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Original

Osteoinductive Activity of Bone Scaffold Bioceramic Companied with Control Release of VEGF Protein Treated Dental stem cells as A New Concept for Bone Regeneration: Part II

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Abstract: In bone tissue engineering, angiogenesis is closely associated with osteogenesis where reciprocal interactions between endothelial and osteoblast cells play an important role in bone regeneration. Over-expression of the angiogenesis-related gene due to a higher dose of vascular endothelial growth factor (VEGF) protein can inhibit osteogenesis process at mRNA level. To study the effect of controlled released of the VEGF protein incorporating fibrin glue (FG) treated with fabricated porous biphasic calcium phosphate (BCP) on osteogenesis gene (BMP-2) and angiogenesis gene (VEGF) on dental stem cells (DSCs) at mRNA level. DSCs were treated with two different modalities; VEGF protein incorporated FG, and VEGF protein incorporated FG added-BCP treated media. 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 the RNA extraction kit. This was followed by performing one step-reverse transcriptase-PCR (RT-PCR) to amplify the osteogenesis BMP-2 gene, angiogenesis VEGF gene and the osteoblast- specific transcription factor expression Osterix (Osx) with and without the controlled release of VEGF protein. The RT-PCR products were then electrophoresed. The gel image was captured using Image Analyser. Controlled release of VEGF protein using FG as a natural delivery system, using a single growth factor, show a significantly enhanced osteogenesis BMP-2 gene and angiogenesis VEGF gene with a high expression of Osx compare with non-delivered free VEGF protein treated groups. FG is a biocompatible material that could be employed as a delivery vehicle for controlled release of VEGF protein single or dual release in bone tissue engineering strategy and design of the study. Application of this method for using FG is mixing with a porous ceramic scaffold loading with the growth factors is a convenient and promising strategy for improving osteogenesis and angiogenesis processes of reconstruction critical-sized bone defects and might change the scope of modern surgery.

Key words: Dental Stem Cells, Fibrin Glue, BCP, VEGF, Osterix, Osteogenesis, Angiogenesis

Introduction

Stem cells can be isolated from the pulp of human exfoliated deciduous teeth. These cells induce bone formation and differentiate into other non-dental mesenchymal cells in vitro. DSCs have higher proliferation rates, form sphere-like clusters, differentiate into osteoblasts and endotheliocytes and form adult bone tissue after transplantation *in vivo*[1]. Recently, the specific detector marker for DSCs expression (CD90++ CD271+ CD73 CD105) showed viable markers of high proliferative capacity and multi-potent DSCs populations. Consequently, if such superior DSCs populations are to be fully exploited and can be used as an alternative source of stem cells for regenerative medicine[2]. Successful solutions for identifying and solving problems associated with translating stem cell research into clinical practice are considered a major breakthrough in stem cell biological study[3]. Dental stem cells (DSCs) have great potential in bone tissue strategies because of their high proliferative ability[4]. Another advantage of DSCs is the decreased morbidity during their harvesting since they are derived from extracted teeth[5]. These cells also have the capability of self-renewal and pluripotential differentiation capacity[6]. All these properties make them a good tool extending far beyond regenerative medicine4). DSCs are considered excellent candidate stem cells for hard and soft tissue regeneration and organ transplantation in BTE[4]. Easy isolation, culturing and cryopreservation with retained multipotential differentiation make them an attractive source of stem cell for tissue engineering capability to generating bone beside dental tissue[5, 7].

Diseases that cause loss of substances or volume and tissue damage is now treated effectively with the combination of cell-based therapy and regenerative medicine[8]. DSCs treated with appropriate cues may solve this problem by its capability of differentiation into OBs, ECs, chondroblasts, and neural and smooth muscle cells, which are similar to other types of MSCs[9]. DSCs originating from teeth exhibit higher proliferative rates and can be easily obtained than bone marrow-

