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Development of PLGA-coated β -TCP scaffolds containing VEGF for bone tissue engineering

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Abstract: Bone tissue engineering is sought to apply strategies for bone defects healing without limitations and short-comings of using either bone autografts or allografts and xenografts. The aim of this study was to fabricate a thin layer poly(lactic-co-glycolic) acid (PLGA) coated beta-tricalcium phosphate (β -TCP) scaffold with sustained release of vascular endothelial growth factor (VEGF). PLGA coating increased compressive strength of the β -TCP scaffolds significantly. For *in vitro* evaluations, canine mesenchymal stem cells (cMSCs) and canine endothelial progenitor cells (cEPCs) were isolated and characterized. Cell proliferation and attachment were demonstrated and the rate of cells proliferation on the VEGF released scaffold was significantly more than compared to the scaffolds with no VEGF loading. A significant increase in expression of COL1 and RUNX2 was indicated in the scaffolds loaded with VEGF and MSCs compared to the other groups. Consequently, PLGA coated β -TCP scaffold with sustained and localized release of VEGF showed favourable results for bone regeneration *in vitro*, and this scaffold has the potential to use as a drug delivery device in the future.

Keywords: PLGA, β -TCP, VEGF, Scaffold, Tissue engineering

1. Introduction

Regeneration of bone defects resulting from infection, oncologic resection, congenital malformation or traumatic fractures secondary to aging is one of the most challengeable issues in reconstruction surgeries during recent years. Autologous bone grafting demonstrated high efficacy of bone regeneration, but it is limited in clinical setting due to both its supply and morbidity in donor site. In addition, although both allografts and xenografts are excessively used, the risk of disease transmission is remained unknown.¹

Bone tissue engineering provides promising strategies according to which bone healing without above-mentioned shortcomings and limitations are expected. Over the past decade, many studies have been conducted to design biocompatible and biodegradable porous engineered scaffolds enabling migration, adhesion, proliferation and differentiation of osteoprogenitor cells to osteoblasts in order to enhancement of bone defects reconstruction.^{2;3;4} It has been reported that macropores diameter > 200 μ m are expected to be effective in osteoconduction,⁵ and those up to 500 μ m can lead to vascularized bone; also, well-interconnected structure can accelerate bone regeneration and vascularization. Moreover, appropriate mechanical properties mimicking those of natural bone is necessary at least in range of mechanical strength of human cancellous bone (2–10 MPa).^{6;7}

β -TCP which is from the calcium phosphates family has been extensively used in orthopedics, oral and plastic surgeries and bone tissue engineering because of its excellent osteoconductivity, and similarity to bone mineral ceramic phase, while the application of porous β -TCP is limited because of the brittleness and poor mechanical properties.^{8,9} Coating/composite of the bioceramic scaffolds with biodegradable polymers like poly (lactic-co-glycolic) acid (PLGA), which is biodegradable polymer, is used to enhance the mechanical strength in previous studies. Moreover, in a recent study, it is indicated that PLGA is the most suitable material for coating β -TCP scaffolds in order to provide a more appropriate basis for further biological responses.^{10,11}

However, nutrition supplement of tissue-engineered non-vascularized bone grafts is one of the limitations of the success rate of this technique. The limitation capacity of implanted cells to uptake nutrient and oxygen and slowly ingrowth of blood vessels leads to the necrosis of the cells especially in the central parts of the scaffolds, thus the acceleration of a functional vascular network establishment within newly formed construct is very important in clinical aspect of bone regeneration. As a result, the aim of bone tissue engineering should be based on both seeding osteogenic cells on an osteoconductive scaffold and induction of angiogenesis to support the metabolic needs of the cells.

In drug delivery applications, both applying polymer coating on the drug-adsorbed surface of the bioceramic and impregnating the drug into polymer coating are used to control the drug release behavior.¹² According to the literature, controllable releasing of biological molecules such as fibroblast growth factor-2,¹³ icaritin,¹⁴ dexamethasone and bovine serum albumin,¹⁵ recombinant human GDF-5,¹⁶ tetracycline¹⁷ and recombinant human bone morphogenetic protein-2 (rhBMP-2)¹⁸ from β -TCP/PLGA composite scaffolds is demonstrated in the recent studies.

However, local and systemic delivery of angiogenic growth factors is a most common approach to enhance vascularization. Most important angiogenic factor is vascular endothelial growth factor (VEGF) which plays necessary roles in angiogenesis by not only promotion of vascular endothelial cells mitosis, but also increasing the

permeability of the vessels.¹⁹ Systemic exposure of VEGF may promote vascularization in non-target sites and also enhance the risk of tumor growth in distant areas. So, the release of VEGF must be sustained and localized in the target zone. Also, the dose of VEGF is very important. Davies et al.²⁰ studied the continued administration of VEGF on scaffold vascularization and reported that delivery of $150 \text{ ng} \cdot \text{d}^{-1}$ could increase vascularization whereas administration of $1500 \text{ ng} \cdot \text{d}^{-1}$ induced transient vessel growth.

