Effect of Various Biomaterials on New Bone Formation in the Maxillary Sinus Floor Augmentation Procedure

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Purpose: To assess the effect of different kinds of biomaterials placed with maxillary sinus floor augmentation (MSFA) on bone regeneration. Materials and Methods: Thirty-six New Zealand rabbits were used in the study. A standardized method of surgical approach was used for MSFA under anesthesia in all groups. The procedure was performed for each animal. Six separate groups with 12 cases were created. In group 1, no graft was used in MSFA. Advanced platelet-rich fibrin (A-PRF), absorbable collagen cone (ACC), venous blood, the combination of ACC and platelet-rich plasma (PRP), and the combination of ACC and enamel matrix derivative (EMD) were used in groups 2, 3, 4, 5, and 6, respectively. At the end of 4 and 12 weeks, three rabbits from each group were sacrificed by applying high-dose anesthetic, and samples were examined histologically and immunohistochemically. Results: Groups 2 and 5 showed significantly increased new bone formation compared with groups 1 and 4, 4 weeks after MSFA (P < .05). Twelve weeks after sinus floor augmentation, groups 2, 3, 5, and 6 showed significantly higher new bone formation than group 1 (P < .05). Groups 2 and 5 showed significantly higher hard tissue response than groups 1 and 4 at the end of 4 weeks (P < .05). Groups 5 and 6 demonstrated significantly higher hard tissue response than group 1 at the end of 12 weeks (P < .05). Group 5 also showed significantly higher hard tissue response than group 4 at the end of 12 weeks (P < .05). Immunohistochimical analysis showed a significant difference between the osteocalcin scores of groups 2 and 4 and group 1 at the end of 4 and 12 weeks (P < .05). There was also a statistically significant difference between osteocalcin scores at 4 and 12 weeks for groups 1 and 5 (P < .05). When osteopontin scores were compared, there was no significant difference between groups at 4 and 12 weeks (P > .05). Groups 1, 2, and 5 showed significant changes in osteopontin scores between 4 and 12 weeks (P < .05). Conclusion: The combination of ACC and PRP and the combination of ACC and EMD showed increased new bone formation and hard tissue response. A-PRF also showed promising results in new bone formation at the end of both 4 and 12 weeks. The usage of ACC as a carrier for liquid-form biomaterials may be more beneficial than the usage of ACC alone. Int J Oral Maxillofac Implants 2021;36:1076–1087. doi: 10.11607/jomi.8928

Keywords: absorbable collagen, enamel matrix derivative, platelet-rich fibrin, platelet-rich plasma, sinus augmentation

After tooth loss, vertical or horizontal bone insufficiency occurs due to the alveolar bone resorption and sinus pneumatization in most patients.¹–³ Although the autogenous bone graft was previously considered to be the gold standard, various biomaterials now show similar results in maxillary sinus floor augmentation (MSFA) procedures.⁴⁻⁵ Allografts, xenografts, and alloplastic materials can also be used in combination with autogenous graft to reduce donor site morbidity, blood loss, operation time, and intraoperative or postoperative complications that are likely to occur when using autogenous bone grafts.⁶ In the last two decades, bone graft science has improved considerably by understanding the cellular and molecular level of bone-healing principles.⁷

In graftless MSFA, the presence of blood clot between the maxillary sinus membrane and native bone in maxillary sinus floor elevation is the most important factor affecting success in new bone regeneration.⁸⁻¹⁰ Blood components play a key role in the wound-healing process. Blood derivatives are regenerative products that are produced from blood and used as therapeutic
agents in regenerative medicine. Platelet concentrations were first used to stop bleeding in conditions that cause thrombocytopenia, such as medullary aplasia, acute leukemia, or a significant amount of blood loss after prolonged surgery. Platelet-rich plasma (PRP) is the first generation of blood derivatives and contains a greater amount of platelet concentration than the level in blood. Since PRP is developed from autologous blood, it has no risk of disease transmission such as HIV and hepatitis. Platelet-rich fibrin (PRF) is a second-generation platelet-rich blood derivative developed by Choukroun and used as an alternative to PRP due to its easy preparation without any anticoagulant additives and being completely autologous. In addition, PRF is reported to release increased levels of autologous growth factors compared with PRP. As a result of the last modification of PRF, advanced platelet-rich fibrin (A-PRF) has been produced by applying lower g-force during centrifugation, and it releases more growth factors than PRF. It has also been shown to contain more viable platelets and progenitor cells.

