RESEARCH ARTICLE

Effects of Combination of BMP7, PFG, and Autograft on Healing of the Experimental Critical Radial Bone Defect by Induced Membrane (Masquelet) Technique in Rabbit

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Abstract

Background: Healing of large segmental bone defects can be challenging for orthopedic surgeons. This research was conducted to provide further insight into the effects of BMP7 in combination with autograft and platelet fibrin glue (PFG) on bone regeneration by Masquelet technique (MT).

Methods: Twenty five domestic male rabbits, more than 6 months old, weighing 2.00±0.25 kg were randomly divided into five equal groups as follows: MT-blank cavity (without any biological or synthetic materials) (1), blank cavity (2), MT-autograft (3), MT-autograft-BMP7 (4), and MT-BMP7-PFG (5). A 20 mm segmental defect was made in radial bone in both forelimbs. The Masquelet technique was done in all groups except group 2. The study was evaluated by radiology, biomechanics, histopathology and scanning electron microscopy.

Results: The results showed that Masquelet technique enhanced the healing process, as, the structural and functional criteria of the injured bone showed significantly improved bone healing ($P<0.05$). Treatment by PFG-BMP7, Autograft-BMP7, and autograft demonstrated beneficial effects on bone healing. However, Autograft-BMP7 was more effective than autograft in healing of the radial defect in rabbits.

Conclusion: Our findings introduce the osteogenic materials in combination with Masquelet technique as an alternative for reconstruction of the big diaphyseal defects in the long bones in animal models. Our findings may be useful for clinical application in future.

Level of evidence: V

Keywords: Autograft, BMP7, Masquelet technique, PFG, Rabbit

Introduction

Trauma, pseudoarthrosis, bone infection, bone necrosis, bone tumors and congenital bone diseases are among the commonest causes of bone defects (1). In contrast to the growing understanding of fracture healing processes, delayed healing and non-union formation remain a significant clinical challenge. Segmental bone defects due to traumatic injuries are complicated problems with significant long-term morbidity. Managing segmental long bone defect is difficult, so amputation was the preferred treatment. Using Ilizarov technique, vascularized fibular grafts, and acute limb shortening have been previously used to address defects of various lengths. Even when the recipient site is well vascularized, the usual bone graft techniques are limited by uncontrollable graft resorption (2-4). Among these, induced membrane technique is a

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two-stage technique for bone repair in the metaphyseal and diaphyseal regions of long bones (5, 6). This technique is also used to treat nonunion, segmental bone defects, and congenital pseudoarthrosis of tibia in children (7). In addition, the Masquelet technique (MT) is an option for treatment of larger osseous defects because it is useful for short and long bone defects and is not length-dependent. Short time of reconstruction, high rate of bone union, and low rate of complications are other advantages of the Masquelet technique. Firstly, a cement spacer is placed in the bone defect. It induces the formation of specialized tissue or membrane around it. After one month, the cement spacer is replaced with a bone graft in the tube of the induced membrane. The induced membrane has a range of key growth factors including BMP2, VEGF, and TGF-β1 (8-13). The induced membrane maintains a space for the biologic material. This shiny membrane that is made during this technique by induction of a periosteal membrane prevents graft resorption.

Till now, approximately 20 members of bone morphogenetic protein family (BMP) (member of the transforming growth factor-β superfamily) have been identified (14). BMPs stimulate reproduction and migration of undifferentiated bone cell precursors. They induce new bone formation by stimulating alkalin phosphatase activity and lead the mesenchymal cells to migrate, proliferate, and differentiate into osteoblasts (15). BMPs also induce angiogenesis and inhibit myoblast formation, help undifferentiated pleuripotent cells to differentiate into cartilage and cells that shape bones, and act as a chemoattractant for mesenchymal cells (16-20). They help in bone matrix formation (21). Among these, rhBMP2 and rhBMP7 have been shown to induce bone formation in vivo (15, 20, 22).

BMP7 has osteogenic properties including osteoinduction and osteoconduction and regulates chemotaxis, mitosis, differentiation, and ossification during the bone healing process (17, 23-26). BMP2 and BMP7 promote bone formation when placed in ectopic areas (20). BMP2 appears to be the most potent agent to induce osteoblastic differentiation of mesenchymal stem cells (27). BMP2 was associated with early events of bone healing whereas BMP7 was connected with pre-hypertrophic and hypertrophic chondrocytes, osteoblasts, and osteocytes (28).

Platelet fibrin glue (PFG), also known as “platelet glue” or “cryoplatelet gel,” is a blood-derived biomaterial that is relatively new. Platelets and fibrinogen are contained in a small plasma volume, resulting in differential centrifugation and precipitation (29-31). This technique produces autologous fibrin glue with high platelet and fibrinogen concentrations (29). Some literatures have proposed PFG to be applied as biomaterial that is suitable in bone tissue engineering (29, 32-34). BMP2 has been found in induced membrane. This study was designed to give more insights into the effects of BMP7 in combination with autograft and PFG on bone healing by Masquelet technique.

Materials and Methods

To prepare PFG, the blood was obtained through rabbits’ jugular vein aspiration and it mixed with citrate phosphate dextrose at a ratio of 5:1 v/v (29). The Platelet-Rich Plasma (PRP) was separated from the blood by centrifugation at 327×g for 15 min at ambient temperature. The fibrinogen within the glue was precipitated from the PRP by ethanol precipitation at low temperature. The precipitated fibrinogen was separated by natural process at 3000×g for eight min at 0-4^{0C}. The separated fibrinogen together with the modified PRP was used to enrich it with growth factors. Finally, a mixture of calcium chloride (Merck, Cat. No. 102382) /topical bovine thrombin (T-4648, Sigma-Aldrich) (10 CC of 10% calcium chloride mixed with 10,000 units of topical bovine thrombin) was added to the platelet-enriched fibrinogen solution (35, 36) to organize PFG prior to operation.

In this study, BMP7 purchased from Sigma Company (Sigma b1434 10UG) was used and for Bone graft harvesting, Fragments of organic graft were removed from a dorsally addressed iliac wing, according to the technique by Bojrab (37). Polymethylmethacrylate (PMMA) samples were prepared by adding methylmethacrylate monomer (MMA) to PMMA powder at a ratio of 1.52 g/ml (38).

Animals and operative procedures

Twenty five male domestic rabbits, more than 6 months old, weighing 2.00±0.25 kg were randomly divided into 5 equal groups as follows: MT-blank cavity (without any biological or synthetic materials) (1), blank cavity (2), MT-autograft (3), MT-autograft-BMP7 (4) and MT-BMP7-PFG (5). The animals were kept in separate cages with ambient temperature of 24^{0C}, served a normal diet, and allowed to move freely during the study. All the animals underwent general anesthesia with xylazine 2% (2 mg/kg) for premedication, and anesthetized by intramuscular administration of ketamine 5% (30 mg/kg) and xylazine 2% (3 mg/kg). Both forelimbs in all animals were aseptically prepared for operation. The radius was uncovered by dissecting the underlying muscles after a 3 cm incision was made craniomedially over the skin of the forelimb. In the middle of each radius, a 20 mm segmental defect was formed. After removing 2 cm segmental bone, the bone defect was filled with PMMA cement_in animals of groups one, three, four, and five, simultaneously. The defect was not filled in animals of group two. The muscles were then sutured with Vicryl No 2-0. The skin was closed routinely. In our study 26 µg BMP7 was used in each rabbit. One month after operation, the second operation was performed. At this stage the skin was opened and the PMMA was then removed by cutting the created membrane and the biological material was placed in the position as mentioned and the membrane was sutured and the skin was then closed. In group 2, after one month, an incision was done on the induced membrane and then sutured as other groups.

