Original Article

Effects of Nano-biphasic Calcium Phosphate Composite on Bioactivity and Osteoblast Cell Behavior in Tissue Engineering Applications

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Abstract

In this paper, preparation, bioactivity, and osteoblast cell behavior of cortical bone derived nano-biphasic calcium phosphate (nano-BCP) are presented. The calcined bovine bone samples with the addition of di-ammonium hydrogen phosphate were heated at 700°C for 100 min, and thus nano-BCP with the composition of 63/37 hydroxyapatite (HA)/β-tricalcium phosphate (β-TCP) was produced. Scanning electron microscopy (SEM) images, energy dispersive X-ray spectroscopy (EDS), and X-ray diffraction (XRD) analysis of immersed samples in simulated body fluid (SBF) solution showed that a uniform layer was formed on the surface after 7 days with the chemical composition of HA. The results indicated that the nano-BCP sample developed excellent bioactivity after 28 days. The nano-BCP samples showed better cell proliferation compared to pure HA samples. After 7 days in cell culture, the prepared nano-BCP (HA/β-TCP) exhibited the maximum proliferation of the MG-63 osteoblast cells.

Keywords: *Animals, body fluids, calcium phosphates, cell proliferation, hydroxyapatites, osteoblasts, phosphates, spectrometry, X-Rays*

Introduction

Bioactive calcium phosphate ceramics have been used for augmentation or substitution of damaged parts of musculoskeletal system by virtue of their similarity to the human bones mineral.[1-3] Among calcium phosphate ceramics, hydroxyapatite (HA) shows a variety of biomedical applications, including bone repair and dental applications. In the biological environment, HA forms an apatite layer at the interface with bone tissue surfaces.[4,5] However, its bioactivity, while attractive, is not enough to actively form apatite layers on the surfaces.[4]

Biodegradable ceramic materials such as biphasic calcium phosphate (BCP) composites are used for the repair and regeneration of bone tissues.[6,7] BCP consisting of HA and β-tricalcium phosphate (β-TCP) shows a higher degree of degradation compared to HA, and this is related to the dissolution of β-TCP phase in the biological environment. This character of BCP creates the osteoinductivity property and supports the existent osteoconductivity, which can be attributed to their higher bioactivity,[6,8,9] and accelerates the repair and regeneration of defects. According to the authors' knowledge, BCP synthesis methods such as sol–gel,[10] mechanical mixing,[11] and microwave irradiation[10,12] show some disadvantages, such as being time-consuming, difficulties in quality control, and their cost. However, burning and heating method of bovine bone, alone or by using reactant materials, was used for preparation of BCP, which reduces the mentioned problems significantly.[13,14]

In burning and heating method, HA of bones will be transformed into BCP ceramic with a wide range of HA/β-TCP compositions. Ooi *et al*.[14] used cancellous bovine bone alone, without any additives or reactant material. As a result, they could not produce ceramic with a wide range of chemical composition. Lin *et al*.[13] used reactant materials such as di-ammonium hydrogen phosphate ($(NH_4)_2HPO_4$) to widen the chemical composition of BCP.

In the previous works,[13,14] the cancellous bones have been used to prepare the BCP ceramic with or without reactant materials, but no study has been reported on the

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How to cite this article: Hosseinabadi ME, Etemadifar M, Ashrafizadeh F. Effects of nano-biphasic calcium phosphate composite on bioactivity and osteoblast cell behavior in tissue engineering applications. J Med Sign Sens 2016;6:237-42.

Website: www.jmss.mui.ac.ir

preparation of nano-BCP by burning and heating of cortical bovine bones. Owing to its lower porosity, the mechanical properties of cortical bone should be significantly more than the cancellous bones. In addition, the amount of synthesized nano-BCP ceramic extracted from the cortical bone facilitates the production as granules and reinforcement of composite scaffolds for bone tissue engineering applications.[15,16] In the present study, the cortical tibia bovine bones are burned, soaked in $(NH_4)_2HPO_4$, dried, and heated at 700°C to create nano-BCP ceramics. Then, the structure, composition, and bioactivity were evaluated, and the biocompatibility of nano-BCP samples was compared to nano-HA samples.