In this study we aimed to fabricate the thin layer PLGA coated high porous β -TCP scaffold with controlled releasing of VEGF encapsulated in PLGA. In order to achieve this goal a β -TCP scaffold was produced and coated with a thin layer of PLGA containing of VEGF. Finally, freshly isolated cMSCs and cEPCs were seeded on β -TCP/PLGA scaffold and the bioactivity of released VEGF and cell proliferation and differentiation were evaluated.

2. Experimental procedure

2.1. Preparation of β -TCP powder

In order to obtain calcium phosphate powders synthesized by ethanol based sol-gel method, phosphorus pentoxide (P_2O_5 , MERCK Co.) and four watered calcium nitrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, MERCK Co.) were employed as chemical precursors for phosphorus and calcium, respectively. Aqueous solutions of both phosphorus pentoxide 0.5 M and four watered calcium nitrate 1.5 M in pure ethanol were prepared separately. Then, phosphorus pentoxide solution was slowly poured into the solution containing calcium nitrate which was stirring vigorously with mechanical stirrer (1000 rpm). Then the resulting sol was stirring for 30 min. For obtaining gel, through the process of aging, the resulted transparent sol was kept in a closed container for 48 h at room temperature. After drying in oven with 120°C the resulted powders were calcined at 1100°C for 3 h.

2.2. Fabrication of β -TCP scaffolds and PLGA-coated β -TCP scaffold

β -TCP scaffolds were fabricated by foam casting method in which polyurethane (PU) foam was considered as a template. For ceramic slurry preparation, β -TCP powder and distilled water (in ratio of g/ml = 1) mixed in a ball mill, and carboxymethyl cellulose (CMC, Sigma Co.), three poly phosphates (TPP, Sigma Co.) was used as additives. Appropriate dimensions of PU foam templates were cut. After cleaning and drying, the PU foams were immersed in the β -TCP slurry and compressed slightly in order to improve its absorption; subsequently, excessive slurry was removed by squeezing. The foams were dried at room temperature for 24 h, and fired using electric furnace (Modutemp Furnace) regarding a three stages schedule. In order to burn out the foam, heating continued from room temperature to 600 °C at a rate of 2 °C/min; then for sintering the ceramic, the temperature was raised from 600 °C to 1200 °C at a same rate, and hold at 1200 °C for 3 h.⁸

Poly(lactic-glycolic acid) (PLGA, ratio M/M% 75:25, Wako) pellets were dissolved under stirring in dichloromethane (CH₂Cl₂, Merck, Germany) solvent with the various concentrations of 5%, 10% and 20% w/v respectively. β -TCP scaffolds were dipped into the PLGA solution for 5 min, and excessive solution was removed by centrifugation of the scaffold at 300 rpm for 1 min. The coated scaffolds were dried under a fume hood overnight, and weighed individually before being kept in a desiccator.

2.3. Encapsulation of VEGF within the PLGA coated β -TCP scaffold

At first, 0.1, 0.5 and 3 μ g of VEGF (Sigma Co.) was added to 10% w/v of PLGA solution under vigorous stirring. Then, these various concentrations were loaded into the scaffolds as mentioned in [Section 2.2](#).

2.4. Scaffold characterization

2.4.1. Structure and morphology

Structure and morphology of the scaffolds were analyzed by using scanning electron microscope (SEM, Zeiss, DSM 940A, Germany) at an operating voltage of 15 kV.

By using the image analyzer software (ImageJ 1.44 p), the dimensions of at least 100 pores randomly selected from the samples were measured to obtain the mean pore sizes of β -TCP scaffolds before and after PLGA coating. The below formula determines the porosity of the scaffolds:

$$\text{Porosity} = \left(1 - \frac{\text{Bulk density}}{\beta\text{-TCP theoretical density}} \right) \times 100$$

The weight of the scaffold divided by the volume of it, is equal with bulk density. Electronic balance and a digital electronic micrometer were used to measurement of the weight and the dimensions of each sample, respectively. The theoretical density of β -TCP is 3.156 g/cm³, and 20 scaffolds of each group were used for determination of the porosity.

2.4.2. X-ray diffraction (XRD)

XRD measurement of the sintered scaffolds was performed by X-ray diffraction (XRD, D/MAX 5000, Siemen-Brucker) which had an X-ray source of Cu K α of 50 kV and 200 mA. All X-ray spectra were referenced to pure silicon (99.95%) as an external standard.

