Repair of segmental bone defect using calcium phosphate granules and allogeneic bone matrix gelatin mixture in rabbits

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ABSTRACT: Several methods are used to enhance bone repair and new bone formation. Bone matrix gelatin is recently introduced and calcium phosphate has gained importance in orthopaedics as repair materials for bone defects. The objective of this study was to evaluate of effects of a mixture of calcium phosphate granules and allogeneic bone matrix gelatin on bone repair in rabbit. 30 male rabbits were divided into three groups. After induction of general anesthesia, a segmental bone defect of 5 mm in length was created in the middle of the left radius shaft. In control group defect was left untreated, in group 2, calcium phosphate granules were used and in group 3, a mixture of calcium phosphate granules and bone matrix gelatin were used to fill the bone defect. Rabbits of each group were euthanized at 45 days intervals for evaluation of histopathological and biomechanical changes. Histopathology finding in control group, defect seemed to be filled with connective tissue and bone marrow spaces and in spite of a poor osteogenic activity. In group 2, defect has been filled by newly formed bone. Active osteogenesis seemed. In group 3, young bone trabeculas increased in number and bone trabeculas more organized. Biomechanical results show that the mean load for fracturing in group 3 was significantly higher than group 1 and group 2 (p=0.000). Results was showed significant changes between the group 2 and control group (p=0.000). In this study calcium phosphate bone granules and bone matrix gelatin mixture is intended to be suitable for defects of any shape; this mixture is the osteoinductive property which provides better to the healing site.

Key words: Calcium phosphate granules, bone matrix gelatin, histopathology, biomechanical, bone healing, rabbits

INTRODUCTION

Many types of bone filling materials have played critical roles in bone healing (Bajammal et al., 2008). Calcium phosphate has excellent osteoconduction and resorbability when filling the bone defect (Chazono et al., 2004; Horisaka et al., 1994). Nowadays blocks and granular of calcium phosphate have been available and use for different shapes of bone defects (Ignjatovic et al., 2007). Physical and chemical characteristics of these materials are very similar to natural minerals of bone (Kale and Di Cesare, 1995), and they lack the disadvantage of autografts and allograft (Komaki et al., 2006). Bone matrix gelatin (BMG) contain many of bone constructing factors such as bone morphogenetic protein (BMP) which persuades local mesenchymal cells to differentiate into bone forming cells, a process known as osteoinduction (Komath M et al., 2000). In allogenic bone matrix gelatin (BMG), 95% of non-collagen proteins which would eliminate antigenicmaterials is removed, this process include defatting,
demineralization and extraction, so it weakly immunogenic and more biocompatible with the host (Kondo N et al., 2005). The objective of this study was to evaluate the effect of a mixture of calcium phosphate granules and allogenic bone matrix gelatin on bone healing.

**MATERIALS AND METHODS**

Thirty New Zealand white rabbits were used, of male gender, aged around 24 week and weighing 3.3-3.5 kg. The animals were obtained from the central animal laboratory of Islamic Azad University, Tabriz Branch. All animals were kept in individual cages during the whole experimental period, under similar conditions at 18-24°C in a 12-hr dark-light cycle and maintained with unlimited amounts of standard laboratory pellet diet and water ad libitum. Investigations using experimental animals were conducted in accordance with the internationally accepted principles for laboratory animal use and care, and our ethical committee on animal care approved the protocol. Animals were divided into three groups (control and experiments), with ten rabbits in each, according to the procedure performed.

Calcium Phosphate granules were made of Kasios TCH Co. and were prepared as biphasic and had basis of tri calcium phosphate and hydroxyapatite. The component used for producing these products as given below:

- 75% hydroxylapatite ± 5% Ca₁₀(PO₄)₆(OH)₂
- 25% tricalcium phosphate ± 5%Ca₃(PO₄)₂

Granules used had 2-3 mm length and was provided as cubic. These granules have a porosity of 70% and the size of each pore was 200 to 500 micrometers and had resistance about 1 to 5 MPa according to the manufacturer's brochure.

To produce bone matrix gelatin as allogeneic was used Urist method (9). Two rabbits were euthanized. Diaphyseal shafts of humerus, radius, ulna, femur and tibia were collected and isolated from soft tissues and were placed into liquid nitrogen to avoid possible denaturation of proteins. Then allogenic BMG was performed as follows:

The bones were removed from liquid nitrogen and periosteum was separate then by bone cutter was divided into pieces of 5 mm. The bones lipid was removed by chloroform/methanol (1:1), and then demineralized in 0.6 NHCL, and in order to extract soluble proteins of bone were used CaCl2 (2.0 M), EDTA (0.5 M), LiCl (0.8 M) and water (55°C), then were crushed into smaller pieces in liquid nitrogen and were kept in -60°C refrigerator.

