is a commercially available were purchased from Invitrogen Corporation, (San Diego, California, USA)

DSCs cells were cultured in T-75 cm² tissue culture flask. The culture medium was incubated at 37°C with 95% relative humid atmosphere and 5% CO₂. Any change in medium color indicates depletion of nutrients. The media were changed twice weekly. The cells were observed under an inverted microscope and sub-cultured when cells reached 80% to 90% confluence in a monolayer. DSCs and 5 ml plain culture media was plated in a filtered top culture flask (T- 25cm²). It was incubated at 37°C in 5% CO₂ and 95% humid environment. After every three days the DMEM was changed. Before passage the confluence of the culture was allowed to reach approximately 80-90%. Following harvest by trypsinization, the cells were plated in 75cm² cultured flask. DSCs were expanded by repeated passage, and each T-75cm² flask at 80% to 100% confluence was plated into three flasks. Cell passage was continued according to the demand of the experiment. The cells were counted and cryopreserved in liquid nitrogen until use. DSCs passage and subculture were conducted when the cells reached 80% to 100% confluence. Under an inverted light microscope, the media were checked for contamination (change in color or turbidity). The medium was removed and washed with PBS twice for the cell passage. Up to 2ml trypsin (0.25) was added (T-75cm² flask) and samples were incubated in 37°C and 5% CO₂ for 3 to 5 minutes. The detached cells were observed under a microscope for their attachment. In case cells were still attached, the flask was incubated and gentle movement was applied to dislodge the cells. The flask was checked again until 90% of cells became rounded and were detached from the flask. Pre-warmed medium (5 ml) at 37°C was added to the flask for neutralization of trypsin action. By continuous gentle pipetting the cell were spread into a single 2 culture flask. Morphology of the cells was observed daily under inverted light microscope using 5 x lenses. cell suspension on the surface of the flask. The cell suspension was transferred to a centrifuge tube and centrifuged at 1,000 rpm for 5 minutes.

Supernatant obtained from the centrifuge tube was discarded.

Formed cells pellet was re-suspended in 3 ml culture medium. The solution was pipette up and down for a few min. Fifteen ml DMEM media and 1 ml of cell suspension was added to three T-75 cm² flasks. It was checked under the microscope, and incubated again at 37°C, 5% CO₂, and 95% humid atmosphere. Cells were monitored closely for 24 hours. Cells were washed with PBS two times. After washing the cells were detached from the bottom of the tube with trypsin solution. It was re-suspended in DMEM by centrifugation. Cell suspension was adjusted to 1×10⁴ cells/ml. It was inoculated on a 96-well plate with 200 µl of suspension per well, and incubated for 24 hours for cell adhesion.

Biocompatibility test

Extracts for indirect tests obtained from BCP powder under standardized conditions were based on references from International standards (ISO 10993 part 5, 2009)¹¹). The solvent used for extracting BCP powder was DMEM. DSCs were exposed to varying concentrations of BCP-treated media. Serial dilutions of 75, 50 and 25mg/ml (vol/vol) from the pure extract were prepared, and DSCs of passage 4 were collected. Culture medium was discarded when the primary cell monolayers were grown until 80% to 90% confluence. Cells were washed with PBS two times vigorously. Sub-culturing by cell passage was performed by trypsinization. For detaching the cells from the flask it was incubated at 37°C for 2 min to 3 minutes. Trypsin cleaves the proteins that link the cells to the extra-cellular matrix. Cell counting was performed using haemocytometer by trypan blue Merck, Germany Ajax Finechem Pty Ltd to determine viable (living) vs. nonviable (dead) cells (the dye cannot be absorbed by viable cells). For each experiment, cells were lysed and pipetted into a 96-well plate with standardized cell number. Treated media at 90µl from each concentration and untreated media for the control group were used in each well plate, followed by incubation in 95% humid atmosphere of 5% CO₂ at 37°C. Viability assessment at different time points (1, 3 and 7 days) after incubation was done. For viability assessment, we choice PrestoBlue® cell viability reagent (Gibco, USA) (PB) in our study because it is more rapidly efficient developed a color change compared with other reagents¹²). PrestoBlue reagent protocol 10µl was added for each well under the dark field. Again Incubate with same standardized condition for 2 hrs using aluminum foil to protect it from light. ELISA reader Tecan was used, and absorbance was read at 570 and 600 nm as reference wavelength The experiment was repeated thrice.

