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Research Article

Investigating the Effects of Gamma Irradiation on Osteogenic Properties of OsvehOss Synthetic Bone Graft Substitutes as Orthopedic Implant Materials

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Abstract

Bone graft substitutes are used in bone tissue engineering, orthopedics, and dentistry to help bone repair. The sterility and pyrogenicity of the bone grafts before clinical use are considered part of the regulatory requirements, however sterilization of biomaterials is challenging due to the physicochemical changes resulting from the localized increase in gamma dose during irradiation. The effects of gamma radiation dose on the biological behavior of synthetic bone grafts have not been extensively investigated. The present study aimed to evaluate the effects of gamma radiation sterilization doses on OsvehOss synthetic bone grafts *via* chemical, mechanical, and *in vitro* biological examinations. XRD analysis and compression test were carried out to evaluate the chemical and mechanical changes of synthetic bone grafts induced by the highest gamma radiation dose applied in this study. Human osteosarcoma MG-63 cells were used to assay their osteogenic response while grown on a biphasic bone graft substitute. Cell attachment and proliferation were confirmed *via* scanning electron microscopy on days 3, 7, and 14 of culture. Alkaline phosphatase (ALP) activity was determined to assess osteogenesis. Alizarin red S (ARS) staining was also used to identify calcium deposition in osteocytes developed after differentiation of MG-63 cells. Our results illustrated that gamma irradiation did not cause dose-dependent changes in chemical and mechanical properties of OsvehOss BCP (Biphasic Calcium Phosphate) bone grafts when the doses increased up to 50 kGy. Furthermore, OsvehOss BCP samples demonstrated high osteoconductivity in all irradiation treatment groups. ALP and ARS analyses also indicated that the application of irradiation doses up to 50 kGy for sterilization of OsvehOss BCP grafts had no significant effects on osteogenesis and calcium deposition in osteoblast cells cultured on grafts. In conclusion, OsvehOss biomaterials can be sterilized safely for biomedical applications.

***Keywords*:** biphasic bone graft substitutes, gamma irradiation, MG-63 cells, osteogenesis

Introduction

The emergence of synthetic bone graft substitutes as a viable option for the treatment of a range of bony defects has revolutionized the field of tissue engineering. Bone grafts have been used in a range of clinical applications to mend bone lesions, fill gaps in multi-fragmentary fractures, non-unions, cysts, and voids resulting from tumor removal due to osteosarcoma, and also for traumatic bone defects (Betz, 2002). The biphasic calcium phosphate (BCP: HA (hydroxyapatite)/TCP (tricalcium phosphate)) scaffold is one of the commonly used synthetic bone substitutes, the application of which has been investigated both *in vitro* and *in vivo*. BCP is a biocompatible material with osteoconductive features providing an environment for new bone formation *in vivo* (Arinzeh et al., 2005; Kouhestani et al., 2018). Bone substitute materials are used not only to mechanically deliver the cells to the defect site but also to provide a suitable matrix for stem cells to proliferate and differentiate into osteogenic cells (Malik et al., 1992; Kouhestani et al., 2018). OsvehOss™ BCP (Osveh Asia Medical Instrument Co., Iran) is a synthetic, porous, osteoconductive, and bioactive bone substitute for the filling/or reconstruction of bone defects.

Among the widely used sterilization techniques, exposure to gamma rays is the most commonly used technique to sterilize pharmaceuticals and medical devices because of its high penetrating ability, and uniform and time-dependent delivery of the required doses without any toxic residues (Nieminen et al.,

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2006). Gamma radiation can be used to sterilize synthetic bone graft substitutes for tissue engineering applications after finding and validating the suitable dose. Several types of microorganisms, mainly bacteria and, less frequently, molds and yeasts, have been found on many medical devices and pharmaceuticals (Takehisa, et al., 1998). Complete eradication of microorganisms and achieving SAL (Sterility Assurance Level, 10^{-6} CFU) is essential for the safety of medical devices and products. The sterilization process must be validated to verify that it effectively and reliably kills any microorganisms that may be present on the sterilized products. Radiation sterilization has been widely used in many developed and developing countries for the sterilization of health care products. Earlier, a minimum dose of 25 kGy was routinely applied for many medical devices, pharmaceutical products and biological tissues. Gamma irradiation can inactivate Gram-positive and Gram-negative bacteria (Shahabi, et al., 2014; Rohin, et al., 2023). Gamma radiation is a time and cost-effective sterilization method, which unlike ethylene oxide (EtO) does not produce any residues toxic to human health, and the environment (Türker et al., 2014). In recent years, sterilization with γ-irradiation has become a generalized practice due to its simplicity, reliability, and easy automation; and since this process does not involve heating, it can be conveyed as a continuous process (Suwanprateeb et al., 1998; Costa et al., 2006). Furthermore, the dosimeters enable parametric release, which provides cost and sterility assurance advantages of gamma radiation sterilization.