The membrane had several advantages, some of which are as follows: it prevented bleeding while it was debrided out of the wound, and it saved the graft. The membrane also serves both as a mechanical vehicle (containment the bone graft) and a biological barrier (promotion of both angiogenesis and osteogenesis through secretion

of cytokines and growth factors). The membrane has a range of key growth factors including BMP2, VEGF, and TGF-β1 (8-13) and acts as a maintaining area for biologic material. The shiny membrane that is manufactured through this technique by induction of a periosteal membrane protects the graft from resorption by supplying the growth factors, cytokines, and vascularization as well as preventing soft-tissue interposition. The membrane secretes cytokines and growth factors including vascular and osteoinductive factors. The membrane acts as a barrier to outward diffusion of growth and osteoinductive factors and avoids excessive bleeding throughout the bone healing duration, helps avoid resorption of the graft and promotes its vascularity and corticalization simultaneously (5, 6, 39). The membrane acts as a biological chamber, and contains epithelial-like cells in the inner part, and fibroblasts, myofibroblasts, and type I collagen bundles in the outer part (3, 6, 8). Many of the layers have a lot of vascularization, and its proper blood circulation is essential in enhanced repair of the bone defect (40, 41). Despite its thin structure, the membrane has a certain mechanical strength, and forms a closed biological chamber after removing the cement, which maintains the volume of bone graft and prevents ingrowth of soft-tissue (3, 6, 40, 42).

In the present study, these compounds were added one month after the first surgery giving the wound the opportunity to be disinfected. The materials were then inserted on the operative site during the second surgery.

Postoperative evaluations

After the surgery, the clinical behavior, ability to use forelimbs, pain, swelling and other physical activities were evaluated. Fourteen weeks after the first surgery, the rabbits were euthanized. For this purpose, they received intramuscular injection of xylazine 2% (2 mg/kg) for premedication, anesthetized by intramuscular administration of 30 mg/kg ketamine 5% and 3 mg/kg xylazine 2%. Then, they were euthanazied by intra-cardiac injection of potassium chloride (KCl) (75-150 mg/kg). The samples were prepared for histological, biomechanical, scanning electron microscope (SEM), and radiological evaluations.

Radiological evaluation

Appropriate radiographs of each forelimb were taken at the 8th and 14th weeks after bone injury to evaluate bone formation, union, and remodeling of the defect. Each radiograph was scored according to the modified Lane and Sandhu scoring system [Table 1] (43).

Biomechanical evaluation

Following the cleaning of the bones' soft tissues, they were wrapped in saline soaked gauze and frozen at -20^{0C} until testing. The samples were defrosted at room temperature before testing. The mechanical properties

Table 1. Modified Lane and Sandhu radiological scoring system

parameter	Value	score
Bone formation	No evidence of bone formation	0
	Bone formation occupying 25% of the defect	1
	Bone formation occupying 50% of the defect	2
	Bone formation occupying 75% of the defect	3
	Bone formation occupying 100% of the defect	4
Union (proximal and distal evaluated separately)	No union	0
	Possible union	1
	Radiographic union	2
Remodeling	No evidence of remodeling	0
	Remodeling of medullary canal	1
	Full remodeling of cortex	2
Total point possible per category	Bone formation	4
	Proximal union	2
	Distal union	2
	Remodeling	2
Maximum score		10

Table 2. Emery's scoring system using for Histopathological evaluation

Value	score
gap was empty	0
gap was filled with fibrous connective tissue only	1
more fibrous tissue than fibrocartilage	2
more fibrocartilage than fibrous connective tissue	3
fibrocartilage only	4
more fibrocartilage than bone	5
more bone than fibrocartilage	6
filled only with bone	7

of bones including yield load, stiffness, elastic stress, and ultimate strength were determined using a three-point bending test. The samples were located horizontally on two grips with 30 mm distance and on diaphysis's midpoint, the third bar was lowered. The bones were loaded at a rate of 10mm/min before they fractured. These tests were performed using a universal tensile testing machine (Instron, London, UK) and the load-deformation curve was used to quantify aforementioned factors.

Histopathological evaluation

For microscopic examination, a transverse section containing one centimeter of both sides of the bone defect without any surrounding soft tissue was fixed in 10% neutral buffered formalin and decalcified in 15% buffered nitric acid solution. The specimens were then dehydrated in serial alcohol dilutions, embedded in paraffin, sectioned at five µm thickness, and finally stained with hematoxylin and eosin (H&E) and Masson's trichrome. Two pathologists blindly examined and scored the newly formed fibrous connective tissues, cartilage, and bone in the defect sites using Emery's scoring system [Table 2] (44). Moreover, the number of fibroblast-fibrocyte, osteoblast-osteocyte, chondroblast-chondrocyte, osteoclast, inflammatory cells, and osteons as well as the density of the fibrous connective tissue (FCT), osseous tissues (OT) and cartilaginous (CT) were recorded and analyzed.

SEM evaluation

To investigate the ultrastructure of bone healing in defect areas, SEM was done at 14th week post-operation. Initially, the representative samples were cut into one cm diameter, fixed in cold glutaraldehyde 2.5%, dehydrated in ascending graded sequence of ethanol (50 to 100%), and coated with gold covering. Factors such as degree of matrix calcification, collagen fibers and fibrils ,degree of matrix calcification, and hydroxyapatite crystals were examined by evaluating the high-qualified SEM images (S360, Cambridge, London, UK).

Statistical analysis

The quantitative results were represented as mean±SD and analyzed using a one-way ANOVA followed by a Tukey's post-hoc test. The Kruskal-Wallis H test, non-parametric ANOVA, and, if significant, the Mann Whitney U test were used to evaluate the scored values and biomechanical results. The scores between 8th week and 14th week were tested using paired T test. A *P<0.05* was considered as statistically significant. Statistical analyses were performed, using SPSS software, version 24.0 (SPSS, Inc., Chicago, USA).

Results

Radiological findings

The results obtained from radiographic evaluation of each group at the 8th and 14th weeks after bone injury have been presented in and [Table 3; Figure 1]. At the 8th week, there was a significant difference between the groups (*P=0.001*). Lane and Sandhu's score of MT-autograft-BMP7 group was higher than other groups. This superiority was seen at the 14th week too. At the 8th and 14th weeks, the MT-BMP7-PFG, MT-autograft, MT-blank cavity groups and blank cavity group respectively had lower scores compared to MT-autograft-BMP7. There was no significant difference between the groups in Lane and Sandhu's score at the 14th week after operation. At the 8th and 14th weeks, the Lane and Sandhu's score of MT-autograft group was lower than the MT-autograft-BMP7 and MT-BMP7-PFG groups. There was a significant difference between 8th and 14th week in MT-Autograft-BMP7 group (*P=0.008*),

Table 3. Radiological findings at the 8th and 14th weeks post-operation (Median (min-max)).

Group/Value	MT-blank cavity (1)	blank cavity (2)	MT-Autograft (3)	MT-Autograft-BMP7 (4)	MT-BMP7-PFG (5)	P[a]
8th week	4.25±1.66 4(2-6)	2.25±1.66 2(0-4)	5.12±2.16 5(2-8)	7±1.56 7.5(4-9)	5.3±1.82 5.5(3-8)	0.001
14th week	6.62±1.59 7(4-9)	5.62±2.38 5(3-9)	6.75±3.28 7.5(1-10)	8.6±1.5 9(6-10)	7.5±1.26 7(6-10)	0.06

[a] Kruskal-Wallis non-parametric ANOVA followed by Mannwhitney test post-hoc test. 8th week: *P=0.02*(2 vs. 3), *P=0.007*(2 vs. 5), *P=0.005*(4 vs. 1), *P=0.001*(4 vs. 2), *P=0.05* (4 vs. 3), *P=0.04*(4 vs. 5). Pair T test between 8th week and 14th week in each group: *P=0.12*(group 1), *P= 0.02* (group 2), *P=0.15*(group 3), *P=0.008*(group 4), *P= 0.01*(group 5).

MT-BMP7-PFG group (*P=0.01*) and blank cavity group (*P=0.02*).