Optimizing the VEGF concentration and RT-PCR

The optimal concentration of VEGF for the induction gene expression in DSCs was determined in the Craniofacial Lab, School of Dental Sciences, USM. The final concentrations of VEGF used in this experiment were: 5, 10, 20 and 50 ng/ml. Cells cultured with untreated media were used as a control and followed the manufacturing protocol. RNA extraction kit (Analytik Jena AG, Konrad-Zuse-Strasse 1, 07745 Jena, Germany) was used after preparation and the experiment was repeated 3 times. Because Buffer RPE was supplied as a concentrate, 4 volumes of ethanol (96–100%) were added to Buffer PRE before using for the first time to obtain a working solution as indicated by the company instructions. The samples (the cell pellets) were retrieved from the ices and 350 µl of RLT buffer were added to each sample. Cellular RNA was totally extracted by followed RNA extraction kit provides by the supplier. The concentration and purity of total cellular RNA were evaluated by measuring the absorbance at 260 nm (A260) and 280 nm (A280) using spectrophotometer (Biophotometer, Eppendorf AG, Hamburg, Germany).

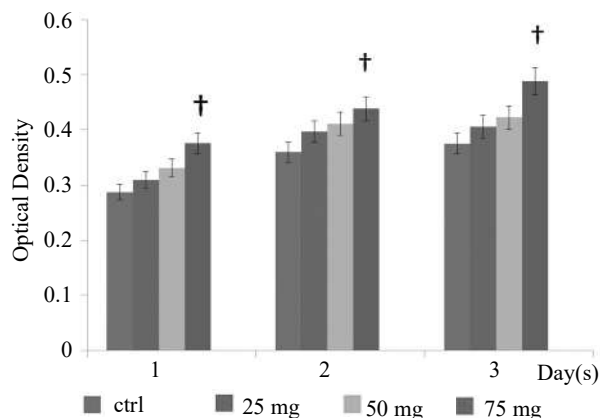


Figure 1. Cytotoxicity test of DSCs treated with BCP at different concentrations. †, a significant difference when compared to other concentrations and control groups ($p < 0.05$). Ctrl, control.

VEGF, bone morphogenetic protein-2 (BMP-2) and osteopontin (OPN) respectively. RT-PCR was performed with specific primers as follows: human GAPDH (F) 5'-GACCACAGTCCATGCCATCAC-3' (R): 5'-TCCACCACCTGTTGCTGTAG-3';¹³⁾, human VEGF (F): 5'-CCCACTGAGGAGTCCAACAT-3' (R): 5'-TTTCTTGCGCTTTCGTTTTT-3';¹⁴⁾, human BMP-2 (F): 5'-GAGTTGCGGCT GCTCAGCATGTT-3' (R): 5'-ACATGTCTCTTGGAGACACCT-3';¹³⁾, human OPN (F): 5'-GGACAGCCAGGACTCCATTGA-3' (R): 5'-CGTTTCATAACTGCCTTCCCA-3';¹⁵⁾. Statistical analyses were performed using statistical software PASW® Statistics 20.0 (SPSS Inc, Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to perform the cytotoxicity analysis of BCP scaffold in DSCs using different concentrations. Comparisons between groups for the cytotoxicity assay data were analyzed followed by using post hoc test to determine the best concentration at a significance level less than 0.05. p-value of less than 0.05 was considered statistically significant.