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mesenchymal stem cells (bone marrow- MSCs)[10]. These cells can be used as an attractive source of autologous stem cells for bone regeneration. In BTE studies, the most widely studied MSC types are bone marrow-MSCs and adipose-derived MSCs, which display similar results[8]. However, pain, morbidity and decreased cell count after harvest are the major problems associated with bone marrow -MSCs, alternative sources such as adipose tissue-derived for MSCs have been explored[5, 6]. Gronthos *et al*.[11] for the first time isolated DSCs in 2000. Stem cells are extracted from pulp tissues very efficiently; they are pluripotent and can result in the differentiation of different cell lineages[12]. DSCs, which are MSCs in origin, are derived from dental pulp tissue that quickly differentiates into osteoblast and endothelial cells[13]. DSCs have the potential of bone formation and repair human bone defects successfully[14]. The favourable interactivity of these cells with biomaterials makes them ideal for tissue regeneration[15]. DSCs exhibit similar differentiation properties as shown during bone differentiation, making them highly interesting models for the study of osteogenesis[16]. The biological relationship of DSCs with different types of scaffolds needs to be analyzed[17]. Oxygen and nutrient supply to the implanted cells is a key for successful tissue engineering[5]. Failure in the rapid formation of blood vessels of the transplanted tissue leads to necrosis[18]. The ability of DSCs to induce angiogenesis and osteogenesis should be investigated to make these cells beneficial for tissue engineering[5]. The use of DSCs is not only limited to the dental field as these cells can also be used as a source of stem cell for general bone regeneration[19]. It is used in the treatment of traumatic injuries of hard and soft tissues[5]. Giuliani *et al*.[20] demonstrated that DSCs seeded on collagen type I scaffolds are conveniently used for the successful repair of human mandibular defects. An observational study demonstrated that biphasic calcium phosphate (BCP) elicits a response from cells similar to that elicited by bone[21]. One of the most prominent growth factors is bone morphogenetic protein-2 (BMP-2), which plays an important role in processes associated with bone repair and is used extensively in both ectopic and orthotopic sites for bone generation[22, 23]. Bone tissues are highly vascularized tissues and vascular endothelial growth factor (VEGF) is regarded as the key molecule for angiogenesis and plays a pivotal role in skeletal development and bone fracture repair[24, 25]. The synergistic contribution and crosstalk between osteogenesis and angiogenesis are crucial in determining the outcome of bone formation[27]. Unlike endochondral ossification, where blood vessels prevent the formation of cartilage, intramembranous bone formation relies on the coupling of angiogenesis and osteogenesis. VEGF has an essential role in this coupling since both vascular and skeletal morphogenesis is VEGF dependent[28, 29]. Bone formation is a complex developmental process involving the differentiation of mesenchymal stem cells to osteoblasts[28]. Osteoblast differentiation occurs through a multi-step molecular pathway regulated by different transcription factors and signaling proteins. Osterix (Osx), a zinc finger-containing transcription factor (also known as Sp[7]) is a master gene controls osteoblast lineage commitment the only osteoblast-specific transcriptional factor identified so far which is essential for osteoblast differentiation and bone formation[30]. The Osx was discovered as a bone morphogenic protein-2 (BMP-2) induced gene in mouse pluripotent mesenchymal cells, encoding a transcription factor that is highly specific to osteoblasts[31]. Incorporation of porous scaffolds with carrier materials for drug-controlled release is a potential solution for the aforementioned problems[28]. This approach could improve the bioactivity of an implant and allow for the sustained release of growth factors. In general, the localized and sustained release of growth factors allows for lower effective dosage than a single injection[31]. VEGF protein has been identified to have positive effects on osteogenesis and angiogenesis[28]. Appropriate dosage and release profiles are very important for optimized biomolecule delivery. In addition, the application of excessive dose may provoke adverse effects or even toxic reactions, such as inhibition of blood vessels and bone formation[31]. Similarly, the slow and sustained release of VEGF protein can produce well-functioning blood vessels, whereas uncontrolled release of VEGF protein leads to malformed and non-functional blood vessels[32]. Therefore, optimizing and controlling the release of VEGF protein and is one of the primary concerns in bone tissue engineering[33]. Growth factors (GFs), also known as cytokines, are endogenous proteins that act on a wide variety of cells and direct their actions via cell-surface receptor binding and activation. The actions of GFs on cells are long-lasting, so that a single exposure of cells to GFs results in long-term (weeks to months) cellular effects[34]. In this study, we investigated the influence of Osx on the osteogenic differentiation of DSCs in response to in situ delivery of VEGF protein treated BCP. Through this study, we try to demonstrate the effect of BCP scaffold treated media with the controlled release of the VEGF protein and the Osx gene -modified DSCs on bone formation at mRNA level, which may advance our understanding of DSCs fate and facilitate the clinical translation of DSCs mediated bone therapies beyond dental regeneration.

Material and Methods

DSCs and all chemicals, drugs, reagents, analytical kits and all materials used in cell culture were described in (Table 1). Preparation of BCP powder which 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 ß-TCP with Ca/P ratio 1.50. For this study CaP ratio 1.52 has been chosen. In this study, HA/b-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 was commercially purchased. The optimal concentration of BCP powder treated media were 75 mg/ml, determined by the protocol of the study design and procedures35). Preparation of the extracts of BCP powder for indirect tests were prepared using standardized conditions as per international standards (ISO/EN 10993-5. (2009)36). Extracts were used at 7 days and at 37°C (duration of extraction) and temperature. A total of 12 g of the BCP powder extract was used for treated of DSCs with a different concentration. The solvent used for extracting the sample was DMEM, samples was added to a centrifuge tube at 50ml and loaded with 20ml solvent. All tubes were labelled properly, and the cover was sealed using paraffin. Finally, the tubes were incubated at 37°C on a shaking plate at low rotational speed (50rpm) for 7 days. The supernatant of the tube was filtered through a filter membrane of 0.2μm and transferred into a 50 ml centrifuge tube. All tubes were rotated in a centrifuge device at 2,000 rpm for 4 minutes, and the supernatant was carefully aspirated until 0.5ml was left at the bottom of each tube. This process was necessary because the supernatant may contain harmful microparticles released from the BCP formula during extraction. The pure extract was prepared through the above-mentioned process, and serial dilutions (vol/vol) were conducted using the DMEM media.