Materials and Methods

Materials and sample preparation

For production of nano-BCP, raw tibia cortical bovine was boiled in distilled water to remove visible tissues and substances on the bone surfaces for 12 h. Before the burning cycle, the cortical blocks of tibia bone were sliced about 7 mm × 7 mm × 4 mm. Then, the samples were burned at 700°C for 60 min to remove the organic substances. After that, the calcined (removing the organic substance by a burning process) bovine bone samples were soaked in 1 mol/l $(NH_4)_2HPO_4$ solution for 24 h. Because the surface of the immersed bone blocks contained superfluous immersion solution, a filter paper was used to sop up the redundant ammonium phosphate solution.[13] Then, the immersed samples were dried at 80°C for 24 h, placed in alumina crucibles, and heated at 700°C for 100 min in a muffle (Carbolite, UK) furnace.

In addition, for production of nano-HA that was used in biocompatibility tests in comparison with nano-BCP, the calcined bovine bone samples were placed in crucibles separately, and heated at 700°C for 100 min in a muffle (Carbolite, UK) furnace without any $(NH_4)_2HPO_4$ agent.

Characterization of heat-treated bovine bone

For evaluation of sample structures, the cubic samples were crushed by milling to produce uniform powders. The chemical composition of the powders was determined by X-ray diffraction (XRD) (Philips, X'pert) using Raynaud method.[17] Phase analysis was performed at room temperature using Cu Kα at a scan speed of 3°/min with 40 kV, 30 mA, $\lambda = 0.154056$ nm, and $2\theta = 15$–70. The structure of the nano-BCP ceramic and nano-HA was characterized by TEM (JEOL, JSM) equipment. Composition analysis of the samples was determined by Fourier Transform Infrared Spectroscopy (FT-IR) (Bruker, Tensor 27, Germany) tools. The FT-IR spectra were obtained over the region 400–4000 cm^{-1}.

Bioactivity evaluation

For evaluation of bioactivity, the powders change to discs with dimensions of 13 mmΦ × 2.5 mm by uniaxially pressing at 550 MPa in a cylindrical mould. The simulated body fluid (SBF) was prepared according to the Kokubo procedure.[18] For *in vitro* bioactivity evaluation, the nanostructured BCP discs were immersed in SBF at 37°C. After 7, 14, 21, and 28 days, the samples were removed from the SBF, gently washed by deionized water, and dried at room temperature. For examination of morphology samples, scanning electron microscopy (SEM) (JEOL JSM) at accelerating voltages in the range of 5–15 kV was used; gold or platinum was deposited on the surface of scaffolds to make them conductive. The energy dispersive X-ray spectroscopy (EDS) and XRD (Philips, X'pert) were used to monitor the morphology and the chemical composition of formed apatite layer on the nano-BCP ceramic surfaces.

Cell culture study

Proliferation rate of MG-63 osteoblast-like (ATCC, USA) on the nano-BCP (37:63 (HA/β-TCP)) discs (with dimensions of 13 mmΦ × 2.5 mm) was studied and compared with nano-HA samples by MTS assay. Briefly, MG-63 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% antibiotics (Penicillin, Streptomycin, Amphotericin, and Gentamycin) under standard cell culture conditions (i.e., 37°C, humidity, and 5% CO_2/95% air environment). After reaching 80–90% confluency, the cells were detached by a 0.25% solution of trypsin–ethylene diamine tetra-acetic acid (EDTA) and viable cells were counted by trypan blue assay.

Prior to cell seeding, the nano-HA and nano-BCP samples were cleaned in 75% ethanol solution, sterilized for 30 min under ultraviolet light, washed two times with PBS, and incubated with DMEM for 12 h. After the samples were placed in a 24-well polystyrene (as a negative control) culture plate, cells were further seeded on to the top of the samples at a density of 20×10^3 cells/well and cultured with medium at 37°C, 5% CO_2, and 95% humidity. During the cell culture, the medium was replaced every other day. For investigation of proliferation after 1, 4, and 7 days of cell seeding in 24-well plate, cells were washed with PBS to eliminate nonviable cells, and incubated with 10% of MTS reagent containing fresh medium. After 4 h of incubation at 37°C in 5% CO_2, the cells were pipetted into a new 24-well plate. The absorbance of the content of each well was measured at 490 nm using a multiwell microplate reader (Infinite, TECAN, USA).

Statistical Analysis

All experimental data were expressed as average ± standard deviation (SD). Statistical analysis was performed using single-factor analysis of variance (ANOVA). A value of $P \leq 0.05$ was considered statistically significant.