Biomechanical findings

As shown in, the ultimate load in the MT-autograft-BMP7 group was higher than that of the other groups, but, the differences were not significant (*P=0.69*) [Table 4]. The Yield load in the MT-BMP7-PFG was greater than the MT-autograft-BMP7, blank cavity, MT-autograft and MT-blank cavity groups, respectively, but, the differences were not significant (*P=0.88*). The stress in the MT-blank cavity group was higher than blank cavity, MT-BMP7-PFG, MT-autograft and MT-autograft-BMP7 groups, respectively, but, there was no significant difference between them (*P=0.44*). The stiffness in the blank cavity group was higher than the MT-autograft-BMP7, MT-BMP7-PFG, MT-blank cavity and MT-autograft groups, respectively, but, there was no significant difference between them (*P=0.31*).

Histopathological findings

Qualitative report

Microscopically, the least amount of new bone formation, fibrous connective tissue with a few newly formed hyaline cartilage foci were observed in the defect sites of the blank cavity and MT-blank cavity groups. In the MT-autograft and MT-autograft-BMP7 groups, a nonhomogeneous mixed matrix of fibrocartilage tissue, hyaline cartilage, and woven bone replaced the defect sites. In the MT-BMP7-PFG group, new bone formation was notable in the defect sites. The radial bone edges were regenerated towards the center of the defect sites by hyaline and fibrous cartilage. The middle part of the defect area was filled with the newly woven bone. Also, these histopathological changes were observed in the Masson's trichrome stained tissue sections [Figure 2]. No marked inflammatory response was evident in all groups at the 14th week after injury, although it may have been present earlier.

Quantitative report

In histomorphometric analysis, the MT-blank cavity group had the highest numbers of fibroblast-fibrocyte and the lowest osteoblast-osteocyte cells [Table 5].

The MT-BMP7-PFG group demonstrated higher osteoblast-osteocyte cells compared with the MT-blank cavity group (*P=0.03*) and blank cavity group (*P=0.03*). There were no statistically significant differences between the MT-autograft and the other groups in all analyzed parameters, aside from the number of osteoclasts that were superior in the MT-autograft-BMP7 group (*P=0.014*). The MT-BMP7-PFG group had significantly lower number of osteoclasts compared with the MT-autograft-BMP7 group (*P=0.009*), at this stage of bone healing. In addition, there were no significant differences between all groups in fibroblast-fibrocyte cells (*P=0.40*), osteons (*P=0.33*) and inflammatory cell counts (*P=0.09*). No statistically significant differences were seen between the blank cavity group and the other groups in all analyzed parameters, with the exception of the number of osteoclasts which were superior in the MT-autograft-BMP7 group (*P=0.008*) and the number of chondroblast-chondrocytes that were higher in the blank cavity group compared to MT-BMP7-PFG group (*P=0.03*). There were no statistically significant differences between the MT-blank cavity group and the other groups in all parameters analyzed, with the exception of the number of osteoclasts which were superior in the MT-autograft-BMP7 group (*P=0.008*) and the superior number of osteoblast-osteocytes in the MT-BMP7-PFG group (*P=0.03*).

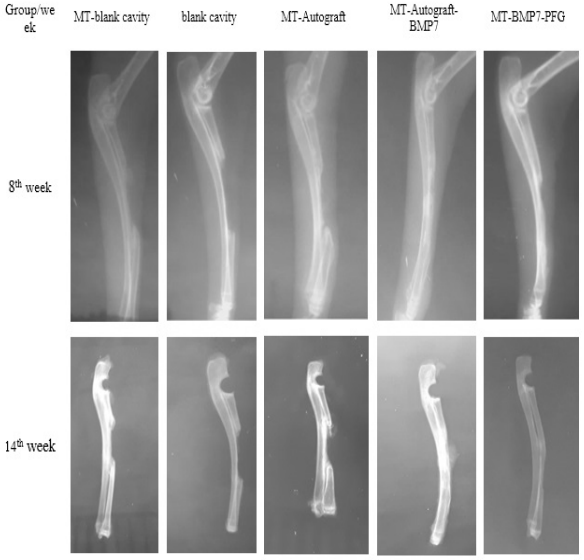

Figure 1. Radiographs of bone healing in the defected area of different groups at 8th and 14th weeks postoperative.

Table 4. Biomechanical performance of the healing radial bone defects from the three-point bending test at the 14th post-operative week (Mean±SD)

Group/biomechanical performance	MT-blank cavity	Blank cavity	MT-Autograft	MT-Autograft-BMP7	MT-BMP7-PFG	p[a]
Ultimate load (N)	69.75±16.62	69±19.36	62.37±30.58	85.4±28.49	64.6±24.95	0.69
Yield load (N)	43.75±30.83	53.7±32.9	44.5±32.7	59.4±39.44	61.1±22.73	0.88
Stress (N/mm^2)	4.24±1.48	2.69±2.23	2.58±0.62	2.33±0.89	2.59±1.57	0.44
Stiffness (N/mm)	4.4±3.43	7.09±2.98	3.30±0.82	5.08±3.77	4.59±2.16	0.31

[a] Kruskal-Wallis non-parametric ANOVA.

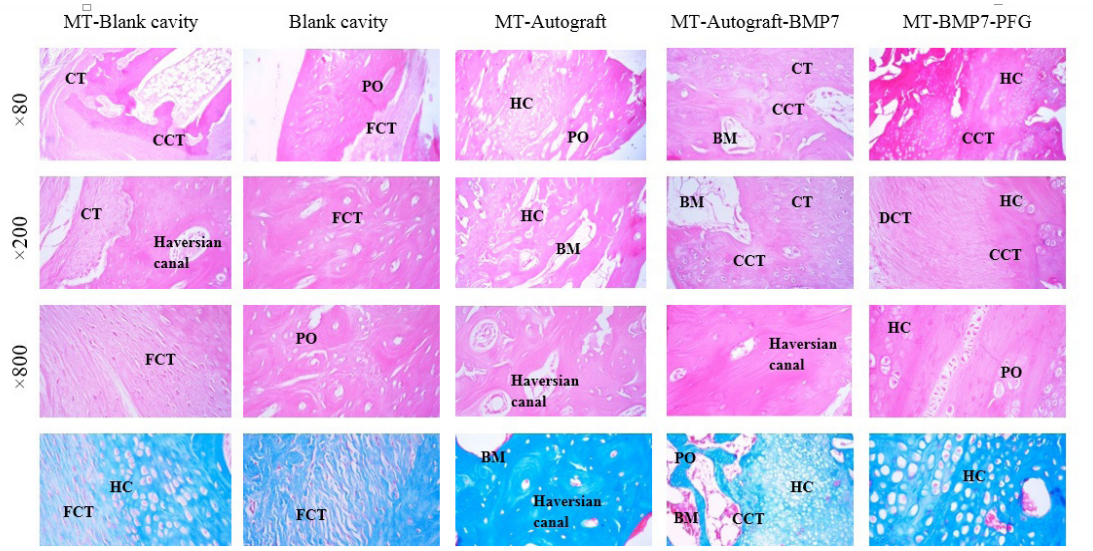

Figure 2. Histological views of the longitudinal sections of the radial bone defects in rabbits after 14 weeks of injury. The defect areas of all rabbits contained various content of fibrous connective tissue, fibrocartilage and hyaline cartilage tissue and new bone tissue. The defect sites of the MT-autograft and MT-Autograft-BMP7 groups were replaced by a nonhomogeneous mixed matrix consisted of fibrocartilage, hyaline cartilage, and woven bone tissues. The blank cavity and the MT- blank cavity groups contained the least amount of new bone and often were filled with a mixture of fibrous connective tissue and cartilage. New bone formation was considerable in the defect sites of the MT-BMP-PFG group. Abbreviations: LACT: Loose areolar connective tissue; FCT: Fibrous connective tissue; CT: Connective tissue; CCT: Calcified cartilaginous tissue; BM: Bone marrow; HC: Hyaline cartilage; PO: primary osteon; WB: Woven bone; DCT: Dense connective tissue.

Table 5. Histomorphometric characteristics of the healed bone defects at the 14th post-operation weeks (Mean±SD) (Median (Min-Max)).

