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Original Article

Reconstruction of mandibular defects using synthetic octacalcium phosphate combined with bone matrix gelatin in rat model

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ABSTRACT

Background: Regeneration of bone defects remains a challenge for maxillofacial surgeons. The objective of this study was to assess the osteogenic potential of octacalcium phosphate (OCP) and bone matrix gelatin (BMG) alone and in combination with together in artificially created mandibular bone defects.

Materials and Methods: In this experimental study Forty-eight male Sprague–Dawley rats (6–8 weeks old) were randomly divided into four groups. Defects were created in the mandible of rats and filled with 10 mg of OCP, BMG, or a combination of both (1/4 ratio). Defects were left unfilled in the control group. To assess bone regeneration and determine the amount of the newly formed bone, specimens were harvested at 7, 14, 21, and 56 days postimplantation. The specimens were processed routinely and studied histologically and histomorphometrically using the light microscope and eyepiece graticule. The amount of newly formed bone was quantitatively measured using histomorphometric methods. Histomorphometric data were analyzed using SPSS software. Mean, standard deviation, mode, and medians were calculated. Tukey HSD test was used to compare the means in all groups. $P < 0.05$ was considered as statistically significant (i.e., 5% significant level).

Results: In the experimental groups, the new bone formation was initiated from the margin of defects during the 7–14 days after implantation. By the end of study, the amount of newly formed bone increased and relatively matured, and almost all of the implanted materials were absorbed. In the control group, slight amount of new bone had been formed at the defect margins (next to the host bone) on day 56. The histomorphometric analysis revealed statistically significant differences in the amount of newly formed bone between the experimental and the control groups ($P < 0.001$).

Conclusion: Combination of OCP/BMG may serve as an optimal biomaterial for the treatment of mandibular bone defects.

Key Words: Bone matrix gelatin, octacalcium phosphate, osteogenesis

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INTRODUCTION

In oral and maxillofacial surgery, it is important to recover impaired bone tissue, such as bone defects originating from extirpation of cysts and tumors and atrophic alveolar processes.[1,2] Implantation of

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autogenous bone that includes osteogenic cells and cytokines is the standard method for bone repairment. Although a second surgery is necessary to collect bone, they cannot be used for large bone defects due to limited quantity. Therefore, various types of synthetic materials are used as substitutes.[3-5] These synthetic materials include calcium phosphate, bone matrix gelatin (BMG), and demineralized bone matrix (DBM).[5-9] Evidence showed increased bone formation by calcium phosphate derivatives, especially octacalcium phosphate (OCP).[10] OCP ($Ca_8H_2[PO_4]_6.5H_2O$) is a salt that has been suggested to be a precursor of biological apatite crystal in bone and tooth.[11] If OCP were implanted into a bone defect, it would be irreversibly converted into the apatite phase, and it was shown to promote osteoblastic cell differentiation.[12] Furthermore, the implanted OCP can serve as a nidus for initiating bone formation and shows the osteoinductive ability as well as the osteoconductive if implanted in the critical-sized bone defects of rats.[13-15] Moreover, since OCP has osteoinductive and osteoconductive properties, whether alone[16,17] or in combination with other biomaterials such as BMG[18] or bone morphogenetic protein (BMP)[19,20] which shows promising results in repairing bone defects.

On the other hand, many investigators have confirmed the successful repair of bone defects after using DBM or BMG and have stated that they can be suitable substitutes for bone grafts.[5,20,21] BMG is an ideal material that is easily prepared and used with no negative effect on the bone formation process at the site of bone defects. It contains a complex insoluble substance composed of type I collagen and a heterogeneous group of noncollagenous proteins.[22,23] One of these proteins is BMP, which induces local mesenchymal cells to differentiate into bone-forming cells, a process known as osteoinduction. This protein is suggested to be responsible, in part, for the osteoinductive properties of BMG.[9,24] The mutual reaction of BMG in combination with most calcium phosphate derivatives has shown promising results in repairing bone defects.[18,19,25,26]

The rate of biodegradation for materials with distinct osteoconductivity can affect the rate of tissue regeneration and the bone quantity. There have been few studies focused on the relationship between the quality of regenerated bone and biodegradability in distinct bone substitute materials. The objective of this study was to compare the biodegradation rate and quantity of regenerated bone among OCP and BMG implanted alone or in combination with together in mandibular bone defects of rats.

