Original Article

# Effects of Calcium Sulfate Combined with Platelet-rich Plasma on Restoration of Long Bone Defect in Rabbits

**Hua Chen[1], Xin-Ran Ji[1], Qun Zhang[1], Xue-Zhong Tian[2], Bo-Xun Zhang[1], Pei-Fu Tang[1]**

[1]Department of Orthopaedics, Chinese People's Liberation Army General Hospital, Beijing 100038, China
[2]Department of Orthopaedics, People's Hospital of Tianjin, Tianjin 300131, China

## Abstract

**Background:** The treatment for long bone defects has been a hot topic in the field of regenerative medicine. This study aimed to evaluate the therapeutic effects of calcium sulfate (CS) combined with platelet-rich plasma (PRP) on long bone defect restoration.
**Methods:** A radial bone defect model was constructed through an osteotomy using New Zealand rabbits. The rabbits were randomly divided into four groups ($n = 10$ in each group): a CS combined with PRP (CS-PRP) group, a CS group, a PRP group, and a positive (recombinant human bone morphogenetic protein-2) control group. PRP was prepared from autologous blood using a two-step centrifugation process. CS-PRP was obtained by mixing hemihydrate CS with PRP. Radiographs and histologic micrographs were generated. The percentage of bone regenerated bone area in each rabbit was calculated at 10 weeks. One-way analysis of variance was performed in this study.
**Results:** The radiographs and histologic micrographs showed bone restoration in the CS-PRP and positive control groups, while nonunion was observed in the CS and PRP groups. The percentages of bone regenerated bone area in the CS-PRP (84.60 ± 2.87%) and positive control (52.21 ± 4.53%) groups were significantly greater than those in the CS group (12.34 ± 2.17%) and PRP group (16.52 ± 4.22%) ($P < 0.001$). In addition, the bone strength of CS-PRP group (43.10 ± 4.10%) was significantly greater than that of the CS group (20.10 ± 3.70%) or PRP group (25.10 ± 2.10%) ($P < 0.001$).
**Conclusion:** CS-PRP functions as an effective treatment for long bone defects through stimulating bone regeneration and enhancing new bone strength.

**Key words:** Bone Restoration; Calcium Sulfate; Long Bone Defect; Platelet-rich Plasma

## Introduction

Long bone defects are a major problem in regenerative medicine.[1] Approximately, 5–10% of all long bone fractures are bone defects, which delay union or develop into nonunion.[2] Patients with long bone defects are afflicted by pain, surrounding joint stiffness, motor function loss, and possible disability.[3] Therefore, it is imperative to identify an effective treatment for this disease.

Recently, several useful methods and drugs, such as platelet-rich plasma (PRP) and calcium sulfate (CS), are shown to play crucial roles in restoring long bone defects. PRP is a modification of fibrin glue from autologous blood and plays a key role in stimulating and accelerating bone and soft tissue healing.[4,5] PRP is considered an appropriate approach for treating bone defects when used in combination with certain specific biomaterials, such as bovine-derived hydroxyapatite and autogenous cancellous bone grafts,[6,7] but its success has been limited in bone regenerative therapy. In addition, CS is used as a bone graft substitute primarily in orthopedics and dentistry.[8,9] CS can fill bone voids and prevent fibrous tissue ingrowth, which accelerates bone healing in an osteoconductive manner.[2] However, CS is confined to sites without substantial compressive loads due to its limited osteoinductivity.[10] Despite considerable advances in discerning the potential roles of PRP and CS, little is known about the combined effect of PRP and CS on long bone defect restoration.

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**Address for correspondence:** Dr. Pei-Fu Tang, Department of Orthopaedics, Chinese People's Liberation Army General Hospital, Beijing 100038, China
E-Mail: tangpf301@163.com



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In this study, we constructed a long bone defect rabbit model using an osteotomy. Next, the long bone defect rabbits were treated with CS-PRP, CS, PRP, and recombinant human bone morphogenetic protein-2 (rhBMP-2), respectively. We evaluated radiographs, histological data, and bone strengths for the rabbits in each group. Our study aimed to investigate the therapeutic effect of CS-PRP on long bone defect restoration in rabbits. Our findings will provide a theoretical basis for future treatment of long bone defect.