Group/Factor	MT-Blank cavity (1)	Blank cavity (2)	MT-Autograft (3)	MT-Autograft-BMP7 (4)	MT-BMP7-PFG (5)	P[d]
Fibroblast -fibrocyte (n)	16.62±24.46 1.5(5-62)	5.62±14.8 0(0-60)	15.06±24.64 1(0-70)	7.44±9.72 4.5(0-34)	8.75±18.53 0(0-57)	0.40
Chondroblast -chondrocyte(n)	6.69±11.04 0(0-32)	21.75±25.21 11(0-70)	12.06±15.59 4.5(0-55)	14.38±24.15 2.5(0-78)	2.75±6.47[a] 0(0-25)	0.041
Osteoblast - osteocyte (n)	22.88±13.14 22(0-50)	24.25±7.47 24(5-34)	27.19±16.64 24(0-56)	27.06±21.69 28(0-64)	39.12±15.42[b] 38(13-63)	0.03
Osteoclast (n)	0	0	0.56±1.78 0(0-7)	9.69±17.64[c] 0(0-47)	0.12±0.5 0(0-2)	0.002
Osteon (n)	0	0.6±0.25 0(0-1)	0.12±0.34 0(0-1)	0.25±0.77 0(0-3)	0.38±0.88 0(0-3)	0.33
Inflammatory cells (n)	0	0.56±1.54 0(0-5)	0	0	3±8 0(0-30)	0.09
Density of OT (%)	49.5±24.2 39(14-81.2)	42.7±27.94 35.3(19.2-73.6)	69.8±10.01 69.8(62.6-77)	52.67±18.75 51.05(34.7-73.9)	58±15.04 59.1(42-79.1)	0.66
Density of FCT (%)	0	0	0	10.64±1.23 4(0-28)	1.4±3.13 0(0-7)	0.13
Density of CT (%)	0	0	18.65±26.37 18.65(0-37.3)	22.85±24.6 22(0-60)	22.72±14.24 26.2(0-35.4)	0.16

dOne-way ANOVA followed by Tukey post-hoc test. a *P=0.03*(5 vs. 2); b *P=0.03* (5 vs. 1); c *P= 0.014* (4 vs. 3) .*P= 0.009* (4 vs. 5), *P= 0.008* (4 vs. 2), and *P=0.008* (4 vs. 1).

In MT-autograft, the density of osseous tissue (OT) was higher than the other groups (*P=0.66*), while there was no significant difference among the groups. The MT-autograft-BMP7 group showed higher density of the fibrocartilaginous tissue (FCT) than the other groups (*P=0.13*). Although the cartilage density (CT) in the MT-autograft-BMP7 group was highest, there was no significant difference between the groups (*P=0.16*). The MT-BMP7-PFG and MT-autograft groups were ranked as the 2nd and 3rd groups.

The histopathological scores (Emery's score) of all groups showed no significant difference (*P=0.17*) at 14 weeks after the surgery [Table 6]. In addition, at week 14, when the histopathological scores of the MT-autograft group were compared to each other, no significant differences were found (*P=0.17*) but its score was higher than the blank cavity and the MT-blank cavity groups was lower than the MT-autograft-BMP7 and MT-BMP7-PFG groups. Emery's score in group MT-blank cavity was lowest among all the groups.

SEM findings

After 14 weeks of bone injury, in the MT-autograft-BMP7 group, a hard callus and cartilaginous matrix with various degrees of calcification and enormous amounts of hydroxyapatite crystals filled the defect. The defect in the MT-autograft group was replaced with fibrocartilage tissue with high calcified matrix. In some areas, a loose connective tissue consisting of immature collagen fibrils filled the defect that was oriented irregularly with no hydroxyapatite crystals. A highly calcified bone matrix and hard callus filled the defect site in the MT-BMP7-PFG group. Irregular and regular collagen fibers filled the defects in blank cavity group. Low density of collagen fibers was seen in MT-blank cavity group. There are three pictures for each group in [Figure 3].

Table 6. Histopathological scores for healing of the bone defects after 14 weeks of injury (Mean±SD) (Median (Min-Max)).

	MT- Blank cavity	Blank cavity	MT-Autograft	MT-Autograft-BMP7	MT-BMP7-PFG	P[a]
Emery's score	2.75±3.2 2.5(0-6)	4.25±2.98 5(0-7)	6±0.81 6(5-7)	6.25±0.5 6(6-7)	6.25±0.95 6.5(5-7)	0.17

[a] Kruskal-Wallis non-parametric ANOVA.

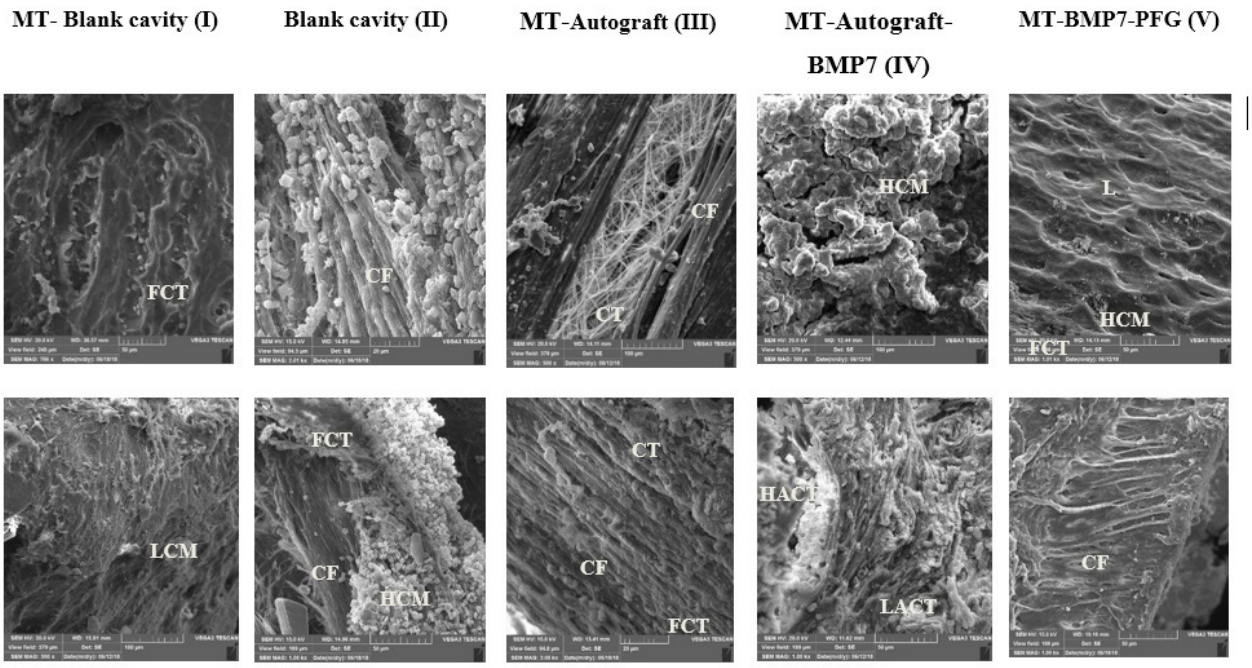

Figure 3. Scanning ultramicrographs of the radial bone defects at the 14th week after bone injury. I: Low density of collagen fibers was seen in the MT-blank cavity group. II: Irregular and regular collagen fibers filled the defects in the blank group. III: The defects in the MT-autograft group were replaced with fibrocartilage tissue with high calcified matrix and in some areas with a loose areolar connective tissue. IV: The defects were filled with hard callus and cartilaginous matrix containing various low to high degrees of calcification. Large amounts of hydroxyapatite crystals were seen in the defect sites of MT-Autograft-BMP group. V: A highly calcified bone matrix and hard callus filled the defect site in the MT-BMP7-PFG group. Abbreviations: CF: Collagen fibrils; CT: Connective tissue; HCM: Highly calcified matrix; HC: Haversian canal; L: Lacuna; FCT: Fibrocartilaginous tissue; LCM: Low calcified matrix

Discussion

Healing of major traumatic bone loss is a major challenge in orthopedic issue due to the risk of infection, long-term healing, and poor long-term clinical results (9, 45, 46). Bone defect etiology can be multifactorial, including acute bone loss, bone debridement after infection, non-union or avascular bone secondary to prior radiotherapy and tumor excision (47). Surgical reconstruction of segmental skeletal defects that may result from great trauma after debridement of osteomyelitis or tumor resection is one of the hardest problems in orthopedic surgery. Many options are available to manage such problems with varying degrees of success and failure (48-50). Masquelet *et al.* (5)) identified a technique that allows soft tissue regeneration, reduces the risk of infection, and avoids graft resorption (51).

