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ORIGINAL ARTICLE

# Comparison of recombinant human bone morphogenetic protein-2-infused absorbable collagen sponge, recombinant human bone morphogenetic protein-2-coated tricalcium phosphate, and platelet-rich fibrin-mixed tricalcium phosphate for sinus augmentation in rabbits

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**KEYWORDS**
absorbable collagen sponge;
BMP-2;
PRF;
sinus augmentation;
TCP

**Abstract** *Background/purpose:* Numerous grafting materials have been used in the bone regeneration of maxillary sinus to obtain a sufficient amount of new bone in implant dentistry. The objective of this study was to compare the potentials of Type I absorbable collagen sponge (ACS) impregnated with recombinant human bone morphogenetic protein (rhBMP)-2, rhBMP-2-coated tricalcium phosphate (TCP), platelet-rich fibrin-mixed TCP for enhancing bone regeneration in sinus augmentation in rabbits.

*Materials and methods:* The sinus defects were grafted with rhBMP-2+ACS (Group A), rhBMP-2-coated TCP (Group B), and platelet-rich fibrin-mixed TCP (Group C). The specimens underwent decalcification, and were stained for histomorphometric analysis.

*Results:* There were no significant differences in inflammatory features among the groups 1-week postoperation. In a histomorphometric analysis, the new bone formation ratio showed significant differences between groups at 2 weeks. rhBMP-2+ACS showed a larger and more rapid bone formation area at 2 weeks than those of Groups B and C.

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*Conclusion:* Our histological evaluation demonstrates that Type I ACS can be used as a carrier of rhBMP-2, and rhBMP-2+ACS showed rapid bone formation, remodeling, and calcification at Week 2 in rabbit.
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## Introduction

After tooth loss, edentulous posterior maxillae often present with insufficient alveolar bone quantity and quality, with maxillary sinus pneumatization.[1] To overcome these structural deficiencies and ensure successful implant surgery, most surgeons used to perform maxillary sinus augmentation.

Autogenous bone, known as the *gold standard*, is a well-established material used to fill insufficient maxillary sinus, because it has osteogenic, osteoinductive, and osteoconductive characteristics.[2] However, some disadvantages and systemic limitations, such as the need for a second surgical site and postoperative morbidity, are well documented.[3] Owing to these limiting characteristics, recent studies have investigated an ideal bone substitute and growth factors to reduce surgical morbidity, and the addition of growth factors and numerous grafting materials to promote bone formation has set high expectations for their clinical potential. Similarly, some studies have focused on early bone formation for early implant loading[4,5]; reduced sinus augmentation operating time and cost are greatly desired by surgeons for successful implantation and meeting patient expectations.

We previously reported the usefulness of tricalcium phosphate (TCP) as a carrier for recombinant human bone morphogenetic protein (rhBMP)-2 and platelet-rich fibrin (PRF) in sinus augmentation in rabbits, and demonstrated the early bone formation capacity of rhBMP-2-coated TCP and PRF-mixed TCP.[6] However, the use of TCP as a carrier for rhBMP-2 still has limitations because particulate artificial bone can remain in the maxillary sinus surrounded by the Schneiderian membrane, and act as a focus for unwanted infection during the healing period, especially in the event of Schneiderian membrane tearing; if sinus infection is detected after augmentation, the surgeon must remove all of the infected grafted materials.[7] Likewise, PRF has early and good bone formation potential, but its use is limited by the recommendation of additional venous blood sampling.

Type I collagen is one of the best rhBMP-2 carriers because of its versatility, high biocompatibility, low immunogenicity, ease of use, and relatively low cost. The first rhBMP-containing products approved by the Food and Drug Administration for the treatment of several spinal disc diseases and open tibial fractures were absorbable collagen sponge (ACS)-based devices impregnated with rhBMP-2.[8,9] Tripplet et al[10] conducted a multicenter, randomized, prospective clinical trial and demonstrated the effectiveness and safety of rhBMP-2/ACS compared with bone graft for sinus floor augmentation.