2.4.3. Mechanical properties

Uniaxial compression tests were performed using a HCT-25/050 Zwick/Roell mechanical tester with a 5 kN load cell at 25 °C. The β -TCP and β -TCP/PLGA at various concentrations were compressed as per ASTM standard (ASTM D5024-95a),²² and compressive strength were recorded. The crosshead speed was set at 1 mm/min, and loading was applied until the scaffold cracked. The dimensions of all samples were measured at four places in the samples by a digital

electronic micrometer and averaged. All experiments were performed for 10 independent runs.

2.4.4. Kinetic of VEGF release

The VEGF concentration was determined by the enzyme-linked immunosorbent assay ELISA kit. Quantizing ELISA kit (R&D Systems) was utilized according to the manufacturer's instructions. Briefly, VEGF loaded scaffolds were immersed in 25 ml phosphate buffer solution (PBS) and incubated at 37 °C, the concentration of VEGF in each sample was then measured at 0, 8, 24, 48, 72, 120, 168 and 240 h after incubation by compared with standards of known concentration and positive and negative controls. The concentration of VEGF was calculated as nanogram per milligram total protein. Based on the resealing amount and previous study²⁰ the 3 µg amount of VEGF was selected for cell culture assay.

2.5. In vitro biocompatibility

2.5.1. Monolayer (2D) cell culture

The present study was conducted in accordance with the approval of the Institutional Animal Care and Use Committee of the Tehran University of Medical Sciences, and conformed to standards of Association for Assessment and Accreditation of Laboratory Animal Care. Bone marrow aspirate (about 10 ml) of humerus of mongrel dogs with average weight of 20 to 25 kg was drawn, and collected into a 50-ml tube containing 7500 unit's heparin.

2.5.2. Isolation and characterization of canine mesenchymal stem cells (cMSCs)

The cMSCs were isolated according to the literature with some modifications.²¹ Polystyrene 72 cm² flask containing Dulbecco modified Eagle medium (DMEM; Gibco-BRL, Life Technologies, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS; Dainippon Pharmaceutical, Osaka, Japan) and 100 µg/ml penicillin-streptomycin (Gibco-BRL, Life Technologies) was used to culture bone marrow suspensions. After 2 days, non-adherent cells were removed by both a

series of phosphate buffered saline (PBS) washing and subsequent medium changing. Adherent cells were expanded as monolayer culture at incubation temperature of 37 °C containing 5% CO₂ in 95% humidity. Medium was changed every two days.

2.5.3. Differentiation ability of isolated cMSCs

Alizarin red staining and oil red staining were used for passage 3 cMSCs cells cultured for 3 weeks in the osteogenic, adipogenic, and chondrogenic differentiation mediums, respectively. Mediums were changed twice per week. The osteogenic differentiation medium contained the control medium supplemented with 0.2 mM ascorbic2-phosphate (Sigma, St. Louis, MO), 10 mM Na-b-glycerophosphate (Sigma Co.), and 10⁻⁸ M dexamethasone (Sigma). The adipogenic differentiation medium contained the control medium supplemented with 0.2 mM ascorbic3 phosphate (Sigma, St. Louis, MO), 100 µM indomethacin (Sigma Co.), and 10⁻⁵ M dexamethasone (Sigma). The chondrogenic differentiation medium contained the control medium supplemented with 10 ng TGF-β3 (Transforming Growth Factor-β3, Sigma), 10 ng BMP-6(Bone Morphogenetic protein-6, sigma Co.), 50 ng ITS + premix (Insulin-Transferin-Selenium, Sigma) and 5.35 mg Bovin Serum Albumin (Sigma). After 3 weeks, cell layers were washed twice with PBS, fixed with 4% formalin for 15 min, and then washed with distilled water. Subsequently, cells were incubated for 2 min with either alizarin red or oil red, and evaluated by light microscope.

2.5.4. Cell isolation of cEPCs

The canine EPCs were isolated by using a modification of a method,²³ accordingly, 5 ml of bone marrow was centrifuged with Ficoll at 1500 rpm for 30 min. The isolated cells were washed twice with PBS. 1 ml of growth medium consist of EGM-2 BulletKit medium (Clonetics, San Diego, CA) supplemented with 5% fetal bovine serum (FBS) was used for cell culture and placed into an incubator with 37 °C and 5% CO₂ for 3 days. The medium was changed every 3 days. The confluent cells were trypsinized with tryosinazer/EDTA and re-plated at 6 × 10⁴ cells/cm².

2.5.5. Cell culture and cell seeding

The scaffolds with dimensions of $10 * 10 * 10 \text{ mm}^3$ were decontaminated by soaking them three times in 70% ethanol for 15 min, then rinsed three times with potassium phosphate buffer solution (PBS) for 15 min before drying overnight under a sterilized hood. The sterilized scaffolds were sealed in a 24-well plate and kept in a refrigerator at 4 °C for later use. Before cell loading, the scaffolds were washed with PBS.