Preparation and measurement of sustained delivery of VEGF protein from FG matrix

The efficacy of the smart FG matrix used to the controlled release of the VEGF protein was measured before running the main experiment. FG matrix was prepared in accordance with the manufacturer's recommendations. The long-term sustained release of VEGF protein using FG was determined by *in vitro* test.

FG consists of two components: (a) a complex of fibrinogen, fibronectin, FXIII and plasminogen and (b) thrombin. These solutions

Table 1: List of cell line, media, buffers, reagents, drugs, antibiotics and analytical kits were used in cell culture

Name	Supplier
Dental Stem Cells (DSCs)	AllCells, LLC Company, Emeryville, CA, USA
Dulbecco's modified Eagles medium (DMEM) high glucose(4.5g/L)	Invitrogen, (GIBCO), Faraday Ave, Carlsbad, CA, USA
Fetal bovine serum (FBS)	Invitrogen, (GIBCO), Faraday Ave, Carlsbad, CA, USA
Dulbecco's PBS (without Ca+2 and Mg+2)	Invitrogen, (GIBCO), Faraday Ave, Carlsbad, CA, USA
Penicillin/streptomycin, , liquid	Invitrogen, (GIBCO), Faraday Ave, Carlsbad, CA, USA
Triple™ Express Stable Trypsin Replacement Enzyme without Phenol Red	Invitrogen, (GIBCO), Faraday Ave, Carlsbad, CA, USA
Dimethyl Sulphoxide (DMSO)	Ajax Finechem Pty Ltd – Australia
PrestoBlue® Cell Viability Reagent	Invitrogen Corporation, (San Diego, California, USA)
Vascular endothelia growth factor (VEGF)10µg/pk	Invitrogen, USA (GIBCO)
Biphasic calcium phosphate (BCP)	School of Materials and Mineral Resources, Engineering Campus, Universiti Sains Malaysia (USM)
Primer Sysnthesis	BioSUN Biotechnology,Malaysia
Human vascular endothelial cells growth factor (VEGF), enzyme-linked immunosorbent assay (ELISA) KIT,96T	(Cusabio Biotech Co., Ltd, Wuhan, China)
Tisseel Fibrin Sealant (FG) KIT	Baxter Healthcare Corporation, Westlake Village, CA-91362 USA, US License No.140
one-step Reverse Transcriptase Polymerase Chain Reaction RT-PCR Kit	QIAGEN, USA
innuPREP RNA Mini Kit-850-KS-2040050	(Analytik Jena AG, Konrad-Zuse-Strasse 1, 07745 Jena, Germany)
spectrophotometer	(Biophotometer, Eppendorf AG, Hamburg,Germany)

Table 2: Amount of vascular endothelial growth factor released from fibrin Glue delivery device in different time points was measured in pg/ml.

Time points	Mean	X (pg/ml)
1 hr	3.086625	2762.840909
23 hrs	3.082556	2759.141414
48 hrs	3.023333	2705.30303
72 hrs	1.573111	1386.919192
96 hrs	0.891667	767.4242424
120 hrs	0.808889	692.1717172
>120 hrs	0.450111	366.010101
--	Total	25439.81061

were diluted and varying composition of FG was prepared. Briefly, FG was prepared following the protocol from the supplier, the potency of thrombin and aprotinin which were used in the test in high concentration. Components of kits were prepared and reconstructed by preheating in a water bath. Prior to reconstruction of FG components, the rubber stoppers of all vials were cleaned carefully with 70% alcohol, and all components were kept at room temperature for 1 hour before their heating in a water bath.

TISSEEL powder was prepared by preheating the vial that contains the aprotinin solution and powder for 10 minutes in the water bath at 37°C. The aprotinin solution after dilution from 3000KIU/ml to 100KIU/ml was used. The new concentration of aprotinin was obtained by diluting 0.2 ml of aprotinin with 5ml of sterilized distal water. This mixture was transferred to the TISSEEL vial with the blue-scaled syringe and needle provided with the kit used for reconstruction.