Collagen is the most abundant protein in animals. It has become an important biomaterial in bone tissue engineering due to its properties of biocompatibility, high porosity, hydrophilicity, low antigenicity, and absorption in the body. Absorbable collagen cone (ACC) is a porous material that comprises tight collagen fibers, and it is used to control bleeding in oral surgery with an anti-bleeding effect and preserve the original structure of the area via accelerating bone defect healing. Placement of ACC induces guided cell migration following the organization and orientation of the collagen matrix. ACC can also be effective in induction of guided cell migration following the organization and orientation of the collagen matrix and new bone regeneration in MSFA.

More than 90% of enamel matrix derivative (EMD) consists of amelogenins and stimulates periodontal regeneration. As a result of regenerative periodontal surgeries using EMD, the acceleration of periodontal ligament regeneration and growth was observed. Moreover, the attachment of periodontal ligament cells to EMD provides intracellular cyclic adenosine monophosphate signaling, cell proliferation, increasing of general metabolism, and various autocrine growth factor secretion. EMD also provides attachment of periodontal ligament fibroblasts, increases alkaline phosphatase activity, and provides the release of transforming growth factors from the periodontal ligament and gingival fibroblasts.

The search for new bone grafting materials in oral augmentation procedures always continues to acquire better results. There is a need for new grafting materials that may invoke more sound bone regeneration between the sinus membrane and the bony floor, especially in border cases with vertical bone height < 3 mm in MSFA. In this context, the investigation and comparison of the efficacy of grafting materials in different compositions and forms on new bone regeneration in MSFA becomes a necessity. With improvement in the biomaterial technology, the use of blood derivatives or biologic scaffolds without any solid component has been proposed in MSFA. Nonmineral and nonsolid grafts may be alternatives to mineralized bone grafts in bone regeneration procedures. Yet, there are limited numbers of experimental and clinical studies to obtain overall knowledge regarding the efficacy of nonsolid grafts on new bone regeneration in MSFA. Therefore, the experimental investigation of the efficacy of nonsolid grafting materials on new bone regeneration in MSFA may be a starting point. This study aimed to evaluate the effect of the graft materials of PRP, A-PRF, ACC, EMD, and their combinations on new bone formation in MSFA in rabbits.

**MATERIALS AND METHODS**

The ARRIVE guidelines were used to increase the reliability of the findings in the study. The study was approved by the local Clinical Research Ethics Committee, with approval number 17.087, and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Thirty-six male New Zealand rabbits 9 to 12 months of age and 3,500 to 4,500 g in weight were used in this study. All animals were kept in physiologic conditions with 22°C to 24°C temperature, and diets were controlled daily. Seventy-two MSFAs were performed in 36 rabbits, and 6 groups were created with a power of 95% (confidence interval: 95%, f = 1,212). The sample size was 12 for each group. Three rabbits with six MSFAs from each group were sacrificed with high-dose anesthetic at the end of 4 and 12 weeks for histologic and immunohistochemical evaluation. The groups were arranged as follows:

- **Group 1:** no graft
- **Group 2:** A-PRF
- **Group 3:** ACC (Collacone, Botiss Biomaterials/Zossen)
- **Group 4:** venous blood
- **Group 5:** ACC and PRP combination (Collacone + PRP)
- **Group 6:** ACC and EMD (Emdogain, Straumann) combination (Collacone + Emdogain)

**Surgical Procedure**

Rabbits were anesthetized intramuscularly with 35 mg/kg ketamine HCL (Alfamine 10%, Atafen) and...
The samples were fixed in 10% neutral formalin solution for the gellation procedure. Blood was obtained by the same procedure prior to the 12 minutes at 2,700 rpm. The same amount of venous blood was taken carefully with the pipetting technique, and it was used without adding any clot activator. PRP was taken after centrifugation for 10 minutes at 1,000 rpm (Process for PRF), it was separated into three layers as platelet-poor plasma, PRP, and erythrocyte. PRP was taken carefully with the pipetting technique, and it was used without adding any clot activator.