The suspensions of 5×10^5 passage 3 cMSCs in 25 μl DMEM medium were prepared and were placed on the top surfaces of the scaffold cubes located in a 12-well culture plate. Before the cultures were provided with the medium, they were pre-incubated at 37 °C for 2 h during which the drop disappeared owing to its penetration into scaffold porosity. The cultures were provided with DMEM containing 15% FBS, and antibiotics were loaded over the scaffolds. They were incubated for 15 min at 37 °C. After that, the medium containing the non-adhering cells was removed, rinsed once again, and incubated for another 15 min at 37 °C. This procedure was repeated three times. Then, the scaffolds were transferred to another well.

For differentiation assays the cultures were provided with DMEM containing 50 $\mu\text{g/ml}$ ascorbic acid 2-phosphate (Sigma; USA), 10 nM dexamethasone (Sigma, USA) and 10 mM β -glycerol phosphate (Sigma; USA), 100 IU/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. All cultures were incubated at temperature of 37 °C in an atmosphere of 5% CO_2 .

2.5.6. Cell morphology, penetration and attachment

Samples were immediately washed three times with PBS after growth medium was pipetted out. Then they were fixed with a 3% glutaraldehyde solution, soaked in an osmium tetroxide solution for 1 h, and dehydrated through a series of ethanol solutions with graded concentrations, followed by two changes of 100% amyl acetate for 15 min each. The scaffolds were dried with the use of supercritical point dryer (Denton Vacuum critical point dryer), and cell morphology and attachment were evaluated by SEM. For evaluation of cell

penetration into the scaffold, cross-sections of scaffolds were used to indicate the depth of cell penetration.

2.5.7. MTT assay

Cell proliferation was analyzed by using [3-(4,5-dimethylthiazol-2-yl)-1,5-diphenyltetrazolium bromide] (MTT, Sigma, USA) mitochondrial reaction. This assay was based on the ability of live cells to reduce a tetrazolium-based compound, MTT, to a purplish formazan product. Briefly, 5×10^5 cEPCs or cMSCs cells were cultivated in composite scaffolds for a period of 2 weeks. Scaffold/cells constructs either from day 7 or 14 of 3D culture were then washed with PBS, transferred into new 24-well plates containing 5:1 ratio of media and MTT solution (5 mg/ml in PBS), respectively and incubated for 2 h at 37 °C. After removing the solution, 0.5 ml of extraction solution (dimethylsulphoxide: DMSO) was added. The constructs were washed extensively by pipetting out repeatedly to allow total colour release. The absorbance of the supernatant was read by a microplate reader (BioTekEL × 800, USA) at 540 nm. Cell number was determined through a standard curve which was established by using a known number of cells counted by a Neubauer-counting chamber.

2.5.8. Activity of VEGF released from scaffolds

To determine the biological activity of VEGF at the dose of 3 µg released from the BTCP/PLGA scaffolds, a cEPCs proliferation assay of 7th day was carried out.

2.5.9. Real-time polymerase chain reaction (real time-PCR)

Total RNA was extracted from the collected cells by using the TRIzol reagent (Invitrogen, Germany), RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Canada) was used to cDNA synthesis and real-time PCR reactions were performed in 96-well optical reaction plates in a 7500 real-time PCR system (Applied Biosystems, USA). In each PCR reaction, 12 ng of cDNA and corresponding primers ([Table 1](#)) was mixed with 1 × SYBR® Green PCR Master Mix (Applied Biosystems, USA) in a total volume of 20 µM.

Table 1. Sequences of primers and RT-PCR conditions.

Name	Size bond	Sequence
Coll1	293 bp	F: 5' CTA CCT ACC ACT GCT AGA AC 3' R: 5' TGA AAC AGA CTG GGC CAA CG 3'
RUNX2	118 bp	F: 5' ATG ACA CTG CCA CCT CTG A 3' R: 5' ATG AAA TGC TTG GGA ACT GC 3'
OSTEOCALCIN	195 bp	F: 5' GGC AGC GAG GTA GTG AAG AG 3' R: 5' CAG CAG AGC GAC ACC CTA GAC 3'
KDR	275 bp	F: 5' AAG TAT GTG ACC CCA AAT TCC 3' R: 5' AGA ACA ACA CTT GAA AAT CTG 3'
VEGF	391 bp	F: 5' CTA CCT CCA CCA TGC CAA GT 3' R: 5' CCT CGG CTT GCT ACA TTT TT 3'
vWF	150 bp	F: 5' CAT TCA GCT AAG AGG AGG AC3' R: 5' TTG TGT TCA TCA AAG GGT GG 3'

2.6. Statistical analysis

Data were analyzed by SPSS 21.0 and reported as mean and standard deviation. If it was necessary, a two-tailed student's *t*-test was used for comparing the results between the scaffolds.