Depending on the pharmacopeias, a minimum dose of 25 kGy was routinely applied for many medical devices, but now, as recommended by the International Organization for Standardization (ISO), the sterilization dose must be set for each type of product depending on its bioburden (Türker et al., 2014). This is because sterilization with γ-irradiation can induce chemical and microstructural modifications that may result in changing the properties of the materials. Previous works have shown that irradiation sterilization can change the color of some bioceramics (Costa et al., 2006). There is evidence that the activity of osteoclasts is reduced when they are cultured onto irradiated bone slices, and that peroxidation of marrow fat increases apoptosis of osteoblasts (Nguyen et al., 2007). This issue has become controversial and has resulted in the application of doses ranging from 15 to 35 kGy. Irradiation is suspected as the most damaging factor for the mechanical properties of bone allografts during processing and sterilization (Triantafyllou et al., 1975). For example, frozen bovine cortical bone specimens (sized 8 x 0.5 x 0.5 cm), irradiated at a dose of 30 ± 3 kGy, displayed a 25-50% reduction in their strength compared to control bone; and similar reductions in maximum torque and stress of rabbit tibiae as 23 and 25%, respectively, have been observed (Godette et al., 1996). Currey et al. reported that the standard dose of 25 kGy significantly reduces bone strength (Currey et al., 1997; Nguyen et al., 2007). Gamma radiation adversely affects the mechanical and biological properties of bone allografts by degrading the collagen in the bone matrix. More specifically, gamma rays break down polypeptide chains. In wet specimens, irradiation causes the release of free radicals *via* radiolysis of water molecules that induce cross-linking reactions in collagen molecules. These effects are dose-dependent and give rise to a decrease in mechanical properties of allograft bone when the gamma dose is increased above 25 kGy for cortical bone or 60 kGy for cancellous bone. At doses between 0 and 25 kGy (standard dose), a clear relationship between gamma dose and mechanical properties has yet to be established (Nguyen et al., 2007).

Some structural changes in bioceramics such as hydroxyapatite may occur after gamma irradiation including an increase in their enthalpy (Kubisz et al., 2003). Biocompatibility and bioactivity of biomaterials can be affected, and it is shown that the bioconductivity and absorption of these materials are dependent upon irradiation doses (Kawasaki et al., 2010). Despite the fact that 25 kGy (standard dose) is considered a gold standard, the local increase of radiation dose during sterilization can lead to adverse effects. The mechanical and physicochemical properties of the bone graft biomaterials must be stable after sterilization.

In summary, previous works have shown that sterilization with γ-irradiation can induce chemical and microstructural modifications that may result in variations in their chemical, physical, and mechanical properties. Despite the vast clinical use of bone substitute materials, there are limited *in vitro* studies evaluating the response of cells towards these modifications, especially the effects of gamma sterilization dose. The aim of this study was to critically analyze the effects of gamma radiation on the chemical, mechanical, and biological properties of OsvehOss™ synthetic bone grafts.

Materials and Methods

The bone graft samples used in this study are listed in Table 1. OsvehOss BCP bone grafts were

produced by the wet precipitation method with the 60:40 weight ratio of HA and β-TCP.

Table 1. The specifications of used OsvehOss and control samples

No.	Trade Name	Chemical Composition	Company	Lot No.	Gamma Sterilization Dose	Sample Code
1	OsvehOss	BCP (60HA/40TCP)	Osveh	171225A1B900	25	BCP25
2	OsvehOss	BCP (60HA/40TCP)	Osveh	171225A1B900	50	BCP50
3	MBCP	BCP (60HA/40TCP)	Biomatlante	0115F215	25	MBCP

At first, the phosphate solution was dropped into calcium solution and the chemical reaction was maintained at room temperature. After the precipitation process, it was filtered many times by distilled water to remove any remaining ammonia solution. Then the aqueous suspension was transferred into the oven and dried following the calcination process.

After calcination and packaging, the samples were sterilized by irradiation in a sealed container and the ^{60}Co γ-ray isotope source was used at 25 and 50 kGy doses. MBCP (Biomatlante, France) bone grafts which were supplied as sterilized particles (25 kGy γ-ray) were used as control samples.

The microstructural characterization of the samples was performed by X-ray diffraction (XRD), (XRD; Geigerflex, Rigaku Co, Akishima, Japan) with CuK_{α} radiation. The induction of mechanical changes in irradiated bone grafts is a controversial issue. The effects of gamma radiation on the compressive strength of bone grafts were documented through the standard pressure test (ISO 13175-3).

MG-63 cells, a human osteoblast-like cell line (obtained from Ferdowsi University of Mashhad, Iran), were used to evaluate the cellular responses to the applied biomaterials. Cells were cultured in an alpha-modified minimal essential medium (α-MEM) (Biowest) supplemented with 10% fetal bovine serum (FBS) (Biowest). $5x10^5$ cells were suspended in 50 µl α-MEM and seeded onto 50 mg of each scaffold, and then incubated for 2 h to allow cell adhesion. After 2 h, the scaffolds were transferred to a 24-well plate to be incubated in 1 ml α-MEM per well, at 37 °C in a humidified atmosphere of 5% CO_2. The medium was changed twice a week.