To prepare PRP, 5 mL of blood was taken from the femoral artery with the aid of a 5-mL tube, which contained citrate phosphate dextrose. After centrifugation for 10 minutes at 1,000 rpm (Process for PRF), it was separated into three layers as platelet-poor plasma, PRP, and erythrocyte. PRP was taken carefully with the pipetting technique, and it was used without adding any clot activator.

To prepare A-PRF, 5 mL of blood was taken similarly and placed with the aid of a 5-mL tube, which contained no anticoagulant material. Then, it was centrifuged for 12 minutes at 2,700 rpm. The same amount of venous blood was obtained by the same procedure prior to the grafting and applied to the defect without any centrifugation procedure.

Histologic Procedures
The samples were fixed in 10% neutral formalin solution with a pH of 7.0 for 72 hours. Then, they were decalcified in 10% ethylenediaminetetraacetic acid (EDTA), and the solution was changed every 48 hours. After the completion of decalcification, they were dehydrated in graded series of ethanol. Transparency was performed with xylene, and paraffin blocking was performed by applying paraffin infiltration for 4 hours. Samples, embedded in paraffin blocks, were cut with 5-µm-thick sections using a microtome, and these samples were placed on slides coated with poly-L-lysine. Samples were stained with Masson's trichrome. Each sample was evaluated via light microscopy (Zeiss Axio-SCOPE A1, Zeiss) by two separate, experienced blinded examiners (D.B., P.B.). The investigators were calibrated by examining histologic slides obtained from a pilot study prior to the present study. All samples were scored by each examiner in terms of quantity of bone formation and hard tissue response within the defect semiquantitatively. The three sections from the apical, coronal, and middle parts of each sample were evaluated with the aid of light microscopy (Zeiss Axio-SCOPE A1, Zeiss) by each examiner. The final decision for the scores was made in consensus between examiners. The scoring system for new bone formation and hard tissue response was used as in the study by Sezer et al and is shown in Table 1.

Immunohistochemistry Procedure
Tissue sections taken on poly-L-lysine–coated slides were deparaffinized with xylene for 20 minutes. Then, they were hydrated for 2 minutes with graded series of ethanol. Samples were then washed with distilled water, and antigen-releasing processes were carried out with citrate solution (pH 6.0) under heat and pressure. The tissues were drawn around with a marker pen. They were washed for 3 × 5 minutes with phosphate-buffered saline (PBS) and kept in 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. Then, they were washed for 3 × 5 minutes with PBS again. They were kept in Ultra "V" Block solution for 5 minutes to stop nonimmunologic reactions and incubated with osteocalcin (Osteocalcin Polyclonal Antibody, Bioss) and osteopontin (Osteopontin, Polyclonal Antibody, Bioss) primary antibodies overnight at +4°C. They were washed again with PBS for 3 × 5 minutes. The samples were kept in secondary antibody (UltraVision LP Detection System HRP polymer & DAB Plus Chromogen, Thermo Scientific) for 10 minutes. They were washed again in PBS for 3 × 5 minutes and kept in DAB (3,3′-diaminobenzidine) for 1 to 3 minutes. They were washed with distilled water for 3 × 5 minutes. After staining with Mayer's Hematoxylin (1 to 2 minutes), the sections were washed in distilled water and kept in graded ethanol series. They were transparent with xylene for 20 minutes, then covered with Entellan (Sigma-Aldrich). Slides were examined from all the three levels of the apical, coronal, and middle parts of each case,
similar to the histologic analysis, and photographed via light microscopy (Zeiss Axioscope A1, Zeiss). Staining intensities for each antibody were evaluated semi-quantitatively considering the scores from “0” to “3” (0 = absent, 1 = mild, 2 = moderate, 3 = intense). The final decision was made with the consensus between examiners.

Statistical Analysis
Data analysis was performed with IBM SPSS V23 software. The Shapiro-Wilk test ($P < .05$) showed that the measurement scores were not normally distributed. The Kruskal-Wallis test was used to compare scores between groups. The Wilcoxon test was used for the comparison of bone regeneration, hard tissue response, osteopontin, and osteocalcin stainings between the two time frames of 4 weeks and 12 weeks. Analysis results were presented as mean ± SD and median (minimum–maximum) for quantitative data. A $P$ value < .05 was considered statistically significant.