Thus, the aim of this histological study was to compare the potential of Type I ACS impregnated with rhBMP-2, rhBMP-2-coated TCP, or PRF-mixed TCP to enhance bone regeneration, and to evaluate the usefulness of Type I ACS as a carrier for sinus elevation in rabbits.

## Materials and methods

### Animals and group design

Thirty-six New Zealand white adult female rabbits, aged > 6 months and weighing 2.5–3.5 kg, were used in this study. The animals were housed individually in standard rabbit cages at an ambient 20°C. All of the sinus procedures were performed under general anesthesia, using intramuscular ketamine HCl (50 mg/kg; Ketara; Yuhan, Seoul, Korea) and xylazine (10 mg/kg; Rumpun; Bayer, Seoul, Korea) in a mixture ratio of 5:1 under sterile conditions.

The dorsal area of each rabbit's cranium was shaved before surgery, and the surgical field was prepared with an iodine solution. A midline skin incision was made on the skull, and the periosteum was reflected laterally, exposing the maxilla. Two symmetric ovoid bone defects were then created in the anterior maxilla wall using a round bur under constant irrigation. Special care was taken to avoid injury to the sinus membrane. The defects were grafted with Type I ACS (Ateloplug; Bioland, Chungbuk, South Korea) impregnated with rhBMP-2 (Group A), rhBMP-2-coated TCP (Group B), or PRF-mixed TCP (Group C; Table 1). Each group included 12 rabbits. After obtaining adequate hemostasis, the periosteum was closed with a 4-0 Vicryl suture, and the skin was closed with a 4-0 nylon suture. The animals were given 5 mg/kg gentamycin (Kookje, Seoul, Korea) postoperatively to prevention infection. The postoperative course in all of the cases was uneventful (Figure 1).

The rabbits were killed at 1 week, 2 weeks, 4 weeks, and 6 weeks after surgery, and the six sites of the sinus area were harvested and subjected to histologic examination. All of the experiments were conducted in accordance with the Dong-A University Medical Research Institute's ethics

**Table 1** Group design.

| | Group A | Group B | Group C |
|---|---|---|---|
| Graft material | rhBMP-2+ACS | rhBMP-2+TCP | PRF+TCP |
| No. of rabbits | 12 | 12 | 12 |
| Sinus lift site | 24 | 24 | 24 |

ACS = Type I absorbable collagen sponge; rhBMP-2: = recombinant human bone morphogenetic protein-2; PRF = platelet-rich fibrin; TCP = tricalcium phosphate.

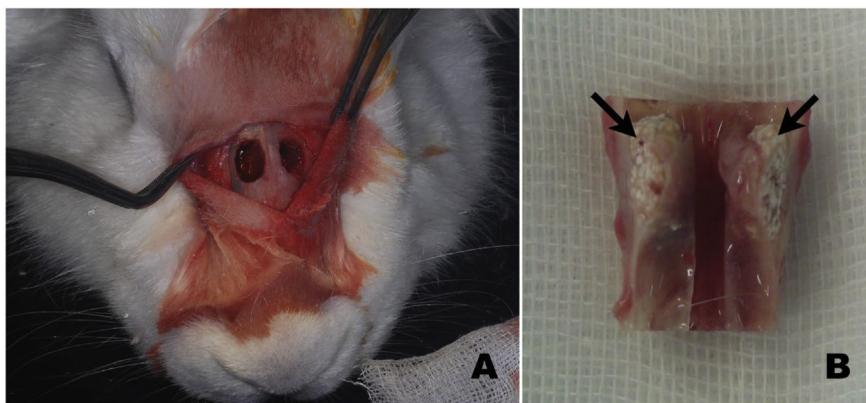


**Figure 1** (A) Two symmetric ovoid bone defects were created, and both sinus membranes were depressed by the periosteal and sinus membrane elevator for grafting. (B) Grafted maxillary sinus site was harvested and grafted materials are stable (black arrow).

guidelines for the treatment and welfare of experimental animals.