3. Results

3.1. Structural and mechanical characteristics of the scaffold

[Fig. 1](#) shows the XRD patterns of the sintered synthesized β -TCP powder. As it can be seen in this figure, the main characteristic peaks of β -TCP are the major phase (β -TCP = 77.9%, HA = 20.4% and CaO = 1.7%).

Fig. 1. XRD patterns of the sintered synthesized β -TCP powder.

The β -TCP porous scaffolds were fabricated by replicating the porous structure of the PU foams. The scaffolds, like the PU foams, had a highly interconnected structure, with open macropores ([Fig. 2A](#)). The average pore size of the macro-pores of the scaffolds was about 500 μm , estimated by the macro-pore sizes taken from the SEM ([Fig. 2B](#) and [C](#)). It was observed that the β -TCP scaffolds had more crack-like defects on and within the ceramic struts than those coated with PLGA. ([Fig. 2D–F](#)) However, the crack-like defects in the β -TCP struts could be filled with the polymer after the polymer solution dipping plus centrifugation. The β -TCP scaffolds when coated with PLGA at low concentration of 5% ([Fig. 2D](#)) and 10% ([Fig. 2E](#)) is covered partially with PLGA and maintains the initial framework structure. Whereas the increase of PLGA concentration to 20%, more surface of β -TCP scaffold gets covered with PLGA coating, the stems becomes thicker and some pores can partially be clogged, as shown in [Fig. 2F](#). The total porosity of the β -TCP scaffolds was determined to be approximately 84.23%. The macroporosity of the scaffolds after infiltration and coating with the various concentration of PLGA was slightly decreased due to the observed thin polymer coating present on the strut surfaces, the macro-pores were not made significantly smaller by the polymer coating ([Fig. 3A](#)).

Fig. 2. SEM micrographs of β -TCP without coating (A–C) and with coating at concentration of 5% PLGA (D), 10% PLGA (E) and 20% PLGA (F).

Fig. 3. VEGF release amount from the scaffolds encapsulated with 0.1, 0.5, and 3 μ g of VEGF.

Compression testing was performed on both the β -TCP scaffolds and the β -TCP scaffolds coated with the different concentration PLGA

polymer. It was found that the β -TCP scaffolds were able to withstand average compressive 0.42 MPa, while those coated with 5%, 10% and 20% PLGA showed a compressive strength of 3.10, 4.95 and 6.62 MPa, respectively (Fig. 3B). Obviously the compressive strength of the scaffolds coated with PLGA was significantly higher than those without the PLGA polymer coating, and were also comparable to that of human cancellous bone (2–10 MPa).⁶

Also, the toughness of the scaffolds after and infiltration and coating with the various concentration of PLGA has been given in Table 2. As it can be observed in this table, with increasing the PLGA concentration, the toughness increased.

Table 2. The toughness of the scaffolds after infiltration and coating with the various concentration of PLGA (p value < 0.05).

Sample	Toughness (MPa)
β-TCP (PLGA 0%)	0.06 $\pm$ 0.004
PLGA 5%	0.94 $\pm$ 0.03
PLGA 10%	1.73 $\pm$ 0.11
PLGA 20%	2.18 $\pm$ 0.17

According to the mechanical properties test and SEM observations, the optimum PLGA concentration for biological assessment and VEGF releasing was 10% w/v.

3.2. The release of VEGF from PLGA-coated β -TCP scaffolds

The release kinetics for VEGF incorporated was subsequently analyzed using specific ELISA kit. VEGF was released from β -TCP scaffolds coated with PLGA, VEGF composition coating including an initial burst release followed by a relatively slow sustained release. An initial burst in the first 24 h was around 2.17, 14.82, and 59.64 ng/ml for the samples encapsulated with 0.1, 0.5, and 3 μ g of VEGF, respectively. Then the protein release was sustained during the next 10 days. The average release amount was 6.87, 70.11 and 165.92 ng/ml, respectively. The release amount was related to the loading concentration, that is, larger release amounts are for higher initial loading concentrations of VEGF (Fig. 4). The result showed that the protein release was at almost the same rate, for all scaffold during

10 days. In contrast, VEGF release from the scaffolds with only PLGA coating were significantly different and burst in nature. Moreover, about 90% of loaded VEGF was released within the first 24 h.

Fig. 4. The macroporosity (A) and compressive strength (B) of the scaffolds after infiltration and coating with the various concentration of PLGA.