RESULTS

New Bone Formation
In the histologic examination after the end of 4 weeks, it was observed that the defect area in group 1 was filled with inflammatory cells, and there were large hemorrhagic areas (Fig 2a). In group 2, it was observed that most of the defect area was filled with mature fibrous tissue and newly developing bone formations (Fig 2b). In group 3, mostly immature and mature fibrous tissues were observed in the defect area (Fig 2c). It was noted that the defect area in group 4 was mostly filled by immature fibrous tissue, and new vascular formations were abundant in the area defined as fibrovascular areas (Fig 2d). It was observed that most of the defect area in group 5 was filled with newly developed bone trabeculae (Fig 2e). The circumference of these trabeculae was extensively surrounded by osteoblasts and osteoclasts, which are easily recognized by their multinucleated forms. In group 6, it was observed that the defect area contains mature fibrous tissue rich in vessels, and there were trabecular structures in addition to new bone (Fig 2f).

In the histologic examination after the end of 12 weeks, group 1 showed the presence of large spaces filled with erythrocytes limited to fibrous tissue in the defect area (Fig 3a). In group 2, it was seen that the defect area was mostly filled with lamellar bone and bone marrow, and the new bone formation was located in a very limited area under the submucosa (Fig 3b). In group 3, it was observed that there were new bone formations in the area of the defect, especially near the submucosal area (Fig 3c). In group 4, it was seen that the defect area contained large spaces with erythrocytes, and the cavity was limited by lamellar bone containing osteon structures (Fig 3d). In group 5, the defect area was completely filled with lamellar bone structures containing bone marrow (Fig 3e). In group 6, new bone development was observed slightly less than the observation made for group 3, whereas lamellar bone development was observed to be denser than group 3 (Fig 3f).
Groups 2 and 5 showed significantly increased new bone formation compared with groups 1 and 4 at the end of week 4 (Table 2; \( P < .05 \)). At the end of week 12, groups 2, 3, 5, and 6 showed significantly higher new bone formation than group 1 (Table 2; \( P < .05 \)). There was no statistical significance for bone formation between the time periods of 4 and 12 weeks for all groups (Table 2; \( P > .05 \)). At the end of weeks 4 and 12, the group with the highest quantity of bone formation within the defect was group 5.
Hard Tissue Response
Groups 2 and 5 showed significantly higher hard tissue response than groups 1 and 4 at the end of week 4 (Table 3; P < .05). Groups 5 and 6 demonstrated significantly higher hard tissue response than group 1 at the end of week 12 (Table 3; P < .05). Group 5 also showed significantly higher hard tissue response than group 4 at the end of week 12 (Table 3; P < .05). Hard tissue response at the end of week 12 for group 6 was significantly higher than the hard tissue response at the end of week 4 (Table 3; P < .05).

Immunohistochemistry Findings
Immunohistochemical staining with osteocalcin and osteopontin primer antibodies showed varying degrees of staining density in all groups at the end of weeks 4 (Figs 4 and 5) and 12 (Figs 6 and 7).

DISCUSSION
Pneumatization of the maxillary sinus and the following vertical bone deficiency in the alveolar crest is one
Fig 4  Immunohistochemical staining with osteocalcin primer antibody showed positive brown staining for (a) no graft, (b) advanced platelet-rich fibrin (A-PRF), (c) absorbable collagen cone (ACC), (d) venous blood, (e) ACC+platelet-rich plasma (PRP), and (f) ACC+enamel matrix derivative (EMD) groups at the end of week 4 (immunohistochemistry x200).

Fig 5  Immunohistochemical staining with osteopontin primer antibody showed positive brown staining for (a) no graft, (b) advanced platelet-rich fibrin (A-PRF), (c) absorbable collagen cone (ACC), (d) venous blood, (e) ACC+platelet-rich plasma (PRP), (f) ACC+enamel matrix derivative (EMD) groups at the end of week 4 (immunohistochemistry x200).
Immunohistochemical staining with osteocalcin primer antibody showed positive brown staining for (a) no graft, (b) advanced platelet-rich fibrin (A-PRF), (c) absorbable collagen cone (ACC), (d) venous blood, (e) ACC+platelet-rich plasma (PRP), and (f) ACC+enamel matrix derivative (EMD) groups at the end of week 12 (immunohistochemistry x 200).

