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# Effect of different calcium phosphate scaffold ratios on odontogenic differentiation of human dental pulp cells

Sarah Talib AbdulQader [a,d], Thirumulu Ponnuraj Kannan [a,b,*], Ismail Ab Rahman [a], Hanafi Ismail [c], Zuliani Mahmood [a]

[a] School of Dental Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
[b] Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
[c] School of Materials and Minerals Resource Engineering, Universiti Sains Malaysia, 14300 Penang, Malaysia
[d] Department of Pedodontic and Preventive Dentistry, College of Dentistry, University of Baghdad, Baghdad, Iraq

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ABSTRACT

Calcium phosphate (CaP) scaffolds have been widely and successfully used with osteoblast cells for bone tissue regeneration. However, it is necessary to investigate the effects of these scaffolds on odontoblast cells' proliferation and differentiation for dentin tissue regeneration. In this study, three different hydroxyapatite (HA) to beta tricalcium phosphate (β-TCP) ratios of biphasic calcium phosphate (BCP) scaffolds, BCP20, BCP50, and BCP80, with a mean pore size of 300 μm and 65% porosity were prepared from phosphoric acid ($H_2PO_4$) and calcium carbonate ($CaCO_3$) sintered at 1000 °C for 2 h. The extracts of these scaffolds were assessed with regard to cell viability and differentiation of odontoblasts. The high alkalinity, more calcium, and phosphate ions released that were exhibited by BCP20 decreased the viability of human dental pulp cells (HDPCs) as compared to BCP50 and BCP80. However, the cells cultured with BCP20 extract expressed high alkaline phosphatase activity and high expression level of bone sialoprotein (BSP), dental matrix protein-1 (DMP-1), and dentin sialophosphoprotein (DSPP) genes as compared to that cultured with BCP50 and BCP80 extracts. The results highlighted the effect of different scaffold ratios on the cell microenvironment and demonstrated that BCP20 scaffold can support HDPC differentiation for dentin tissue regeneration.



## 1. Introduction

Tissue engineering approaches have gained great importance to restore or replace tissues that were damaged or lost. This involves the regeneration of new functional healthy tissues using progenitor cells with scaffold in response to appropriate signals [1]. In clinical dentistry, dentin tissue regeneration is a desirable goal that allows the formation of new healthy dentin to be integrated with the pre-existing dentin to overcome the drawbacks of the conventional dental treatments [2–4]. Recent advances in stem-cell biology have revealed the possibility of dentin regeneration using human adult dental pulp cells (HDPCs) as progenitor cells that have the ability to form a dentin/pulp-like complex [5,6] and possess self-renewal [7] and multi-lineage differentiation capability [8–10]. These cells can be carried by scaffolds that play an important role in providing physical support for the cells and maintaining space for the regenerated tissue. These scaffolds guide new tissue growth before degradation and are eventually replaced by new tissues [11].

Scaffold biological properties depend primarily on the chemical composition of the biomaterial [12,13], and its porous structure: pore shape, pore size, porosity percentage and pore interconnection pathway [14,15]. Microporosity of diameter <10 μm permits body fluid circulation and macroporosity of diameter >100 μm is necessary for migration and proliferation of cells and tissue formation [16,17]. The total porosity of 65% is efficient for total protein production and alkaline phosphatase (ALP) activity that were taken as indicators of growth/matrix production and for the comparison of cell differentiation [18].

Among many types of scaffolds that have been used for the regeneration of hard tissues, biphasic calcium phosphate (BCP) scaffolds have shown to induce appropriate osteoblastic differentiation of stem/progenitor cells in vitro and bone formation in vivo [19–21] due to their desirable properties including similarity in composition to the bone mineral, bioactivity and osteoconductivity [22,23]. They consist of an intimate mixture of hydroxyapatite (HA) [$Ca_{10}(PO_4)_6(OH)_2$], and beta-tricalcium phosphate (β-TCP) [$Ca_3(PO_4)_2$] crystals of varying phase compositions (HA/β-TCP ratios) that have been reported to be more effective than pure HA or β-TCP alone [24–26]. They exhibit different biological behaviors of their components in which HA is bioactive and β-TCP is resorbable [27]. The effects of different HA/β-TCP ratios of several modified BCP scaffolds have been well studied for bone tissue

* Corresponding author at: School of Dental Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
*E-mail address:* kannan@usm.my (T.P. Kannan).

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regeneration [15,28–31]. It was reported that BCP of 20/80 HA to β-TCP ratio stimulated the osteogenic differentiation of human mesenchymal stem cells (HMSCs) in vitro and fastest rate of bone induction in vivo as compared to 100% HA, HA/β-TCP (76/24, 63/37, 56/44), and 100% β-TCP [24]. However, these effects are not well known for odontogenic cell differentiation and dentin tissue regeneration. Although odontoblasts and osteoblasts show almost identical genetic profiles, there is a clear difference between the process of odontogenesis and osteogenesis and fundamental differences in cellular morphology between bone and dentin. These differences can affect the convenience and appropriateness of the scaffold used in tissue regeneration [32].

In spite of all improvements on the solubility and bioactivity obtained with BCPs, it is important to consider the possible impact of their composition on the processes linked to cell proliferation and differentiation, and to ensure the proportional amounts of each component do not impoverish the final composite properties. Hence, it would be of interest to evaluate the effect of different HA/β-TCP ratios of BCP scaffolds on HDPC odontogenesis for dentin tissue regeneration. The aim of the present study was to determine the optimum microenvironment and the ratio of HA to β-TCP that supported the viability and the differentiation of HDPCs. The influence of BCP characteristics of different ratios of HA to β-TCP and their degradation products on the microenvironment, viability, ALP activity, and gene expression of HDPCs has been investigated. The null hypothesis was that there would be no differences in the microenvironments and in HDPCs' odontogenesis due to the different ratios of HA to β-TCP.

