RESEARCH ARTICLE

Histomorphometric Analysis of Newly-formed Bone Using Octacalcium Phosphate and Bone Matrix Gelatin in Rat Tibial Defects

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Abstract

Background: Repair of bone defects is challenging for reconstructive and orthopedic surgeons. In this study, we aimed to histomorphometrically assess new bone formation in tibial bone defects filled with octacalcium phosphate (OCP), bone matrix gelatin (BMG), and a combination of both.

Methods: A total of 96 male Sprague Dawley rats aged 6-8 weeks weighing 120-150 g were randomly allocated into three experimental (OCP, BMG, and OCP/BMG) and one control group (n=24 in each group). The defects in experimental groups were filled with OCP (6 mg), BMG (6 mg), or a combination of OCP and BMG (6 mg, 2:1 ratio). No material was used to fill the defects in the control group and the defect was only covered with Surgicel. Samples were taken on days 7, 14, 21, and 56 after the surgery. The sections were stained with hematoxylin-eosin (H&E) and assessed using light microscopy.

Results: In our experimental groups, bone formation was started from the margins of the defect towards the center with an increasing rate during the study period. Moreover, the formed bone was more mature. Bone formation in our control group was only limited to the margins of the defect. The newly formed bone mass was significantly higher in the experimental groups (*P=0.001*).

Conclusion: OCP, BMG, and OCP/BMG compound enhanced osteoinduction in long bones.

Level of evidence: III

Keywords: Bone formation, Bone matrix gelatin, Octacalcium phosphate, Rat, Tibia

Introduction

Repair of bone defects caused by various reasons such as tumors, infections, agenesises, and trauma has been a challenging field in orthopedics and reconstructive medicine (1). Currently, various methods have been identified and are being used for reconstruction and repair of lost bone tissues (2). However, most of these methods are accompanied by several clinical risks (3-5). One of these methods is the use of autogenous or

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allogeneic bone grafts. This method is expensive and sometimes leads to bone deformities, scar formation, and mortality as well as increased risk of hemorrhage, inflammation, infection, and chronic pain, since they require two surgeries. Moreover, autogenous grafts are less effective in case of extensive bone defects (6). Allogeneic grafting is the second most common method for bone transplantation. These grafts are obtained from cadavers and are available in different forms. Despite their biocompatibility, they can also trigger immune response and cause infection (7, 8). Currently, to overcome the limitations of allogeneic and autogenous bone grafts such as post-operative complications (hemorrhage, inflammation, infection, and chronic pain), scars, and sometimes death, as well as preventing secondary surgery, various types of synthetic materials are used as substitutes (6, 9). These synthetic materials include calcium phosphates, bone matrix gelatin (BMG), and demineralized bone matrix (DBM) (10-13). Evidence suggests increased bone formation by calcium phosphate derivatives, especially octacalcium phosphate (OCP) (14). Octacalcium phosphate [$Ca_8H_2(PO_4)_6.5H_2O$] has been suggested to be a precursor of biological apatite crystal in bone and tooth (15). When OCP is implanted into a bone defect, it is irreversibly converted into the apatite phase that has been shown to promote osteoblastic cell differentiation (16). Furthermore, the implanted OCP can serve as a core for initiating bone formation and shows the osteoinductive and osteoconductive abilities if implanted in the critical-sized bone defects in rat models (17). Moreover, due to its osteoinductive and osteoconductive properties, alone (18) or in combination with other biomaterials such as BMG, transforming growth factor beta (TGFβ), or bone morphogenetic protein (BMP); it has shown promising results in repair of bone defects (19, 20).

On the other hand, many investigators have confirmed successful repair of bone defects after using DBM or BMG and have stated that they can be suitable substitutes for bone grafts (11, 21, 22). Bone Matrix Gelatin (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 (23). One of these proteins is bone morphogenic protein (BMP) which persuades 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 (3). The mutual reaction of BMG in combination with most calcium phosphate derivatives has shown promising results in repairing bone defects (20, 24-26).