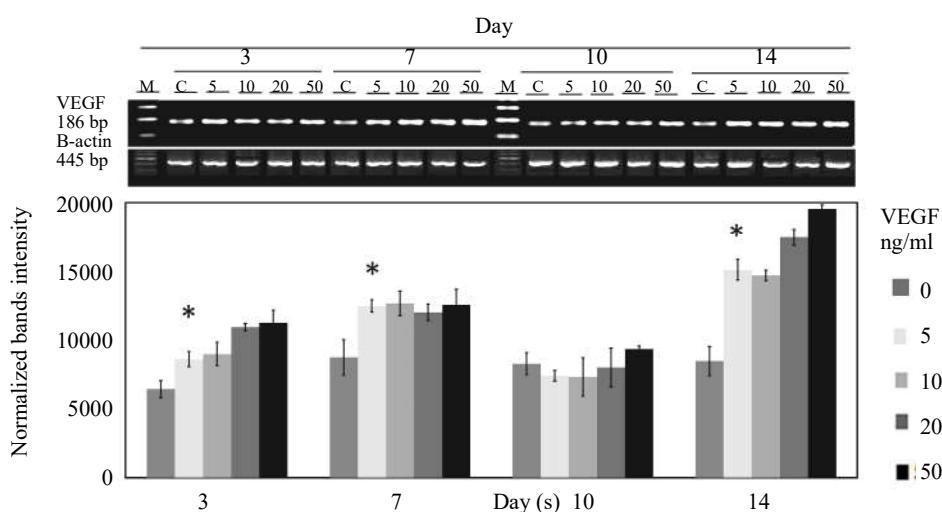


Figure 2. Optimizing VEGF concentration by Reverse Transcriptase-PCR, 5ng/ml was considered an optimal concentration

Osteogenesis and angiogenesis gene expressions experiments

After determining the optimal concentration of BCP and VEGF, DSCs were treated with three different modalities; BCP only, VEGF only, and VEGF-BCP combined. The cells were collected on days: 3, 7, 10 and 14. Different initial numbers of cells were used for different days. The cells numbers were: 300×10^3 cells for day 3, 25×10^3 for day 7, 10×10^3 cells for day 10 and 10×10^3 cells for day 14. The cells were harvested at four different time intervals (day 3, 7, 10 and 14) and subjected to RNA isolation using RNA extraction kit.

This study was conducted to evaluate the specific biological activities within the DSCs cultured in media treated with BCP with and without the addition VEGF. Gene expressions were analyzed using RT-PCR technique. Angiogenic and osteogenic gene expressions were assessed at mRNA level. Amplification of osteogenesis and angiogenesis target gene was performed by one-step RT-PCR, which was prepared according to the recommendations by the manufacturers (RNeasy®, Qiagen, USA). RT-PCR was performed using specific primers; the housekeeping gene used in this study was GAPDH. One-step RT-PCR master mix preparation was prepared, and the total volume per RT-PCR reaction was 25 μ l of the total RNA, 0.2 μ g was used in the RT-PCR reaction. The protocol followed for one-step RT-PCR was in accordance with the recommended procedure. Angiogenic and osteogenic gene expressions were assessed at mRNA level including A

The optimal concentration of VEGF, growth factor protein on DSCs based on VEGF gene expression was determined by using Kruskal-Wallis test. The comparison was between 5, 10, 20, 50 ng/ml and control (0 concentration) at different time points. A p-value of less than 0.05 was considered statistically significant.

Results

Viability test for BCP powder at different concentrations was expressed as relative cell viability that is a percentage of the readings (or activities) in the absence of BCP. The concentration of 75mg/ml BCP treated media showed more proliferation of DSCs compared with other concentrations and control ($p < 0.05$), and 100% cells viability (Fig. 1). The results of DSCs treated with VEGF showed that 5ng/ml was considered an optimal concentration ($p < 0.05$) (Fig. 2).

Osteogenesis and angiogenesis-regulated gene expression analysis

Angiogenesis and osteogenesis genes were clearly expressed in DSCs in response to treatment with BCP, VEGF and VEGF-BCP combined. Angiogenesis gene, VEGF was a marker for DSCs if cultured more than 80% confluence. VEGF gene was expressed in all groups but highly expressed in VEGF and VEGF/BCP groups. In BCP group, it was highly expressed only at day 14 (Fig. 3). Osteogenesis gene (BMP-2 and OPN) was shown to be affected by both BCP and VEGF. BMP-