## Methods

This study was conducted using a protocol approved by the Institutional Animal Care and Use Committee and performed in accordance with the Guide for Care and Use of Laboratory Animals.

### Experimental groups

Forty skeletally mature male New Zealand rabbits (8-month-old) weighing 3.5–4.0 kg were obtained from the Laboratory Animal Center of Chinese People's Liberation Army General Hospital and used in this study. The 40 rabbits were randomly assigned to the four groups: CS-PRP group, CS group, PRP group, and positive (rhBMP-2) control group. Notably, the commercially available powder form of CS is CS hemihydrate ($CaSO_4 \cdot 1/2H_2O$). When the hemihydrate is mixed with water in the correct proportions, the hemihydrate become a fluid suspension and then completely precipitates until crystals of dehydrated solid CS are formed, in which 1 g of hemihydrate CS combines with 186 μl of water,[11] suggesting that the volume of water must be more than 186 μl to form a workable fluid CS. Moreover, the previous study has been demonstrated that the powder (CS) to liquid (PRP) ratio was adjusted to 1 g of CS to 240 μl of PRP because of the viscosity of plasma and the platelet's volume.[11] Considering that dissolution of CS hemihydrate needed a different volume of solvent, our preliminary experiments confirmed that it could be good contrast when 1000 mg of CS was dissolved in 300 μl of double distilled sterile water (CS group) while 1000 mg of CS was dissolved in 240 μl of PRP (PRP group). Therefore, rabbits in the CS-PRP group were implanted with 1000 mg of CS and 240 μl of PRP; rabbits in the CS group were implanted with 1000 mg of CS and 300 μl of double distilled sterile water; rabbits in the PRP group were implanted with 240 μl of PRP that was activated by thrombin and $CaCl_2$; and rabbits in the positive control group were implanted with 5 μg rhBMP-2 (obtained from the Academy of Military Medical Science, Beijing, China) and 6 mg decalcified bone matrix (obtained from Institute of Orthopaedics, Chinese People's Liberation Army General Hospital, Beijing, China).

### Platelet-rich plasma preparation

The PRP was prepared from autologous blood using a two-step centrifugation process. Briefly, 3 ml of peripheral blood was collected from each rabbit's ears into tubes containing ethylene diamine tetraacetic acid ([EDTA], Gibco, Grand Island, NY, USA) as an anti-coagulant (1.5 mg/1 ml blood). The blood was immediately centrifuged at 1130 g to separate the red blood cells from the platelets and plasma. The supernatant composed of the platelets and plasma was collected and again centrifuged at 1130 g to pellet the platelets. The pellet was then re-suspended in a suitable volume of plasma for a platelet concentration equal to 8–10-fold greater than physiological levels. The concentrations of platelets in the whole blood and PRP were determined using an automatic cytometer (BC-3000, Shenzhen Mindray Bio-Medical Electronics CO., Ltd., Shenzhen, China).

### Calcium sulfate-platelet-rich plasma preparation

Dehydrated solid CS was prepared by mixing an α-hemihydrate powder at a water-powder weight ratio ranging from 0.27 to 0.30. Next, CS-PRP was obtained by mixing hemihydrate CS with PRP, and the powder (CS) to liquid (PRP) ratio was 1 g of CS to 240 μl of PRP. An available paste was obtained. Approximately 30 min later, the CS-PRP crystallized into a solid and homogeneous compound. The materials were sterilized using 25 kGy of $^{60}Co$ irradiation before use in this study.

### Operative technique

As described previously,[12] the rabbits received an intramuscular injection of ketamine (1 ml/kg) at the buttocks and lidocaine-induced local anesthesia to maintain respiration. After they were shaved, each rabbit received a standard sterilization preparation at the radius. A 4 cm vertical skin incision was made at the mid-anteromedialis radius. Mucoperiosteal flaps were gently raised to preserve the periosteum; a 12 mm long bone defect was then created via an osteotomy at the left radius. After implanting the materials, the residual periosteum was meticulously closed with 4–0 vicryl sutures using routine procedures. During the test period, the rabbits' general behaviors were constantly observed.

### Radiography

Radiographs were immediately collected following implantation and at postoperative 4 and 10 weeks. An anteroposterior and lateral views of the defected radius were produced using X-ray to compare the serial changes of the healing callus and bone regeneration at the bone defect sites.