Results and Discussion

Heat-treated bovine bones

Figure 1 represents the XRD pattern of nano-BCP after heating of cortical bovine bone. The composition of this nano-BCP was estimated to be 63/37 HA/β-TCP by Raynaud method.[17] This composition has demonstrated a higher efficiency in the repair of bone defects as compared to either pure HA or pure β-TCP ceramic.[13,19] For all samples, the crystalline HA transformed to HA and β-TCP mixture. The bovine bone (deficient HA) was represented by the formula: $Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$ $(0 < x < 2)$, with a Ca/P ratio between 1.33 and 1.67.[13,20] The deficient HA has the same crystalline structure as HA, but its decomposition temperature is lower than that of stoichiometric HA. When bovine bone is heated at or above 700°C, it yields deficient HA decomposition into β-TCP and HA. The reactions are summarized in the following equation:[13,17]

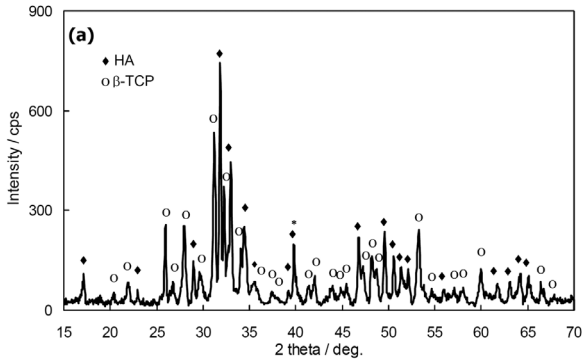

Figure 1: XRD pattern of nBCP powders after heating at 700°C

$$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x} \rightarrow 3xCa_3(PO_4)_2 + (1-x)Ca_{10}(PO_4)_6(OH)_2 + xH_2O \quad (1)$$

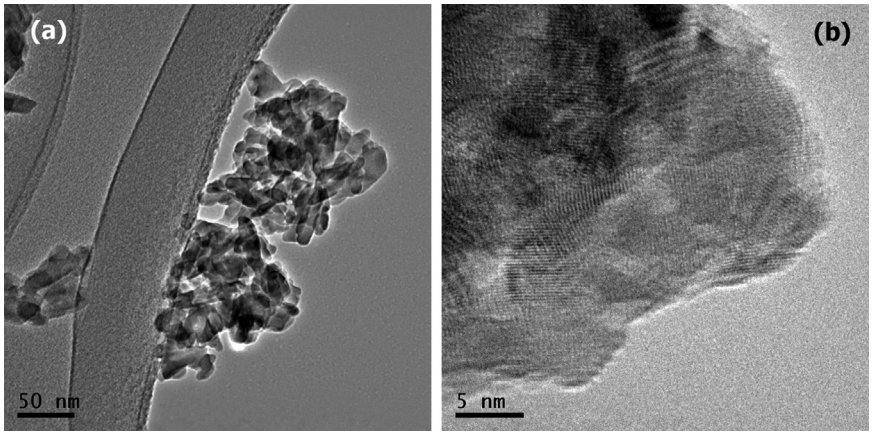

Figure 2: (a) TEM micrograph of nBCP powder and (b) TEM micrograph of nBCP crystallites