MATERIALS AND METHODS

Preparation of materials

In this experimental study synthetic OCP ($Ca_8H_2[PO_4]_6.5H_2O$) was prepared by mixing a calcium and phosphate solution as described previously.[11,27] The granules (consisting of an OCP crystal aggregate) were prepared by lightly grinding the dried OCP cake using a pestle and mortar and then passing through a standard testing sieve. Granules with diameters ranging from 300 to 500 μm were used for implantation. The sieved granules were sterilized by heating at 120°C for 2 h in an oven before implantation. BMG was prepared according to the method previously described by Urist *et al*., with some modification.[22,28,29] Briefly, diaphyseal shafts of femur and tibiae from 6-week-old male Sprague–Dawley rats were cut into chips. Liquid nitrogen was used to freeze bone shafts after their removal and while they were being cut into chips to avoid possible denaturation of proteins. The bone chips were extracted in chloroform-methanol (1:1) for 4 h at 25°C, demineralized in 0.6 N of hydrochloric acid at 4°C for 72 h, and gelatinized in 6 M of lithium chloride at 2°C for 24 h. The bone chips then were autodigested at 37°C for 48 h in phosphate buffer (pH 7.4) with 10 mM of sodium azide and 5 mM of iodoacetic acid as a protease inhibitor. The bone chips then were pulverized with a sample chamber and sifted. Particles sized 75–500 μm were collected by the testing sieve, lyophilized, sterilized in ethylene oxide and stored in sterile glass containers at −70°C until use.

Animals and surgical procedures

This experimental study was conducted on 48 adult (6–8 weeks old) male Sprague–Dawley rats, with a mean weight of 120–150 g. The animals were randomly allocated to the three experimental (OCP, BMG, and OCP/BMG) groups and one control group and kept in standard conditions with light/dark cycles of equal duration. The principles of laboratory animal care, as well as national laws for animal experimentation, were followed. All procedures were approved by the Ethics Committee for Animal Experiments of Zahedan University of Medical Sciences (IR.ZAUMS.REC.1396.35).

Animals were anesthetized by intraperitoneal injection of 60 mg/kg ketamine hydrochloride (Ketalar,

Trustech Pharma Care, Bayern, Germany) and 20 mg/mL xylazine (Pantex Holland B.V., Duizel, Netherlands) in 2/1 ratio. Diethyl ether was used for anesthesia maintenance. After the induction of general anesthesia, the animals were fixed on the operating table in a supine position. The respective area on the body of the mandible was shaved and disinfected using 10% betadine (Tolid Darou, Tehran, Iran). Using a sterile surgical scalpel, a 1.5-cm incision was made on both sides of the mandible and a full-thickness periosteal flap was elevated. Using a dental drill, a critical-sized defect measuring 5 mm in diameter and 2 mm in depth[30] was drilled in the mandible close to the alveolar crest (in-between the first molar and canine teeth) under copious irrigation with cold saline solution.

In the first experimental group, 10 mg of OCP (previously prepared and packed) was implanted in the defect. In the second experimental group, 10 mg of BMG, and in the third experimental group, 10 mg of OCP/BMG with a ¼ ratio were implanted in defects. As a control group, animals were processed in the same way as experimental, with an only exception of implantation after the defects were created. The skin and the underlying connective tissue at the surgical site were sutured in two layers using a 4/0 absorbable chromic suture (Catgut, Wei Gao Group Kanglida Medical Products Co., Ltd., Heze, China) and disinfected. After completion of the operation and recovery of the rats from anesthesia, they were transferred to hygienic cages and kept there until sacrifice at the end of the time table.

Tissue preparation

In all experimental and control groups, tissue samples were harvested at 7, 14, 21, and 56 days (from six rats sacrificed at each time point) to prepare histological sections. At the mentioned time points, general anesthesia was induced by intraperitoneal injection of ketamine hydrochloride. The chest was opened, and 10% buffered formalin (fixative) was perfused through the heart to achieve *in situ* fixation of tissues. The respective area along with a margin of host bone was resected and stored in 10% buffered formalin at room temperature for 1 week for complete fixation. Next, the tissue specimens were rinsed with cold distilled water for several times and decalcified by immersion in a decalcifying solution containing 10% formic acid, 2.9% citric acid, and 1.8% trisodium citrate dihydrate at room temperature for 4 weeks.[31] After performing the routine tissue preparation stages and preparing paraffin blocks, 5 µ sections were serially obtained for histological and histomorphometric examinations. The sections were stained with hematoxylin-eosin (H and E). Sections obtained from all four groups on days 7, 14, and 21 postimplantation were histologically assessed using light microscopy (Zeiss, Carl Zeiss Microscopy GmbH., Goettingen, Germany) and those obtained on the 56th postimplanted day were assessed both histologically and histomorphometrically.