## Preparation of graft materials

### ACS impregnated with rhBMP-2 and TCP coated with rhBMP-2

In order to apply the same amount of rhBMP-2 on the two different carriers, we used the rhBMP-2 product that manufactured by Cowellmedi Co. The rhBMP-2 was produced in *Escherichia coli* by genetic engineering. U2OS cells, which express high levels of BMP-2, were used to produce the activated rhBMP-2. The mature forms of rhBMP-2 were cloned from U2OS cells. Finally, dimerized rhBMP-2 was purified by affinity chromatography in a heparin column. Type-I absorbable collagen sponge was soaked with 2 mL 100 μg/mL rhBMP-2 solution by simple injection and divided into four pieces each of 7.6 mm × 12.7 mm in size. By the same pattern, 2 mL 100 μg/mL rhBMP-2 solution was adsorbed on 0.25 g TCP and divided into 0.065 g each for grafting of sinus defects. The final rhBMP-2 concentration of each grafted experimental defect sites was 50 μg/mL.

## Preparation of PRF

After administering local anesthesia, 9-mL venous blood was drawn from the visible venous vessels in the rabbit ears. Immediately afterward, the dried monovettes (without anticoagulant) were centrifuged at 400*g* for 10 minutes in a laboratory centrifuge (Gyro 400g; Dong-seo Science Co., Seoul, Korea) in accordance with the manufacturer's instructions. Thereafter, the PRF clot was cut to a suitable size by scissors.

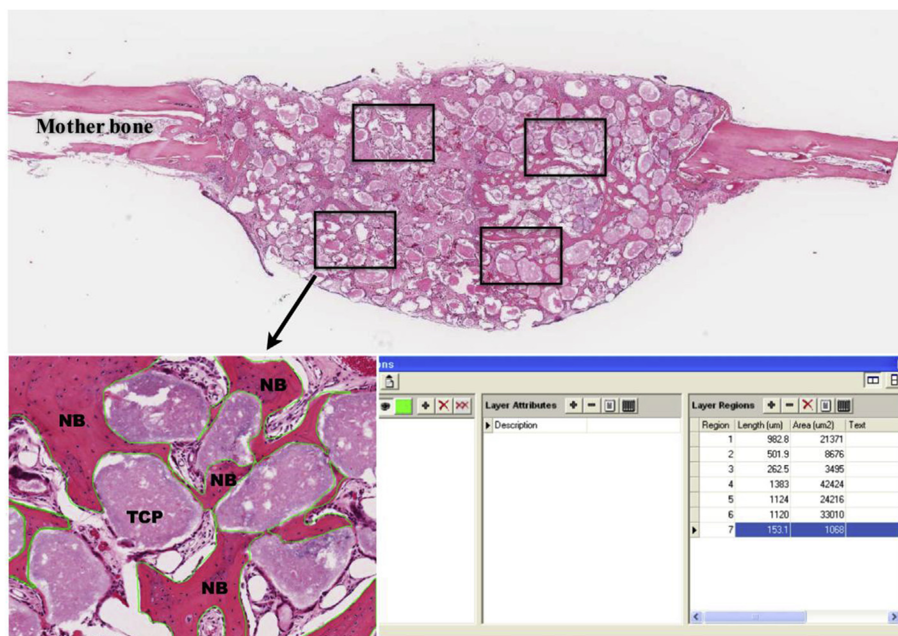


**Figure 2** Traces of the newly formed bone outline using Aperio Technologies Scanscope. NB = newly formed bone; TCP = tricalcium phosphate.

## Histology

Each specimen was fixed in 10% formaldehyde solution, decalcified in formic acid for 48 hours, and embedded in paraffin. Serial cross-sections (5 μm) were cut through the larger diameter of the defect and stained with hematoxylin—eosin (HE). The slides were photographed in a virtual slide system (Scanscope CS system; Aperio Technologies, Vista, CA, USA).

## Immunohistochemistry and histomorphometric analysis

For detection of the new bone formation property, we performed immunohistochemistry by using osteopontin in Week 4 of each group.