According to a study by Anada and colleagues on the effect of OCP on bone marrow stromal cells, OCP was reported to be able to differentiate stromal cells to bone-forming cells, especially in initial stages of maturation (27). Moreover, after evaluating the effect of calcium phosphate on osteoblast activity and bone regeneration by OCP, Suzuki et al. concluded that OCP has a more prominent bone-forming activity than other calcium derivatives (28). In another study, Kim and co-investigators studied the bone-forming effects of DBM in rat skull defects and found that DBM effectively increased bone repair at the graft site (29). In a study on bone-forming process induced by BMG in parietal bone defects in rats, the researchers concluded that BMG induces bone formation through intramembranous and endochondral ossification in repairing skull defects, especially through intramembranous ossification (30). In another study on repairing bone defect in the calvarial bone of rats using the OCP/BMG combination, the researchers found that this combination could stimulate bone defect repair in calvarial bone (19).

Materials and Methods

Preparation of materials

Synthetic OCP [$Ca_8H_2(PO_4)_6.5H_2O$] was prepared following the methods previously described (31, 15). In brief, 250 ml of 0.04 M $Ca(CH_3CO_2)2.H_2O$ solution was slowly added to 250 ml of 0.04 M NaH_2PO_4.2H2O solution over a period of one hour. The solution was stirring at 400 rpm at 67.5 ˚C. Ground granules of OCP between 32 and 48 mesh (particle size: 300–500 µm) were used for implantation. The sieved granules were sterilized by heating at 120˚C for 2 hours. A previous study showed that such heating does not affect physical properties such as the crystalline structure or specific surface area of OCP granules (32, 33). Bone matrix gelatin (BMG) was prepared using the method previously described by Urist et al. which was modified by the method of Wei-Qi Yan et al. (22, 23). In brief, diaphyseal shafts of femora and tibiae from 6-week-old male Sprague dawley rats were cut into chips. Liquid nitrogen was used to freeze the bone shaft chips to avoid possible denaturation of proteins. The bone chips were extracted in chloroform-methanol (1:1) for 4 hours at 25 ˚C, demineralized in 0.6 N of hydrochloric acid at 4 ˚C for 72 hours, and gelatinized in 6 M of lithium chloride at 2 ˚C for 24 hours. The bone chips were then auto-digested at 37 ˚C for 48 hours in phosphate buffer saline (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

In this study, a total of 96 male Sprague Dawley rats aged 6-8 weeks with a weight of 120-150 g were enrolled. The experimental animals were obtained from the Animal Research Center of Zahedan University of Medical Sciences. The rats were randomly allocated into three experimental groups (OCP, BMG, and OCP/BMG) and one control group (n=24 in each group) and kept in standard conditions with light/dark cycles of equal duration. The principles of laboratory animal care, as well as national low, were followed. All procedures were approved by the Ethics Committee for Animal Experiments of Zahedan University of Medical Sciences (IR.ZAUMS.REC.1394-350). The rats were anesthetized

with an intraperitoneal injection of ketamine hydrochloride (60 mg/kg) and xylazine (20 mg/kg) with a ratio of 2:1 using a pre-planned schedule during consecutive days. Then, a cutaneous incision was made on the inside leg and the periosteum was scratched and removed and a defect was made using a dental drill (diameter: 3 mm, thickness: 2 mm) with a critical size (34) on the medial surface of the upper end of the tibia. The defect was filled with OCP (6 mg), BMG (6 mg), or a combination of OCP and BMG (6 mg, 2:1 ratio). Surgicel was used to prevent the displacement and dispersion of particles. No material was used to fill the defects in the control group rats and they were only covered with Surgicel. The surgical site was sutured with absorbable sutures (number 04) and disinfected. Samples were taken on days 7, 14, 21, and 56 after the surgery and implanted materials.