Histological evaluation

Histological analysis of stained specimens was done blindly under a light microscope by a histologist who was blinded to the type of bone substitutes used. Photomicrographs were taken using a photomicroscope (Leica DM500, Leica Microsystems, GmbH. Wetzlar, Germany) at 7, 14, and 21 days of the experiment.

Histomorphometric assessment

For histomorphometric assessment and for determining the volume of the newly formed bone in all groups, sections obtained on the 56th day were used so that, from each group, six sections were randomly selected (two sections from the surface, two sections from the middle section, and two sections from the deep section of the created defect) and placed on three slides (three slides and six sections for each sample). Ultimately, 18 slides and 36 sections were selected for histomorphometric evaluation in each group. The sections were stained by H and E and were evaluated using the light microscope equipped with eyepiece graticule at ×40 according to the point-counting technique. The mean volume of newly formed bone was calculated in all groups and expressed in percentages.[32]

Statistical analysis

Histomorphometric data were analyzed using SPSS software (version 20, IBM Corp. Armonk, NY, USA). Mean, standard deviation, mode, and medians were calculated. Tukey HSD test was used to compare the means in all groups. $P < 0.05$ was considered as statistically significant (i.e., 5% significant level).

RESULTS

Histological assessments

In the control group on the 7th day after surgery, the defect area was filled with young connective tissue [Figure 1a]. On the 14th day, the connective tissue filling the defect area had a greater organization

[Figure 1b]. On the 21st day, the volume of the newly formed bone increased and showed highly organization in vicinity of host bone [Figure 1c]. On the final day of the study, newly formed bone showed full structural organization with strong attachment to the host bone [Figure 1d].

The most important findings in the OCP group on the 7th day after implantation were the ability of OCP particles in differentiation and adherence of the connective tissue cells to the implanted particles. The cuboidal shape of these cells and their basophilia suggests differentiation of the first osteoblast-like cells in this group during this period [Figure 2a]. In the 2nd week after implantation, the OCP-implanted particles had greater ability to induce bone, which showed more tissue organization [Figure 2b]. On the 21st day after implantation, the first Haversian systems appeared with vascular connective tissue between newly formed bone spicules. In addition, during this period, active absorption of the implanted particles by multinuclear preosteoclast cells was observed adjacent to the implanted particles [Figure 2c]. On the final day of the study, the newly formed bone, while having a remarkable volume increase, exhibited a high degree of tissue organization [Figure 2d].

In BMG experimental group on the 7th day after implantation, the implanted BMG particles showed distinct eosinophilic masses at the defect area. The spaces in-between BMG particles had been filled with a relatively dense fibrous connective tissue [Figure 3a]. One of the important findings of the BMG group was induction of cartilage tissue in the connective tissue surrounding the implanted particles with mature chondrocytes and cartilage matrix on the cartilage islands [Figure 3b]. On the 14th day after implantation, signs of intramembranous ossification were seen close to the defect margin and adjacent to the host bone. The appearance of this bone tissue in the periphery and near the defect's margin reflects the quick differentiation of osteoblastic cells and their tendency to form new woven bone tissue [Figure 3c]. The ability of induced basophilic osteoblast-like cells around BMG particles and their ability to interconnect with each other on the surface of these particles indicate the high ability of bone formation of these particles in this experimental group in the 3rd week after implantation [Figure 3d]. In other specimens in this time point, most induced osteoblastic cells displayed multilayered pseudostratified appearance, adjacent and attached to the newly formed bone