Immunohistochemical staining with osteopontin primer antibody showed positive brown staining for (a) no graft, (b) advanced platelet-rich fibrin (A-PRF), (c) absorbable collagen cone (ACC), (d) venous blood, (e) ACC + platelet-rich plasma (PRP), and (f) ACC+enamel matrix derivative (EMD) groups at the end of week 12 (immunohistochemistry x 200).
of the most important difficulties encountered when placing dental implants in patients with edentulous posterior maxillae. MSFA provides adequate new bone required for implant placement and subsequent prosthetic restoration.\textsuperscript{29}

The gold standard for the determination of the quality of new bone formation in MSFA is the histologic assessment of the sinus bone graft after healing.\textsuperscript{30} The osteotomy for biopsy in the augmented sinuses may sometimes be a clinically difficult procedure. Animal studies gain importance at this point.\textsuperscript{31} The rabbit maxillary sinus is similar to the human maxillary sinus because of its characteristics, such as having a prominent ostium and being wide and easily accessible.\textsuperscript{32} In addition, rabbits have positive features, such as being friendly, calm, and easy to handle when taking blood samples.\textsuperscript{33} Rabbits are cheap, and their breeding and anesthesia are very easy.\textsuperscript{31} Therefore, rabbits were used as experimental animals in the present study.

Recently, graftless sinus membrane elevation has gained popularity. In computed tomography analysis, no difference was observed in bone density between MSFA using allogeneic graft materials and sinus membrane elevation without graft material.\textsuperscript{9} The human sinus membrane has been produced in culture, and it has been shown to produce osteoprogenitor cell markers and induce osteogenic differentiation.\textsuperscript{34} Sohn et al\textsuperscript{35} used screws in an animal study to prevent the downward movement of the membrane in the first group after membrane elevation and left it empty. In the second

### Table 4: Intergroup and Intragroup Comparisons of Immuno histochemical Staining Scores of Osteocalcin After Maxillary Sinus Floor Augmentation

<table>
<thead>
<tr>
<th>Groups</th>
<th>Time period</th>
<th>Mean ± SD</th>
<th>Median (Min–Max)</th>
<th>Mean ± SD</th>
<th>Median (Min–Max)</th>
<th>Test statistics (Z)</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>4 wk</td>
<td>12 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Group 1</td>
<td>1.17 ± 0.41</td>
<td>1.83 ± 0.41</td>
<td>1 (1–2)b</td>
<td>–2.000</td>
<td>.046</td>
<td></td>
<td></td>
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<tr>
<td>Group 2</td>
<td>2.33 ± 0.52</td>
<td>2.83 ± 0.52</td>
<td>2 (2–3)a</td>
<td>–1.342</td>
<td>.180</td>
<td></td>
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<tr>
<td>Group 3</td>
<td>1.33 ± 0.52</td>
<td>2.33 ± 0.52</td>
<td>2 (2–3)ab</td>
<td>–1.857</td>
<td>.063</td>
<td></td>
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<tr>
<td>Group 4</td>
<td>1.33 ± 0.52</td>
<td>2.17 ± 0.41</td>
<td>3 (3–3)a</td>
<td>–0.890</td>
<td>.059</td>
<td></td>
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<tr>
<td>Group 5</td>
<td>2.33 ± 0.52</td>
<td>3 ± 0</td>
<td>2.5 (2–3)ab</td>
<td>–2.000</td>
<td>.046</td>
<td></td>
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<tr>
<td>Group 6</td>
<td>2 ± 0.63</td>
<td>2.5 ± 0.55</td>
<td>4 (3–4)ab</td>
<td>–1.342</td>
<td>.180</td>
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Test statistics $\chi^2 = 18,562$ $\chi^2 = 18,002$

P .002 .003

Group 1 = no graft; group 2 = advanced platelet-rich fibrin (A-PRF); group 3 = absorbable collagen cone (ACC); group 4 = venous blood; group 5 = ACC + platelet-rich plasma (PRP); group 6 = ACC + enamel matrix derivative (EMD). $\chi^2$: Kruskal Wallis test statistics, Z: Wilcoxon test statistics, a-b: There was no statistically significant difference between groups sharing same letter.