The TISSEEL powder vial was kept in a water bath for 10 minute with gentle swirling for complete dissolution and full reconstruction with avoided frothing. The vial was kept at 37°C until used within 4 hours. Whenever un-dissolved particles were observed vial was incubated for an extra 10 minutes in the water bath at 37°C. The solution was agitated or stirred until complete dissolution. Under sterile conditions, TISSEEL solution was drawn from the vial for reconstruction.

Preparation of the 2_{nd} component: Thrombin and calcium chloride (40µmol/ml) solution were mixed for the preparation of thrombin solution. In this study, the concentration of thrombin used was 4 vials rather 500 vials because slow solidification was used. The vials were preheated in a water bath for 10 minutes at 37°C. The calcium chloride solution was transferred to the vial using another needle. The lyophilized material was swirled for dissolution and kept at 37°C until used. Thrombin solution was drawn under sterile conditions using the double sterile syringe. It was used within 4 hours after preparation. VEGF protein was prepared in a lyophilized form, stored at 2°C to 8°C, preferably desiccated. Optimal concentration was evaluated by a dose response assay for the application. The preparation was started according to the recommended protocol by briefly centrifuging the vial before opening to let the contents settled at the bottom.

Lyophilized VEGF protein was reconstituted in sterile distilled water or in an appropriate buffered solution, such as RNase-free water. These stock solutions were divided into working solutions in the form of aliquots and stored at ≤ –20°C. Reconstituted lyophilized VEGF was prevented from repeated freezing and thawing. Upon FG complex solution prepared, VEGF protein was injected within 1 minute before the clot formation. For the determination of the quantity of the released growth factors, phosphate-buffered saline PBS 1,000-µl solution; FG solution to VEGF was used [VEGF (0.25 µg/ml)]+ (FG once) [200 µl].

To prevent lysis of the FG clot, 1 ml of PBS and Aprotinin bovine (3000 KIU/ml) was added. It was kept in a 15 ml centrifuge tube and incubated on a shaking plate at low rotational speed (50 RPM) at 37°C. After centrifugation of the samples, supernatant of the tube was collected. Fresh PBS and aprotinin were added again to the same tubes at 5 definite time points 1, 23, 48, 72, 120 hours and above. One thousand microlitres of phosphate buffer saline was harvested. It was replaced with 1,000-µl fresh PBS solution. At 120 hour, the fibrin matrices were lysed with trypsin to determine the residual VEGF protein content in the fibrin matrix. After collection, the PBS, samples was stored at −80°C. Harvested samples were analysed using VEGF-specific enzyme-linked immunosorbent assay ELISA kit according to the manufacturer's instructions. For statistical

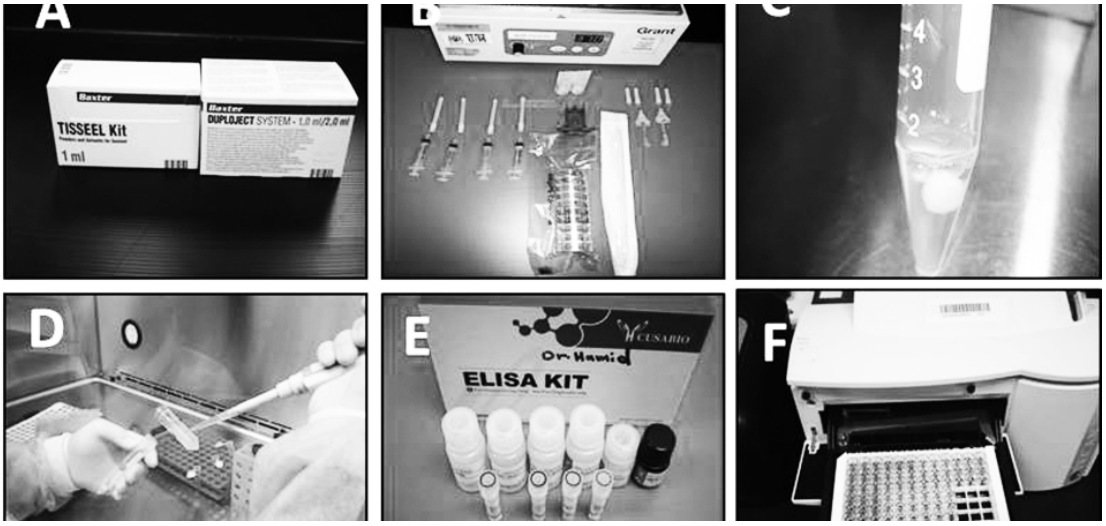

Figure 1: A, FG kit from Baxter company, B, preparation of FG is using a water bath, C, fibrin matrix inside 15 ml centrifuge tube contain 1 ml phosphate buffered saline, D, collected the old phosphate buffered saline after incubation and replaced by a fresh one. E, Vascular endothelial growth factor-specific enzyme-linked immunosorbent assay (ELISA) kit was prepared before using, F, insertion of the plate inside the spectrophotometer.