For investigation of osteogenesis, the cell-loaded scaffolds were then immersed in osteogenic medium (α-MEM; 0.1 µM dexamethasone; 0.2 mM ascorbate-2-phosphate; 10 mM β-glycerophosphate) for 21 days to induce osteogenic differentiation. The medium was changed every other day. The samples were analyzed for alkaline phosphatase (ALP) activity on days 7 and 14 of induction. At the time of evaluation, the medium was discarded and cells were washed twice with phosphate-buffered saline (PBS) before the addition of lysis buffer (0.1% (v/v) triton X-100 in 0.2 M Tris buffer). The pellets obtained after centrifugation at 10000 *g* for 10 min, were transferred to a 96-well plate. ALP activity was assessed by the addition of 1 mg/ml *p*-nitrophenyl phosphate (*p*NPP) (Sigma) solution in 0.2 M Tris buffer. Optical absorbance was assessed after 20 min of incubation at room temperature, at 405 nm.

Experimental groups were evaluated for mineralization on days 14 and 21 of osteogenesis by alizarin red S (ARS) staining. Briefly, after washing the samples with PBS, they were fixed in 4% paraformaldehyde for 60 min and then washed with distilled water. Next, a 2% ARS solution (Sigma) was added for 30 min. After the removal of the staining solution, each well was washed several times with distilled water. To quantify the staining, 500 µl of 10% acetic acid was added to each well and incubated for 20 min to elute the stain. The eluted stain was then assayed at 405 nm using a spectrophotometer (BioTek, USA).

Evaluation of cell growth and morphology of MG-63 cells on the scaffold surfaces was examined using scanning electron microscopy (SEM). The cell-loaded scaffold specimens were assayed on days 3, 7, and 14. The cell–scaffold constructs were rinsed twice with PBS and fixed in 3% glutaraldehyde for 3 h. After rinsing in deionized water, they were dehydrated with increasing concentrations (50%, 70%, 90%, and 100%) of ethanol and incubated for 10 min at each concentration. Applying a SEM coating system (Bio-Rad), the samples were covered with gold and then examined by SEM (Leo 1450VP, Germany).

Results and Discussion

Characterization

To characterize the physicochemical properties of bone substitutes before and after irradiation, some chemical and physical methods including XRD and SEM were employed. Primary visual observation of the samples showed that the BCP bone grafts submitted to sterilization at 50 kGy dose (BCP50) presented no change of physical properties, especially in the color as compared with the 25 kGy ones (BCP25). XRD spectra of OsvehOss BCP after gamma radiation with 25 and 50 kGy doses are shown in Figure 1.

The diffractograms of the BCP25 and BCP50 samples were slightly different; however, these changes were not significant. It can be observed that all samples present a high degree of crystallinity. No impurity phase was created by a change in the chemical composition of the materials due to an increase in gamma radiation dose. As it is evident, similar spectra indicate that no changes in the crystal phase have been induced by gamma irradiation. For example, all diffraction peaks of HA and β-TCP in both BCP25 and BCP50 composite biomaterials appear at almost the same angle and have similar intensity before and after irradiation (single cell symmetry and crystallite size). Since the BCP phase remains stable with increasing gamma dose up to 50 kGy, therefore biocompatibility and bioactivity of grafts will not be affected by changing chemical composition. Indeed, the bioactive features of calcium phosphate (CaP) are related to its degradation properties and this characteristic is dependent on Ca/P ratios (Liu et al, 2016; Liu et al, 2019). Any change in the CaP chemical composition of biomaterials results in variations in *in vitro* and *in vivo* calcium and phosphate ion release. Consequently, the pH of the local microenvironment of bone is affected by the released calcium and phosphate ions, which then influence the viability of osteoblasts and osteoclasts. Therefore, based on the XRD test results, it can be said that the increase in the gamma dose did not lead to a change in the chemical composition and creation of impurities or the difference in Ca/P ratio, and the bioactivity and biocompatibility of the OsvehOss bone grafts were not affected by the change in the chemical composition.

Mechanical strength

The mechanical properties (compressive strength) of the samples were measured using a universal testing machine (SANTAM, Iran) at a crosshead speed of 0.5 mm/min according to ISO 13175-3 standard (Figure 2). Table 2 shows the mechanical properties of the OsvehOss biphasic calcium phosphates with different sterilization doses. As indicated, the mean compressive strength of the BCP50 decreased slightly when compared with BCP25. The compressive strength of OsvehOss BCP showed irradiation dose-independence, with a negligible decrease in the 50 kGy treatment group.