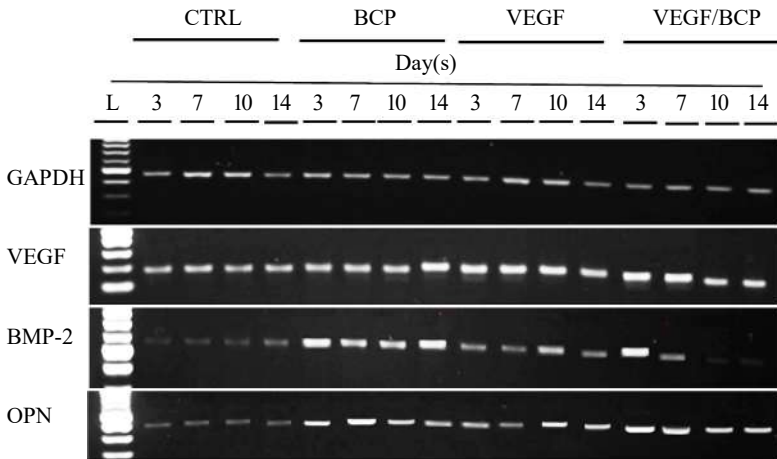


Figure 3. Electrophoresis images of RT-PCR product of osteogenesis genes, bone morphogenetic proteins-2 (BMP-2) and osteopontin (OPN) and angiogenesis gene vascular endothelial growth cells (VEGF) expressed in dental stem cells (DSCs) treated with biphasic calcium phosphate (BCP) and VEGF. VEGF gene was expressed in all treatment groups but highly expressed in VEGF only and VEGF-BCP combined groups. BMP-2 gene was expressed at all time points of BCP only and VEGF only group. The expression was highest in BCP only group, but lightly expressed in VEGF only group. In VEGF/BCP group, it was highly expressed on day 3 and 7, but repressed afterward. In control, it was very lightly expressed. OPN gene was expressed in all treatments groups with a higher expression on day 3, 7 and 10 of BCP only group, day 10 and 14 of VEGF only group, and all time points of VEGF/BCP group. It was lightly expressed in the control group. CTRL, control. L, ladder. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

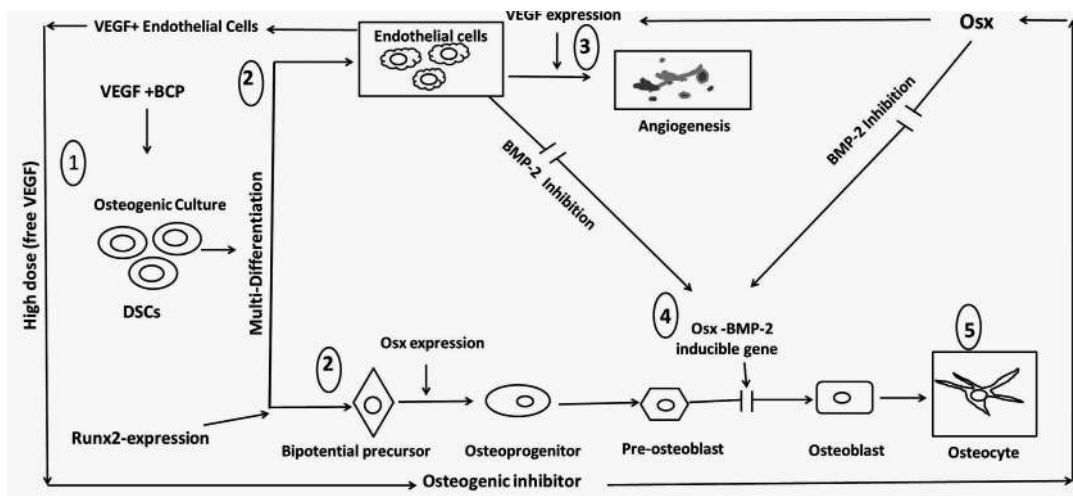


Figure 4. A simplified schematic diagram representation of the intracellular signaling pathway (molecular) with different stages of the differentiation of dental stem cells (DSCs) into osteoblasts and endothelial cells. The effect of exogenous vascular endothelial growth factor (VEGF) may have the ability to arrest osteoblastic differentiation in a preosteoblastic stage by inhibiting Osterix (Osx) gene expression bone morphogenetic proteins-2 (BMP-2 inducible gene) for the pre-angiogenesis process. Runt-related transcription factor- 2 (Runx2).