To evaluate the bone healing potential of autograft, PFG, BMP7, and their combinations as well as Masquelet technique, a defect model was developed in rabbit radial bone. The segmental defect was created in the middle part of the radius approximately 20 mm in order to cause a non-union defect as a critical size defect and to avoid spontaneous and quick healing.

Different segmental bone defect from 5mm to more than 20 mm has been used by the researchers. Also, there are different ideas about the gap as a critical size for the bone (52-58). In our opinion, preferably 15 mm gap for the rats and 15 mm or more for the rabbits are suitable.

More weight-bearing is on the hind limbs in rabbits. The fore limbs also bear weight. Especially the rabbits use their hands for feeding. Radius has been used for fracture healing in different studies (59-69). Actually the ulna supports the radius, so, no need for extra fixation of radius. The fixation (any technique) has some effects and interferes on healing process. We didn't use fixation for the radius in this study, but, the undue effects of fixation technique has been removed.

The theory was based on the fact that both BMP7 and PFG may have some positive effects on bone healing and Masquelet facilitates bone healing.

No statistically significant differences was seen in histomorphometry between the blank cavity group and the other groups for any of the analyzed parameters, with the exception of the number of osteoclasts that was higher in the MT-autograft-BMP7 group (*P=0.008*) and the number of chondroblast-chondrocytes that was higher in the blank cavity group compared to MT-BMP7-PFG group (*P=0.03*). Therefore, it seems the Masquelet technique enhances the healing processes. Advanced stages of bone healing were evident in groups with Masquelet technique while the chondrocytes in the defect field were converted into osteoblasts. Compared to many of the previously published investigations that used Masquelet technique with autograft, we used this technique with autograft and other biological materials (40, 41).

The Masquelet technique was used in limitary and was mostly reported as case reports, but, although it is time-consuming in large defects, we found that it can be done routinely if it is costly. Combination of Masquelet technique with using other biological materials has not been tried in other studies. In other studies bone biological materials have been added to the operative site in first surgery. In the present study, these compounds were added one month after the first surgery and this makes it possible for the wound to have a chance to be disinfected and then the materials can be inserted on the operative site during second surgery.

Limited studies have been done on the Masquelet technique in animal models. A study on mid-diaphyseal metatarsal bone defects (25 mm) of sheep with plate and PMMA (for first step) and morcellized autologous corticocancellous graft and external coaptation for 6 months (for second step) resulted in proper bone formation (40). Another study on diaphyseal femoral defects (3 cm) of sheep showed that after 16 weeks, the group with induced membrane and graft was filled with the new bone tissue compared with the graft, induced membrane and no graft-no induced membrane group (41). Another study performed on rabbits showed that after implantation of ceramic implants and loading with BMP7 in heterotopic sites like in a subcutaneous and in a membrane previously subcutaneously induced, no bone formation was seen at 4 and 16 weeks groups with untreated implants. However, less bone formation and resorption (80%) was seen when the implants were placed in an induced membrane (70). Miniplate and PMMA implanted after segmental osteotomy of mandible in rabbits (first step) and triphasic calcium phosphate and cancellous autograft hydroxyapatite (second step), demonstrated that VEGF was positive in the induced membranes and higher number of capillaries were formed at one, three, and six months post-operation (71). These trials were performed in situations similar to our study on experimentally induced bone defect in the rabbit model.

Histomorphometry showed no statistically significant differences between the MT-blank cavity and other groups for any of the parameters analyzed in any of the areas of interest; however, the number of osteoclasts was higher in the MT-autograft-BMP7 group (P=0.008) and the number of osteoblast-osteocytes was higher in the MT-BMP7-PFG group (P=0.03). Therefore, the presence of biological materials resulted in enhanced bone healing. The results of the radiological, histological, biomechanical analysis and SEM evaluation showed that regardless of the MT-autograft group, the best results were gained from the PFG-BMP7 treated defects followed by autograft-BMP7 treated ones. Healing of the MT-blank cavity group was associated with slight dominance to the blank cavity group but the difference between them was not significant. Really, the healing process in the MT-blank cavity group stayed in the early stages and fibrous connective tissue was the main constituent in this group, while, healing was more advanced in the MT-BMP7-PFG, MT-autograft-BMP7 and MT-autograft groups and some signs of cartilage formation was seen in the blank cavity group. Also, these changes were seen in radiological results at the 8th week.

A comprehensive history in the literature has proposed the application of BMP7 as an admitted biomaterial in bone tissue engineering in different animal models (52, 72-75). However, there are a lot of controversies in the

application of BMPs in the healing of different bone defects and non-unions (76). Here we showed that BMP7 combined with autograft and PFG has a positive role in bone regeneration in vivo. Also, it has been shown that BMP7 has similar effects on intact control group (52).

It has been shown that rhBMP7 is as effective as autograft ,when used with intramedullary rod fixation, in treatment of tibial nonunion and in femoral fracture nonunions (19, 47). Since angiogenesis and penetration of vascular endothelial cells into the soft callus is stimulated by proangiogenic factors such as VEGF, BMPs, TGF-β, FGF, and angiopoietins BMPs play a main role in bone healing (77, 78). Our results showed that BMP7 was more effective than autograft in treatment of radial defect in rabbits. In another study on 395 patients with nonunion, the success rate of using a single application of 3.5 mg rhBMP7 with proper surgical fixation was up to 82% and no complications was reported after application of rhBMP7 (47, 79). Compared with the MT-blank cavity group and the blank cavity group with spontaneous healing process, the PFG-BMP7, BMP7-autograft and autograft treated groups had higher bone and cartilaginous cells than the other groups and the healing was more advanced, in the initial steps of bone healing.

In line with our findings, a massive background in the literature has proposed PFG to be applied as an suitable biomaterial in bone tissue engineering (29, 32, 33). This study was performed to give more insight on the effect of BMP7 combination with autograft and PFG on bone regeneration by Masquelet technique. The results of the present research confirmed a variety of clinical and laboratory trials and demonstrated the positive effect of PFG in bone substitute materials on bone healing (34, 80-82).

However, in some other researches, the graft materials did not increase bone healing when completed with the platelet derived products (83-85). In our study, the PFG-BMP7 and autograft-BMP7 treated groups had higher cartilaginous and bone cells and these treatment regimens resulted in more advanced bone healing and were as good as the MT-autograft group and they were also better in some criteria than the MT-autograft group. In addition to other results, we could state that PFG-BMP7 and Autograft-BMP7 have some osteoinductive properties because some signs of new bone formation were seen after 14 weeks of injury in both groups. New bone formation was not limited to both defect edges and bone formation was induced in the middle of the defect area with these components and thus, they displayed high osteoconductive properties.

While in most cases the differences were not significant, the new bone formation and the volume of bone tissue present in the defect sites of the MT-BMP7-PFG and MT-BMP7-autograft groups were more than the MT-autograft group and were higher than that in the MT-blank cavity and the blank cavity groups. Based on the histological results, the volume of bone tissue in the MT-autograft group, MT-BMP7-PFG and MT-autograft-BMP7 was higher than other groups. The MT-blank cavity group and the blank cavity group had lower volume of bone tissue. The highest volume of chondral tissue was seen in the MT-BMP7-autograft, MT-BMP7-PFG and MT-autograft group, respectively. The MT-autograft-BMP7 group had lower bone tissue but higher chondral tissue and connective tissue than the MT-BMP7-PFG group. Based on these results, this group was in initial stage of bone healing as the cartilage tissue can be converted to bone and the defect can be filled completely later. The MT-BMP7-PFG group had lower bone and higher fibrous connective tissue volume and chondral tissue than the MT-autograft group. It seems that these cartilagineous tissues can be converted to bone tissue in near future. The density of the fibrous connective tissue in the MT-autograft-BMP7 was more than the MT-BMP7-PFG group. This factor in MT-BMP7-PFG group is higher than MT-autograft group. The volume of healing tissue in these groups is considerable.