Surgical procedure: Under general anesthetics by intramuscular injection of Xylazine/Ketamine (Ketamine 10%, Alfasan, Woorden-Holland, 50mg/kg and Xylazin 2%, Alfasan, Worden Holland, 5mg/kg), left radius was routinely prepared for surgery. A 4-cm longitudinal skin incision was made. The space between extensor and flexor muscles groups was dissected, providing a wide view of radius, of which periosteum was fully dissected. A segmental bone defect was created in the middle of the radius shaft, 5-mm in length, using a delicate orthopedic motor saw. In control group defects were left empty. In group 2, the bone defect was filled with calcium phosphate granules. In group 3, the bone defect was filled with a mixture of calcium phosphate granules and BMG. The muscle attachment was sutured with simple stitches using Vicryl 4.0 suture with nontraumatic needle, and skin was sutured with silk suture 4.0, and the surgical sites were fixed with plaster bandages. The animals received antibiotics Enrofloxacin 2.5% intramuscular in the dose of 2.5ml/Kg of body weight for five days after surgery, and anti-inflammatory Banamine (Flonexin Meglumine) injected in the dose of 1.1 mg/Kg of body weight for three days after surgery by applications in the muscle.

Histopathology evaluation: Rabbits were euthanized with an intravenous injection of an over dosage of Thiopental sodium, causing a quick and painless death, at 45days postoperative. Five radiiuses of each group were removed and fixed in 10% neutral buffered formalin during five days, for fixation; then dehydrated in 10% EDTA. Finally, they were embedded in paraffin. Serial sections were cut and stained with Haematoxylin and Eosin (H&E) method and used for light microscopic examination under a Nikon microscope (ECLIPSE E200, Japan) to histopathology evaluation.

Biomechanical analysis: After euthanasia of rabbits, five radiiuses of each group were removed, wrapped in saline-soaked gauze, frozen, and stored. Specimens were thawed at room temperature in a saline bath prior to mechanical testing. All mechanical testing were performed in biomechanics laboratory of Polymer Engineering Department of Sahand University of Technology, using a Zwick/Roell Z010 with a crosshead speed of 3 mm/min. A load-distance curve was recorded to determine mechanical properties. The central load support was applied over the radius bone. Maximum compression force, maximum displacement and young modulus were calculated.
Statistical analysis: Statistical evaluation of data was performed using the software package SPSS version 18 (SPSS Inc., Chicago, IL). Data are reported as mean±standard deviations (SD). The significant level was set at p<0.05. Statistical comparisons were used analysis of variance (ANOVA).

RESULTS

Histopathology findings of induced defect in control group showed that defect was completely filled with connective tissue containing thin relatively parallel collagen fibers undergoing mineralization, some osteoblasts could already be seen, in spite of a poor osteogenic activity (Fig 1). Results obtained from group 2 shows that defects have been filled by newly formed bone. The amounts of emerged bone in this group are more than control group. Active osteogenesis with calcification in this group yields to compaction in the new bone mass (Fig 2). In group 3, young bone trabeculas increased in number and were more evident, observing of lot of lamellar bone in the healing area indicates a regenerative process. Increase in remodeling of newly formed bone results in formation of hovers system (Figure 3&4).

![Figure 1](image1.png)
Figure 1. Microscopic view from healing area in control group. Filling of the bone defect with mature connective tissue and intramembraneous ossification is obvious, the major part of the defect is filled with connective tissue. H&E, 40x.

![Figure 2](image2.png)
Figure 2. Microscopic view from healing area in group 2 (calcium phosphate granules group). New immature woven bone is observed and active osteoblasts are present within the spicules. H&E, 40x.
Results of biomechanical tests obtained in this study show that maximum compression force for fracturing in group 3 (calcium phosphate granules and allogeneic bone matrix gelatin mixture) is significantly higher than group 1 (control) and group 2 (calcium phosphate granules) (p=0.000). Comparisons between the study groups were showed significant changes between the group 2 and control group (p=0.000). A comparison evaluation between the groups 2 and 3 was showed significant changes (p=0.000). These studies show that the maximum displacement in group 3 is significantly higher than groups 2 and 3 (p=0.003). Young modulus in group 3 also significantly higher than other groups (p=0.029). The mean±SD of mechanical test results for each group are provided in table 1. Figures 5 and 6 shows maximum compression force and Young modulus in study groups.