### Histological evaluation

At 10 weeks after implantation, the rabbits were euthanized with an overdose of pentobarbital (40–50 mg/kg) for histological observations. Five rabbits in each group were randomly used for histological observations. Bone specimens were harvested from these rabbits, including the defective portion of the radius and the adjacent ulna bone. The resected radius was demineralized in 14% EDTA for 10 days, processed to produce tissue sections (4-μm sections), and stained with hematoxylin and eosin. Five middle bone defect sections were extracted from bone specimens in each group. Four visual fields were observed microscopically, which consisted of 1 random visual field from each group. The percentage of new bone formation was calculated as the new bone/total cross-sectional area (scaffold areas and

new bone areas) using imaging analysis software (IPP 5.1, Olympus, Tokyo, Japan).

### Bone strength analysis

The bone strength was measured using a 3-point bending test on 5 cm long radial bone segments containing sites with defects. The bone segments were harvested from the other 5 rabbits in each group until the rabbits were sacrifice 10 weeks after surgery. The measurements were collected using a powerful compression instrument (MTS 858 Mini Bionix II: MTS, Albany, New York, USA). A harvested bone segment was positioned between two supporting jigs mounted on the instrument. Each jig was placed 10 mm from the resected margin. A 3-point bending test was performed using a 1 mm/min crosshead speed and 1000 kg load cell. Both the defect and contralateral sides were measured. A numeric value was obtained at the point of failure. The defect bone strength was quantitatively compared with the contralateral radius.

### Statistical analysis

The data of the percentage of the newly regenerated bone area and bone strength were expressed as mean ± standard error (SE). One-way analysis of variance (ANOVA) was performed followed by a Fisher's protected least significant difference *post-hoc* test using a commercially available software package (SPSS 10.0; SPSS Inc., Chicago, Illinois, USA). Statistically significant was defined as $P < 0.05$, two-sided.

## Results

### General observation

During the test periods, the rabbits appeared in good spirits, moving freely, and eating a good diet. Local red swelling, edema, and incision splitting were not observed 1 week after surgery. The incisions healed completely, and the sutures automatically fell off 2 weeks after surgery. No infections or inflammatory responses in the surrounding tissues were observed. The implants remained *in situ* through the rabbits' sacrifice.

### Radiographs

In the immediate postoperative radiographs, the CS-PRP and CS groups exhibited a radiopaque shadow in the bone defect [Figure 1a and 1d], but no radiopaque shadows were observed in the PRP and positive control groups [Figure 1g and 1j]. At 4 weeks, the CS-PRP group exhibited substantial callus formation at the bone defect site as well as bridging at the defect site and host bone fragments [Figure 1b]. These phenomena were similar to the positive control group [Figure 1k]. At 10 weeks, the CS-PRP implant stimulated the cortical bone formation and partial development of the bone marrow cavity [Figure 1c]. Implanting with a positive control yielded a bony consolidation and complete projection of the bone marrow cavity by 10 weeks [Figure 1l]. The CS and PRP groups showed moderate callus formation, but bridging at the defect site was not observed at 4 weeks [Figure 1e and 1h]. Likewise, by 10 weeks, nonunion of the defect sites remained in the two groups [Figure 1f and 1i].