Osteoclasts are the main cells involved in resorption of the calcified bone. As several growth factors and cytokines such as BMPs, ILs, TNF-α and TGF-β promote osteoclastogenesis (86). The number of osteoclasts in the MT-autograft-BMP7 group was significantly higher than other groups in our study. The number of osteoblast-osteocyte in the MT-BMP7-PFG group was significantly higher than the MT-blank cavity group. Some studies have pointed out that the proliferation of fibroblasts and chondrocytes is particularly due to TGF-β, FGF, PDGF, IGF and BMP growth factors (77, 78, 87, 88), while in our study, we showed that the MT-BMP7-PFG group had the least chondroblast-chondrocyte cells. chondroblast-chondrocytes were significantly superior in the blank cavity group compared with the MT-BMP7-PFG group. Therefore in histopathology sections of MT-BMP7-PFG group, chondroblast- chondrocytes were converted to osteoblast-osteocyte cells and MT-BMP7-PFG group was at a more advanced stage in bone repair. The percentage of OT in MT-BMP7-PFG group was higher than the blank cavity group and confirms the above description.

The difference between the histology scoring results and cell counting is perhaps due to the differences in the sections selected. In this study, the number of inflammatory cells was low because of the presence of induced membrane. The membrane has several advantages, for example, it prevented bleeding while it is debrided out of the wound, and saved the graft. The membrane serves both a mechanical (containment of the bone graft) and a biological function (promotion of both angiogenesis and osteogenesis through secretion of growth factors).

In this study, because of the use of Masquelet technique the healing process was more rapid. The induced membrane prevents bone graft resorption by providing growth and vascularization factors and preventing soft tissue interposition. It secretes vascular and osteoinductive factors and acts as a barrier for outward diffusion of growth and osteoinductive factors during bone healing and avoids excessive bleeding (6, 39). It also helps to avoid early resorption of the graft and simultaneously promotes its vascularity and corticalization (5). The membrane acts as a biological shiny chamber, and consists of epithelial-like cells in inner part and myofibroblasts, fibroblasts and type I

collagen bundles in the outer part (3, 6, 8). In all its layers, it is richly vascularized, and blood vessels refer to the formation of bone defect (40, 41). Despite the thin structure, it has mechanical strength, and forms a closed biological chamber after removing the cement, which maintains the volume of the bone graft and prevents ingrowth of soft-tissue (3, 6, 40, 42).

This study showed that autograft-BMP7 and PFG-BMP7 could promote bone healing in the long bone defects in the rabbit model better than using only autograft. Combinations of these materials with Masquelet technique are useful for big segmental defects. This finding introduces the osteogenic materials in combination with Masquelet technique as a favorable alternative for the reconstruction of large diaphyseal defects in long bones in animal models and the findings of our study may be useful for human clinical use in the future, as rabbits have similar Haversian systems to humans. (89).

Ethics and consent to participate: All animals received humane care in accordance with the guide for care and use of laboratory animals published by the National Institutes of Health (NIH publication No. 85-23). The study was approved by the local ethics committee of "Regulations for using animals in scientific procedures" in our school of veterinary medicine. We obtained written informed consent to use the animals in our study from the owners of the animals.

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References

1. Motsitsi N. Masquelet's technique for management of long bone defects: from experiment to clinical application. East and Central African Journal of Surgery. 2012;17(2):43-7.
2. Hertel R, Gerber A, Schlegel U, Cordey J, Rüegsegger P, Rahn B. 10. Cancellous bone graft for skeletal reconstruction Muscular versus periosteal bed—Preliminary report. Injury. 1994;25(1):SA59-SA70.
3. Viateau V, Bensidhoum M, Guillemin Gv, Petite H, Hannouche D, Anagnostou F, et al. Use of the induced membrane technique for bone tissue engineering purposes: animal studies. The Orthopedic clinics of North America. 2010;41(1):49-56.
4. Tong K, Zhong Z, Peng Y, Lin C, Cao S, Yang Y, et al. Masquelet technique versus Ilizarov bone transport for reconstruction of lower extremity bone defects following posttraumatic osteomyelitis. Injury. 2017; 48(7):1616-22.
5. Masquelet A, Fitoussi F, Begue T, Muller G, editors. Reconstruction of the long bones by the induced membrane and spongy autograft. Annales de chirurgie plastique et esthetique; 2000;45(Suppl 3):346–353.
6. Masquelet AC, Begue T. The concept of induced membrane for reconstruction of long bone defects. Orthopedic Clinics. 2010;41(1):27-37.
7. Gouron R, Deroussen F, Juvet M, Ursu C, Plancq M-C, Collet L-M. Early resection of congenital pseudarthrosis of the tibia and successful reconstruction using the Masquelet technique. The Journal of bone and joint surgery British volume. 2011;93(4):552-4.
8. Pelissier P, Masquelet A, Bareille R, Pelissier SM, Amedee J. Induced membranes secrete growth factors including vascular and osteoinductive factors and could stimulate bone regeneration. Journal of orthopaedic research. 2004;22(1):73-9.
9. Giannoudis PV, Faour O, Goff T, Kanakaris N, Dimitriou R. Masquelet technique for the treatment of bone defects: tips-tricks and future directions. Injury. 2011;42(6):591-8.
10. Karger C, Kishi T, Schneider L, Fitoussi F, Masquelet A-C. Treatment of posttraumatic bone defects by the induced membrane technique. Orthopaedics & Traumatology: Surgery & Research. 2012;98(1):97-102.
11. Aho O-M, Lehenkari P, Ristiniemi J, Lehtonen S, Risteli J, Leskelä H-V. The mechanism of action of induced membranes in bone repair. JBJS. 2013;95(7):597-604.
12. Yin Q, Sun Z, Gu S. Progress of Masquelet technique to repair bone defect. Zhongguo xiu fu chong jian wai ke za zhi= Zhongguo xiufu chongjian waike zazhi= Chinese journal of reparative and reconstructive surgery. 2013;27(10):1273-6.
13. Christou C, Oliver RA, Yu Y, Walsh WR. The Masquelet technique for membrane induction and the healing of ovine critical sized segmental defects. PLoS One. 2014;9(12).
14. Cecchi S, Bennet SJ, Arora M. Bone morphogenetic protein-7: Review of signalling and efficacy in fracture healing. Journal of orthopaedic translation. 2016;4:28-34.
15. Lane JM. Bone morphogenic protein science and studies. Journal of orthopaedic trauma. 2005; 19(10):S17-S22.
16. Epstein NE. Complications due to the use of BMP/INFUSE in spine surgery: the evidence continues to mount. Surgical neurology international. 2013; 4(Suppl 5):S343-52.
17. Bowler D, Dym H. Bone Morphogenic Protein. J Dental Clinics of North America. 2015;59(2):493-503.
18. Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N,