Table 1. The mean and standard deviation of biomechanical data in groups study

<table>
<thead>
<tr>
<th>Group</th>
<th>Max Compression Force (N)</th>
<th>Max Displacement (mm)</th>
<th>Young modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>144.78±5.32^a</td>
<td>0.31±0.02^a</td>
<td>165.42±6.86^a</td>
</tr>
<tr>
<td>Calcium Phosphate</td>
<td>209.81±20.65^b</td>
<td>0.99±0.23^b</td>
<td>137.61±11.16^b</td>
</tr>
<tr>
<td>Calcium Phosphate &amp; BMG</td>
<td>472.49±36.15^c</td>
<td>1.52±0.08^c</td>
<td>205.6±18.61^c</td>
</tr>
</tbody>
</table>

a,b,c: Dissimilar letters indicate significant differences in each column.
Figure 5. Statistical analysis graphs of maximum compression force in control and experiments groups

Figure 6. Statistical analysis graphs of bone young modulus in control and experiments groups
DISCUSSION

Autogenous bone graft has been the implant of choice for most of the orthopaedic procedures. However, autogenous and allogenic bone grafts have several limitations, such as donor-site infection, pain, and disease transfer (Mathias et al., 2005). Nowadays, many researchers are trying to find materials that can improve bone healing. Many types of bone filling materials have been developed and have played critical roles in bone repair. It has been reported that calcium phosphate has biocompatibility, excellent osteoconduction and resorbability material (Bajammal et al., 2008; Mousavi et al., 2012; Mousavi et al., 2012; Mousavi and Rezaie, 2011). With regard to shape, both block and granular of calcium phosphate have been available. Considering that used BMG contains BMP, we can conclude that the active osteogenesis in the experimental samples is due to BMP (Mousavi et al., 2010). Assessment of histopathological results, show that the majority of the defect in control group was filled by immature bone and connective tissue, and its healing has not more severity and extension because of absence materials having growth promotion effects, the amount of lamellar bone in this group is very low. In the second group, that the defect was filled by calcium phosphate granules, because of used biomaterial cause accelerating of histological process, bone trabeculae was formed more regular and organized than control group and defect was filled by newly formed bone. In the third group, that the defect was filled by the mixture of calcium phosphate granules and BMG, a lot of mature bone trabeculae have been filled defect area which is changing into the lamellar bone. It seems that the addition of BMG can induce the osteogenesis, so that, at the end of the study, osteoblasts were observed in the active form. In this group, woven bone was more organized and compared than two other groups. The biomechanical results also show that the highest mechanical results were obtained in group of calcium phosphate granules and BMG mixture. Furthermore, the lowest results were obtained in control group. It seems this mixing, due to its high osteoinductive, become stronger on load bearing as the remodeling process. Histopathological and biomechanical results are consistent with each other.

Komaki and et al. showed that segmental bone defects were healed with cortical bone 12 weeks after implantation of the complex of β-TCP granules and 5% collagen with rhFGF-2. They showed that the resorption of β-TCP is important for bone formation (Nilsson et al., 1986). In a study by Ignjatovic et al., 2007, they used of Poly-D-, L- Lactide-co-Glycolide as bone replacement and they found that calcium phosphate and hydroxyapatite have most important effect on aggregation of fibroblasts and their union, which provides situation for healing in the defect area (Ogose et al., 2002). A composite matrix when embedded with human-like osteoblast cells showed better osteoconductive properties compared to monolithic calcium phosphate and produced calcification of identical bone matrix (10). Mousavi and et al. showed that the addition of collagen to calcium phosphate cement can induce ossification processes in an early stage (Bajammal et al., 2008). The principal element of BMG was bone morphogenetic protein (BMP) (Ohgushi et al., 1990). BMPs play a role in the differentiation, proliferation, growth inhibition and arrest of maturation of a wide variety of cells, depending on the cellular microenvironment and the interactions with other regulatory factors (Ozturk et al., 2006). BMPs play an important role in the process of bone modeling and remodeling. The morphogenetic activity of bone matrix is apparent only after its demineralization, which occurs with the controlled action of osteoclasts. Insulin-like growth factors (IGF-I, IGF-II), TGFβ-1, TGFβ-2, PDGF, basic and acidic fibroblast growth factors, BMPs and other molecules are produced and become incorporated into the forming bone matrix that serves as a reservoir (Ozturk et al., 2006; Sykaras and Opperman, 2003; Urist, 1973). Ohgushi and colleagues showed that, bone morphogenetic protein present in the BMG, causes the cell progressive differentiation to the osteoblast cell and provide more osteogenesis (Yang et al., 2010). The results achieved in this investigation indicate that this mixture really stimulates a favorable reaction of long bones and the best fracture healing was observed in the mixture of calcium phosphate granules and BMG group. Our findings were found to be similar to some of the earlier studies. Indeed, both BMG and calcium phosphate were found to enhance osteoblast differentiation, but mixture together; they were shown to accelerate osteogenesis.

CONCLUSION

As result, mixtures of synthetic biomaterials and osteoinductive organic agents to achieve better results. Due to the availability of calcium phosphate granules and considering the possibility of preparation the bone matrix gelatin and osteogenic properties of these two materials, the mixture of these can be effective in healing of bone defects.
REFERENCES