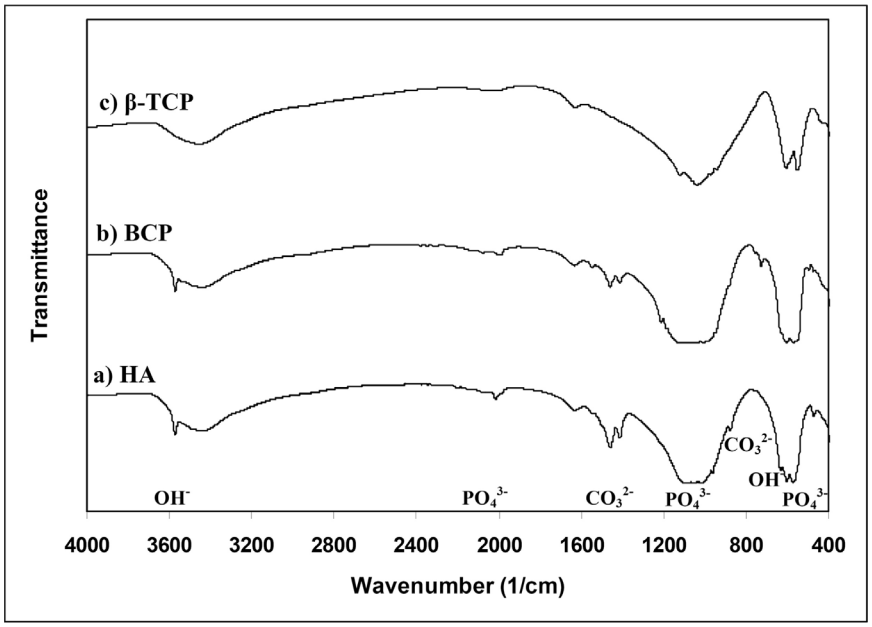

Figure 3: FT-IR spectra of bovine bones after burning and heating process. (a) HA, (b) BCP, and (c) β-TCP

From the above reaction, it is known that HA and β-TCP are the main residual crystalline phases when the bones were heated at high temperatures.

Transformation of HA to β-TCP depends on time and temperature, and by increasing the temperature, the created ions ($P_2O_7^{4-}$ and PO_4^{3-}) from $(NH_4)_2HPO_4$ can provide better conditions for the transformation reactions [Eq. 1].[13]

Figure 2(a) shows a congeries of the nano-BCP powders that were agglomerated with a nonspherical morphology. The size of the nanoparticles was estimated to be less than 100 nm. In addition, the crystallite size of the nanoparticles was characterized by high-resolution TEM micrograph; according to Figure 2(b), the crystallite size is about 20–40 nm. According to the last result, the HA extracted from bovine bones for creation of nano-BCP (after burning) has nanostructure, and it was used for cell culture tests in the next experiments.

Figure 3 shows the FT-IR spectra for 3 samples of bovine bones after burning and heating treatment. Figure 3(a) shows the FT-IR spectrum of burning sample at 700°C (calcined