### Table 5: Intergroup and Intragroup Comparisons of Immuno histochemical Staining Scores of Osteopontin 4 and 12 Weeks After Maxillary Sinus Floor Augmentation

<table>
<thead>
<tr>
<th>Groups</th>
<th>Time period</th>
<th>Mean ± SD</th>
<th>Median (Min–Max)</th>
<th>Mean ± SD</th>
<th>Median (Min–Max)</th>
<th>Test statistics (Z)</th>
<th>P</th>
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<tr>
<td></td>
<td>4 wk</td>
<td>12 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Group 1</td>
<td>2 ± 0</td>
<td>3 ± 0</td>
<td>3 (3–3)b</td>
<td>–2.449</td>
<td>.014</td>
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<tr>
<td>Group 2</td>
<td>2.33 ± 0.52</td>
<td>3 ± 0</td>
<td>3 (3–3)b</td>
<td>–2.000</td>
<td>.046</td>
<td></td>
<td></td>
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<tr>
<td>Group 3</td>
<td>2.33 ± 0.52</td>
<td>2.83 ± 0.41</td>
<td>3 (2–3)b</td>
<td>–1.342</td>
<td>.180</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 4</td>
<td>2 ± 0</td>
<td>2.5 ± 0.55</td>
<td>2.5 (2–3)b</td>
<td>–1.732</td>
<td>.083</td>
<td></td>
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<tr>
<td>Group 5</td>
<td>2.33 ± 0.52</td>
<td>3 ± 0</td>
<td>3 (3–3)b</td>
<td>–2.000</td>
<td>.046</td>
<td></td>
<td></td>
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<tr>
<td>Group 6</td>
<td>2.67 ± 0.52</td>
<td>3 ± 0</td>
<td>3 (3–3)b</td>
<td>–1.414</td>
<td>.157</td>
<td></td>
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Test statistics $\chi^2 = 9,154$ $\chi^2 = 12,031$

P < .103 < .051

Group 1 = no graft; group 2 = advanced platelet-rich fibrin (A-PRF); group 3 = absorbable collagen cone (ACC); group 4 = venous blood; group 5 = ACC + platelet-rich plasma (PRP); group 6 = ACC + enamel matrix derivative (EMD). $\chi^2$: Kruskal Wallis test statistics, Z: Wilcoxon test statistics, a-b: There was no statistically significant difference between groups sharing same letter.
A-PRF accelerates the natural healing mechanism in sinus membrane elevation. When PRF and A-PRF were compared, it was seen that A-PRF contains more vital progenitor cells and platelets. A-PRF also provides earlier soft tissue development, more BMP release, more and faster vascularization, and more cytokine release compared with PRF. Clark et al showed that A-PRF produced a significantly higher amount of vital bone than allografts in extraction sockets. In the present study, the A-PRF group showed significantly higher new bone formation and hard tissue response than the graftless and venous blood groups at the end of week 4. It also demonstrated significantly higher osteocalcin staining density than the graftless group at the end of weeks 4 and 12. However, there was no significant change in the osteopontin staining density between the A-PRF and other groups.

Collagen is used as a local anti-bleeding material in surgery. It has been reported that major bleeding can be prevented in patients receiving anticoagulant therapy by placing collagen material in extraction sockets. Liu et al placed ACC into the calvarial defect in rats and showed that it accelerates the new bone formation and collagen maturation. Sohn et al showed that using an absorbable collagen sponge in MSFA provides new bone formation without bone grafts. Ahn et al used ACC in maxillary sinus augmentation, and histologic examination showed that only 2 of the 13 samples provided a small amount of woven bone. In the present study, the ACC group showed higher new bone formation than the graftless group at the end of week 12. However, there were no significant changes in hard tissue response and immunohistochemical stainings of osteocalcin and osteopontin between the ACC group and other groups.

PRP provides the secretion of an increased quantity of growth factors in the first 15 to 60 minutes following application compared with PRF and A-PRF. Tseung et al performed bilateral MSFAs in rabbits and showed that more dense bone was formed in the PRP group. Collagen has the feature of being used as a carrier of regenerative proteins and activators. PRP was used by mixing with ACC in the present study both to benefit from this feature and because PRP is not a solid material that cannot withstand sinus membrane pressure. The new bone formation within a defect was the highest in the ACC and PRP combination group at the end of weeks 4 and 12. However, this group was found to be significantly different from the no-graft and venous blood groups at the end of week 4 and from only the no-graft group at the end of week 12. For the hard tissue response within the defect, ACC and PRP combination showed significantly increased scores compared with the no-graft and venous blood groups at the end of weeks 4 and 12. The ACC and PRP combination also showed higher significant osteocalcin and osteopontin staining at the end of week 12 compared with week 4.