The Aperio Technologies Scanscope CS system is useful for calculating new bone formation areas on HE-stained slides. The simple calculation involves only drawing the newly formed bone outlines. Slides in each group were photographed by virtual slide system microscopy (×100), and six slides from each group were selected in Week 2, Week 4, and Week 6. To calculate the new bone formation area, four sites were randomly selected for each slide, and 0.884 mm × 0.684 mm photographs were collected (Figure 2). In this study, we applied two statistical methods to the significance testing of each group. The dependent variables of the control and experimental groups were averages and standard deviations. The difference between the dependent variables in each group for Week 2, Week 4, and Week 6 was analyzed by two-way analysis of variance and the Kruskal—Wallis test. The collected data were analyzed using SPSS Win 22.0 (SPSS Inc., Chicago, IL, USA).

# Results

## Gross observations

All of the animals recovered from the operation and healed uneventfully until the end of the experiment. Group A (rhBMP-2+ACS), Group B (rhBMP-2+TCP), and Group C (PRF+TCP) were all well adapted in critical bone defects. Generally, Groups A and C at Week 2 showed more rigid and well-osteointegrated graft material. However, after 4 weeks, there was no apparent differentiation of rigidity among the groups.

## Histological findings

### HE staining

One week after grafting, there was normal inflammatory reaction and no new bone formation in any of groups (Figure 3). At Week 2, Groups A, B, and C showed slight aggregations of collagen fibers with new bone formation, with greater effects in Groups A and C. Groups A and C exhibited osteoblast proliferation occurring in the early stage of new bone formation, and Group A showed more new bone formation than Group B or C (Figure 4). By Week 4, each experimental group manifested advanced bone formation and calcification; in Group B, bone formation was observed but was relatively small in volume. By Week 6, all of the experimental groups showed more advanced calcification or complete calcification. Thus, Groups A and C showed early initiation of bone formation and remodeling in Week 2.

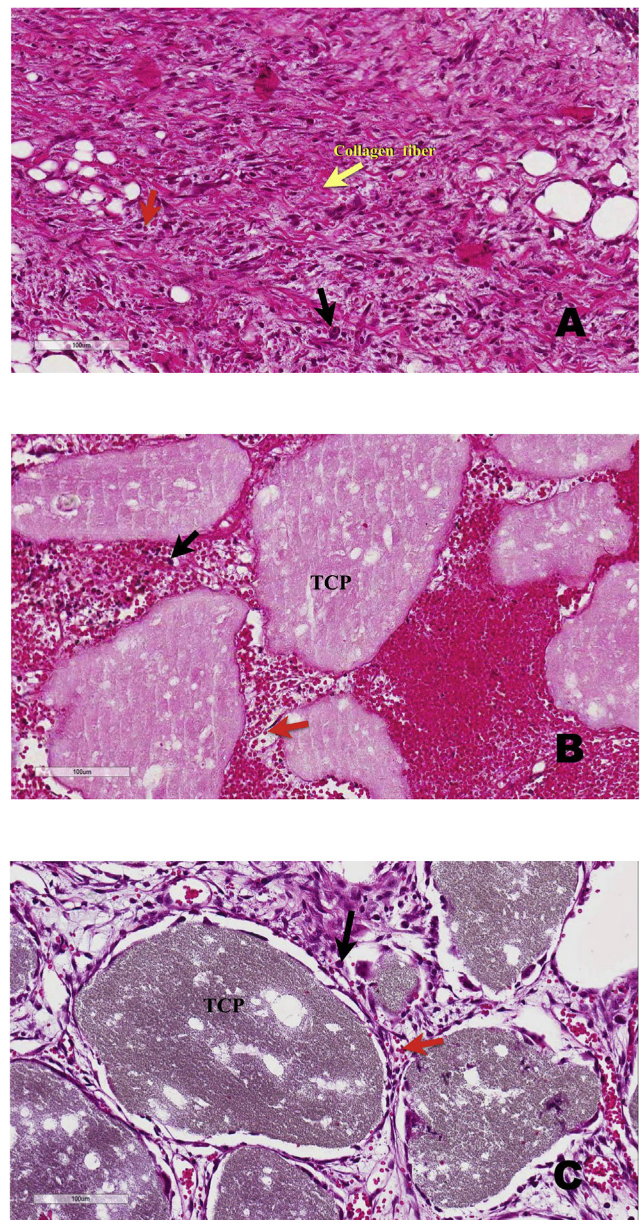


**Figure 3** Comparison of the inflammatory reactions at Week 1 in (A) Group A, (B) Group B, and (C) Group C. HE staining revealed normal red blood cell (red arrow) with slight acute inflammatory cell infiltration (black arrow) but not remarkable inflammatory activity in all groups. HE = hematoxylin and eosin; TCP = tricalcium phosphate.