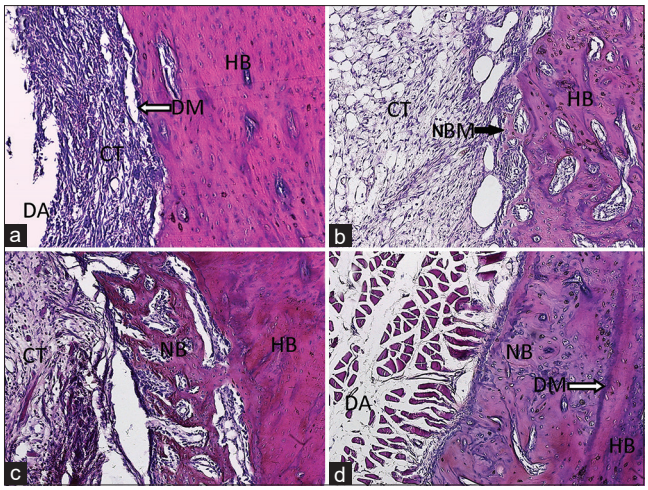

Figure 1: Histological micrograph of the control group at 7-day (a), 14-day (b), 21-day (c), and 56-day (d) postimplantation. These photomicrographs clearly show that there is no any evidence of new bone in defect area in control group except the photomicrograph d that belong to 56th day of the experiment. Connective tissue (CT), host bone (HB), newly formed bone (NB), defect margin (DM), defect area (DA). H and E staining, magnification = a, b, c, d (10X).

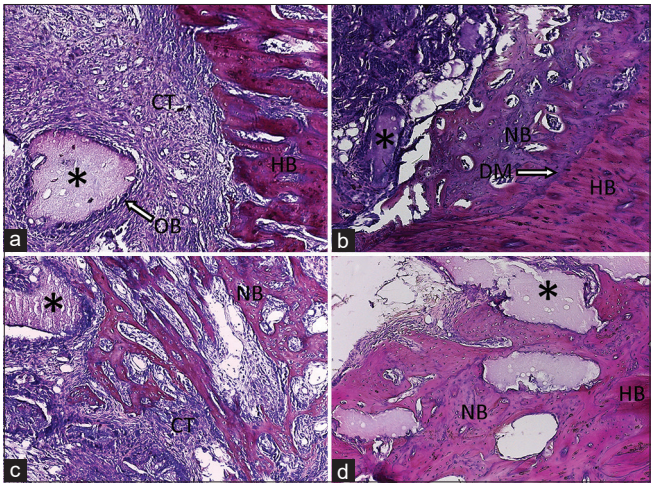

Figure 2: Histological micrograph of the octacalcium phosphate group at 7-day (a), 14-day (b), 21-day (c), and 56-day (d) postimplantation. These photomicrographs clearly show the induction of new bone especially near the defect margin in vicinity of host bone. Octacalcium phosphate particle (*), connective tissue (CT), host bone (HB), newly formed bone (NB), Defect margin (DM), osteoblast cell (OB). H and E staining, magnification = a, b, c, d (10X).

[Figure 3e]. On the final day of the study, it was observed that newly formed bone enclosing some of the retained and unabsorbed BMG particles have a high degree of organization [Figure 3f].

The different nature of OCP- and BMG-implanted particles to induce connective tissue in OCP/BMG

experimental group showed that OCP particles had been absorbed to a lesser degree than the BMG particles on the 7th day after implantation [Figure 4a]. On the 14th day after implantation, new bone formation was started under the inductive effects of the implanted particles adjacent to the defect margin. The presence of cartilage cells on the surface of new bone spicules adjacent to BMG particles showed the pattern of endochondral ossification that is dependent on the nature of these organic particles [Figure 4b]. On the 21st day after implantation, it was observed that the amount of newly formed bone, with greater organization, increased [Figure 4c]. The remaining nonabsorbed OCP particles within the newly formed bone were the most important finding of this experimental group on the 56th day after implantation [Figure 4d]. The greater ability of BMG particles to absorb enabled this experimental group to have a larger volume of new bones formed on the final day of the study that occupied the major part of the defect [Figure 4e].