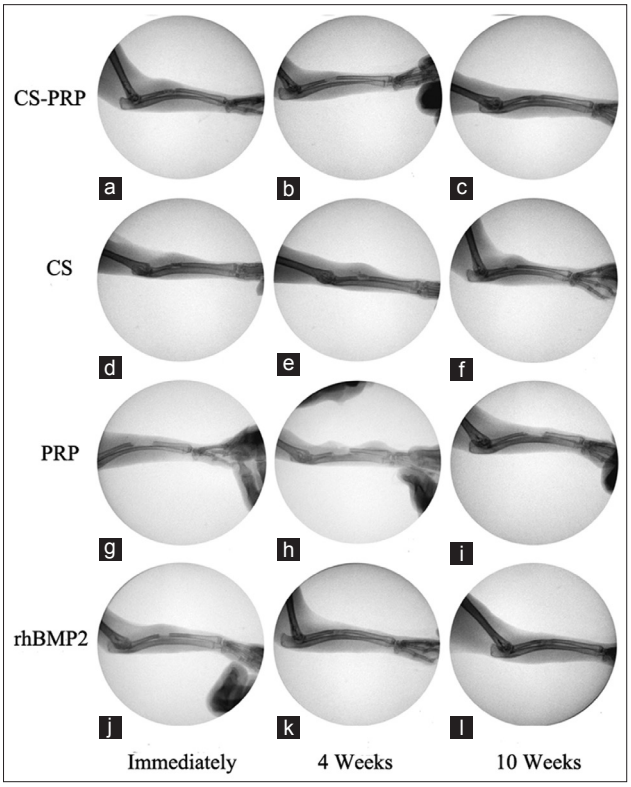


**Figure 1:** Radiographs of radial defects in rabbits. (a-c) The bone restoration process in the calcium sulfate-platelet-rich plasma group. (d-f) The changes in bone defect for the calcium sulfate group. (g-i) The radius after treatment for the platelet-rich plasma group. (j-l) Restoration of bone defects in the positive control group.

### Histological evaluation

At 10 weeks, all of the materials implanted in the rabbits were completely absorbed. In the CS-PRP group, newly formed woven bone connected the host bone edges of the defect site [Figure 2a], and the bone marrow cavity was partially formed [Figure 2b], which corresponds to the radiographs. In the CS group, we observed substantial fibrous connective tissue (FCT) in the defect portion with only marginal woven bone formation [Figure 2c and 2d]. After treatment with PRP alone, new woven bone formed within certain regions of the bone defect [Figure 2e]. Further, a substantial quantity of FCT was observed around the islands of the newly formed bone [Figure 2f]. Cortical continuation, which exhibited a similar morphology to mature bones, was observed in the positive control group [Figure 2g], and the bone marrow cavity was thoroughly formed [Figure 2h].

The percentage of the newly regenerated bone area was 84.60 ± 2.87% in the CS-PRP group, 12.34 ± 2.17% in the CS group, 16.52 ± 4.22% in the PRP group, and 52.21 ± 4.53% in the positive control group. The results of one-way ANOVA showed that statistically significant differences existed among the four groups ($F = 89.62$, $P < 0.001$). The pairwise comparisons between groups showed the CS-PRP and positive control groups displayed more bone formation than the rabbit groups treated with CS or PRP alone ($P < 0.001$). Interestingly, the CS-PRP group exhibited more stimulated