## Immunohistochemistry and histomorphometric analysis

In Week 4, all of the groups were represented a large amount of osteopontin staining around grafted TCP and newly formed bone that indicates osteoconductive and

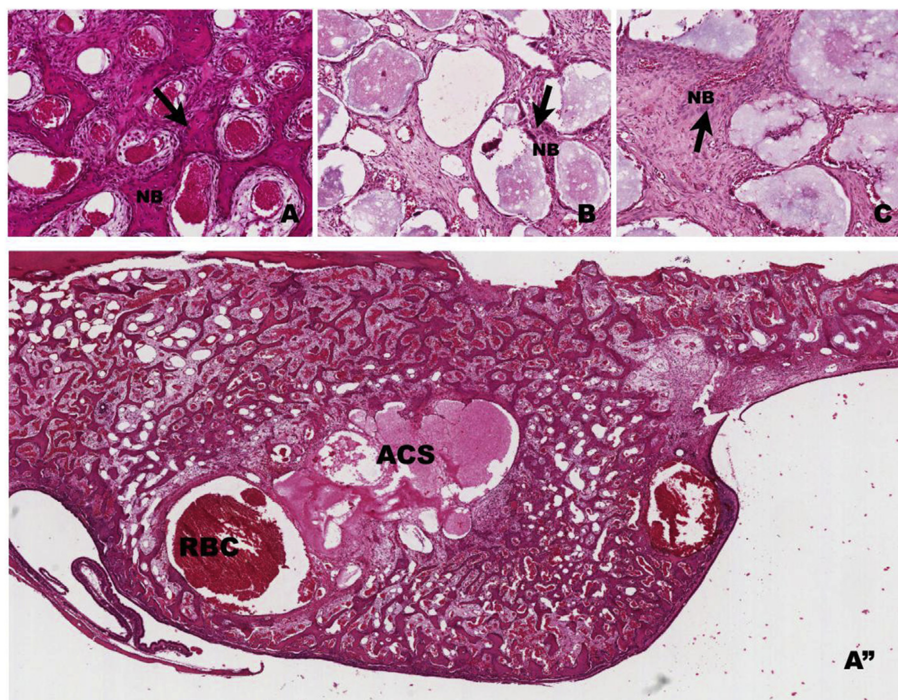


**Figure 4** Histologic view at Week 2 in (A) Group A, (B) Group B, and (C) Group C. Early osteoblast proliferation (black arrow) and new bone formation was observed in Group A rather than Groups B and C. (A″) The total hematoxylin and eosin view of Group A (rhBMP-2+ACS) showed abundant bone formation around ACS after 2 weeks. ACS = absorbable collagen sponge; NB = newly formed bone; RBC = red blood cell.

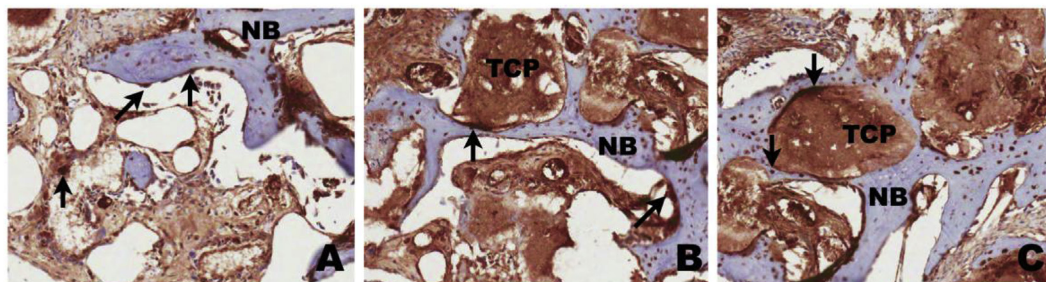


**Figure 5** Osteopontin immunohistochemical staining at Week 4 in (A) Group A, (B) Group B, and (C) Group C. All of the groups demonstrated large amount of osteopontin staining around grafted materials and newly formed bone (black arrow). NB = newly formed bone; TCP = tricalcium phosphate.

osteoinductive properties of the grafted materials (Figure 5).