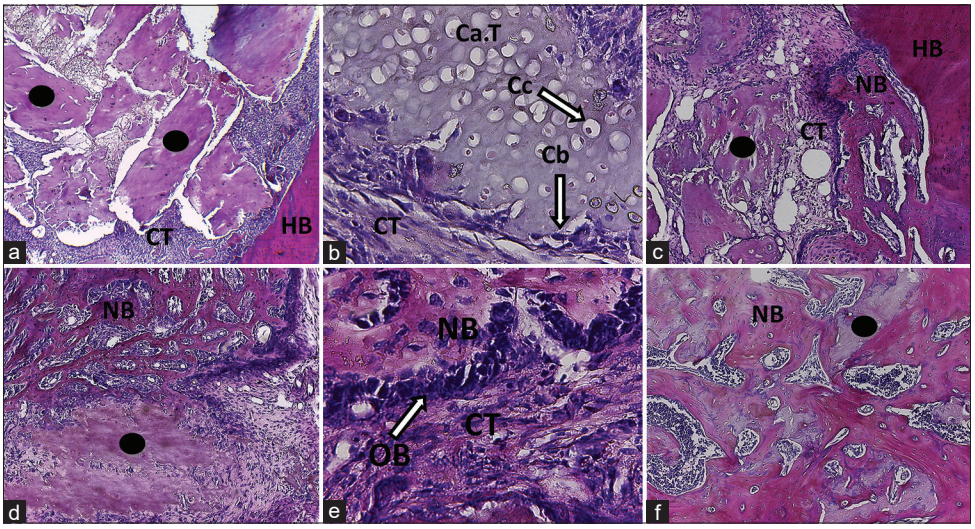

Figure 3: Histological micrograph of the bone matrix gelatin group at 7-day (a and b), 14-day (c), 21-day (d and e), and 56-day (f) postimplantation. These photomicrographs clearly show the induction of cartilaginous tissue and also direct differentiation of osteoblastic cells by bone matrix gelatin particles. Octacalcium phosphate particle (*), bone matrix gelatin particle (●), connective tissue (CT), host bone (HB), newly formed bone (NB), cartilage tissue (Ca. T), chondrocyte (Cc), chondroblast (Cb), osteoblast (OB). H and E staining, magnification = a (4X), b, e (40X), c, d, f (10X).

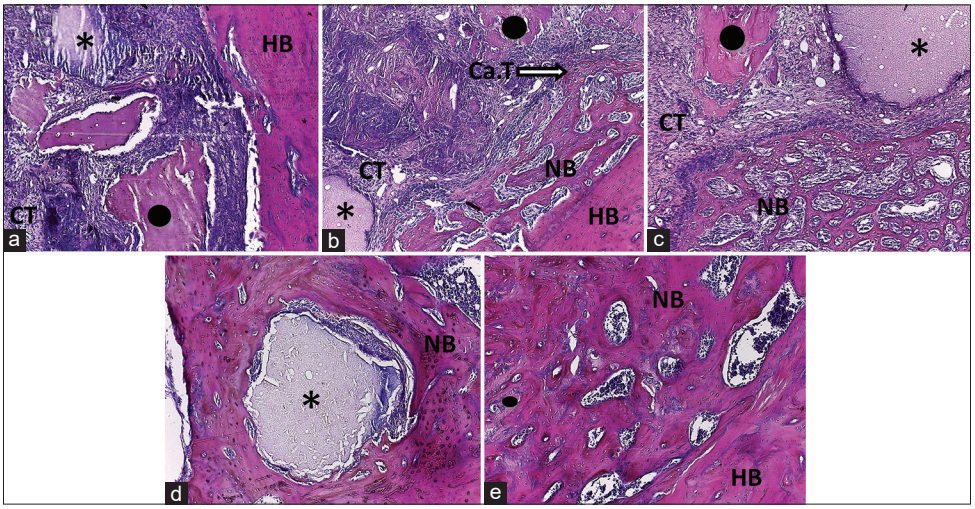

Figure 4: Histological micrograph of the octacalcium phosphate/bone matrix gelatin group at 7-day (a), 14-day (b), 21-day (c), and 56-day (d and e) postimplantation. These photomicrographs clearly show that octacalcium phosphate/bone matrix gelatin have a greater ability for induction of osteogenesis and also well organization of new bone in defect area. Bone matrix gelatin particle (●), octacalcium phosphate particle (*), connective tissue (CT), host bone (HB), newly formed bone (NB), cartilage tissue (Ca.T). H and E staining, magnification = a, b, c, d, e (10X).