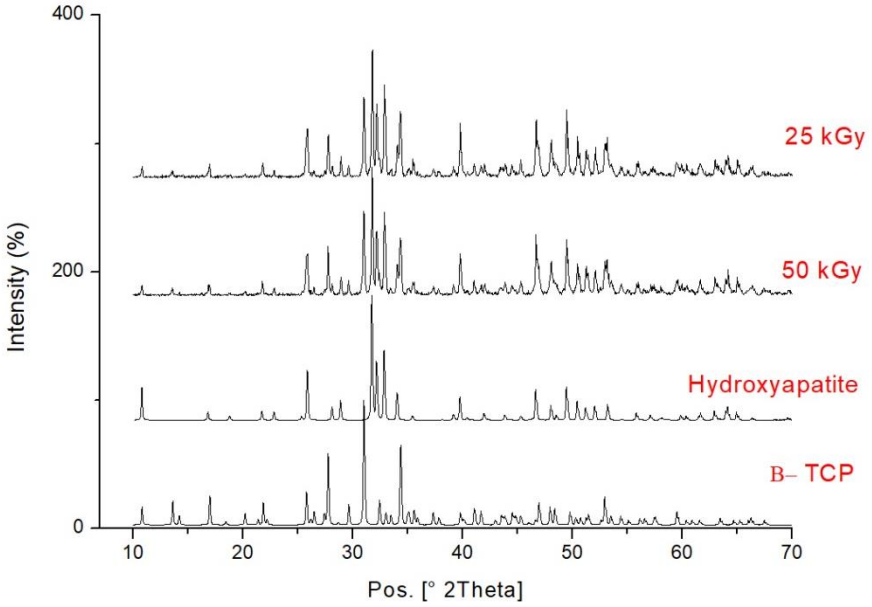

Figure 1. X-ray diffraction analysis of OsvehOss BCP sterilized with 25 and 50 kGy gamma rays (BCP25 and BCP50). BCP: biphasic calcium phosphate; TCP: tricalcium phosphate.

Table 2. Mean compressive strength of OsvehOss bone grafts irradiated at different gamma-ray doses

Sample code	Compressive strength (N/mm), mean of 5 samples
BCP25	84±3
BCP50	82±4

Gamma irradiation did not affect cell attachment and survival *in vitro*

After attaching to the surface of the bone grafts, MG-63 cells enter a proliferation state. The surface roughness and hydrophilicity of biomaterials have been reported to positively influence the proliferation potential of MG-63 cells (Deng et al., 2015). SEM analysis was performed to examine the influence of gamma irradiation on the proliferation of cells cultured on the bone graft biomaterials, qualitatively.

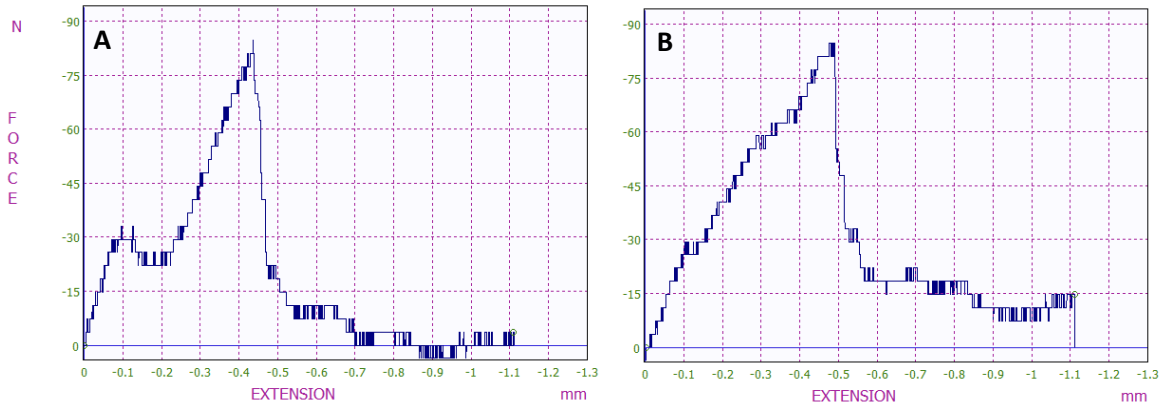

Figure 2. Force-extension curve of compression test for OsvehOss bone grafts: A) BCP25 and B) BCP50

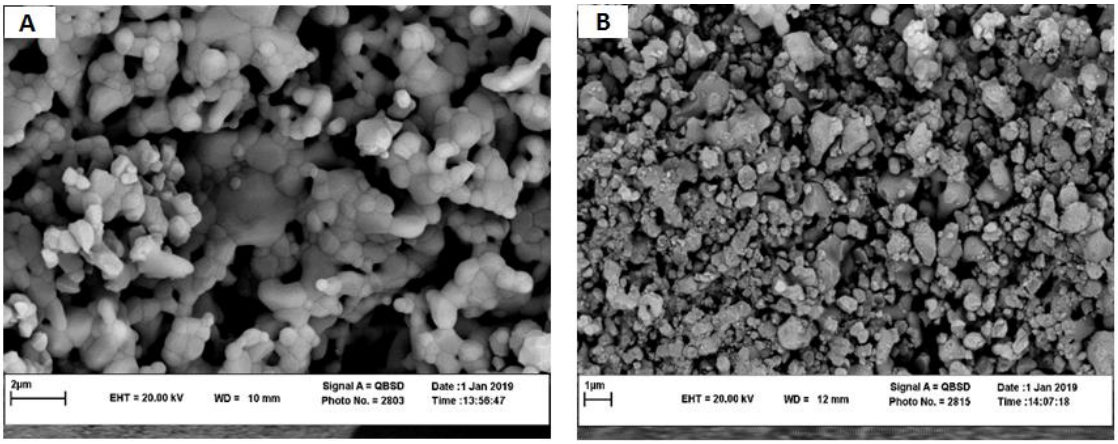

Figure 3. Microstructural differences of OsvehOss BCP (A) and MBCP (B) bone grafts, 20000x magnification

Considering the effects of surface structure on the bioactivity of bone grafts, the microstructure difference between the two samples before cell culture was also verified as shown in Figure 3.

