Advances in Bioresearch
Adv. Biores., Vol 8 (1) January 2017: 17-24
©2017 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.8.1.1724

Advances in Bioresearch

ORIGINAL ARTICLE

Bone Regeneration by Octacalcium Phosphate (OCP)-Gelatin Composites in Rat Mandibular Bone Defects - A Qualitative Study

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ABSTRACT

This study aimed to histologically assess the process of regeneration of mandibular bone defects in rats following implantation of octacalcium phosphate (OCP) alone and in combination with gelatin. Standard defects were created in the mandibles of 36 Sprague Dawley adult male rats(6-8 weeks old, weighing 120-150g) and filled with OCP, gelatin or OCP/gelatin combination. Tissue samples were obtained after seven, 14 and 21 days of experiment. Following standard preparation, 5µ-thick tissue slices were sectioned and evaluated under a light microscope after hematoxylin and eosin staining. In OCP and gelatin groups, defects were filled with woven and lamellar bone at 21 days. In OCP/gelatin group, new bone had been formed by intramembranous and endochondral ossification processes. In the control group, slight amount of bone had been formed at the defect margins (next to host bone) on day 21, which was prominent lower than the amount of newly formed bone in the experimental groups. Implantation of a combination of OCP and gelatin was more effective than each of them alone for bone regeneration at the defect site and this combination can be efficiently used for regeneration of mandibular bone defects.

***Key words**: Octacalcium phosphate, Gelatin, Osteogenesis*

Received 07/10/2016 Revised 30/11/2016 Accepted 11/12/2016

How to cite this article:
E A Saberi, F S Aval, M R Arab, N Sargolzaei, H Shahraki, F Sargolzaei Aval. Bone Regeneration by Octacalcium Phosphate (OCP)-Gelatin Composites in Rat Mandibular Bone Defects - A Qualitative Study. Adv. Biores., Vol 8 [1] January 2017: 17-24.

INTRODUCTION

Regeneration of bone defects due to trauma, infection or tumor resection is a major challenge in maxillofacial and endodontic surgeries. Small defects may be successfully managed by the application of autogenous bone grafts, which are the gold standard for this purpose; but large defects require the use of biocompatible bone graft substitutes [1]. Several types of synthetic bone substitutes are available in the market. These materials are either osteoinductive or serve as a carrier for maintenance and gradual release of bone morphogenetic proteins (BMPs) [2-5].

Calcium phosphate derivatives are among the most frequently applied synthetic biomaterials to induce the regeneration of hard tissues [6]. These materials are divided into different subtypes depending on the ratio of calcium and phosphate in their composition. In terms of physical and chemical properties, they highly resemble the natural mineral content of bone and teeth [7,8].

Different compositions of calcium phosphate have variable potentials for induction of regeneration of bone defects. Some of them serve as carriers for BMPs [9,10] while others are osteoinductive [11,12].

Octacalcium phosphate is an osteoinductive bone substitute serving as a direct precursor of bone and tooth hydroxyapatite [13]. Compared to other calcium phosphate derivatives, OCP has greater osteoinductive and osteoconductive potential. It is gradually absorbed and replaced by the newly formed bone [14]. Evidence shows that OCP alone or in combination with other biomaterials may efficiently enhance regeneration of bone defects [15-18].

Despite various advantages of calcium phosphate derivatives especially OCP, application and handling of these materials may be associated with some difficulties. For instance, delivery and compaction of these materials at the site of bone defects are difficult [19]. To overcome these problems, polymer materials such as chitosan, alginate and gelatin have been recently introduced for addition to bone substitutes in order to enhance their handling [20-22].

Gelatin is a natural polymer procured from bovine skin by thermal denaturation or physical and chemical degradation of collagen. It has extensive applications in tissue engineering (19). Moreover, gelatin is a biocompatible polymer. Evidence shows that gelatin is completely resorbed; in combination with cement, it is capable of forming both organic and non-organic phases for bone regeneration [22]. Moreover, gelatin forms electrostatic bonds with drugs and thus, it is added to many medications to enable their sustained release [23].

Considering all the above regarding OCP and bovine gelatin, the need to enhance regeneration of bone defects in endodontic surgery as well as the paucity of in vivo studies on the efficacy of application of these materials alone and in combination especially in mandibular bone defects, this study sought to histologically assess the process of regeneration of mandibular bone defects in rats following implantation of OCP alone and in combination with gelatin in order to search for a new strategy in this regard.