Histomorphometric assessments

The volume of the newly formed bone in all experimental and control groups was calculated on the 56th day after implantation using a point-counting technique. The mean for each group was reported as a volume percentage with up to two decimals for each group, the results of which are summarized in Table 1 and Figure 5. The mean volume of newly formed bone was highest in the OCP/BMG group, followed by the BMG and OCP groups. The control group had the lowest volume of the newly formed bone. Based on Tukey HSD test, the mean bone volume formed in the control group was significantly different from all experimental groups ($P < 0.001$). The experimental OCP/BMG group, with the highest volume of newly formed bone, had a statistically significant difference with OCP ($P < 0.001$) and BMG groups ($P < 0.002$). The volume of the newly formed bone was not significantly different between the two groups of OCP and BMG ($P < 0.836$).

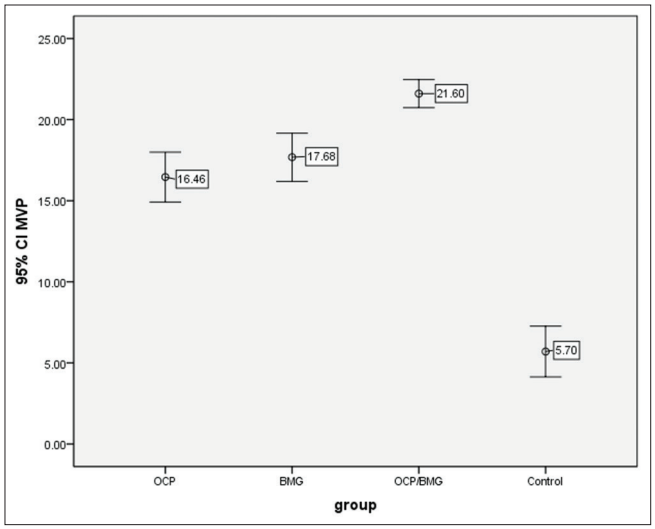

Figure 5: Comparing mean volume percent of new osseous tissue among the experimental and control groups.

DISCUSSION

The efficacy of synthetic bone substitutes alone or in combination for bone regeneration has been evaluated in several previous studies.[4,20,31] The current study aim was to evaluate the osteogenic potential of OCP- and BMG-implanted alone and in combination in artificially created bone defects in the mandible of rats. The results showed that implantation of OCP induced intramembranous bone formation. Kamakura *et al*. reported similar reaction to OCP implantation on the parietal bone.[16] In our study after 3 weeks, irregular bone trabecula was seen at the defect site. Osteoblasts and osteocytes were clearly seen on the bone trabecula, which were indicative of the organization of the newly formed bone and their remodeling. Most researchers found similar results in the 4th week after implantation.[11,33] The difference between the mentioned study and our results could be attributed to the role of implanted material in the deeper areas of the defect.

The enhancement of bone formation and OCP biodegradation, followed by a remodeling-like mechanism, has been reported in various bone defects when OCP particles are implanted in the medullary canal of rabbit femur[34] or rat tibia[35] and in the

Table 1: Comparing the volume percent of new osseous tissue among the experimental and control groups

Groups	Number of microscopic field	Mean	SD	Median	Mode (%)	Minimum	Maximum	*P*
OCP								
BMG	144	17.66	9.02	21	25 (43.1)	0	25	0.836
OCP/BMG	144	21.60	5.28	21	25 (50.7)	0	25	<0.001
Control	144	5.70	9.49	0	0 (65.3)	0	25	<0.001
BMG								
OCP	144	16.45	9.35	21	25 (37.5)	0	25	0.836
OCP/BMG	144	21.60	5.28	21	25 (50.7)	0	25	0.002
Control	144	5.70	9.49	0	0 (65.3)	0	25	<0.001
OCP/BMG								
OCP	144	16.45	9.35	21	25 (3.7.5)	0	25	<0.001
BMG	144	17.66	9.02	21	25 (43.1)	0	25	0.002
Control	144	5.70	9.49	0	0 (65.3)	0	25	<0.001
Control								
OCP	144	16.45	9.35	21	25 (37.5)	0	25	<0.001
BMG	144	17.66	9.02	21	25 (43.1)	0	25	<0.001
OCP/BMG	144	21.60	5.28	21	25 (50.7)	0	25	<0.001