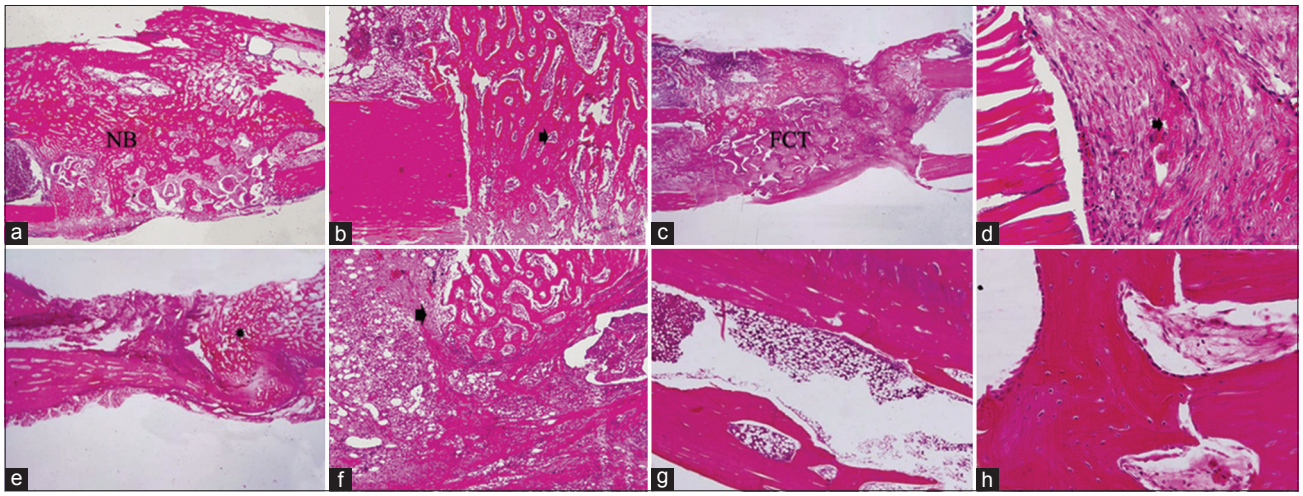


**Figure 2:** Histological micrographs for all groups at 10 weeks. (a and b) Newly formed woven bone and bone marrow cavity in the calcium sulfate-platelet-rich plasma group (the black arrow indicates the bone marrow cavity). (c and d) Substantial FCT and marginal woven bone in the calcium sulfate group (the black arrow indicates newly formed woven bone). (e and f) Little woven bone and substantial FCT in the platelet-rich plasma group (the black star points toward newly formed woven bone; the black arrow shows the FCT). (g and h) Cortical continuation and bone marrow cavity in the positive control group. FCT: Fibrous connective tissue; NB: Newly formed bone. a, c, e and g: HE staining, original magnification ×1.25; b, d, f and h: HE staining, original magnification ×200.

bone formation than the positive control group ($P < 0.001$). The bone formation between the CS and PRP groups did not differ significantly ($P = 0.42$) [Figure 3].

### Bone strength

The bone strengths were 43.10 ± 4.10% in the CS-PRP group, 20.10 ± 3.70% in the CS group, 25.10 ± 2.10% in the PRP group, and 49.50 ± 3.90% in the positive control group at 10 weeks. The results of one-way ANOVA also showed that statistically significant differences existed among the four groups ($F = 15.82$, $P < 0.001$). The pairwise comparisons between groups showed the CS-PRP and positive control group radiuses presented greater bone strengths than the CS and PRP groups ($P < 0.001$). The bone strength in the CS-PRP group did not differ significantly from the positive control groups ($P = 0.21$) or between the CS and PRP groups ($P = 0.33$) [Figure 4].

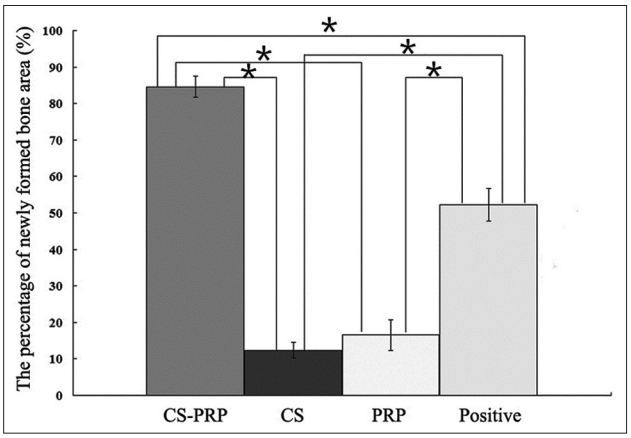


**Figure 3:** The percentage of new bone formed in the bone defect in different groups. *$P < 0.05$.

## Discussion

Currently, treating long bone defects is a hot topic in the medical field.[13,14] The radiographs and histologic micrographs in this study exhibited better bone restoration at the bone defect sites in the CS-PRP and positive control groups compared with the CS and PRP groups. The CS-PRP group exhibited the greatest percentage of the newly regenerated bone area among the groups. The radius bone strength in the CS-PRP group was significantly greater than that in the CS or PRP groups.

Our results showed that the newly regenerated bone area percentages were greater in the CS-PRP group compared with the CS and PRP alone groups, which indicated the acceleration of bone regeneration by CS-PRP in long bone detect restoration. Intini *et al.* showed bone regeneration in a rat calvarial critical-size defect for a CS-PRP group, but no bone regeneration was observed in the CS and PRP groups.[11] In addition, better bone regeneration in teeth has been shown in a canine model after treating with CS-PRP compared with CS alone.[15] In addition, Cui *et al.* exhibited abundant callus, bridging, new woven bone, and bone marrow cavity at bone defect sites in an experimental study using rabbits, which indicates effective bone healing.[12] Another study demonstrated complete defect bridging with callus and full cortical bridging with medullary cavity development in a rabbit radius defect after treatment, which resulted in fully mature bone restoration.[16] The radiograph and histologic micrograph results in our study suggest that CS-PRP may promote bone restoration through stimulating generation of callus, bridging, woven bone, and bone marrow cavity. Moreover, research shows that CS-PRP promotes controlled release of osteogenic factors, which accelerates bone regeneration.[17] Based on our results, we speculate that CS-PRP may treat long bone defects through releasing osteogenic factors to induce bone regeneration and may be superior to CS or PRP alone for long bone defect therapy.