Tissue preparation

General anesthesia was induced by intraperitoneal injection of ketamine hydrochloride (60 mg/kg) in all experimental and control groups. Formalin buffer 10% was perfused for fixation though the heart and in situ fixation was immediately done. For a better fixation, the respective area along with a margin of host bone was resected and stored in 10% buffered formalin at room temperature for one week. The samples were decalcified using formic acid 10%, citric acid 2.9%, and trisodium citrate 1.8% over four weeks at room temperature (35).

After performing the routine tissue preparation stages and preparing paraffin blocks, 5 micron sections were serially obtained for histological and histomorphometrical examinations. The sections obtained from all four groups on days 7, 14, and 21were stained with hematoxylin-eosin (H&E) and histologically assessed using light microscopy (Zeiss, Germany). The sections obtained on the 56th post-operative day were assessed both histologically and histomorphometrically.

Histomorphometric assessment

For histomorphometric assessment and determining the volume of the newly formed bone in all groups, sections obtained on the 56th day were used; so that, from each group 6 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&E and were evaluated by using the light microscope equipped with eyepiece graticule at X40 magnification according to the point counting technique. The mean volume of newly formed bone was calculated in all groups and expressed in percentages (36-37).

Statistical analysis

Histomorphometric data were analyzed using SPSS software (version 20). Mean, SD, mode and medians were calculated. Dunnet T3 test was used to compare the means in all groups. $P<0.05$ was considered as statistically significant.

Results

Histological evaluation

In the OCP group, on the 7th post-implant day, bone formation was started from the margin of the defect with osteoblast and osteocyte activity around the OCP particles [Figure 1a]. On day 14, these particles were more absorbed with the activity of the osteoclasts around the implanted particles [Figure 1b]. On day 21, the amount of newly formed bone was increased so that in situ bone formation was observed in parts far from the margin defects accompanied by the absorption of the material [Figure 1c]. On day 56, the site of the defect was almost completely filled with primary and lamellar bone similar to the histology of the host bone [Figure 1d].

In the BMG group, on day 7, new bone mass with high density of osseous cells was observed, indicating that bone tissue (non-lamellar bone) was being directly formed [Figure 2a]. On day 14, a dual pattern of bone formation (intramembranous and endochondral) was observed between the remnant particles [Figure 2b]. On day 21, most of the implanted particles were absorbed and the induced bone formation had a lamellar pattern [Figure 2c]. On the 56th day, the defect was almost completely filled with the newly formed bone which was repaired with disorganized haversian systems that differed from the host bone with respect to the staining reaction [Figure 2d].

In the OCP/BMG group, on day 7, bone tissue differentiation was seen and osteoclasts and osteoblasts were seen in the vicinity to the implanted particles, similar to the previous samples, leading to new bone formation of woven type [Figure 3a]. On day 14, the induced bone tissue was penetrated into the central bone cavity because of the absorption of the implanted material [Figure 3b]. On day 21, the induced bone had enough time to repair, thus, mature osteons were seen [Figure 3c]. On day 56, the absorption rate of implanted particles, especially BMG particles, was higher than previous groups and induced histologically a considerable amount of lamellar bone type [Figure 3d]. In the control group, the newly formed bone was observed at the margins of the defect with adhesions to the host bone and most of the defect was filled with connective tissue [Figure 4].

Histomorphometric evaluation

The volume of the newly formed bone in the experimental and control groups on day 56 was calculated using the point counting technique and the mean obtained amounts were expressed as volume percentage up to 2 decimals for each group. Table 1 and Figure 5 summarize the related results. Since the volume of the newly formed bone between the control and experimental groups did not have a normal distribution, Dunnett T3 tests was used.