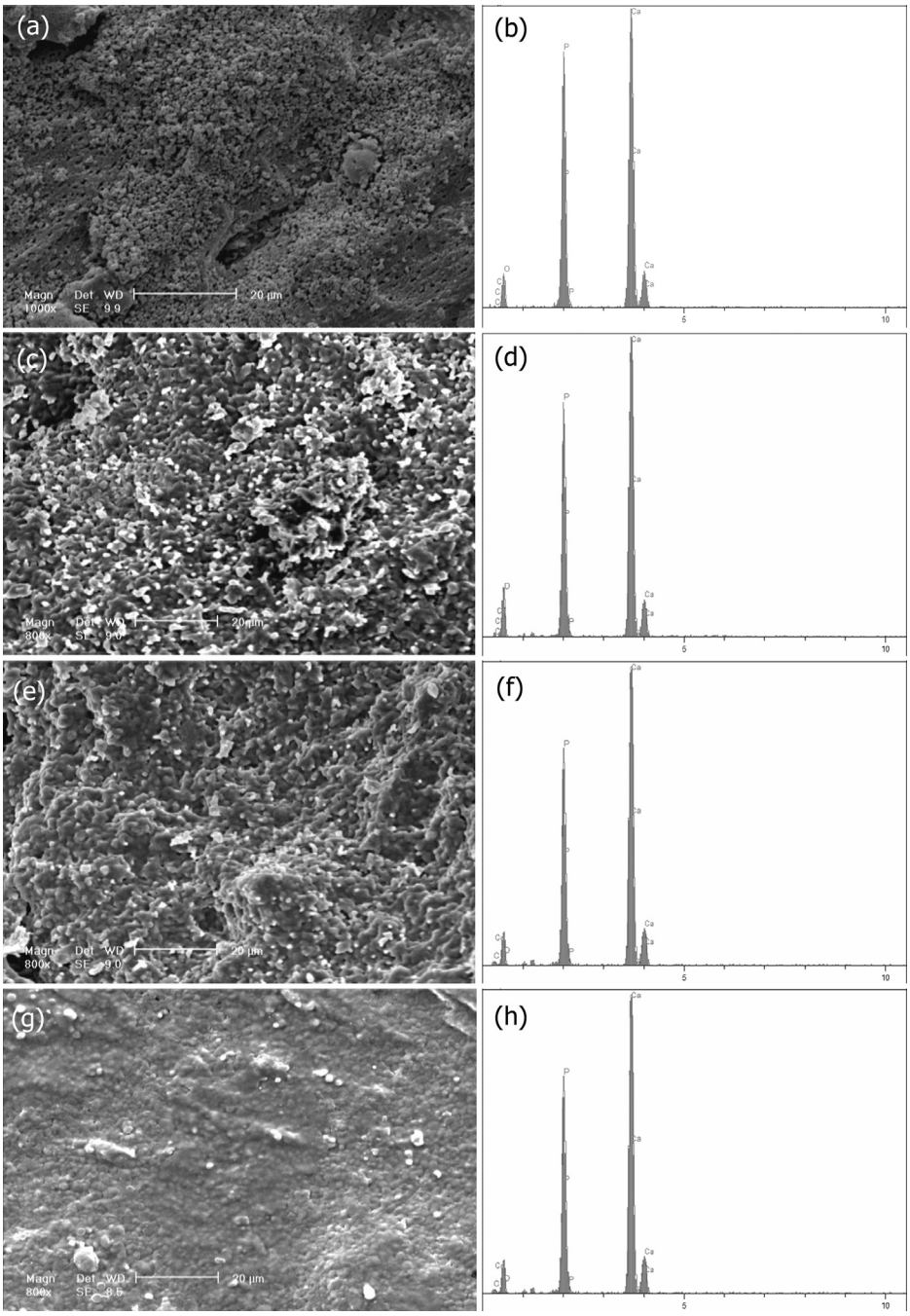

Figure 4: SEM and EDS image surfaces of cortical bovine bone with 40:60 (HA/β-TCP) composition after 7, 14, 21, and 28 days immersion in SBF solution. (a) and (b) 7 days, (c) and (d) 14 days, (e) and (f) 21 days, and (g) and (h) 28 days

bovine bone) that is similar to pure HA spectrum.[12,21] As can be seen, with heating process, the vibrational band of OH^- at 464, 633, and 3570 cm^{-1} (related to HA) decreases in intensity [Figure 3(b)], which is attributed to prepared BCP ceramic. The spectra confirm that addition of di-ammonium hydrogen phosphate was heated at 700°C and the nano-BCP with composition of 63/37 HA/β-TCP was produced.

Bioactivity and cell behavior

Nano-BCP ceramics including 30–40 wt% β-TCP have demonstrated more efficiency in the repair of bone defects than pure HA or pure β-TCP ceramics.[13,19] Therefore, nano-BCP ceramics with 63:37 (HA/β-TCP) ratio were selected for evaluation of *in vitro* bioactivity assay.

Figure 4 shows the SEM micrographs and EDS spectrums of the typical morphology of apatite formed on the surface of BCP samples after immersion in SBF for 7, 14, and 28 days. After 7 days, the SEM image [Figure 4(a)] confirmed that the morphology of the surface has changed and many congeries have been formed. The EDS spectrum showed that the layer on the surface is a mixture of Ca and P, and this attributed to calcium-apatite layer on the surface. The Ca/P atomic ratio was estimated to be about 1.56. With an increase of immersion time, the apatite layer [Figure 4(c)] was more uniform after 14 days and showed a ball-like morphology.[22] For this sample, the EDS showed the Ca and P peaks, as the Ca/P atomic ratio is about 1.60. After 21 and 28 days, the apatite layer [Figure 4(e) and (g)] was completely uniform, and "cauliflower" morphology of apatite formed on the surface of BCP samples. The EDS showed the Ca and P peaks and that the Ca/P atomic ratio is about 1.7, in which the composition of apatite can be HA.

Figure 5 shows the XRD patterns of the surface of the samples before and after soaking in SBF solution for 7, 14, 21 and 28 days. As can be seen, the XRD pattern showed HA and β-TCP peaks on the 7th day, but the intensity of β-TCP and HA peaks were lower and higher in comparison to non-soaked sample, respectively. In fact, the apatite layer is thin and the intensity of peaks is related to substrate (BCP) and HA layer. After 14 days, the β-TCP peaks of substrate had disappeared and the remaining peaks were related to HA. In this stage, the apatite layer thickness had increased and the XRD pattern showed the composition of apatite layer. By increasing the immersion time in SBF, the peaks of the crystalline HA gradually became sharper. It was explained in terms of the growth of apatite grains by taking calcium and phosphate ions from the SBF solution. These broad peaks may indicate that the apatite layer is composed of superfine crystalline structure.[22]