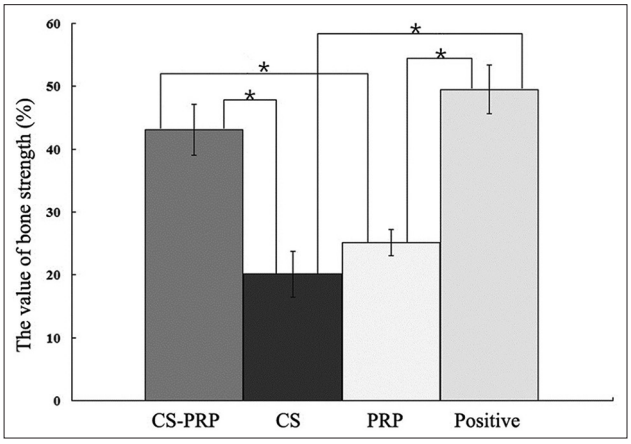


**Figure 4:** The results of 3-point bending tests at 10 weeks for all groups. *$P < 0.05$.

In addition, our bone strength results for the CS-PRP group showed enhanced strength in the newly formed bone compared with the CS and PRP groups. Certain investigators have noted that increased bone strength indicates a good level of bone regeneration within a segmental bone defect area.[18] CS-PRP exerts a positive effect on bone strength after gradual reabsorption and is considered a suitable candidate for restoring segmental bone defects.[19] Hence, we surmise that CS-PRP may exert a therapeutic effect on long bone defects via enhancing new bone strength. However, our results showed no significant difference in bone strength between the CS-PRP and positive groups [Figure 4]. Research shows that BMP-2 can stimulate bone mesenchymal stem cell formation and differentiation.[20] Luca *et al.* showed incomplete regeneration of a rabbit radius defect treated with rhBMP-2 at 8 weeks with composite leakage from the defect.[13] Hence, rhBMP-2 may play a key role and the role same as CS-PRP in determining bone strength during long bone defect restoration.

In conclusion, our study indicates that CS-PRP acts as an effective treatment for long bone defects by stimulating bone regeneration and enhancing new bone strength. Furthermore, the therapeutic effect of CS-PRP is superior to that of treatment with CS or PRP only. CS-PRP may be a promising and suitable candidate therapy for restoring long bone defects. Nevertheless, further studies are warranted to verify the effects of CS-PRP on long bone defects in clinical applications.

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## Conflicts of interest
There are no conflicts of interest.