The results indicated that the MBCP bone graft had a finer grain structure and a higher surface roughness compared to OsvehOss BCP. Moreover, in the microscopic image, the interconnected pores in the OsvehOss BCP sample were more visible. This discrepancy can affect the behavior of the two materials, which is discussed in the following part.

The suitability of the BCP50 for cell culturing was indicated by cell morphology and possible proliferation using SEM images and comparison with BCP25. Similar cell attachment and proliferation on the BCP25 and BCP50 can be observed in SEM images suggesting the favorable physico-chemical properties and cytocompatibility of both scaffolds, confirming that increasing gamma dose had no effects on their properties (Figure 4).

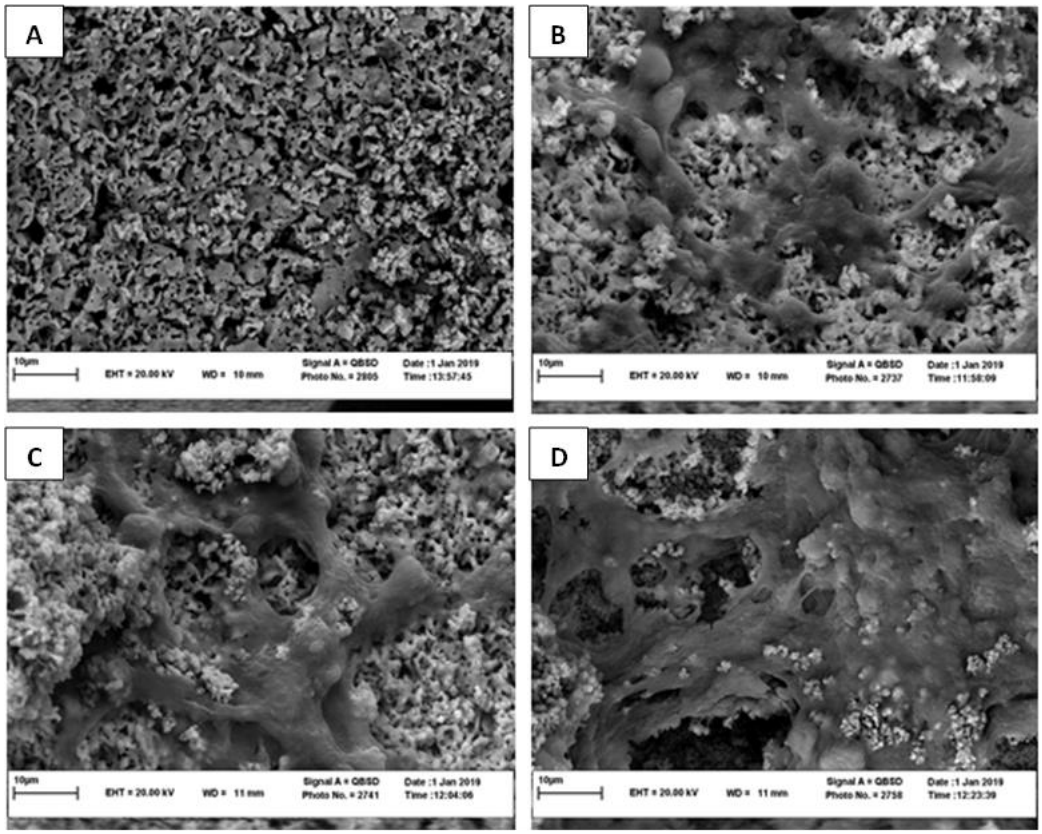

Figure 4. A) SEM micrograph of OsvehOss BCP before sterilization and without cells, B) A typical SEM micrograph of MG-63 cells after 3 days of culture on BCP25, C) SEM micrograph of MG-63 cells after 3 days of culture on BCP50 scaffold and D) SEM micrograph of MG-63 cells after 3 days of culture on MBCP, 5000x magnification.

In addition, it is well known that biphasic bone grafts facilitate the attachment and growth of osteoblastic cells owing to their high hydrophilic property. The cell adhesion and proliferation are strongly influenced by the hydrophilicity of the scaffold and efficient cell adhesion occurs on hydrophilic surfaces. The lack of significant changes in cell adhesion with increasing sterilization radiation dose indicates that the hydrophilicity of OsvehOss was not affected, however, more experiments are required to prove this.

SEM imaging results on day 7 of MG-63 culture on the OsvehOss™ BCP grafts showed cell attachment and colonization of the microsphere surfaces, confirming these formulations as promising candidates for regenerative medicine strategies addressing compromised musculoskeletal/orthopedic diseases.

Cells appeared to be spindle-like with lamellipodia and filopodia extending to neighboring cells in all studied grafts. In a qualitative comparison, a greater proliferation occurred after 7 days relative to day 3 of culture, and some areas were completely covered by cells and their extracellular matrix.