Figure 2: A, FG kit (step of cover removal), B, FG inside the water bath (step of preparation), C, fibrin matrix inside the small petri dish (step of matrix formation), D preparation of the VEGF protein. E, step of VEGF injection inside the clot before set, F, FG inside the prepared flack contain DSCs treated BCP scaffold.

analysis, this experiment was repeated three times (Figure 1& Figure 2).

Summary of assay ELISA procedure; reagents, samples and standards were prepared as instructed in the protocol provided by the supplier. One hundred µl of prepared standard or sample was added to each well followed by incubation for 2 hours at 37°C. Liquid of each well was removed; washing was not done in this step.

One hundred µl of Biotin-antibody (1x) was added to each well. It was incubated at 37°C for 1 hour. The Contents of the wells were aspirated and washed 3 times with wash buffer (1x). One hundred µl horseradish peroxidise (HRP-avidin, 1x) was added to each well. It was incubated at 37°C for 1 hour. It was aspirated and washed five times with wash buffer. To each well, 90µl of tetramethylbenzidine was added. It was incubated at 37°C for 30 minutes and protect the samples from the light. After incubation in the dark, to each well 50 µl of stop solution was added. The plate was read at reference point of 450 nm within 5 minutes (Fig. 1).

Osteogenesis process with and without control released of the VEGF protein

After Preparation of BCP powder and determine the optimal concentration for both BCP scaffold and the VEGF protein concentrations were 75 mg/ml and 250 ng/ml respectively. VEGF protein were injected inside the FG matrix before set within one minute after preparation. To avoid burst release of VEGF protein, the matrix was kept for 2 days in PBS before starting the main experiment. DSCs were treated with two different modalities; VEGF only and VEGF-BCP combined with and without control release manner of VEGF protein (Fig. 2). The cells were

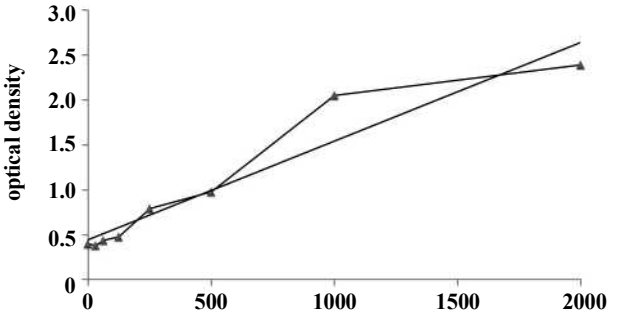

Figure 3: Cumulative release of vascular endothelial growth factor of the FG delivery device in vitro. Measurement of standard concentration of vascular endothelial growth factor against optical density (OD).

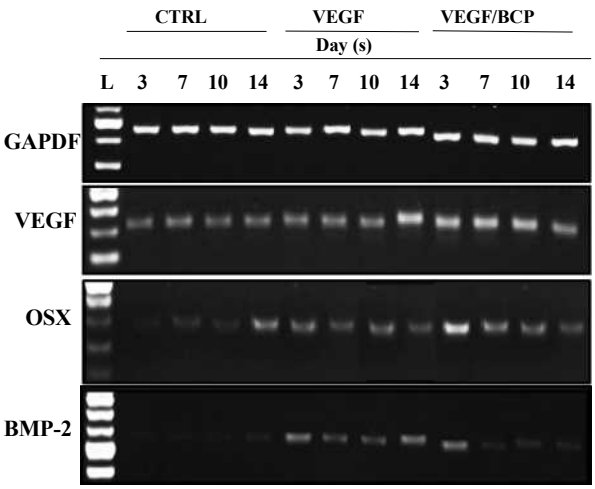

Figure 4: Electrophoresis images of RT-PCR product of osteogeneis gene bone morphogenetic proteins-2 (BMP-2) and angiogenesis gene vascular endothelial growth cells (VEGF) expressed in dental stem cells (DSCs) treated with biphasic calcium phosphate (BCP) and VEGF free dose. VEGF gene was expressed in treatment groups. Osx gene showed mild expression in both groups of treatment. The BMP-2 gene was expressed slightly in VEGF only group. In VEGF/BCP group, it was expressed on day 3 and slightly on day 7, but repressed afterwards. In control, it was very lightly expressed. CTRL, control. L, ladder. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

collected on days: 3, 7 10 and 14. Different initial numbers of cells were used for different days. The cell numbers were; 300 x 103 cells for day3, 25 × 103 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 the RNA extraction kit.