In vitro cell culture assay is the first step for evaluation of cell growth and interaction between bioceramics and cells. On the basis of bioactivity behavior, the nano-HA and nano-BCP with 63:37 (HA/β-TCP) ratio samples were selected. Figure 6 shows the results of MTS assay on nano-HA and nano-BCP samples by MG-63 osteoblast-like cells. As it is shown, the osteoblast cells can attach and grow on nano-HA and nano-BCP samples, but nano-BCP samples improve and accelerate the cell proliferation compared to the cell growth on nano-HA samples; after 1 day of incubation, it was found that cells adhered and spread on the surface of the scaffolds. Using statistical analysis, however, the samples did not show significant difference for cell proliferation on day 1. Nevertheless, after 4 and 7 days, cell proliferations on nano-BCP samples were considerably ($P < 0.05$) higher in comparison to nano-HA samples. In nano-BCP samples, with existence of β-TCP, cell proliferation was raised, thus confirming that the presence of β-TCP besides HA in the nano-BCP increased cell growth and enhanced cellular proliferation.

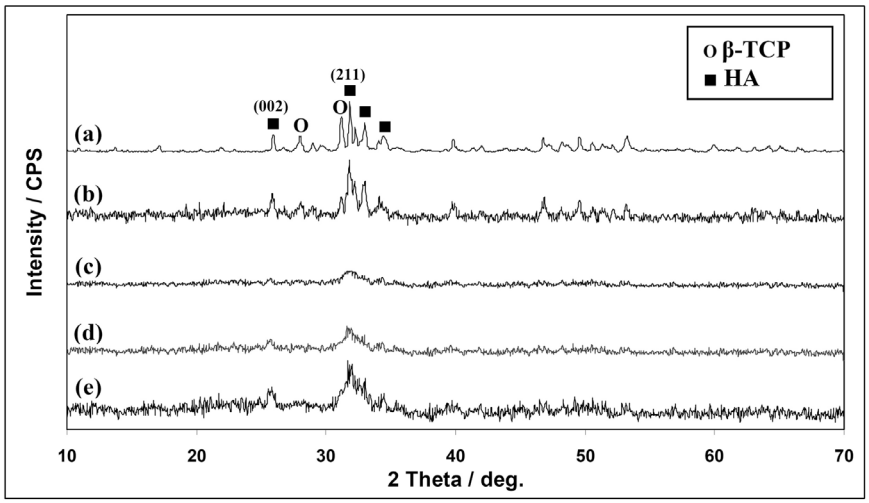

Figure 5: XRD patterns of the surfaces of BCP samples soaked in SBF solution. (a) 0 day, (b) 7 days, (c) 14 days, (d) 21 days, and (e) 28 days

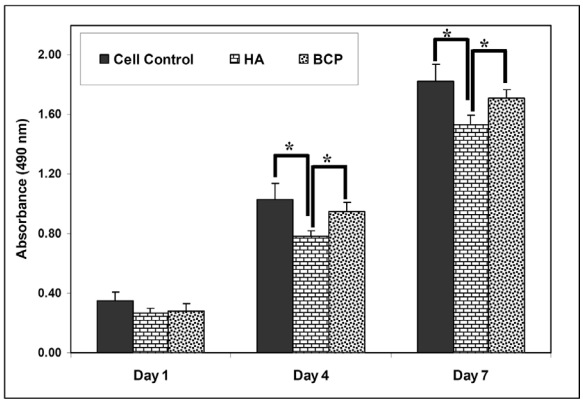

Figure 6: MTS assay results of MG-63 osteoblast-like cells on HA and BCP samples after 1, 4, and 7 days (*$P \leq 0.05$, the rest $P > 0.05$)

Conclusion

After burning of cortical bovine bones, immersion of bones in $(NH_4)_2HPO_4$ solution and heating at 700°C caused the nano-HA to transform into nano-BCP with the crystallite size being about 20–40 nm. The nano-BCP sample with 63:37 (HA/β-TCP) compositions showed an excellent bioactivity in SBF after 28 days. By an increase in the immersion time at SBF, the HA layer gradually became thicker. All nano-BCP samples showed better cell proliferation compared to pure nano-HA samples. After 7 days in cell culture, the ceramic with 63:37 (HA/β-TCP) compositions exhibited the maximum proliferation of the MG-63 osteoblast cells.

Acknowledgements

The authors acknowledge the support by Isfahan University of Technology (IUT) in Iran and Professor Subbu S. Venkatraman, from School of Materials Science and Engineering, Nanyang Technological University (NTU) in Singapore.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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