3.3. Characterization of cMSCs and cEPCs

Formation of a number of colonies consisting of a few adherent fibroblastic cells was demonstrated regarding regularly observations of bone marrow cell cultures with a phase contrast invert microscope. The colonies had grown till 10 days after which fibroblastic cells covered all the available surfaces of the culture dishes. Passage 3 fibroblastic cells were used to differentiate into osteoblasts in monolayer culture. After 3 passages, MSCs morphology changed from fusiform monolayer to multilateral shaped. Moreover, multilayer small colonies and EPCs demonstrated the characteristic cobblestone morphology.

It was noticed that higher proliferation rate was observed even after passages 4 or 5. According to the flow cytometry analysis, bone

marrow stromal cells consist of mesenchymal (positive for CD90, CD44 and negative for CD146, CD34, CD45), endothelial (positive for CD34, VEGFR2, and CD45 and negative for CD133 and CD146), and hematopoietic (positive for CD45) lineages.

Although most hematopoietic cells were lost after 7 days, the similar proportion of cells with mesenchymal and endothelial phenotypes was observed. For instance, in mesenchymal-lineage cells, the percentage of CD90 and CD44 was 95.5% and 94%, respectively, whereas in endothelial-lineage cells, the percentage of CD34 and CD45 was 17% and 1.35% respectively. After passage 3 EPCs, in endothelial progenitor-lineage cells, the percentage of CD34 and VEGFR2 was 72.5% and 64.8% respectively, whereas the percentage of CD133 and CD 146 was 18.9% and 16.2%, respectively.

A strong alizarin red staining demonstrated the ability of freshly isolated MSCs to deposit abundant mineralized matrix when cultured in osteogenic medium for 3 weeks. Furthermore, as a strong oil red and safranin staining were indicated, MSCs were able to differentiate toward the adipogenic and chondrogenic lineage when cultured in adipogenic and chondrogenic medium, respectively.

3.4. Initial cell penetration into and attachment onto the PLGA-coated β -TCP scaffolds

Since cell suspension was added drop-wise to the surface of the scaffolds for seeding of MSCs and EPCs, the cells were not homogeneously seeded across the surface of the scaffolds. With the use of cross-sections of the scaffolds, the penetration of the cells into the scaffolds was investigated. According to SEM observations ([Fig. 5A](#)), the cells could penetrate to a depth of 5.5 mm with adding a drop in one side, density of cells was not homogeneous along the depth direction, for instance, even though cells were most plentiful on the surface, the cell density gradually diminished with regard to increasing the depth. Due to the highly interconnected pores of the β -TCP scaffolds coated with PLGA, significant cell penetration was demonstrated. Cells retaining spindle morphology was revealed 7 days after culture of MSCs on the scaffolds ([Fig. 5B](#)). Where osteoblast projections and pseudopodia were evident, fibrous extracellular matrix

(ECM) was observed between cells. After a week of culture, SEM images indicated that the scaffolds were entirely covered with a layer of EPCs (Fig. 5C). High cell-to-cell contact and a typical cobblestone-like appearance was retained by EPCs. Moreover, pseudopodia extensions were observed on the cells surface (Fig. 5D). Cell adhesion, spread and proliferation on the scaffolds indicated normal cell metabolism; therefore, β -TCP scaffolds coated with PLGA showed favourable biocompatibility *in vitro*.

Fig. 5. SEM micrograph of MSCs (A and B) and EPCs (C and D) seeded on the scaffolds.

3.5. Cell proliferation and activity of VEGF released from scaffolds

The viability of cells grown on the scaffold was determined by MTT assay. There were no statistical differences between the β -TCP and β -TCP scaffolds coated with PLGA (10%) ($p > 0.05$) (Fig. 6A).

Fig. 6. Proliferation of MSCs on the β -TCP and β -TCP coated with PLGA scaffolds (A) and EPCs on the β -TCP coated with PLGA scaffolds with and without VEGF (B) at 7 days by MTT assay.

Following demonstration and confirmation of the release of VEGF from the β -TCP scaffolds coated with PLGA, the bioactivity of the released VEGF was determined by its proliferative induction of cEPCs *in vitro*. Cell number as determined by MTT assay at day 7 conclusively indicated that releasing of VEGF into the medium from the β -TCP scaffolds coated with PLGA statistically increased the proliferation of cEPCs in culture ($p < 0.05$) (Fig. 6B).

3.6. Cell differentiation

To thoroughly examine and determine the level of osteogenic differentiation and vascularization in the different groups, real time-PCR analysis was performed in each groups (PLGA coated β -TCP with and without VEGF) (Fig. 7). A culture of MSCs on the VEGF loaded scaffolds demonstrated the highest expression of the osteogenic markers ($p < 0.05$). The scaffolds loaded with VEGF and MSCs showed a statistical increase in expression of COL1 and RUNX2 compared to the other groups ($p < 0.05$).