As shown, the mean volume of newly-formed bone was highest in the OCP group, followed by the OCP/BMG and BMG groups, respectively ($P=0.999$). The control

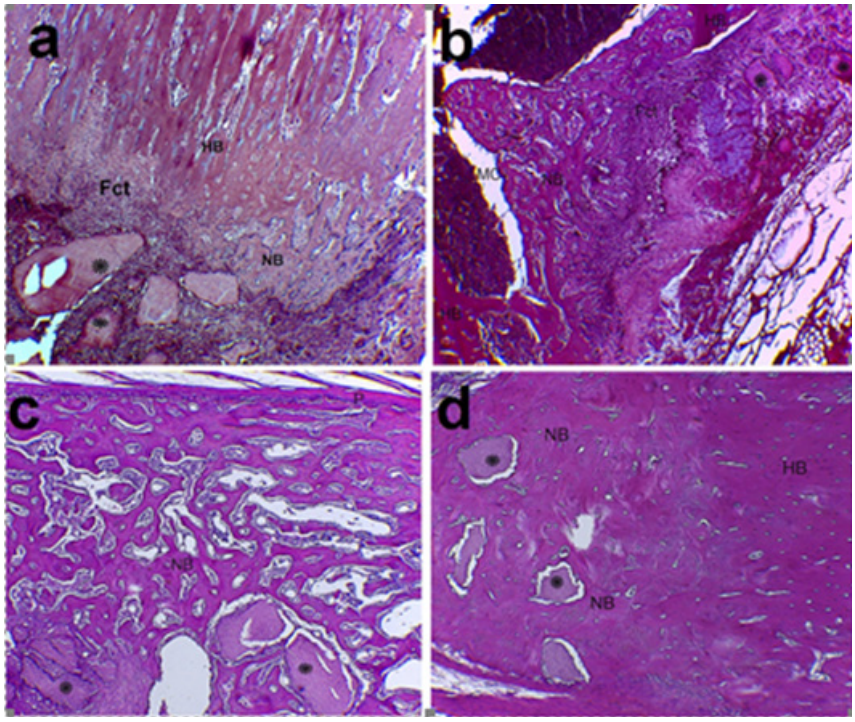


Figure 1. The OCP group: day 7 (a), day 14 (b), day 21 (c), day 56 (d). Newly formed bone (NB), implanted OCP particles (*), fibrous connective tissue (Fct), host bone (HB), H&E staining: magnification= X4.

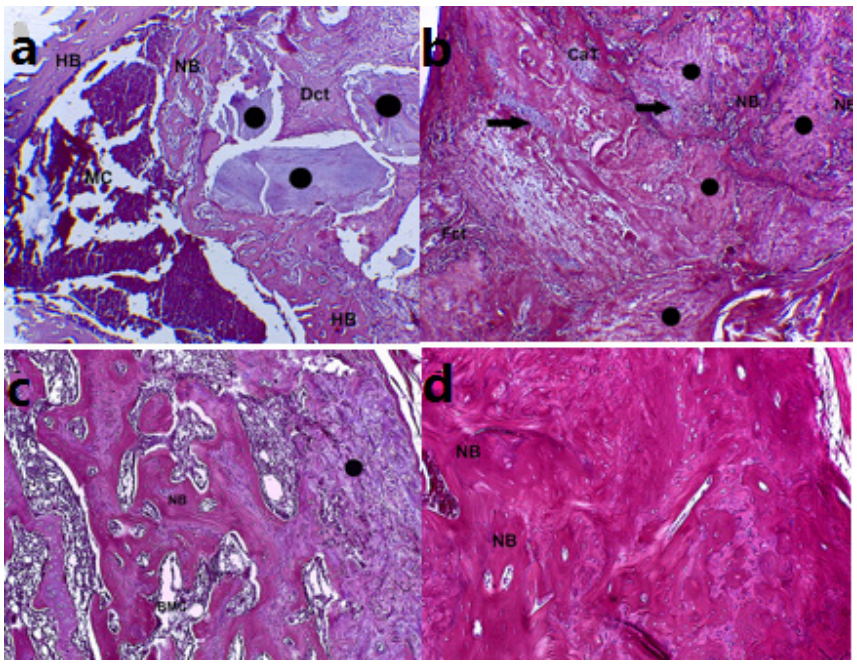


Figure 2. The BMG group: day 7 (a), day 14 (b), day 21 (c), and day 56 (d). newly formed bone (NB), implanted BMG particles (●), fibrous connective tissue (Fct), host bone (HB), induced cartilage tissue (arrows),cartilage tissue (Cat), H&E staining: magnification=a and b X4, c and d X10.