SEM photographs revealed that OsvehOss™ BCP provided a good environment for the adhesion and proliferation of osteoblast cells. In addition, we can observe that the difference between OsvehOss BCPs and MBCP samples in attaching and proliferation of cells is minimized 7 days after culture (Figure 5). This can be attributed to the microstructural difference between the samples (Figure 3). During the first days after cell culture, the cells adhered well to MBCP with higher surface roughness, but after 7 days, the rate of cell proliferation on both grafts was similar. After 7 days of culture, the rate of cell proliferation increased on BCP25 and BCP50 grafts in a competitive manner with MBCP. The SEM micrographs with higher magnification (Figure 6) also revealed similar results. The rate and quality of bone integration can be dependent on pore size, porosity volume fraction, and interconnectivity, both as a function of structural permeability and mechanics (Hing, 2005; Hannink and Arts, 2011).

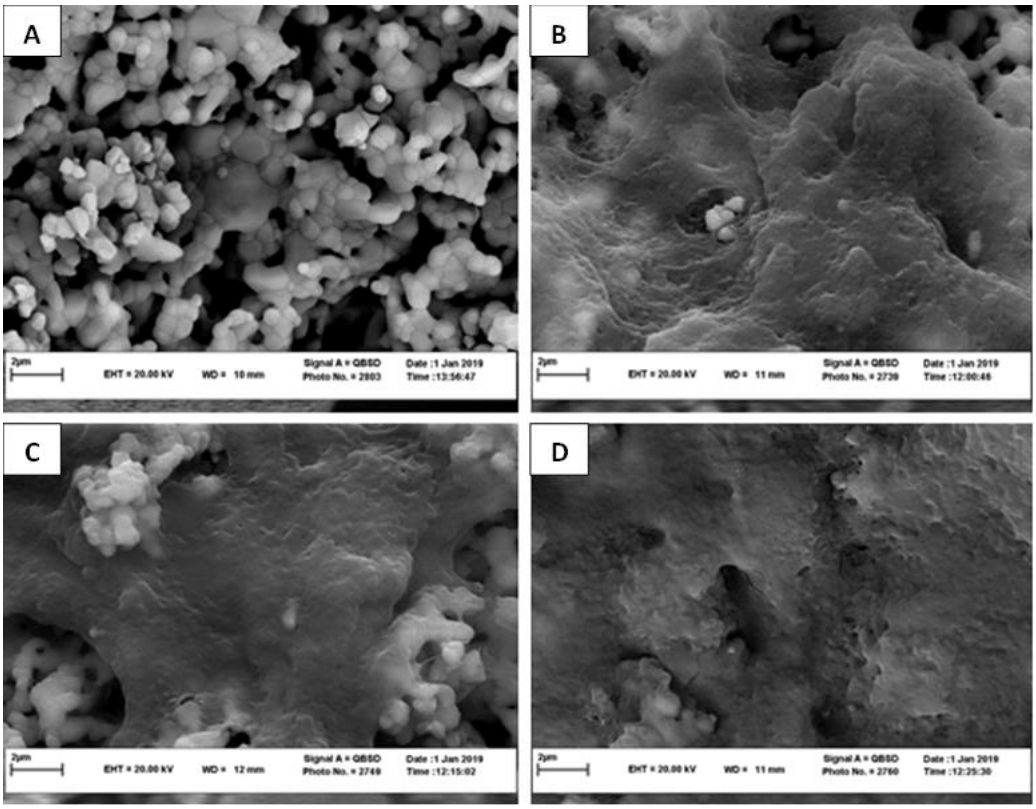

Figure 5. A) SEM image of OsvehOss BCP before cell culture/without cells, B) typical SEM micrograph of MG-63 cells after 7 days of culture on BCP25, C) SEM micrograph of MG-63 cells after 7 days of culture on BCP50, and D) SEM micrograph of MG-63 cells after 7 days of culture on MBCP, 20000x magnification

Bone regeneration following the transplantation of a scaffold *in vivo* requires the recruitment and penetration of cells from the surrounding bone tissue, as well as vascularization. Relatively larger pores favor direct osteogenesis since they allow vascularization and high oxygenation (Hannink and Arts, 2011; Kuboki et al., 2011). Cell adhesion, proliferation, and detachment strength are sensitive to surface roughness and increase as the roughness of bone graft increases (Deligianni et al., 2001).

MG-63 osteoblast cell line was chosen for this research to study the interactions between cells and materials *in vitro*. Alkaline phosphatase activity was measured 7 and 14 days after cell seeding. Figure 7 (A and B) reveals the intracellular ALP activity of MG-63 cells grown on BCP samples after 7- and 14-day incubation in osteogenic medium. The results showed no significant differences in ALP activity among different groups of bone grafts including BCP25, BCP50, and MBCP on day 14. However, the enzyme activity in the gamma-irradiated group was significantly higher than MBCP, 7 days after induction ($P < 0.05$). Moreover, in all groups, the ALP activity was doubled on day 14 compared to day 7. Alkaline phosphatase is the marker of choice for assessing the maturity of osteogenic cells (Golub et al., 1992).