Ikeda T, et al. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. The Journal of cell biology. 1994;127(6):1755-66.
19. Friedlander GE. OP-1 clinical studies. J Bone Joint Surg Am. 2001;83(1):160-1.
20. Derner R, Anderson AC. The bone morphogenic protein. Clinics in podiatric medicine and surgery. 2005;22(4):607-18.
21. Kaur S, Grover V, Kaur H, Malhotra R. Evaluation of bone morphogenic proteins in periodontal practice. Indian journal of dentistry. 2016;7(1):28-37.
22. Reddi AH. Bone morphogenetic proteins: from basic science to clinical applications. JBJS. 2001;83(1_ suppl_1):S1-S6.
23. Cook SD. Preclinical and clinical evaluation of osteogenic protein-1 (BMP-7) in bony sites. Orthopedics. 1999;22(7):669-71.
24. Sciadini MF, Johnson KD. Evaluation of recombinant human bone morphogenetic protein-2 as a bone-graft substitute in a canine segmental defect model. Journal of Orthopaedic Research. 2000;18(2):289-302.
25. Pluhar GE, Manley PA, Heiner JP, Vanderby Jr R, Seeherman HJ, Markel MD. The effect of recombinant human bone morphogenetic protein-2 on femoral reconstruction with an intercalary allograft in a dog model. Journal of orthopaedic research. 2001;19(2):308-17.
26. Badlani N, Oshima Y, Healey R, Coutts R, Amiel D. Use of bone morphogenic protein-7 as a treatment for osteoarthritis. Clinical Orthopaedics and Related Research®. 2009;467(12):3221-9.
27. Spanjol J, Dordevic G, Markic D, Fuckar Cupic D, Krpina K, Bobinac D. Bone Morphogenetic Protein 7 and Pyelonephritis. Collegium antropologicum. 2010;34(2):61-4.
28. Lories RJ, Luyten FP. Bone morphogenetic proteins in destructive and remodeling arthritis. Arthritis research & therapy. 2007;9(2):207.1-7
29. Thorn J, Sørensen H, Weis-Fogh U, Andersen M. Autologous fibrin glue with growth factors in reconstructive maxillofacial surgery. International journal of oral and maxillofacial surgery. 2004; 33(1):95-100.
30. Burnouf T, Su CY, Radosevich M, Goubran H, El-Ekiaby M. Blood-derived biomaterials: fibrin sealant, platelet gel and platelet fibrin glue. ISBT Science Series. 2009;4(1):136-42.
31. Burnouf T, Goubran HA, Chen T-M, Ou K-L, El-Ekiaby M, Radosevic M. Blood-derived biomaterials and platelet growth factors in regenerative medicine. Blood reviews. 2013;27(2):77-89.
32. Zhu S-J, Choi B-H, Jung J-H, Lee S-H, Huh J-Y, You T-M, et al. A comparative histologic analysis of tissue-engineered bone using platelet-rich plasma and platelet-enriched fibrin glue. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2006;102(2):175-9.
33. Lee H-J, Choi B-H, Jung J-H, Zhu S-J, Lee S-H, Huh J-Y, et al. Maxillary sinus floor augmentation using autogenous bone grafts and platelet-enriched fibrin glue with simultaneous implant placement. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2007;103(3):329-33.
34. Gholipour H, Meimandi-Parizi A, Oryan A, Sadegh AB. The effects of gelatin, fibrin-platelet glue and their combination on healing of the experimental critical bone defect in a rat model: radiological, histological, scanning ultrastructural and biomechanical evaluation. Cell and tissue banking. 2018;19(3):341-56.
35. Butterfield KJ, Bennett J, Gronowicz G, Adams D. Effect of platelet-rich plasma with autogenous bone graft for maxillary sinus augmentation in a rabbit model. Journal of Oral and Maxillofacial Surgery. 2005;63(3):370-6.
36. Findikcioglu K, Findikcioglu F, Yavuzer R, Elmas C, Atabay K. Effect of platelet-rich plasma and fibrin glue on healing of critical-size calvarial bone defects. Journal of Craniofacial Surgery. 2009;20(1):34-40.
37. Bojrab MJ. Enxertamento osseo. Técnicas atuais em cirurgia de pequenos animais 3a ed Sao Paulo: Roca. 1996:786-93.
38. Fini M, Cadossi R, Canè V, Cavani F, Giavaresi G, Krajewski A, et al. The effect of pulsed electromagnetic fields on the osteointegration of hydroxyapatite implants in cancellous bone: a morphologic and microstructural in vivo study. Journal of orthopaedic research. 2002;20(4):756-63.
39. Mühlhäusser J, Winkler J, Babst R, Beeres FJ. Infected tibia defect fractures treated with the Masquelet technique. Medicine. 2017;96(20):1-17.
40. Viateau V, Guillemin G, Calando Y, Logeart D, Udina K, Sedel L, et al. Induction of a barrier membrane to facilitate reconstruction of massive segmental diaphyseal bone defects: an ovine model. Veterinary Surgery. 2006;35(5):445-52.
41. Klaue K, Knothe U, Anton C, Pfluger DH, Stoddart M, Masquelet AC, et al. Bone regeneration in long-bone defects: tissue compartmentalisation? In vivo study on bone defects in sheep. Injury. 2009;40(4):S95-S102.
42. Viateau V, Guillemin G, Bousson V, Oudina K, Hannouche D, Sedel L, et al. Long-bone critical-size defects treated with tissue-engineered grafts: A study on sheep. Journal of orthopaedic research. 2007;25(6):741-9.
43. Lane JM, Sandhu H. Current approaches to experimental bone grafting. The Orthopedic clinics of North America. 1987;18(2):213-25.
44. Emery SE, Brazinski MS, Koka A, Bensusan JS, Stevenson S. The biological and biomechanical effects of irradiation on anterior spinal bone grafts in a canine model. The Journal of bone and joint surgery American volume. 1994;76(4):540-8.
45. Papakostidis C, Kanakaris NK, Pretel J, Faour O, Morell DJ, Giannoudis PV. Prevalence of complications of open tibial shaft fractures stratified as per the Gustilo–Anderson classification. Injury. 2011;42(12):1408-15.
46. Calori G, Colombo M, Mazza E, Ripamonti C, Mazzola S, Marelli N, et al. Monotherapy vs. polytherapy in the treatment of forearm non-unions and bone defects. Injury. 2013;44(1):S63-S9.
47. Kanakaris NK, Tosounidis TH, Giannoudis PV. Surgical