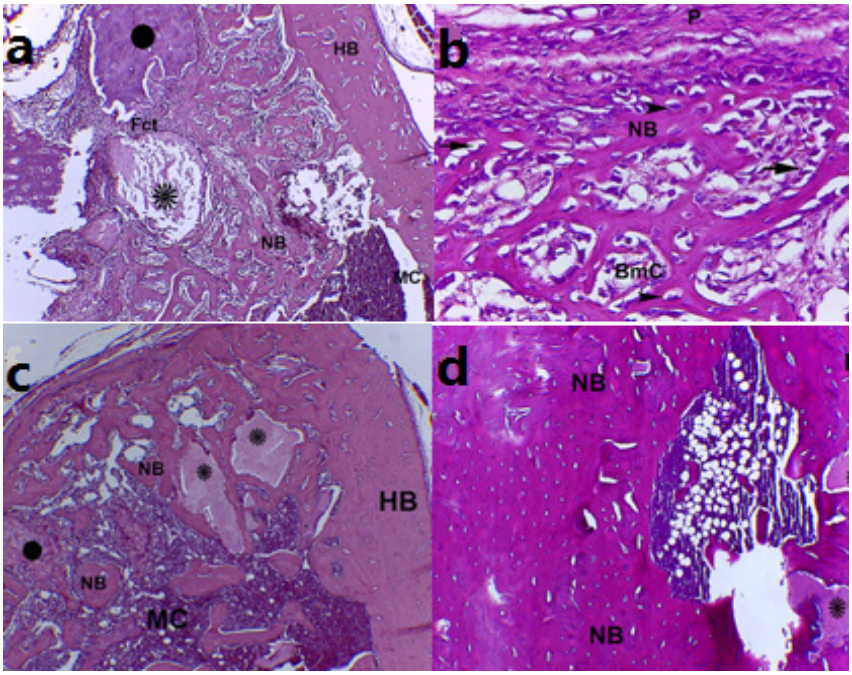


Figure 3. The OCP/BMG group: day 7 (a), day 14 (b), day 21 (c), and day 56 (d). Newly formed bone (NB), implanted OCP particles (*) and BMG particles (●), Periosteum (P), host bone (HB), bone marrow cavity (BmC), osteocyte cells (arrow head), osteoblast cells (short arrows), H&E staining: magnification=a and b X4, c and d X140.