In the current study, the ALP activity of MG-63 cells cultured on all scaffolds increased during the 14 days of the experiment. This finding confirms the *in vitro* osteoinductive property of these bone substitutes. However, in the osteogenic medium, there was no significant difference in ALP activity of OsvehOss™ BCP25 and BCP50 with MBCP groups. This could indicate that the osteoinductivity of OsvehOss™ BCP grafts was not affected by gamma sterilization dose up to 50 kGy.

Alizarin red S is an anthraquinone derivative that was used to identify calcium-containing osteocytes in the differentiated culture of MG-63 cells. To evaluate the impact of different tested scaffolds on the osteogenic differentiation of MG-63 cells, ARS staining was performed 14 and 21 days after culturing in osteogenic medium. Figure 7 (C-D) shows the quantitative analysis of ARS stain for mineral deposits in the MG-63 cells loaded on the scaffolds. The results of ARS staining of the samples indicated no significant difference between the studied groups at day 14. However, at day 21, a significant ($P < 0.05$) higher calcification ability is observed for MG-63 cells seeded on the BCP25 scaffold, in comparison with BCP50. Mineralization of samples has increased from day 14 to day 21 of the induction process.

Alizarin red staining is commonly used to detect the formation of calcium nodules (mineralization) formed by osteoblasts in the late stages of differentiation. In the present study, the culture of MG-63 cells on BCP50 exhibited no adverse effects in ALP activity and calcium nodule formation compared with those cultured on MBCP as the control group, indicating that the increasing gamma dose up to 50 kGy has no significant adverse effects on osteogenic differentiation potential of MG-63 cells cultured on OsvehOss™ BCP.

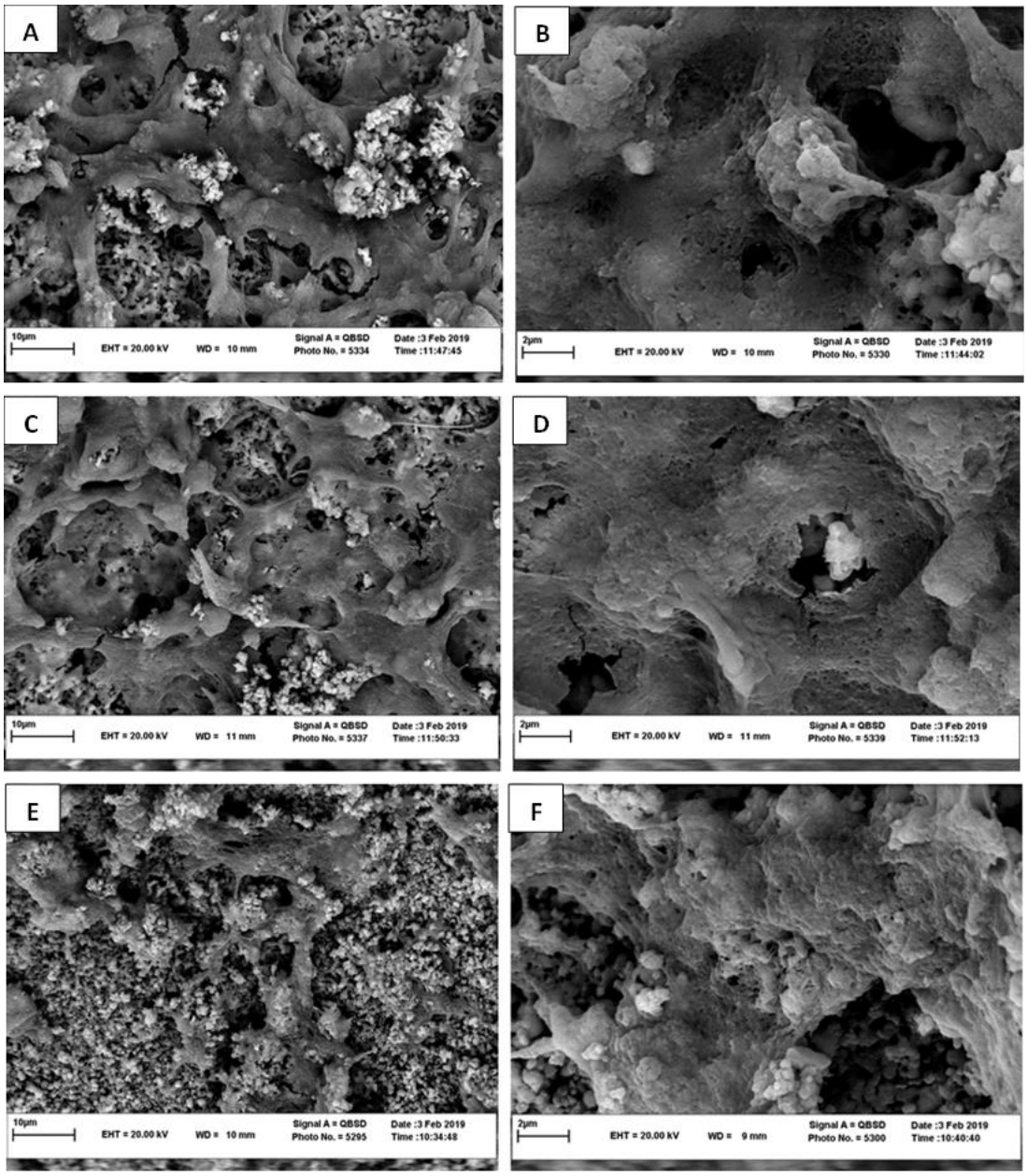

Figure 6. Typical SEM micrographs of MG-63 cells after 7 days of culture on A) BCP25, 5000x, B) BCP25, 20000x, C) BCP50, 5000x, D) BCP50, 20000x, E) MBCP, 5000x, and F) MBCP, 20000x