## References
1. Wojtowicz AM, Shekaran A, Oest ME, Dupont KM, Templeman KL, Hutmacher DW, *et al.* Coating of biomaterial scaffolds with the collagen-mimetic peptide GFOGER for bone defect repair. Biomaterials 2010;31:2574-82. doi: 10.1016/j.biomaterials.2009.12.008.
2. Borrelli J Jr., Prickett WD, Ricci WM. Treatment of nonunions and osseous defects with bone graft and calcium sulfate. Clin Orthop Relat Res 2003;411:245-54. doi: 10.1097/01.blo.0000069893.31220.6f.
3. Shanmugarajan S, Swoboda KJ, Iannaccone ST, Ries WL, Maria BL, Reddy SV. Congenital bone fractures in spinal muscular atrophy: Functional role for SMN protein in bone remodeling. J Child Neurol 2007;22:967-73. doi: 10.1177/0883073807305664.
4. Sánchez AR, Sheridan PJ, Kupp LI. Is platelet-rich plasma the perfect enhancement factor? A current review. Int J Oral Maxillofac Implants 2002;18:93-103.
5. Marx RE. Platelet-rich plasma (PRP): What is PRP and what is not PRP? Implant Dent 2001;10:225-8. doi: 10.1097/00008505-200110000-00002.
6. DurmuSlar MC, Alpaslan C, Alpaslan G, Çakir M. Clinical and radiographic evaluation of the efficacy of platelet-rich plasma combined with hydroxyapatite bone graft substitutes in the treatment of intra-bony defects in maxillofacial region. Acta Odontol Scand 2014;72:948-53. doi: 10.3109/00016357.2014.926023.
7. Chen TL, Lu HJ, Liu GQ, Tang DH, Zhang XH, Pan ZL, *et al.* Effect of autologous platelet-rich plasma in combination with bovine porous bone mineral and bio-guide membrane on bone regeneration in mandible bicortical bony defects. J Craniofac Surg 2014;25:215-23. doi: 10.1097/SCS.0000000000000420.
8. Thomas MV, Puleo DA. Calcium sulfate: Properties and clinical applications. J Biomed Mater Res B Appl Biomater 2009;88:597-610. doi: 10.1002/jbm.b.31269.
9. Kutkut A, Andreana S, Kim HL, Monaco E Jr. Extraction socket preservation graft before implant placement with calcium sulfate hemihydrate and platelet-rich plasma: A clinical and histomorphometric study in humans. J Periodontol 2012;83:401-9. doi: 10.1902/jop. 2011.110237.
10. Wang ML, Massie J, Allen RT, Lee YP, Kim CW. Altered bioreactivity and limited osteoconductivity of calcium sulfate-based bone cements in the osteoporotic rat spine. Spine J 2008;8:340-50. doi: 10.1016/j.spinee.2007.06.003.
11. Intini G, Andreana S, Intini FE, Buhite RJ, Bobek LA. Calcium sulfate and platelet-rich plasma make a novel osteoinductive biomaterial for bone regeneration. J Transl Med 2007;5:13. doi: 10.1186/1479-5876-5-13.
12. Cui X, Zhang B, Wang Y, Gao Y. Effects of chitosan-coated pressed calcium sulfate pellet combined with recombinant human bone morphogenetic protein 2 on restoration of segmental bone defect. J Craniofac Surg 2008;19:459-65. doi: 10.1097/SCS.0b013e31815ca034.
13. Luca L, Rougemont AL, Walpoth BH, Boure L, Tami A, Anderson JM, *et al.* Injectable rhBMP-2-loaded chitosan hydrogel composite: Osteoinduction at ectopic site and in segmental long bone defect. J Biomed Mater Res A 2011;96:66-74. doi: 10.1002/jbm.a.32957.
14. Zhang M, Wang GL, Zhang HF, Hu XD, Shi XY, Li S, *et al.* Repair of segmental long bone defect in a rabbit radius nonunion model: Comparison of cylindrical porous titanium and hydroxyapatite scaffolds. Artif Organs 2014;38:493-502. doi: 10.1111/aor.12208.
15. Shi B, Zhou Y, Wang YN, Cheng XR. Alveolar ridge preservation prior to implant placement with surgical-grade calcium sulfate and platelet-rich plasma: A pilot study in a canine model. Int J Oral Maxillofac Implants 2006;22:656-65.
16. Lyons F, Gleeson J, Partap S, Synnott K, O'Byrne J, O'Brien F. The healing of a novel collagen based scaffolds compared to autogenous bone graft and rhBMP2-soaked collagen scaffolds in a long bone defect. J Bone Joint Surg Br 2012;94:162.
17. Bateman J, Intini G, Margarone J, Goodloe S, Bush P, Lynch SE, *et al.* Platelet-derived growth factor enhancement of two alloplastic bone matrices. J Periodontol 2005;76:1833-41. doi: 10.1902/jop.2005.76.11.1833.
18. Cui X, Zhang BX, Zhao DW. Restoration of segmental bone defect by calcium sulfate pellet: Experiment with rabbit (in Chinese). Natl Med J China 2009;89:777-81. doi: 10.3760/cma.j.issn.0376-2491.2009.11.014.
19. Simson J, Crist J, Strehin I, Lu Q, Elisseeff JH. An orthopedic tissue adhesive for targeted delivery of intraoperative biologics. J Orthop Res 2013;31:392-400. doi: 10.1002/jor.22247.
20. Tadokoro M, Matsushima A, Kotobuki N, Hirose M, Kimura Y, Tabata Y, *et al.* Bone morphogenetic protein-2 in biodegradable gelatin and ß-tricalcium phosphate sponges enhances the *in vivo* bone-forming capability of bone marrow mesenchymal stem cells. J Tissue Eng Regen Med 2012;6:253-60. doi: 10.1002/term.427.