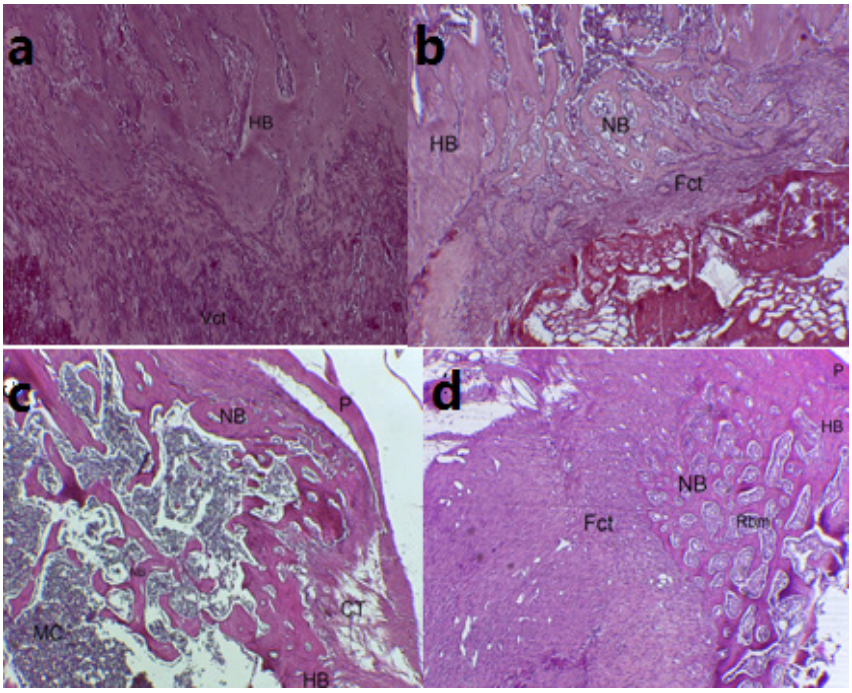
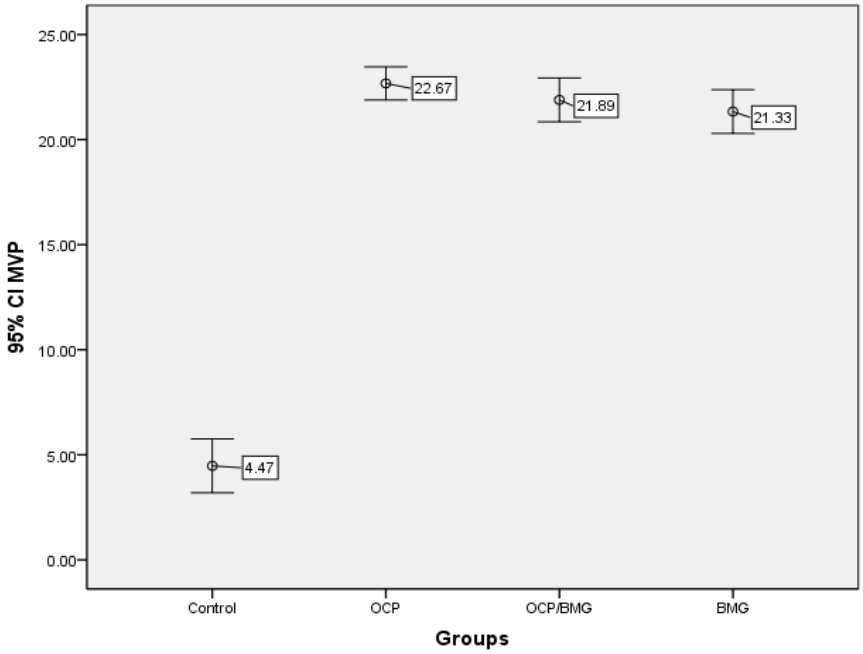


Figure 4. The control group: day 7 (a), day 14 (b), day 21 (c), and day 56 (d). newly formed bone (NB), fibrous connective tissue (Fct), host bone (HB), Periosteum (P), bone marrow cavity (MC), connective tissue (CT), H&E staining: magnification= a X4, b, c and d X10.

Table 1. Comparing the percentage of formed bone among the experimental and control groups based on Dunnett T3 test

Groups		Number of microscopic fields	Mean of volume percent of new osseous tissue	Standard Deviation	Median	Mode of volume percent of new osseous tissue (Percent)	Min	Max	*P value*
BMG	OCP	144	22.67	4.78	25	25 (71.5%)	0	25	0.234
	OCP/BMG	144	21.88	6.32	25	25 (72.2%)	0	25	0.974
	Control	144	4.47	7.81	0	0 (68.8%)	0	25	<0.001
OCP	BMG	144	21.33	6.32	25	25 (62.5%)	0	25	0.234
	OCP/BMG	144	21.88	6.32	25	25 (72.2%)	0	25	0.799
	Control	144	4.47	7.81	0	0 (68.8%)	0	25	<0.001
OCP/ BMG	BMG	144	21.33	6.32	25	25 (62.5%)	0	25	0.974
	OCP	144	22.67	4.78	25	25 (71.5%)	0	25	0.799
	Control	144	4.47	7.81	0	0 (68.8%)	0	25	<0.001
Control	BMG	144	21.33	6.32	25	25 (62.5%)	0	25	<0.001
	OCP	144	22.67	4.78	25	25 (71.5%)	0	25	<0.001
	OCP/BMG	144	21.88	6.32	25	25 (72.2%)	0	25	<0.001



MVP: Mean of volume percent of new osseous tissue

Figure 5. Comparing mean percentage of bone volume between the experimental and control groups.

group had the lowest volume. Therefore, the three groups differed significantly with the control group in this regard, showing the positive effect of the implanted material on bone defects.