## 2. Materials and methods

### 2.1. Scaffold preparation and characterization

Calcium phosphate powders were prepared by a wet precipitation method using $CaCO_3$ and $H_2PO_4$ as starting materials as mentioned previously [33]. Briefly, the reagents were mixed at Ca/P ratios of 1.517, 1.568, and 1.619 corresponding to BCP of 20/80 HA to β-TCP ratios (BCP20), 50/50 HA to β-TCP ratio (BCP50), and 80/20 HA to β-TCP ratio (BCP80) respectively. The precipitate was heated at 80 °C for an hour with stirring, aged for 48 h, washed, filtered, and dried at 100 °C. The ground powder was then added to polyethylene (PE) spherical particles as pore-former agents of 300–350 μm (Cospheric, USA) at a ratio of 4:2.5 v/v for total porosity of 65% and pore size range of 300 μm. The powder mixtures were compressed with a uniaxial press of 24 MPa in a 32 mm die to form 6 mm thickness pellets. Subsequently, pellets were submitted to thermal treatment of 400 °C for 2 h and sintered at 1000 °C for 2 h. Then, the pellets were crushed and sieved to obtain granules with the desired particle size range of 0.5–1 mm as shown in Fig. 1.

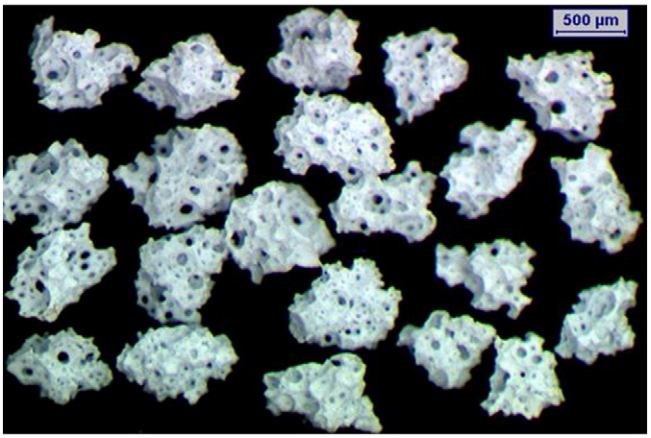


**Fig. 1.** Particle size of the prepared scaffolds after sieving as visualized by image analyzer.

The phases of HA and β-TCP and the ratios of HA/β-TCP of the scaffold were analyzed qualitatively and quantitatively by X-ray diffraction (XRD) analysis using Eva and X'Pert HighScore Plus software, respectively. The registered patterns were compared with the International Centre for Diffraction Data (ICDD) powder diffraction file (PDF) to determine the crystalline phases.

Field emission scanning electron microscopy (FESEM) (Zeiss Supra 55VP, Germany) was used to evaluate the surface morphology of the scaffolds. The scaffolds' surfaces were first sputter-coated with a layer of platinum alloy (150 Å thick). The total porosity of the scaffolds was assessed using Archimedes' method.

### 2.2. Preparation of scaffold extract

The particles thus obtained were sterilized by autoclave before use. The extract of each scaffold sample (BCP20, BCP50, and BCP80) was prepared according to international standards for medical devices evaluation ISO standard (10993-12) [34]. After sterilization, 1 g of each scaffold material (in the form of particles) was incubated in 10 ml alpha minimum essential medium (Alpha-MEM, Invitrogen, USA) for 1, 3 and 7 days at 37 °C. After incubation, the extract was collected by filtration using 0.2 μm filter.

### 2.3. Measurements of calcium and phosphate ions, and pH values of the extracts

The content of calcium and phosphate ions in each extract at each time was quantified colorimetrically using Calcium and Phosphate Colorimetric Assay Kits (BioVision, USA) according to the manufacturer's protocols. Briefly, for $Ca^{2+}$ measurement, a chromogenic complex formed between $Ca^{2+}$ and 0-cresolphthalein was measured at 575 nm using an ELISA reader (Sunrise, Tecan, Austria). For phosphate ion measurement, malachite green and ammonium molybdate that form a chromogenic complex with phosphate ions was measured at 405 nm. The intensity of the color was directly proportional to the concentration of ions in the samples. The difference between amounts of ions in the media exposed or not to particles, in the absence of cells, corresponded to the release of ions from the particles. Changes in the pH were measured at pre-determined time intervals as a function of time using a pH meter (Hanna 210, Japan).

### 2.4. Culture of HDPCs

Immortalized human dental pulp cells (HDPCs) that was kindly provided by Professor Takashi Takata (Hiroshima University, Hiroshima, Japan) were used in this research [35]. Cells were cultured in Alpha-MEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 5% $CO_2$ and 95% relative humidity at 37 °C. Culture medium was changed every three days until confluence. Cells were trypsinized, suspended in complete culture medium of Alpha-MEM, and subcultured at a cell density of $1 \times 10^4$ cells/well in 96-well culture plates (Corning, USA) with 200 μl of culture medium. After 24 h, the medium was removed from each well and replaced by 180 μl of each scaffold extract plus 20 μl of fetal bovine serum (final concentration of 10%). The extracts of BCP20, BCP50, and BCP80 scaffolds incubated in the medium for 3 days were chosen for the rest of the study's tests.

### 2.5. Cell viability assay

The viability of HDPCs exposed to the different scaffold extracts was evaluated by MTT assay. After 1, 3, 5, and 7 days of incubation of cells with different scaffold extracts, the extracts were removed and the cells were tested for viability. The 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich, USA) assay was used as previously described [36] to determine cell viability. This assay