The concentration and purity of total extracted cellular RNA was evaluated by measuring the absorbance at 260 nm (A260) and 280 nm (A280) using spectrophotometer. BMP-2 and VEGF are the most well-known regulators of osteogenesis and angiogenesis process in bone healing. Amplification of osteogenesis and angiogenesis target gene was performed by one-step RT-PCR at molecular level (mRNA level), which was prepared according to the recommendations by the manufacturers. RT-PCR was performed with specific primers as follows: human GAPDH (F) 5'-GACCACAGTCCATGCCATCAC-3' (R):5'-TCCACCACCCTGTTGCTGTAG-3';[37],human VEGF(F):5'-CCCACTGAGGAGTCCAACAT-3'(R):5'- TTTCTTGCGCTTTCGTTTTT -3';[38] human BMP-2 (F):5'-GAGTTGCGGCTGCTCAGCATGTT-3'(R):5'-ACAT GTCTCTTGGAGACACCT- 3';[13] Osx (F) 5'-GCAAAGCAGGCACAAAGAAG-3'(R) :5'-AGGGAATGAGTGGGAAAAGG-3'[39].

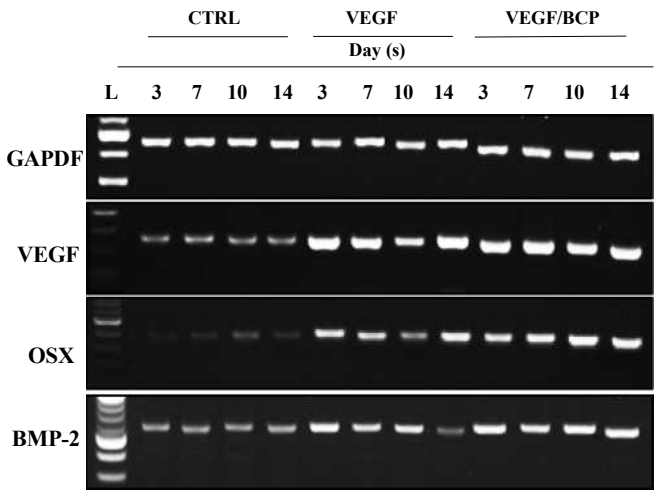

Figure 5: Electrophoresis images of RT-PCR product of osteogenesis gene, bone morphogenetic proteins-2 (BMP-2) and angiogenesis gene vascular endothelial growth cells (VEGF) expressed in dental stem cells (DSCs) treated with biphasic calcium phosphate (BCP treated media) and controlled release of the VEGF protein. VEGF gene was expressed in treatment groups and slightly in control. Osx gene showed higher expression in both treatment groups. The BMP-2 gene was highly expressed in VEGF only group and the VEGF/BCP group at all time points. CTRL, control. L, ladder. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Results

The amount of the VEGF protein released using a fibrin matrix that was present in the release medium was measured at multiple time points up to 120 hours. VEGF was rapidly released from the FG matrix starting within the first hour of incubation and later was slower releases at lower concentrations maintained. An initial burst release of about 15–20% of the VEGF was observed during the first 48 hours (Table 2). From 1 hour to 48 hours, the cumulative measured amount of VEGF release from FG clots was approximately 20% of the initial VEGF concentration. Lysate of the remaining FG matrix showed residual VEGF as it was serially measured over 120 hours (Fig. 3). Results showed that most of the VEGF release occurred in the first 48 hours. For plating cells, it is better to delay the plating that would minimize the effect of released soluble factors. Because the maximum release of VEGF occurs in the overlying media in the first 48 hours with minimal release occurring thereafter. The VEGF content was estimated from the standard curve (Fig. 3). Our study suggested that the diffusion-like process governed protein release could be used in in vitro research work with warranted impressive results.

Expression of bone-associated genes

To evaluate the effects of the in situ control release of VEGF protein treated bone scaffold bioceramic BCP on DSCs on osteogenic and angiogenic gene expression and the role of Osx. RT-PCR analyses were performed for bone associated genes in DSCs cultures either grown on VEGF and BCP with or without controlled release at different time points after culture on day (3, 7, 10 and 14). One step-RT-PCR analyses (Fig. 4) revealed that no differences for Osx expression level in all groups were detectable in DSCs seeded on the BCP scaffold treated media with free release VEGF protein, VEGF gene was expressed in both groups of treatment VEGF only and VEGF added BCP. The expression of BMP-2 gene in VEGF/BCP group was higher only on day 3 but showed a reduction on day 7 and totally repression afterward. Osx gene showed

minimum expression in VEGF group and VEGF added BCP group. Although, VEGF gene expressed from day 3 to day 14 in both groups of the treatment. Conversely, in DSCs treated with in-situ control release of the VEGF protein using FG as a delivery system natural polymer, the result was impressive compared with non-delivered VEGF protein by mean free release.