2 gene was expressed in all groups. In BCP group, it was expressed at all time points. The expression of BMP-2 gene in VEGF/BCP was higher only on day 3 but showed a reduction on day 7 and repression afterward. However, in VEGF group the BMP-2 gene expression is higher compared to the control group. OPN gene was expressed at all time point in all groups. OPN gene was affected positively by BCP and VEGF protein. This is because OPN gene in BCP group was expressed higher in day 3 and day 7 and in VEGF group in day 10 and day 14. A combination of VEGF and BCP showed that the OPN gene was highly expressed in day 3 and moderately expressed in the subsequent days afterward, compared to the control group (Fig. 3).

Discussion

The goal of developing novel approach strategies for bone replacement with bone substitutes are focused on stimulation of osteointegration, osteoconduction, osteoinduction as well as induction of angiogenesis and vascularisation¹⁶. Dental sources MSCs can be applied to obtain suitable autologous DSCs for tissue replacement therapies of both bone and cartilage¹⁷. DSCs are MSCs in origin in which have a capacity to differentiate into many cell lineages. Runt-related

transcription factor-2 (Runx2) is the principal osteogenic master switch, which is involved in the differentiation of MSCs into preosteoblasts stage. Expression of Runx2, which then regulates the expression of Osterix (Osx) in osteoblastic differentiation¹⁸. Osx is an osteoblast-specific transcription factor required for osteoblast differentiation and bone formation. A Recent investigation into the molecular pathway of the growth factor signaling via the BMP-2 has shown BMP-2 dependent activation of Osx. Osx was first discovered as a BMP-2 inducible gene in MSCs¹⁹. Another study established that VEGF transcription in osteoblasts occurs downstream from transcription factor Osx²⁰. They established that Osx directly targets VEGF expression and controls osteoblast marker gene. Thus, osteoblasts secrete VEGF in the process of their terminal differentiation, which clearly highlights the crucial role of angiogenesis for bone regeneration. Osx coupled with VEGF regulates expression in osteoblasts and considered a master regulator essential for the commitment of preosteoblastic cell differentiation into mature osteoblasts²¹.

Osx sciption factor is an osteogenic transcription factor vital to the process of bone formation. DSCs are differentiating and have the potential to commit to an osteoblastic lineage. On the other hand, direct

cell-cell contact is another way ECs and osteoblasts communicate. In addition, VEGF and ECs specifically have an inhibitory effect on dexamethasone-induced bone marrow-MSCs differentiation (osteogenic inhibitor) into osteoblast, by inhibiting the expression of *Osx*. The rate of differentiation of bone marrow-MSCs to osteoblasts maybe controlled by ECs and VEGF, by initiating the recruitment of osteoprogenitor cells at sites of bone remodeling and maintaining them in a pre-osteoblastic stage, to avoid mineral deposition within the vessel^{22, 23}. *Osx* plays multiple functional roles, the most important of which includes the up-regulation of several genes like BMP-2 and type 1 collagen, these genes encoding for proteoglycans involved in bone mineralization. *Osx* is osteogenic marker express in osteoblast cell in response to BCP treatment and controls both angiogenesis and osteogenesis²¹. *Osx* directly targets VEGF expression, whereas *Osx* controls osteoblast marker gene and coupled with VEGF by regulates expression in osteoblasts. It is considered a master regulator essential for the commitment of preosteoblastic cell differentiation into mature osteoblasts^{24, 25}. VEGF is an important mediator of angiogenesis and osteogenesis²⁴. Many cytokine genes are up-regulated in the events of bone repaired. It is thought that both the increased expression of regulatory genes, as well as the later decreased expression of these same genes, are choreographed to promote sequential steps in the healing process (Fig. 4).