OCP: Octacalcium phosphate; BMG: Bone matrix gelatin; SD: Standard deviation

subperiosteal region of mouse calvaria.[12] Based on these results, it seems likely that the enhancement of bone formation by OCP is accompanied by simultaneous activation of osteoclastic resorption of OCP.[10] On the other hand, the mechanism to enhance these cellular activities has been proposed to be stemmed from the intrinsic chemical property of OCP, which can be progressively converted to Ca-deficient hydroxyapatite under physiological condition. Thus it is logic to purpose which OCP particles can provide a suitable surface for cell attachment and differentiation through inorganic ionic dissolution and protein interactions between tissue fluid and the hydrolyzing surface. Thus, OCP could have a stimulatory capacity on the calcified tissue that could be influencing the involved cells apart from the materials.[36]

We found that in the BMG group on day 7, bone repair was initiated with the formation of cartilage tissue on the BMG particles, but the new bone formation was not clearly visible yet. Presence of intramembranous bone formation was evident at the defect margins in the 2nd week following implantation. The timing of new bone formation in this group was similar to the findings of a previous study.[32] Appearance of osteoblasts and formation of bone tissue occurred latter than the cartilaginous tissue. Hence, the amount of the newly formed bone is directly correlated to osteoblastic differentiation via intramembranous ossification route was greater than the amount of newly formed bone through the endochondral ossification route. Thus, it may be stated that cartilaginous tissue played an insignificant role in osteogenesis in the defects, and formation of new bone was mainly the direct result of osteoblastic differentiation. This finding was in agreement with the results of Wang *et al.*,[37] who reported intramembranous ossification following implantation of DBM in the cranial region. Thus, it can be concluded that in contrast to endochondral ossification, which occurs following implantation of DBM subcutaneously, intramuscularly, or in defects of long bones, implantation of BMG particles in mandibular bone defects of rat results in direct proliferation and differentiation of mesenchymal cells to osteoblasts and consequent induction of new bone formation. Furthermore, the result of the present study indicates that BMG induced formation of bone trabeculae in close approximation to host bone, and in some cases, it was difficult to distinguish between the newly formed bone and host bone. Moreover, histological observation showed that BMG particles decreased with time, presumably via cell-mediated mechanisms. Yamashita and Takagi suggested that multinuclear giant cells that are initially present within the defect, started absorption of BMG particles and provide suitable environment for induction of osteogenesis. It appears that the delay in osteogenesis in BMG group was a temporary process.[38]

In the OCP/BMG group in the current study, mesenchymal cells had differentiated into osteoblast-like cells and covered the implanted materials in a regular and orderly route. Osteoinduction in this group was greater at the margins compared to the center of defects in the 2nd–3rd weeks. On the 56th day, new bone formation significantly increased and the absorption rate of BMG particles was higher than OCP particles. High osteogenesis in this group may be partly because of the delayed absorption of OCP particles, since they induce the release of BMPs from the surrounding tissues and particularly the traumatized bone.[18] Wang *et al.* reported better BMG performance in creating compound scaffolds when BMG is combined with other biomaterials.[39]

Histomorphometric findings and comparison of mean volume of newly formed bone mass among the experimental and control groups showed significantly greater volume of newly formed bone in all experimental groups than the control group ($P < 0.001$), which indicates the positive effect of the implanted materials on induction and formation of new bone at the defect area. It was also observed that among the experimental groups, the highest volume of newly formed bone was in OCP/BMG group and the smallest volume in the OCP group. The OCP/BMG group was significantly different from OCP and BMG groups in terms of the volume of newly formed bone ($P < 0.001$ and $P < 0.002$, respectively).

The effect of BMG together with OCP may accelerate the biodegradation of the OCP particles. Consistent with our results, it seems that OCP, as one of the most effective calcium phosphate derivatives, releases the osteoinductive proteins present in BMG for the differentiation of osteoblasts and in turn increases bone mass. We believe that highly resorbable and osteoconductive biomaterials, such as OCP/BMG combination, have potential use in clinical practice. Further studies are necessary to investigate the relationship between the rapid biodegradability and bone regenerative capacity of the OCP/BMG combination.

CONCLUSION

Regeneration of a functional and living tooth is one of the most promising therapeutic strategies, and the end goal of tissue engineering is to develop products capable of healing diseased or lost tissues and organs.[40] The present study showed that implantation of OCP and BMG particles alone and in combination enhanced the regeneration of bone defects. Combination of OCP/BMG had greater potential for inducing osteoblastic differentiation and new bone formation compared to the application of OCP or BMG alone.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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