MATERIALS AND METHODS

A. Preparation of materials:

Gelatin was purchased from Sigma(Type B, Sigma-Aldrich Co. Louis MO, USA) and the OCP was prepared according to the method described by Legeros [24].

Combining gelatin and OCP*:* One gram of gelatin was added to 10mL of distilled water and heated. The temperature was increased from 40 to 60°C within 30 minutes to obtain gelatin solution; 1mL of this solution was added to 250mg of OCP particles (300-500μ) (4mL/g). The obtained material was dried at room temperature. Blocks measuring 3mm in diameter and 2mm in thickness (depending on the size of lesion) were prepared and autoclave-sterilized(23).

B. Sample size:

This study was conducted on 36 Sprague Dawley adult male rats (6-8 weeks old with a mean weight of 120-150g) obtained from the Animal Research Center of Zahedan University of Medical Sciences. The animals were randomly divided into OCP, gelatin, OCP/gelatin and control groups and were kept under standard conditions. They had free access to food and water with similar light/dark cycles. The study protocol was reviewed by the institutional review board and approved by the ethics committee of Zahedan University of Medical Sciences.

C. Surgical procedure and implantation of materials:

During consecutive days, animals in the four groups were anesthetized by intraperitoneal injection of 60mg/kg ketamine hydrochloride ((Ketalar, Trustech Pharma Care, Bayern, Germany) and 20mg/mL xylazine(Pantex Holland B.V., Duizel, Netherland) in 2/1 ratio. Diethyl ether was used for maintenance of anesthesia. After achieving adequate depth of anesthesia, the animals were fixed on the operating table in a supine position and the respective area in the body of mandible was shaved and disinfected with 10% Betadine(Toliddaru, Tehran, Iran). During the procedure, Bactimide® (Toliddaru, Tehran, Iran) eye drop was used to prevent corneal dryness. Using a sterile surgical scalpel, a 1.5cm incision was made on both sides of the body of mandible and the skin along with the underlying tissue was elevated in the form of two flaps. After cutting and completely elevating the periosteum, defects measuring 3mm in diameter and 2mm in depth were created on both quadrants of the mandible close to the alveolar crest (in-between the first molar and canine) under copious irrigation with cold saline. In the first experimental group 6mg of OCP (previously prepared and packed), in the second experimental group 6mg of gelatin and in the third group a combination of OCP and gelatin were implanted at the defect site and covered with Surgicel®(Altaylar, Medical, Ankara, Turkey) to prevent dissemination of particles. No material was implanted in the control group; the defects were rinsed with cold saline and covered with Surgicel®. The muscle, underlying connective tissue and skin were sutured in two layers using 4/0 chromic cut gut sutures (catgut, Wei Gao Group Kanglida Medical Products Co, LTD. Heze, China). The area was

disinfected and 0.1mL gentamicin was injected intramuscularly for infection prophylaxis. After gaining consciousness, the animals were transferred to cages and received daily care and proper nutrition.

D. Tissue harvesting:

In all groups, tissue samples were harvested at seven, 14 and 21 days (from six rats at each time point). The animals were anesthetized by intraperitoneal injection of ketamine hydrochloride. After opening the chest, fixator (10% buffered formalin) was perfused via the pump to achieve in situ fixation of tissues. The defect site along with a margin of the adjacent host bone was removed and immersed in 10% buffered formalin at room temperature for one week to achieve better fixation. The specimens were rinsed with cold distilled water for a couple of times and decalcified by immersion in a decalcifying solution containing 10% formic acid, 2.9% citric acid and 1.8% trisodium citrate dihydrate for four weeks at room temperature [25]. Tissue specimens were conventionally prepared and paraffin blocks were serially sectioned into 5-micron slices. Selected slices were H & E stained for evaluation under a light microscope.

E. Histological assessment:

Microscopic slides were evaluated under a light microscope (Zeiss, Carl Zeiss Microscopy GmbH. Goettingen, and Germany) by a histologist blinded to the group allocation of samples. Photomicrographs were obtained and evaluated under a photomicroscope((Leica DM 500, Leica Microsystems, GmbH. Wetzler, Germany) at seven, 14 and 21 days.

RESULTS

Osteogenesis was evaluated at seven, 14 and 21 days under a light microscope. The results were as follows:

Control group:

First week (seven days): Hyper-cellular and vascular connective tissue with a regular margin of host bone had filled the defects. This hyper-cellular connective tissue and desmocytes at the defect site were among the most important histological characteristics at this time point (Figure 1a).