In Week 2, osteoblast proliferation was observed in all experimental groups at the early stage of new bone formation. The areas of newly formed bone in each group after 2 weeks, 4 weeks, and 6 weeks were measured. In Week 2, the mean extent of bone formation in the Groups A, B, and C was 29.6%, 12.9%, and 17.6%, respectively, and that in Week 4 was 30.5%, 25.8%, and 31.2%, respectively. After 6 weeks, the results were 42.5%, 31.2%, and 40.6%, respectively (Table 2, Figure 6). Kruskal–Wallis statistical analysis was performed and the results are presented (Table 3, Figure 7). We further compared the bone formation area of the three experimental groups using two-way analysis of variance. There was a statistically significant difference between subject (group:week) effect ($F = 14.894$, $P < 0.001$) and a difference in the marginal means of bone formation area, especially at Week 2, in each group (Table 4).

**Table 2** Quantitative analysis of bone formation area.

| | Group A | Group B | Group C |
|---|---|---|---|
| Wk 2 | 29.6% | 12.9% | 17.6% |
| Wk 4 | 30.5% | 25.8% | 31.2% |
| Wk 6 | 42.5% | 31.2% | 40.6% |

(Unit: %, 1/0.6046 $mm^2$).

## Discussion

The addition of growth factors (rhBMP-2, platelet-derived growth factor, transforming growth factor-β, insulin-like

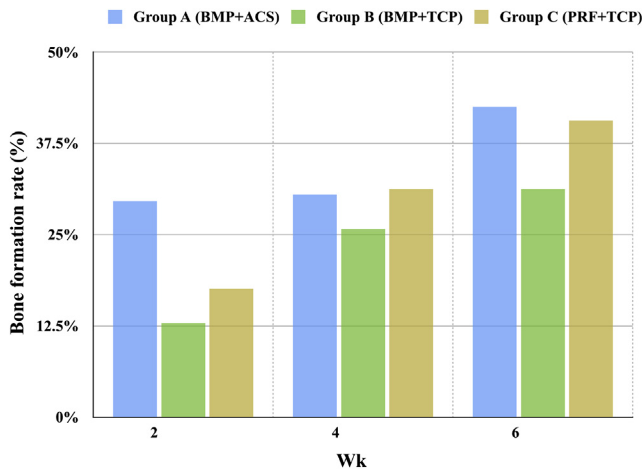


**Figure 6** Quantitative analysis of bone formation area. At Week 2, Group A showed higher bone formation area than Groups B and C. ACS = absorbable collagen sponge; BMP = bone morphogenetic proteins; PRF = platelet-rich fibrin; TCP = tricalcium phosphate.

**Table 3** Kruskal–Wallis test of bone formation area.

| Group | Wk 2 | Wk 4 | Wk 6 |
|---|---|---|---|
| A | 0.179 ± 0.009 | 0.185 ± 0.011 | 0.257 ± 0.014 |
| B | 0.078 ± 0.009 | 0.156 ± 0.017 | 0.189 ± 0.015 |
| C | 0.107 ± 0.012 | 0.189 ± 0.009 | 0.246 ± 0.015 |
| $\chi^2$ | 14.149 | 9.789 | 11.942 |
| P | <0.001 | <0.007 | <0.003 |

Values indicate mean ± standard deviation; unit: $mm^2$.

growth factor, etc.) and use of numerous grafting materials to promote bone formation in sinus augmentation have become routine procedures in implant dentistry. After the first report of sinus floor augmentation by Boyne and James,[11] many researchers reported variable outcomes from numerous grafting materials and techniques.