management of infected non-unions: an update. Injury. 2015;46(5):S25-S32.
48. Ilizarov G, Ledyaev V. The replacement of long tubular bone defects by lengthening distraction osteotomy of one of the fragments. Clinical Orthopaedics and Related Research (1976-2007). 1992;280:7-10.
49. DeCoster TA, Gehlert RJ, Mikola EA, Pirela-Cruz MA. Management of posttraumatic segmental bone defects. JAAOS-Journal of the American Academy of Orthopaedic Surgeons. 2004;12(1):28-38.
50. Levin SL. Vascularized fibula graft for the traumatically induced long-bone defect. JAAOS-Journal of the American Academy of Orthopaedic Surgeons. 2006;14(10):S175-S6.
51. McCall TA, Brokaw DS, Jelen BA, Scheid DK, Scharfenberger AV, Maar DC, et al. Treatment of large segmental bone defects with reamer-irrigator-aspirator bone graft: technique and case series. Orthopedic Clinics. 2010;41(1):63-73.
52. Cook SD, Baffes GC, Wolfe MW, Kuber Sampath T, Rueger DC, Whitecloud Iii TS. The effect of recombinant human osteogenic protein-1 on healing of large segmental bone defects. J Bone Joint SurgA Am. 1994;76(6):827-38.
53. Geiger F, Bertram H, Berger I, Lorenz H, Wall O, Eckhardt C, et al. Vascular endothelial growth factor gene-activated matrix (VEGF165-GAM) enhances osteogenesis and angiogenesis in large segmental bone defects. Journal of Bone and Mineral Research. 2005;20(11):2028-35.
54. Cooper GM, Mooney MP, Gosain AK, Campbell PG, Losee JE, Huard J. Testing the "critical-size" in calvarial bone defects: revisiting the concept of a critical-sized defect (CSD). Plastic and reconstructive surgery. 2010;125(6):1685.
55. Mills L, Simpson A. In vivo models of bone repair. The Journal of bone and joint surgery British volume. 2012;94(7):865-74.
56. Vajgel A, Mardas N, Farias BC, Petrie A, Cimões R, Donos N. A systematic review on the critical size defect model. Clinical oral implants research. 2014;25(8):879-93.
57. Bigham-Sadegh A, Oryan A. Selection of animal models for pre-clinical strategies in evaluating the fracture healing, bone graft substitutes and bone tissue regeneration and engineering. Connective tissue research. 2015;56(3):175-94.
58. Liu F, Chen K, Hou L, Li K, Wang D, Zhang B, et al. Determining the critical size of a rabbit rib segmental bone defect model. Regenerative biomaterials. 2016;3(5):323-8.
59. Oryan A, Alidadi S, Bigham-Sadegh A, Moshiri A. Effectiveness of tissue engineered based platelet gel embedded chitosan scaffold on experimentally induced critical sized segmental bone defect model in rat. Injury. 2017;48(7):1466-74.
60. Oryan A, Bigham-Sadegh A, Monazzah S. The effect of hPRP and autogrfat-PRP on bone healing in the radial defect of rat. Journal of Musculoskeletal Research. 2016;19(01):1650003.
61. Moshiri A, Shahrezaee M, Shekarchi B, Oryan A, Azma K. Three-dimensional porous gelapin–simvastatin scaffolds promoted bone defect healing in rabbits. Calcified tissue international. 2015;96(6):552-64.
62. Shafiei-Sarvestani Z, Oryan A, Bigham AS, Meimandi-Parizi A. The effect of hydroxyapatite-hPRP, and coral-hPRP on bone healing in rabbits: radiological, biomechanical, macroscopic and histopathologic evaluation. International Journal of Surgery. 2012;10(2):96-101.
63. Oryan A, Bigham-Sadegh A, Monazzah S. Fish bone versus fish demineralized bone matrix (vertebra) effects on healing of experimental radial defect in rat model. Comparative Clinical Pathology. 2016;25(5):981-5.
64. Alidadi S, Oryan A, Bigham-Sadegh A, Moshiri A. Role of platelet gel embedded within gelatin scaffold on healing of experimentally induced critical-sized radial bone defects in rats. International orthopaedics. 2017;41(4):805-12.
65. Parizi AM, Oryan A, Shafiei-Sarvestani Z, Bigham A. Human platelet rich plasma plus Persian Gulf coral effects on experimental bone healing in rabbit model: radiological, histological, macroscopical and biomechanical evaluation. Journal of Materials Science: Materials in Medicine. 2012;23(2):473-83.
66. Oryan A, Parizi AM, Shafiei-Sarvestani Z, Bigham A. Effects of combined hydroxyapatite and human platelet rich plasma on bone healing in rabbit model: radiological, macroscopical, hidtopathological and biomechanical evaluation. Cell and tissue banking. 2012;13(4):639-51.
67. Oryan A, Bigham-Sadegh A, Abbasi-Teshnizi F. Effects of osteogenic medium on healing of the experimental critical bone defect in a rabbit model. Bone. 2014;63:53-60.
68. Oryan A, Alidadi S, Bigham-Sadegh A, Meimandi-Parizi A. Chitosan/gelatin/platelet gel enriched by a combination of hydroxyapatite and beta-tricalcium phosphate in healing of a radial bone defect model in rat. International journal of biological macromolecules. 2017;101:630-7.
69. Alidadi S, Oryan A, Bigham-Sadegh A, Moshiri A. Comparative study on the healing potential of chitosan, polymethylmethacrylate, and demineralized bone matrix in radial bone defects of rat. Carbohydrate polymers. 2017;166:236-48.
70. Pélissier P, Lefevre Y, Delmond S, Villars F, Vilamitjana-Amedee J. Influences of induced membranes on heterotopic bone formation within an osteo-inductive complex. Experimental study in rabbits. Annales de chirurgie plastique et esthetique; 2008, 54(1):16-20.
71. Zwetyenga N, Catros S, Emparanza A, Deminiere C, Siberchicot F, Fricain J-C. Mandibular reconstruction using induced membranes with autologous cancellous bone graft and HA-βTCP: animal model study and preliminary results in patients. International journal of oral and maxillofacial surgery. 2009;38(12):1289-97.
72. Cook SD, Wolfe MW, Salkeld SL, Rueger DC. Effect of recombinant human osteogenic protein-1 on healing of segmental defects in non-human primates. J Bone Joint Surg Am. 1995;77(5):734-50.

73. Salkeld SL, Patron LP, Barrack RL, Cook SD. The effect of osteogenic protein-1 on the healing of segmental bone defects treated with autograft or allograft bone. JBJS. 2001;83(6):803-16.
74. Chen X, Kidder LS, Lew WD. Osteogenic protein-1 induced bone formation in an infected segmental defect in the rat femur. Journal of orthopaedic research. 2002;20(1):142-50.
75. Djapic T, Kusec V, Jelic M, Vukicevic S, Pecina M. Compressed homologous cancellous bone and bone morphogenetic protein (BMP)-7 or bone marrow accelerate healing of long-bone critical defects. International orthopaedics. 2003;27(6):326-30.
76. Oryan A, Alidadi S, Moshiri A, Bigham-Sadegh A. Bone morphogenetic proteins: A powerful osteoinductive compound with non-negligible side effects and limitations. Biofactors. 2014;40(5):459-81.
77. Moshiri A, Oryan A. Structural and functional modulation of early healing of full-thickness superficial digital flexor tendon rupture in rabbits by repeated subcutaneous administration of exogenous human recombinant basic fibroblast growth factor. The Journal of foot and ankle surgery. 2011;50(6):654-62.
78. Oryan A, Moshiri A. Recombinant fibroblast growth protein enhances healing ability of experimentally induced tendon injury in vivo. Journal of tissue engineering and regenerative medicine. 2014;8(6):421-31.
79. Giannoudis PV, Tzioupis C. Clinical applications of BMP-7: the UK perspective. Injury. 2005;36(3):S47-S50.
80. Ito K, Yamada Y, Naiki T, Ueda M. Simultaneous implant placement and bone regeneration around dental implants using tissue-engineered bone with fibrin glue, mesenchymal stem cells and platelet-rich plasma. Clinical oral implants research. 2006;17(5):579-86.
81. You T-M, Choi B-H, Zhu S-J, Jung J-H, Lee S-H, Huh J-Y, et al. Platelet-enriched fibrin glue and platelet-rich plasma in the repair of bone defects adjacent to titanium dental implants. International Journal of Oral & Maxillofacial Implants. 2007;22(3):417-422.
82. Trouillas M, Prat M, Doucet C, Ernou I, Laplace-Builhé C, Saint Blancard P, et al. A new platelet cryoprecipitate glue promoting bone formation after ectopic mesenchymal stromal cell-loaded biomaterial implantation in nude mice. Stem cell research & therapy. 2013;4(1):1.
83. Sánchez AR, Sheridan PJ, Kupp LI. Is platelet-rich plasma the perfect enhancement factor? A current review. International Journal of Oral & Maxillofacial Implants. 2003;18(1).
84. Ranly DM, Lohmann CH, Andreacchio D, Boyan BD, Schwartz Z. Platelet-rich plasma inhibits demineralized bone matrix-induced bone formation in nude mice. JBJS. 2007;89(1):139-47.
85. Giovanini AF, Deliberador TM, Gonzaga CC, de Oliveira Filho MA, Göhringer I, Kuczera J, et al. Platelet-rich plasma diminishes calvarial bone repair associated with alterations in collagen matrix composition and elevated CD34+ cell prevalence. Bone. 2010; 46(6):1597-603.
86. Marsell R, Einhorn TA. The biology of fracture healing. Injury. 2011;42(6):551-5.
87. Oryan A, Moshiri A. A long term study on the role of exogenous human recombinant basic fibroblast growth factor on the superficial digital flexor tendon healing in rabbits. J Musculoskelet Neuronal Interact. 2011;11(2):185-95.
88. Moshiri A, Oryan A. Role of platelet rich plasma in soft and hard connective tissue healing: an evidence based review from basic to clinical application. Hard Tissue. 2013;2(1):6.
89. Oryan A, Alidadi S, Moshiri A. Current concerns regarding healing of bone defects. Hard tissue. 2013;2(2):1-12.