EMD is a composition of enamel extracellular matrix proteins and is reported to provide cementogenesis successfully in patients with periodontitis and to completely restore periodontal ligament, cementum, and alveolar bone. It was reported that autocrine production of cAMP signals, transforming growth factor (TGF)-β1, interleukin (IL)-6, and platelet-derived growth factor (PDGF) levels were increased in cells in EMD culture compared with the control group. In a study by Cornelini et al, EMD was placed in bone defects in rabbit tibia, and it was shown that EMD was completely resorbed and did not affect bone formation. However, EMD cannot support the gingiva in periodontal surgery due to its fluid nature. To overcome this situation, it has been proposed that the EMD can be mixed with graft materials such as autogenous bone, allografts, inorganic bovine bone minerals, bioactive glass, or bet-tricalcium phosphate. Considering that EMD is not a solid material and cannot withstand the pressure of the sinus mucosa, a carrier was needed. In the present study, the ACC and EMD combination showed significantly higher new bone formation and hard tissue response than the no-graft group at the end of week 12. At the end of week 12, the ACC and EMD combination also showed significantly higher scores for hard tissue formation than the scores at the end of week 4.

Osteopontin and osteocalcin are extracellular proteins in bone matrix and provide structural integrity to the bone, determining bone size, shape, and strength. They control calcium and phosphate ion anchoring and in this way regulate mineral deposition in the bone matrix. In the present study, there was a significant increase in the osteocalcin levels in the A-PRF and venous blood groups compared with the graftless group at the end of both 4 and 12 weeks. A-PRF also showed a significant increase in osteopontin levels between 4 and 12 weeks. It has been reported that the presence of A-PRF enhances the osteocalcin and osteopontin production in bone and gingival mesenchymal stem cells. Venous blood as a blood derivative similar to A-PRF may also be effective in enhancing the mineral composition and strength in the bone defect. Interestingly, both osteocalcin and osteopontin levels seemed to increase in the no-graft group and the ACC and PRP combination group between weeks 4 and 12. The cavity in the no-graft group was filled with self-capillary blood, and this
result may also be considered as the success of blood being a scaffold for bone regeneration.

Rabbits are considered as the first choice in musculoskeletal research or studies that investigate new bone substitutes due to the ease in availability, housing, and handling. However, there were three main disadvantages regarding the animal model in the present study. The first one was the effect of the increased bone activity and turnover in the rabbit animal model on the bone regeneration in the grafted sinus cavity. This rapid turnover may have affected the quantity and quality of the newly produced bone. As the result of this situation, the bone production in this experiment may not be predictable in the human sinus. The second one is the small sample size due to ethical reasons. The sample size should be as small as the power and effect size can be allowed to be in animal studies. MSFA procedures were performed bilaterally in each animal to overcome this limitation. The third limitation was the usage of a semiquantitative scoring system instead of metric measurement for histologic and immunohistochemical evaluation. Experienced reviewers were selected and calibrated prior to the study to overcome this limitation.

CONCLUSIONS

There was no clear superiority between grafting materials used in this study in MSFA. However, the combination of ACC and PRP and the combination of ACC and EMD showed increased new bone formation and hard tissue response compared with the no-graft group at the end of 12 weeks. The combination of ACC and PRP also showed increased osteopontin and osteocalcin scores between the two time periods. The usage of ACC as a carrier for liquid-form biomaterials may be more beneficial than the usage of ACC alone. MSFA using A-PRF also showed promising results in new bone formation both at the end of weeks 4 and 12. Graftless MSFA and venous blood usage in MSFA may be disadvantageous in new bone formation compared with new-generation biomaterials and their combinations in terms of new bone formation. However, the no-graft group showed increased osteocalcin and osteopontin scores compared with other groups between the two time periods. This may be because of the change in the protein composition in the maturing bone in the late stage of bone formation. More animal and human studies are required for better results in MSFA.

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