Osx expression considerably decreased in non-treated cells (control) compared to treated cells. The Osx was another marker of osteoblast differentiation that we considered in our observations. Actually, its expression showed high expression, but progressive increase more from day 7 to day 14 in both VEGF only and BCP treated media with the control released of the VEGF protein groups. Moreover, we analyzed also BMP-2 and VEGF expression (Fig. 5). Regarding the BMP-2 expression, it was significantly up-regulated in VEGF (controlled release) added BCP treated media and control release VEGF only group. In the control group, there is no significant differences were detectable (Fig. 5).

Discussion

DSCs can differentiate into various cell types and thus have great potential for regenerative medicine. In bone formation, Osx is a transcriptional factor essential for osteogenesis process and for osteoblast lineage, osteoprogenitors undergo a proliferative stage. Osx also express finally in mineralization of the extracellular matrix such as osteocalcin (OC), osteonectin (ON) and osteopontin (OPN)[40]. On the other hand, Osx overexpression upregulated key osteogenesis-related genes, such as special AT-rich binding protein-2, ALP, OC, and OPN, at both mRNA and protein levels. Our result showed that the Osx-expressing DSCs treated with porous BCP scaffold powder with a unique release of entrapped recombinant human VEGF using FG as a delivery system. The controlled release of VEGF promoted osteogenic differentiation capacity by the high expression value of the Osx gene compare with non-delivered VEGF protein group which showed a minimal band expression of Osx gene and failed osteogenesis represented by BMP-2 repression bands after day 7.

Our findings demonstrate that BCP scaffold treated media with in-situ slow release of VEGF protein induced an increase osteoblast-related gene marker expression in DSCs. In fact, we observed an increase in mRNA expression of the bone-associated transcription factors, like Osx a strong upregulation gene of early and late stages gene expression during bone healing, a marker of early-late-stage osteoblast differentiation. These findings suggest that DSCs are differentiating into osteoblasts in DSCs treated with BCP scaffold and FG to deliver the VEGF protein in a sequential way comparing with non-delivered VEGF protein group which shows good angiogenesis but poor osteogenesis. In our results, we suggest that the amount of BMP-2 and VEGF secreted by DSCs treated with BCP scaffold and VEGF protein released using FG were increased with time, with respect to the already differentiated cells, thereby establishing the interaction between osteoblast function and angiogenesis, which are combined, closely associated processes leading to bone formation and although our suggestion agrees with these data representing by RT-PCR analyses. In our study, we used FG as a delivery system to maintain, control growth factor release, protect them from degradation and retain them from excessive initial dose dumping (burst release) to achieve a longer period with low concentration release[33]. Our finding was consistent also with Mangano *et al.*[39] who confirmed that BMP-2 expression was significantly up-regulated in DSCs seeded in the Biocoral scaffold without the addition of VEGF. Some other studies have suggested that adding VEGF exogenously could have an osteoinductive effect on human adipose stem cells (HASCs) with osteogenic medium[41]. Contrary to the results of our study, D'Alimonte *et al.*[42] showed that the addition of VEGF to the DSCs (10-40 ng/ml for 8- day dose- and time-dependently) in osteogenic condition could enhance both osteogenesis and angiogenesis, while results of the current study disagreed with these findings. The work of Tirkkonen *et al.*[43] strongly agreed with our study as he confirmed that using high dose of VEGF protein in free released did not enhance osteogenesis process due to differentiation of HASC by meant BMP-2 inhibition. BMP-2 is a growth factor that has a high ability to induce osteogenesis by inducing the signaling control osteogenesis[44].

VEGF protein release, the levels of VEGF loaded into the FG were determined. The result demonstrated sustained release for up to 120 hours. The cumulative amounts of releasing VEGF protein from the FG matrix were plotted against time to exhibit the VEGF releasing concentration as shown in (Fig. 3). The sustained release may be attributed to the electrical interaction between the positively charged amino acid residues in the VEGF proteins, and the negatively charged sites of the pectin, FG. The release profiles of VEGF from FG also confirmed the strong interaction which was similar with previous study [45]. The results indicated that the VEGF protein was released from the fibrin clots more slowly, and this finding was in agreement with previous studies using different types of delivery system[46, 47]. In all studies using pre-encapsulated VEGF, the initial burst release was followed by an incomplete release. Therefore, the current protein release test was consistent with the previous findings[48]. Similarly, Ennett *et al.*[48] used poly (lactide-co-glycolide) (PLG) scaffolds in his work, he found that the burst release of VEGF protein occurred within the first 48 hours by rapid desorption or diffusion process. The subsequent release most likely resulted from the protein located deeper within the microspheres that diffused through the polymer and/or was released as the polymer degraded. FG is a candidate polymer for use in fabricating composite biomaterials and delivery vehicle of growth factor for bone regeneration. Numerous data showed that effects of growth factors on both angiogenesis and bone formation are dose-dependent. The required dose of growth factor varied with the mode of administration and carrier type. In general, the localized and sustained release of growth factors allows for lower effective dosage than a single injection[31]. VEGF protein has been identified to have positive effects on osteogenesis and angiogenesis. Appropriate dosage and release profiles are very important for optimized biomolecule delivery. In addition, the application of excessive dose may provoke adverse effects or even toxic reactions, such as inhibition of blood vessels and bone formation[31]. Similarly, the slow and sustained release of VEGF protein can produce well-functioning blood vessels, whereas uncontrolled release of VEGF protein leads to malformed and non-functional blood vessels[32]. Therefore, optimizing and controlling the release of VEGF protein and is one of the primary concerns in bone tissue engineering.