OPN gene is known to play an important role in cell attachment and calcification of mineralized tissue. It was confirm to be early and effective markers of bone formation. During the healing process of bone, BMP-2, and OPN mRNA levels, both actively participate during the early stages of bone healing with a peak level at 14 days, indicative of active osteoblastogenesis²⁶. OPN is one of the major noncollagenous proteins in bone synthesized and secreted during the process of osteoblast differentiation and mineralization and expressed at or near the time of mineralization²⁷. BMP-2 expression participated in all phases of bone formation. In our *in vitro* study, a preselection of the most important genes responsible for regulation of the two critical step in bone healing by mean angiogenesis and osteogenesis. Angiogenesis is closely associated with osteogenesis where reciprocal interactions between endothelial and osteoblast cells play an important role in bone regeneration²⁸. It was concluded in the present study, the effect of VEGF/BCP on angiogenic and osteogenic marker production was interesting. BCP group showed upregulation in some gene in different time point but fluctuation occur when added VEGF protein, but the effect of treatment modalities on gene expression still controversial. BMP-2 gene showed expression by VEGF group while, BMP-2 gene was expressed only in day 3 but repression occurs at day 7, 10 and 14 in VEGF/BCP group, besides, BMP-2 showed upregulation in BCP group. However, the BMP-2 were, in general, expressed at too low a level to be detectable by RT-PCR in our samples in VEGF- BCP combined group with down regulation from up to day 3 but repression occurred at day 7, 10, 14 in the VEGF-BCP combined with undetectable, mean that BMP-2 inhibit by VEGF action at mRNA level. However, our results confirm with previous findings of Schönmeier *et al.*²⁹ was showed in his work that using of VEGF at high dosage without control release resulting in over expression of VEGF and inhibit BMP-2. VEGF over-expression has the potential to impair bone formation by unbalancing between angiogenesis and osteogenesis.

D'Alimonte *et al.*³⁰ showed that addition of VEGF to the DSCs in (osteogenic condition) could enhance both osteogenesis and angiogenesis. The current finding showed that only angiogenic enhances with VEGF-BCP combined group but not osteogenic. The work of Tirkkonen *et al.*³¹ strongly agreed with our study findings as

he confirmed that VEGF did not enhance osteogenesis differentiation of human adipose stem cells (HASC) by meant BMP-2 inhibition. From this information, it is, therefore, clear that VEGF-BCP culture conditions had significant effects in these experiments, but a definitive conclusion on angiogenesis and osteogenesis will need to be confirmed by further studies. Extensive studies *in vitro* and *in vivo*, using DSCs with a suitable combination of control release growth factors and scaffold materials, is essential before resorting to human trials.

At least three parameters could be considered in order to devise strategies for coupling of these two important processes in bone formation and to preserve the positive effect on vascularization and high quality osteogenesis, while avoiding the negative effect on bone formation: 1) the duration of VEGF expression; 2) its dose; and 3) the presence of an osteogenic environment. VEGF expression is required only for about 4 weeks in order to generate persistent vessels³². Threshold dose of VEGF level for normal angiogenesis can be induced over a wide range because control expression at moderate levels might induce effective vascularization while limiting osteogenesis achieved³³. Purified growth factors are heat and pH unstable and highly sensitive to proteolytic degradation³⁴. Therefore, the equilibrium between VEGF triggered angiogenesis, osteogenesis needs to be carefully investigated in controlled release manner of VEGF with low concentration by using long term sustained delivery system meanwhile, concentration in pg/ml not in ng/ml with respect to the threshold dose consideration pro-angiogenic potency of VEGF expression. DSCs have the potential to “rephrase” repair craniofacial defects and repair/regenerate teeth³⁵. Protein release is not generally quantifiable for *in vivo* studies, due to the relatively low concentrations of released protein in blood and tissue samples compared to protein produced naturally by cells in the body. Current study has shown that VEGF protein can inhibit BMP-2 expression at the mRNA level. Delivery of VEGF for *in vitro* cell culture purposes in bone tissue engineering therefore requires careful optimization and precise delivery of growth factors for regulation of gene expression control both angiogenesis and osteogenesis processes using MSCs.

We have shown that uncontrolled release of VEGF protein added BCP can inhibit BMP-2 expression at the mRNA level. Therefore, over expression of VEGF gene showed bad osteogenesis process. Control release of VEGF protein for tissue engineering purposes is necessary for further investigation. Extensive studies *in vitro* and *in vivo* using BCP scaffold treated DSCs with control release of biomedical mediator growth factor (*in vitro*), on animals using DSCs with a suitable combination of growth factors and scaffold materials, is essential before resorting to human trials.

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Conflict of Interest

The authors have declared that no COI exists.

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