Second week (14 days): Hyper-cellular and vascular connective tissue with a regular margin of host bone had filled the defects and penetrated into some parts of the defect margin and host bone. The entire defect had been surrounded by osteoblast-like basophilic cells adjacent to the host bone(Figure 1b).

Third week (21 days): More organized connective tissue had further penetrated into the host bone. The connective tissue had characteristics similar to those reported in previous time points with higher cellularity (Figure 1c).

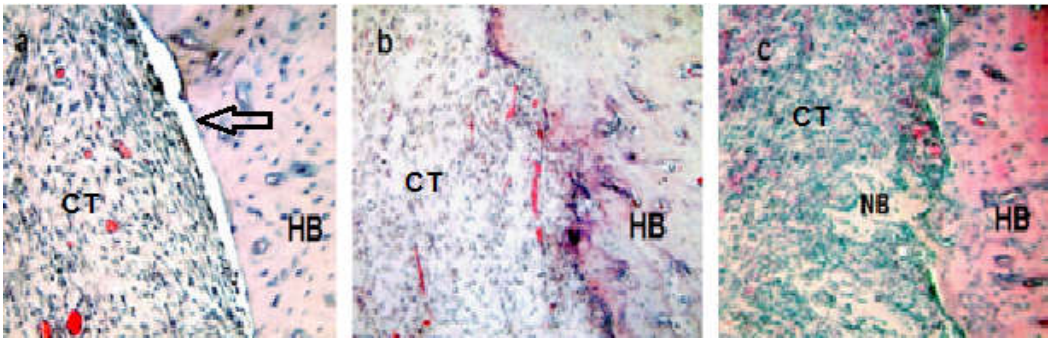

Figure 1. Histological micrograph of the control group at one week(a), 2 week(b) and 3 week(c) post-implantation. CT:connective tissue. HB: host bone. NB: newly formed bone. Defect margin(long Arrow). H&E staining, magnification=20×(a), 10×(b, c).

OCP group:

First week(seven days): The amount of newly formed bone around the implanted particles was similar to the amount of implanted particles. Other characteristics of the connective tissue at the defect site were similar to those reported for the control group; this indicates the differentiation potential of mesenchymal cells around the implanted particles (Figure 2a).

Second week(14 days): Osteoinduction and osteogenic differentiation were noted at the defect site and around the implanted particles. The newly formed bone had been well organized at the defect margins adjacent to the host bone. The newly formed bone was separated from the host bone by a basophilic line. Penetration of connective tissue into the newly formed bone around the implanted particles at the defect margins clearly indicated the osteoinductive potential of these particles resulting in initiation of new bone formation from the periphery towards the center of defects (Figure 2b).

Third week (21 days): Osteoinduction and complete organization of the newly formed bone at the site of defect were noted in a specimen in this group. The newly formed bone had extended from the periphery towards the center of defect with islets of the connective tissue in-between the newly formed bone, which indicated osteoinduction starting from the margins next to host bone towards the deeper areas of defect (Figure 2c). Multinuclear osteoclasts (differentiated from the pre-osteoclastic cells) were also seen adjacent to the margin of implanted particles. The cytoplasm of these cells was acidophilic at the top with a basophilic base (where the nuclei had been accumulated), mimicking osteoclasts (noted in one specimen in this group). These cells had apparently absorbed the implanted particles and created gaps, enhancing the penetration of osteoblast-like cells and initiation of bone matrix deposition (Figure 2d).