There was a consensus conference[12] on the *sinus floor lift* procedure in 1996. Probably the most important conclusion reached was that the most effective grafting material is autologous bone. Nevertheless, the requirement of two surgical areas leads many surgeons and patients to refuse this treatment. Owing to these morbidities, numerous grafting materials were developed, such as freeze-dried bone allograft (FDBA), demineralized FDBA, β-TCP, and xenograft. Currently, PRF and rhBMP-2 are the most widely studied and used materials in implant dentistry for regeneration of bone defect sites.[6,8,13–15]

The bone morphogenetic proteins (BMPs) are members of the transforming growth factor superfamily, which were first described by Urist[16] after observing ectopic bone formation in a rodent model from implanted devitalized cadaveric bone. After Urist,[16] many researchers reported that BMPs are one of the most potent local growth factors for induction or stimulation of bone formation in instances of skeletal defects and fracture.[17,18] In 2004, rhBMP-2 was approved for adjuvant use in open tibia fractures, and in March 2007, it was approved "as an alternative to autogenous bone graft for sinus augmentations, and for localized alveolar ridge defects associated with extraction sockets."[13]

For successful bone regeneration and new bone formation by BMPs, suitable carriers that retain certain amounts of BMPs at application sites are required. An ideal carrier is

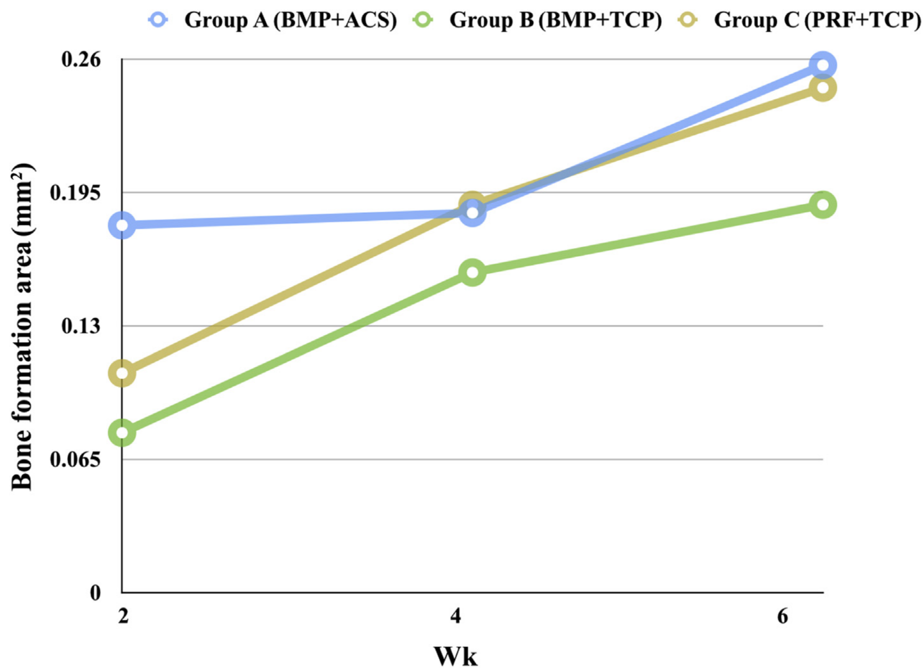


**Figure 7** Kruskal–Wallis test of bone formation area. All groups showed increasing bone formation patterns to Week 6. In particular, Group A (rhBMP-2+ACS) showed earlier peak bone formation than Group B or C. ACS = absorbable collagen sponge; BMP = bone morphogenetic proteins; PRF = platelet-rich fibrin; TCP = tricalcium phosphate.

**Table 4** Two-way ANOVA test of bone formation area.

| Source | Type III sum of squares | DF | Mean square | F | P | Partial $\eta^2$ |
|---|---|---|---|---|---|---|
| Corrected model | 0.160[a] | 8 | 0.020 | 95.898 | <0.001 | 0.945 |
| Intercept | 1.660 | 1 | 1.660 | 7955.258 | <0.001 | 0.994 |
| Group | 0.040 | 2 | 0.020 | 96.548 | <0.001 | 0.811 |
| Wk | 0.107 | 2 | 0.054 | 257.257 | <0.001 | 0.920 |
| Group × wk | 0.012 | 4 | 0.003 | 14.894 | <0.001 | 0.570 |
| Error | 0.009 | 45 | 0.000 | | | |
| Total | 1.830 | 54 | | | | |
| Corrected total | 0.169 | 53 | | | | |