Fig. 7. Real time-PCR analysis for PLGA coated β -TCP with and without VEGF.

Twenty-one days after cultivation, we observed a highest expression of vWF and VEGFR2 gens in the scaffolds that released VEGF and were loaded with MSCs ($p < 0.05$) in comparison to the scaffolds loaded with MSCs.

4. Discussion

Bone tissue engineering aims to use osteoconductive scaffolds together with delivery of osteogenic cells and biological factors. The Scaffolds play a key role in bone regeneration and should have both an interconnected pore structure and good mechanical properties. The scaffold also can be used as delivery vehicle of growth factors. Due to high osteoconductivity and biodegradability of β -TCP which is a calcium phosphate ceramic, it has been used in different clinical situations for bone defects regeneration.^{24;25} In the present research, we fabricated a 3D highly interconnected porous PLGA coated β -TCP scaffold with significantly enhanced mechanical strength as delivery vehicle of VEGF, and demonstrated that its releasing of VEGF

significantly increased both proliferation and differentiation of MSCs and EPCs, and highly expressed osteogenic markers.

The β -TCP porous scaffold was fabricated with template casting method with PU foam. The scaffold had a highly interconnected structure; with the average pores size of about 500 μm . The previous studied has showed that the pore size of about 200 μm and greater is accelerate osteogenesis and the pore size of about 500 μm and greater is good for vascularization. However, the major problem of highly porous β -TCP is poor mechanical properties, the polymer coating expected to improve the mechanical property of the brittle β -TCP.²⁶ In our study a β -TCP/PLGA scaffold with enhanced mechanical strength and highly porous structure were fabricated. Coating with PLGA leads to increase of compressive strength of the β -TCP scaffolds from 0.42 MPa to 3.10, 4.95 and 6.62 MPa for 5%, 10% and 20% PLGA coatings, respectively. Increasing of PLGA concentration resulted in the increase of compressive strength but it was shown that in 20% PLGA concentration some pores was clogged as shown in [Fig. 2F](#). PLGA impregnation leads to fills or blunt the flaw of β -TCP template and increase of mechanical strength. The presence of microcrack of β -TCP template is responsible for poor mechanical strength. Pezzoti and Asmus revealed that ductile polymer ligament could be stretched and consume energy during propagation of microcrack. Moreover, PLGA coatings increased mechanical strength of 3D porous β -TCP scaffold in that of human cancellous bone range (2–10 MPa).⁶ In consistent with our study, Kang et al. demonstrated enhanced mechanical strength of 3D porous β -TCP scaffold by PLGA (10 wt%) coating;⁸ however, their scaffold was fabricated by a template-casting technique used templates comprised of paraffin bead. Also, Miao et al. demonstrated that PLGA coated hydroxyapatite (HA)/ β -TCP scaffolds fabricated by a polyurethane foam replica method showed significant increase in mechanical strength (0.6 MPa) compared to HA/ β -TCP scaffolds (0.05 MPa).⁹

The total porosity of the β -TCP scaffolds was determined to be approximately 86.87%. The macroporosity of the scaffolds after infiltration and coating with the various concentration of PLGA was slightly decreased due to the observed thin polymer coating present on the strut surfaces; however, the macropores were not made significantly smaller by the polymer coating. The coating of PLGA was

result of the decrease in porosity by approximately 2% and it was consistent with the previous study by Kang et al.⁸

The biocompatibility of β -TCP and β -TCP/PLGA scaffold was evaluated with MSCs and EPCs attachment and proliferation. Our results revealed that MSCs could be attached and proliferated on β -TCP scaffold with and without PLGA which is in consistent with the previous study.⁸ The number of MSCs was significantly higher in scaffold groups than positive control, but there were no significant differences between the scaffolds. SEM observation of cross-sections of the scaffolds showed that the cells could penetrate non-homogenously to a depth of 5.5 mm. The results were consistent with the study by Miao et al. according to which bone marrow stromal stem cells (BMSCs) penetration to a depth of 4.5 mm was reported into a HA/ β -TCP coated with PLGA.⁹

The loading capacity of pure β -TCP is limited, and weak physical adsorption is responsible for loading. Our results showed that encapsulation of VEGF in PLGA coating could be a suitable way to control of loading amount, and it was controlled by changing the VEGF concentration in PLGA coating. Localized and sustained delivery of VEGF permits prolonged exposure of regenerating tissue to efficient doses to enhance vascularization, however high levels of VEGF can lead to abnormal vascular function and vascular tumor growth. VEGF should be released as sustained manner during healing process of bone.