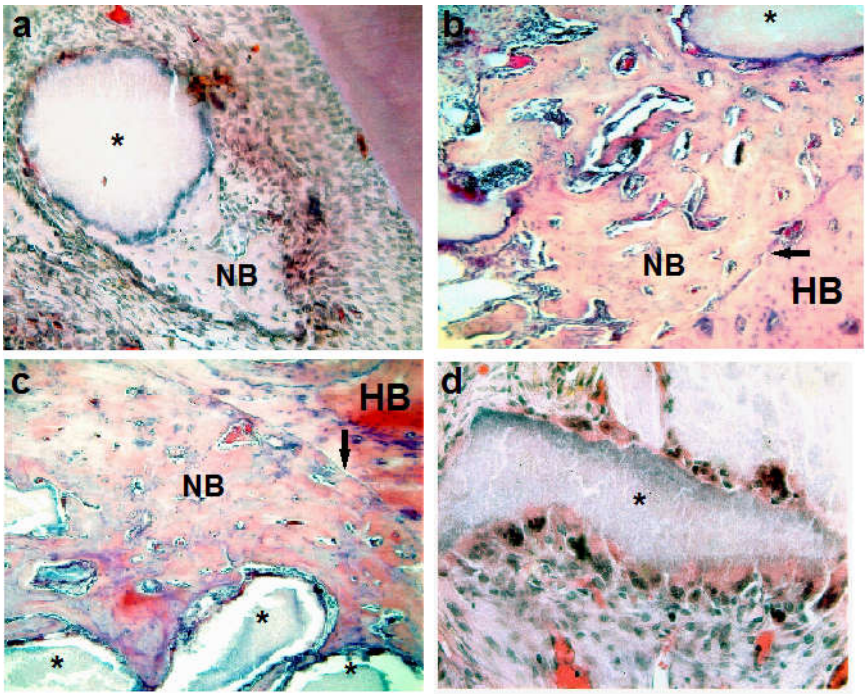

Figure 2. Histological micrograph of the OCP group at one week(a), 2 week(b) and 3 week(c , d) post-implantation. OCP particle(*). HB: host bone. NB: newly formed bone. defect margin(long arrow). Multinuclear osteoclasts(short arrow). H&E staining, magnification=20×(a), 10×(b, c), 40×(d).

Gelatin group:

First week (seven days): The defects had been completely filled with a dense connective tissue. The density of the connective tissue changed from the center towards the periphery of the defect with a tendency to an increase in the amount of fibers. Implanted gelatin particles were seen separately or in the form of aggregates, invaded by the loose connective tissue(micrograph not shown). These particles formed amorphous, porous clumps, and mesenchymal cells showed low affinity for these particles (Figure 3a).

Second week (14 days): Gelatin particles were more dispersed due to the penetration of connective tissue in-between them. Inflammatory cells were seen at the center and more frequently at the peripheral areas. These cells were mostly polymorphonuclears. In part of the defect occupied by the gelatin particles distinct bone tissue in the process of organization of matrix was seen in the form of primary osteons. These osteons, depending on their distance from the host bone (margin of defect) had a central canal with variable depths and shapes (Figure 3b).

Third week (21 days): Out of the central dense connective tissue and adjacent to the host bone, newly formed bone with a woven pattern at the center and a trabecular pattern at the periphery was noted. Rows of osteoblastic cells were noted on the surface of the newly formed bone trabeculae (woven bone). The distance between the woven bone and the more peripheral bone trabeculae had been filled with loose connective tissue. Differentiation of cells in this area next to host bone was noticeable (Figure 3c). Osteoblasts were clearly seen on some of the peripheral bone trabeculae near to the eosinophilic material of bone matrix (Figure 4d).

Octacalcium phosphate/gelatin group:

First week (seven days):.Defects had been filled with fibrous connective tissue with a relatively regular, well-defined margin. At the center, OCP and gelatin particles were visible surrounded by inflammatory

cells and connective tissue fibers. No signs of cell adhesion to particles or differentiation were noted either at the center or at the periphery of defects. Gelatin particles were in the form of porous masses with no clear cell differentiation at their periphery. This reticular pattern indicated possible dissolution of particles at the center or periphery of defect (occurred either in vivo or during passage). The OCP particles were larger and had a porous pattern. Inflammatory cells had mainly accumulated around the OCP rather than gelatin particles(Figure 4a).