ANOVA = analysis of variance.
[a] $R^2$ = 0.945 (Adjusted $R^2$ = 0.935).

not only nonimmunogenic and bioresorbable, but also provides a three-dimensional structure as a scaffold for new bone formation, and is easy to use. Various carriers have been introduced and developed.[19–22] Uludag et al,[23] reported rhBMP-2 pharmacokinetics of various biomaterial carriers in the rat ectopic model. In that study, a gradual loss of rhBMP-2 was subsequently observed dependent on the implanted carrier. Collagenous carriers were observed to lose rhBMP-2 gradually from the implant site, whereas some of the mineral-based carriers retained a fraction of implanted rhBMP-2. These differences among carriers are expected to affect the biological activity.[23] Similar pattern to the above result, we found that bone formation was more rapid and larger in the experimental Group A (BMP+ACS) rather than Group B (BMP+TCP) but, the relationship between bone formation rate and release rate of rhBMP-2 carrier is still unclear.

Owing to its good biocompatibility and osteointegrative property, TCP [$Ca_3(PO_4)_2$], a synthetic bone-promoting biomaterial, has been extensively applied and investigated as a biodegradable bone replacement for repairing various shapes and sizes of bone defects caused by trauma, tumor resection, or skeletal abnormalities.[24] TCP is generally considered highly osteoconductive but not osteoinductive.[25,26] In the present study, TCP was given an osteoconductive property by coating it with rhBMP-2 (Group B) and mixing it with PRF (Group C).

Choukroun et al,[15] the first to report PRF, found that sinus floor augmentation with FDBA and PRF reduces healing time in humans before implantation. They compared an FDBA+PRF-grafted group harvested after 4 months with an FDBA-only grafted group harvested after 8 months. The histological similarities between the two groups (FDBA alone and FDBA+PRF) make sinus floor augmentation surgery with shorter healing period before implantation (4 months instead of 8 months) a real possibility.[15]

We previously reported the usefulness of TCP as a nonimmunogenic and inorganic carrier for rhBMP-2 and PRF in rabbit sinus augmentation and compared bone regeneration capacity.[6] In that study, the bone regeneration capacity using TCP as an rhBMP-2 carrier was lower than that using PRF, and PRF showed more early bone formation capacity in rabbit sinus augmentation. However, the recommendation of additional venous blood sampling limits the clinical use of PRF.[6]

Recently, several studies have investigated the efficacy of PRF as the sole grafting material for sinus bone augmentation; however, the results differed for bone regeneration in maxillary sinus augmentation and remain controversial.[27–29] Nevertheless, Sohn et al[30] reported that placement of a dental implant in the maxillary sinus, with an absorbable gelatin sponge, can be a predictable procedure for sinus augmentation. Generally, Type I ACS is useful for minimizing postoperative complications because it promotes new granulation tissue formation, blood clot stabilization, and wound protection; thus, many maxillofacial surgeons have used bullet-shaped collagen sponges to obtain favorable results.

In the present study, we observed significant differences in new bone formation between the rhBMP-2-coated TCP, the PRF-mixed TCP-grafted, and the rhBMP-2-impregnated ACS groups at 2 weeks and 4 weeks. Finally, we found that rhBMP-2+ACS (Group A) and PRF+TCP (Group C) had improved early bone formation properties compared to rhBMP-2+TCP (Group B). Furthermore, the rhBMP-2+ACS group showed significantly faster and more extensive bone formation areas than the PRF+TCP group at 2 weeks. These results indicate that simply impregnating ACS with rhBMP-2 has significant bone regeneration capacity comparable with that of PRF+TCP.

Consequently, without the need for additional procedures such as blood sampling or the use of particulate grafting materials, simple application of ACS with rhBMP-2 could be an effective choice for sinus augmentation in rabbits.

## Conflicts of interest

All authors have no conflicts of interest to declare.

## Acknowledgments

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