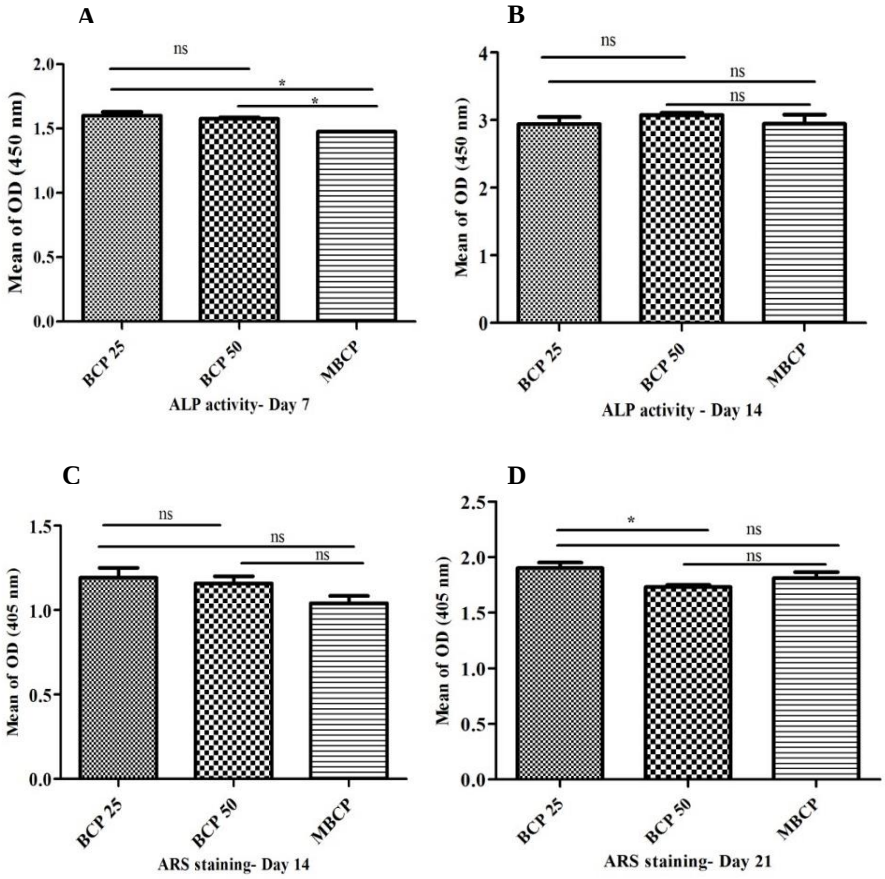

Figure 7. Investigating the osteogenesis of MG-63 cells cultured on different bone grafts (BCP25, BCP50 and MBCP). ALP activity on days 7 and 14 (A, B) as well as ARS quantification performed on days 14 and 21 (C, D) indicated no adverse effects of gamma irradiation on osteogenic potential of OsvehOss BCP. Data are presented as mean ± SEM (n=3 repeats), * indicates $P < 0.05$.

Conclusion

Gamma radiation adversely affects the mechanical and biological properties of bone allografts by degrading the collagen in the bone matrix (Nguyen et al., 2007). A review of published literature shows a consensus that most bone allografts have a dose-dependent response but various results have been obtained for different bone graft substitutes. This study showed that OsvehOss™ biphasic bone grafts promote adhesion and growth of human MG-63 cells. The observations made in this study suggest that there was no difference between BCP scaffolds irradiated with 25 and 50 kGy gamma doses for chemical composition. The compression strength of the BCP grafts was not dose-dependent for up to 50 kGy gamma irradiation used for sterilization. Moreover, the biological behaviors including adhesion, survival, possible proliferation, and differentiation of MG-63 cells cultured on bone grafts irradiated with different doses were not significantly different. Furthermore, the *in vitro* biological behavior of the tested samples was not significantly different from that of the MBCP control grafts, indicating the osteoinductive property of OsvehOss™ BCP as an orthopedic scaffold. A comprehensive evaluation of the biological potential of OsvehOss BCP bone graft substitutes *in vitro* would provide important additional information for further progression toward *in vivo* studies. The chemical and mechanical properties and also biofunctionality of OsvehOss synthetic bone grafts showed biocompatible properties in a dose-independent manner for up to 50 kGy gamma irradiation used for sterilization. In conclusion, the OsvehOss biomaterials could be sterilized safely with validated radiation doses for tissue engineering applications.

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Conflict of Interests

Heidar Khadivi Ayask, Nasrin Sasani and Vahideh Sadat Ebrahimi are directly involved in the Osveh Asia Medical Instrument Company, which provided the OsvehOss bone grafts.

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