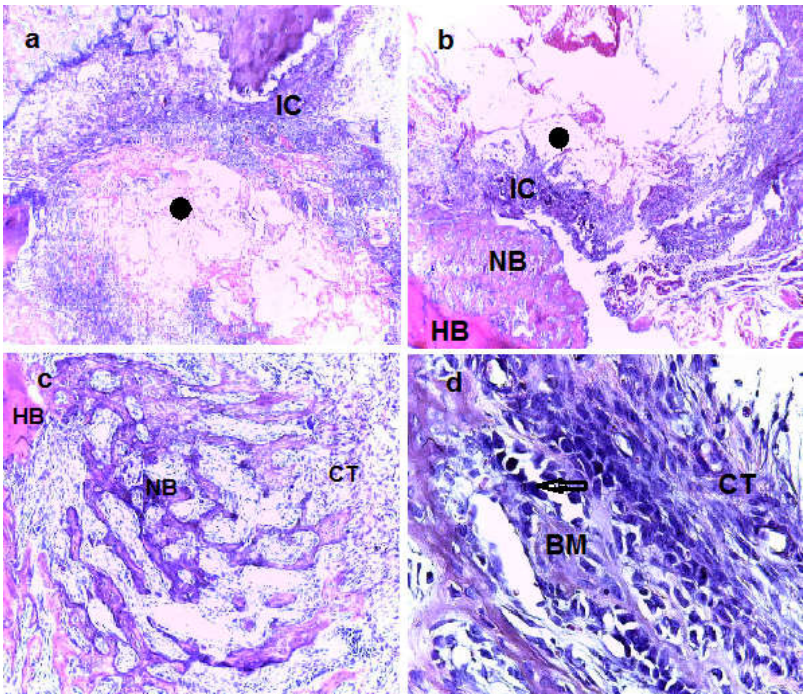

Figure 3. Histological micrograph of the gelatin group at one week(a), 2 week(b) and 3 week(c ,d) post-implantation. Gelatin particle(●). CT: connective tissue. IC: inflammatory cells.. HB: host bone. NB: newly formed bone. osteoblast cell(long arrow). H&E staining, magnification=40×(a, b, c, d).

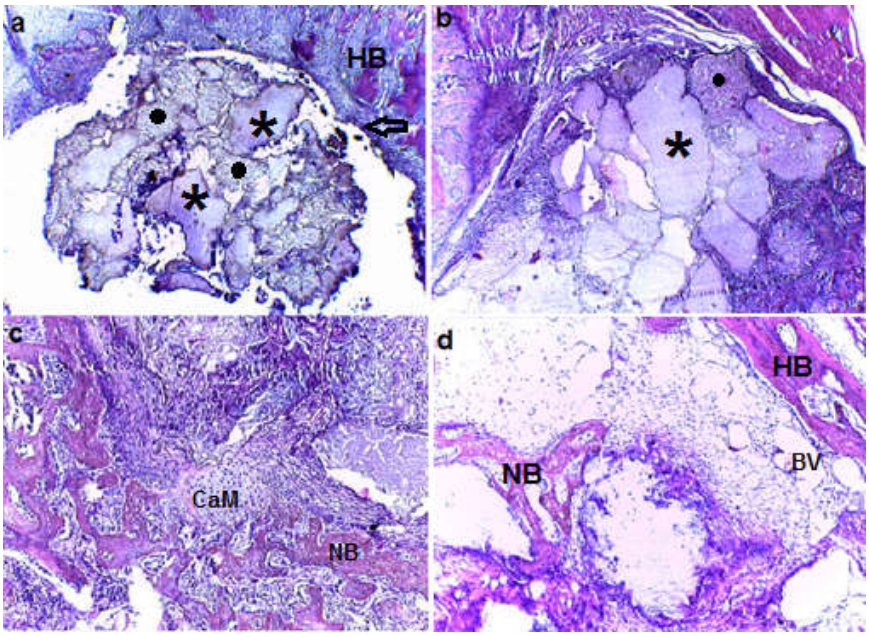

Figure 4. Histological micrograph of the OCP/G group at one week(a), 2 week(b) and 3 week(c , d) post-implantation. gelatin particle(●). OCP particle(*) CT: connective tissue. BV: blood vessels.. HB: host bone. NB: newly formed bone. Cam: cartilaginous mass. defect margin(long arrow). H&E staining, magnification=4×(a, b), 10×(c, d).

Second week (14 days): Defects had been surrounded by fibrous connective tissue, separating them from the host bone. The implanted OCP/gelatin particles had the same morphology as in the first week. Level of organization of some particles, especially the peripheral ones, had increased, resulting in a more intense eosinophilic pattern in peripherally implanted particles. This organization and penetration of connective tissue into particles were indicative of the first phase of differentiation of osteoblasts and formation of new bone by intramembranous ossification(Figure 4b).
Third week (21 days): Defects had been surrounded by relatively dense fibrous connective tissue. In the central areas, cartilaginous mass surrounded by an eosinophilic connective tissue was visible. Morphology of cells at the center was similar to that of chondrocytes and they had been surrounded by a relatively eosinophilic matrix. Classically, this step is the first phase of endochondral ossification due to osteoinductive properties of implanted particles (Figure 4c). In some specimens in this group, close to the implanted materials or in variable distances from the host bone, distinct bone trabeculae were clearly seen (Figure 4d).