In our study, we used the VEGF protein at 0.25 ng/ml which were injected inside the FG matrix before it sets. This suggests, low concentration of VEGF protein loaded might have a negative effect on the adsorption process from the delivery system because the low concentration of ions decreases the electrostatic charge repulsion[49]. The quick release of VEGF protein could potentially be due to repulsive interactions between VEGF protein and FG itself. Higher doses of VEGF protein lead to an efficient quantity of protein released by desorption ionization which is more beneficial for osteogenic efficacy[33]. On the other hand, too low a dose is ineffective, and it has been shown that excessive delivery of particular growth factors can cause adverse effects.

Unregulated overexpression of VEGF gene induced hemangioma formation and fatal vascular leakage whereas the prolonged presence of low concentrations of VEGF resulted in the formation of normal blood vessels[46]. Additionally, VEGF may act synergistically with BMP-2 by indirectly stimulating osteoblast activity through the expression of soluble osteogenic factors by endothelial cells[50].

Recently, BCP scaffold has a strong choice to use it as a bone graft in bone reconstruction in maxillofacial and craniofacial bone defects. BCP synthetic graft elicits a response from cells such as osteoblast, endothelial and fibroblast cells that are similar to those elicited by bone during the bone healing phase. BCP ceramic and its degradation products promoted the secretion of inflammatory cytokines and growth factors from macrophages[51]. Our results showed clear differences in Osx expression, DSCs treated with BCP scaffold with a FG matrix for VEGF protein delivery was a high expression and in all days of treatment compared to the other group of DSCs treated with a free dose of the VEGF protein which shows high VEGF gene expression but minimum BMP-2 gene expression followed by repression. In the group of DSCs treated with free VEGF protein release with high dose show efficient angiogenesis but minimal osteogenesis by the mean of absence of the equilibrium between both necessary processes of bone healing. Consequently, DSCs treated with BCP scaffold treated the media with FG as a matrix to deliver the VEGF protein showed high Osx gene expression which considered the importance marker of osteoblast differentiation that we considered in our observations. Actually, its expression showed progressive increase expression from day 3 to day 14 in DSCs. This result was consistent with previous finding using DSCs[39].

However, the role of VEGF production in osteoblasts has recently been widely discussed[52]. As we discuss in our previous work[35], Schönmeyr *et al*.[53] revealed that VEGF was a potent inhibitor of BMP-2 expression in MSCs, and that supplementation with or overexpression of VEGF inhibited BMP2 mRNA expres¬sion, protein production and MSC differentiation. The result suggested that the overexpression of VEGF gene can inhibit the action of BMP-2 in osteogenesis.

Our data indicate that VEGF over-expression in DSCs treated with non-controlled VEGF protein is an effective strategy to improve the vascularization of osteogenic grafts, but also has the potential to impair bone tissue formation by increasing osteoclast recruitment and bone resorption. Therefore, the equilibrium between VEGF triggered angiogenesis, osteogenesis and bone resorption needs to be carefully investigated in controlled models to devise rational strategies that exploit the pro-angiogenic potency of VEGF expression for a clinical application. Helmrich *et al*.[54] stressed in his work that in minimizing cell expansion, the osteogenic differentiation potential of bone marrow-MSCs was maintained, but a strong reduction in bone formation was observed when the VEGF gene over-expressed. VEGF has been reported to participate in several stages of bone repair and regeneration; however, most studies have primarily examined the consequences for bone repair when VEGF levels were manipulated at local injury sites or systemically[55]. Using a free dose of the VEGF may recruit excess numbers of osteoclasts, resulting in resorption of newly formed bone, since VEGF regulates the differentiation and migration of osteoclasts. Interruption of angiogenesis-osteogenesis coupling occurs when used, the excess VEGF dose without a delivery system by inhibiting the function of pericytes through VEGFR-2 mediated inhibition of PDGFR, a receptor required for pericyte maturation, and this leads to the formation of immature blood vessels and poor osteogenesis process.

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. RT-PCR analyses revealed that VEGF, which is a potent angiogenic stimulator that plays an important role in bone formation, was highly expressed in all the samples. Extensive studies and, using DSCs with a suitable combination of control release growth factors and scaffold materials, is essential before resorting to human trails.

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Conflict of Interest

The authors have declared that no COI exists.

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