Discussion

Various studies have shown the effect of synthetic material on bone defect repair, either alone or in combination with each other (19, 38, 39). In this

study, we assessed bone formation after implanting OCP and BMG alone or in combination. We found that implanting OCP alone induced intramembranous bone formation. It seems that in the created tibial defect, bone tissue differentiation follows a surface to depth pattern under the influence of the implanted particles; so that the initial cancellous bone masses were formed on day 7 in the peripheral regions of the defect near the host bone. It seems that this had occurred using the host's osteoblast cells that were at the defect's margins. With respect to the absorption of OCP particles, on day 14 we found that osteoclast cells were differentiated near the implanted particles, indicating the release of the particles' content and the initiation of osteoblast differentiation. In this group and on day 21, the penetration of the OCP particles led to the formation of new bone tissue in situ and in the medullary cavity near the created defect. Kamakura and colleagues found similar results in the fourth week after implantation of OCP particles (35). The difference between the mentioned study and our results could be attributed to the role of implanted material in the deeper sections of the defect.

Another material that effects bone formation and was used in this study was BMG. This material exerts its osteoinductive mechanisms through releasing endogenous BMPs and growth factors (39). On day 7 after implant, we found direct bone formation of the non-spindle-shaped form with a high density of bone cells, which is consistent with another study showing the differentiation of mesenchymal cells to osteoblasts and osteoclasts during the first week after implant (30). On day 14, we found a dual pattern of direct and indirect bone formation. In this regard, Wang and colleagues reported the existence of chondrocytes in the second week after implant after the use of BMG compound with other biomaterials (40). On the 56th day, the created defect was almost completely repaired. Kim and co-authors reported that after implanting DBM in combination with other biomaterials, bone absorption by osteoclasts and new bone formation was increased on the eighth week after implantation compared with previous time periods (29).

We found that in the OCP/BMG group, on day 7, the extent of bone formation was more than the other two experimental groups and it was seen to be intramembranous. This is while Sargolzaei and colleagues had found that bone repair was initiated with the formation of cartilage tissue in the BMG particles but the new bone formation was not clearly visible (19). This finding is inconsistent with our results. This could be attributed to the younger age of the rats that BMG was prepared from, which had higher inductive capacity. On day 56, the absorption rate of the implanted particles, especially BMG, was higher than the other groups. 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. Wang and colleagues also reported better BMG performance in creating compound scaffolds when BMG is combined with other biomaterials (40).

Histomorphometric findings showed that the newly formed bone mass in the experimental groups significantly differed from the control group, indicating the positive effects of the implanted material at the site of the defect. On the other hand, we found that although bone mass was more in the OCP group compared with the BMG and OCP/BMG, the difference was not significant. Therefore, it seems that the age difference of the rats from which BMG was obtained, the percentage of BMG in the OCP/BMG compound, and the preparation conditions could have affected the early absorption of the implanted BMG particles, before they had enough time to induce more bone formation and therefore, no synergistic effect was seen between OCP and BMG particles. Further study is under way to establish the optimal conditions for an OCP/BMG component, which may lead to the development of a bone substitute material compatible with autogenous bone.

The present study demonstrated that implantation of OCP and BMG alone or in combination in long bone defects can enhance bone repair as well as resorption of implanted materials in early stages of bone formation. Combination of OCP and BMG could be a suitable bone substitute which is expected to be resorbed with time and replaced by newly formed bone simultaneously.

Conflict of interest: There is no Conflict of interest.

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