DISCUSSION

The efficacy of synthetic materials alone or in combination with one another for regeneration of bone defects has been the topic of many previous investigations (3-8). This study aimed to assess the osteoinductive potential of OCP and gelatin alone and in combination after implantation in mandibular bone defects of rats. The osteoinductive and osteoconductive properties of these materials were also evaluated. The results showed that OCP induced intramembranous ossification in the mandible. First, inflammatory cells infiltrated the area around the OCP particles and then the area occupied by OCP was surrounded by organic matrix, which indicates host response to OCP. Such a reaction has also been reported after implantation of OCP in the alveolar crest of the mandible of rats in a study by Handa *et al* [25]. Also, it was reported that at three weeks after implantation, irregular bone trabeculae were seen in the defects. Osteoblasts were seen on the surface of bone trabeculae while osteocytes were found at the center of bone lamellae. This indicates organization of bone trabeculae in their process of formation and shows the continuation of bone remodeling. Most researchers reported this finding at four weeks after implantation [26, 27].
The process of osteoinduction by OCP has not yet been completely elucidated and the biological properties responsible for osteoinduction have yet to be clearly understood. Suzuki and Nakamura believed that glycoconjugates accumulated over the surface of OCP particles were effective for osteogenesis [26, 27]. Also, Suzuki et al. have claimed that the role of OCP in osteoinduction is similar to that of TGF-β with similar biological properties. They added that bioactive factors such as TGF-β in the surrounding tissues accumulate on the OCP particles and may serve as a factor inducing the differentiation of osteoblasts, new bone formation and employment of osteoclasts for bone remodeling [28]. Sasano et al, also assessed the biological properties and the role of OCP in osteoinduction and osteoconduction in comparison with prostaglandin E1. They explained that prostaglandin E1 may accumulate in the tissues surrounding the OCP particles and result in osteoblastic differentiation. Moreover, the release of prostaglandin E1 may continue as long as the OCP particles are present at the site [29].
Osteogenesis seems to be induced by the host bone tissue and starts in the outermost area of defect and then continues towards the center. Histological findings in the gelatin group showed scattered inflammatory infiltrates at the defect site. In the second and third weeks, woven bone formation was noted at the center and bone trabeculae were seen at the periphery of defects. This indicates superior osteogenesis in presence of gelatin, which has not been reported in previous studies [30]. Advancement of osteogenesis over time may be due to the role of gelatin as an organic polymer scaffold that can enhance the adhesion, proliferation and differentiation of host cells [31].
Some other studies have shown that gelatin serves as an extracellular matrix for bone and enables the gradual release of osteogenic factors, increases cell adhesion and enhances the activity of alkaline phosphatase and osteocalcin products [32,33]. Based on the results, it appears that gelatin is capable of serving as an osteoconductive material during the process of bone regeneration. Although this is a key advantage, its exact mechanism requires further investigation to be elucidated.
On the other hand, difficult handing due to mechanical properties is one limitation associated with the application of calcium phosphate compounds. In other words, they are supplied in the form of powder or particulates, which makes their delivery difficult to the site of defect. To overcome this limitation, we used OCP in combination with gelatin to improve its mechanical properties; this finding is in agreement with the results of Bigi *et al*, [34] and Habraken *et al* [23].

In our study, in the OCP/gelatin group, connective tissue fibers and first signs of osteoblastic differentiation were noted in the first and second weeks following implantation. This finding is in accordance with the results of Suzuki *et al*, [30] and Handa *et al* [35]. In the third week, in addition to the woven bone formed at the defect margins, cartilaginous tissue was noted at the center of defects; this finding has not been reported in any of the previous studies. Considering the fact that OCP possesses both osteoinductive and osteoconductive properties [28, 29] and gelatin serves as a carrier and a scaffold [36-38], it seems that the interaction of these two materials induces bone formation by promoting direct differentiation of undifferentiated mesenchymal cells to osteoblasts. Also, the interaction effect of these two may even result in differentiation of some of the mesenchymal cells to chondroblasts or chondroblast-like cells observed between the particles and at the defect margins. This is also a new finding not reported in any previous study.

CONCLUSION

Within the limitations of this study, the results showed that implantation of OCP and gelatin alone caused new bone formation in the mandibular defects. This was a new finding related to the application of gelatin. It was also shown that combination of OCP and gelatin not only induced intramembranous ossification (which is among the properties of OCP), but also resulted in induction of endochondral ossification. This was also a new and interesting finding that can pave the way towards new treatment strategies for more efficient management of bone defects in maxillofacial and reparative surgery.

ACKNOWLEDGMENT

The authors would like to thank the personnel of the animal research institute at the Zahedan University of Medical Sciences.

COMPETING INTERESTS

The authors have declared that no competing interest exists.

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