To investigate the mitogenic potential of VEGF released from PLGA coated β -TCP scaffold, proliferation of EPCs cultured on the scaffolds was indicated after 7 days. According to the study conducted by Silva et al., the rate of endothelial cells proliferation reached a plateau at 50 ng/ml, which demonstrated proliferation of cells 60% more than compared to without discretion of VEGF.²⁷ They also indicated by the 3D sprout assay that higher numbers of sprouts were revealed when cells exposed to higher doses of VEGF in the early days.

The release profile from PLGA in 0.5, 1 and 3 μ g concentration of VEGF displays a sustained manner up to 14 days, nevertheless there was a significantly smaller amount of VEGF released from 0.5 and 1 μ g compared to concentration of 3 μ g. Sustained release of

VEGF is due to distribution of VEGF into polymer coating whereas the initial burst release is due to binding of VEGF to PLGA surface. The bioactivity of released VEGF is very important; our results showed that the dose of 750 ng VEGF per each PLGA (10 wt%) coated β -TCP scaffolds leads to significantly increasing the number of EPCs and MSCs. Using a range of 250 ng to 3 μ g VEGF per each construct is reported for bone tissue engineering in the literature.²⁸ The studies in animal models have revealed that the inhibition of however the main VEGF receptors expressed on endothelial cells, there are many VEGF receptors expressed on osteoblasts.¹⁹

After confirmation of effects of VEGF on MSCs and EPCs proliferation we examined the osteogenic and angiogenic differentiation ability of cells seeded on β -TCP/PLGA scaffolds with and without VEGF. This study indicated the sustained release of VEGF significantly increased marker of osteogenesis and angiogenesis.

VEGF expression and low concentration of oxygen lead to an upregulation of BMP-2. This pathway may play a significant role in bone repair. VEGF is a significant therapeutic agent for bone regeneration which not only promotes angiogenesis but also play an important role in bone growth and repair.²⁹

In summary, it is enticing to hypothesize that the degree of differentiation and thus the maturity of EPCs could play a crucial role in their effect on MSCs osteogenic differentiation.³⁰ This is demonstrated by the work of Loibl et al. that determines EPC differentiation into mature EC by direct cell-cell contact with MSCs.³¹ Additionally, various studies ascertained the presence of PL could promote cell proliferation of EPC in EPC-MSC co-culture supports a pericyte-like differentiation of MSCs in both MSC and EPC-depleted MSC populations.^{31;32} Furthermore, it was displayed that the incorporation of MSC could promoted stable neovascularization in EPC-derived tube formation *in vivo*.³³ Still, the perfect proportion of EPC for early neovascularization is questionably discussed in the literature. Duttenhoefer et al. revealed the ratio of 50/50 of MSC and EPC is ideal.³² These obtained results were confirmed by *in vivo* study, where the highest number of vessels in the center of scaffolds, implanted subcutaneously in the mice, was determined in 50% MSC + 50% EPC proportion.³⁴ In actuality, Fu et al., ascertained that the ratio of 75%

EPC + 25% MSC in modified calcium polyphosphate constructs displayed the highest expression of osteogenic and angiogenic markers, while the level of EPC maturation still stays indistinct.³⁵

Duttenhoefer et al. found that absence of EPCs in MSC population enables higher osteogenic gene expression and matrix mineralization and thus may prompt prior new bone formation. Nevertheless, the utilization of cells in bone tissue engineered scaffolds requests the support of a functional blood supply. In this way, our obtained results may cause to novel approaches in cell seeding to develop vascularized bone tissue engineered scaffolds, such as specific, organized, or time dependent seeding of various cell types. Finally, further evaluations on the mechanisms by which CD34⁺/CD133⁺ EPC effect MSC osteogenic differentiation as well as the effect of EPC maturation in this approach are mandatory.

It is important to point out, although the direct mechanisms of tubular-like structure formation in co-culture of MSCs and EPCs are still obscure, it has been exhibited that mature endothelial cells and osteoblasts influence proliferation, as well as differentiation of each other.³² It has been reported that growth factors such as BMP-2 and IGF released by endothelial cells influence osteoblast proliferation and differentiation.^{36;37} Consequently, osteoblasts can increment endothelial cell viability, proliferation and angiogenesis, by releasing high levels of VEGF.³⁸ Also, Duttenhoefer et al. revealed that without MSCs, number of EPCs reduced after 7 days in 3D culture which it is consistent with the study of Hofman et al.³⁹

5. Conclusion

PLGA coated β -TCP scaffold demonstrated desired results for bone tissue engineering since it showed appropriate mechanical strength and biocompatibility with sustained and localized release of VEGF which affect proliferation rate of EPCs. This scaffold may be utilized as a drug delivery device for other growth factors and biomolecules in the future.

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