Prosthetic rehabilitation of the edentulous alveolar crest due to tooth loss is a common procedure in dentistry. In cases of alveolar insufficiency, bone augmentation procedures are preferred before prosthetic procedures. Clinical and experimental studies have shown that critical-sized bone defects simulating the extraction socket cannot spontaneously heal without the use of any osteopromotive material. Different surgical techniques, new graft materials, biostimulant systems, systemically applied vitamins, hormones and mineral supplements, electrical stimulants, and anabolic agents such as growth factors are used in the rehabilitation of bone defects. Among graft materials, autogenous bone grafts are accepted as the gold standard. Due to their disadvantages, different graft materials and growth factors have gained importance in regenerative treatment of bone defects. Xenografts are used successfully in oral and maxillofacial surgery in bone defects, in various osteotomies requiring grafts, alone or in combination with other materials. Studies in various animal and human models show that the use of a mechanical barrier in bone defects increases bone regeneration. This can be explained by inhibiting the growth of soft tissue into the defect when the barrier membrane is used. Hockers et al concluded that xenografts and autogenous grafts combined with the resorbable collagen membrane increases bone regeneration in dogs. Various plant extracts such as Ankaferd Blood Stopper (ABS; Ankaferd Pharmaceutical Cosmetics) have beneficial effects on wound healing. ABS is a mixture of the plants Urtica dioica, Vitis vinifera, Glycyrrhiza glabra, Alpinia officinarum, and Thymus vulgaris. It is commercially available in spray, buffer, and ampule forms. The use of ABS, mainly known for its hemostatic properties, is approved by the Turkish Ministry of Health, and many studies support this effect. Its hemostatic effect is only physiologic, and coagulation factors are not affected during the process. In addition, Firat et al reported

**Purpose:** To evaluate the effect of bovine-derived anorganic bone graft (ABB) in combination with hemostatic plant extract (ABS) on bone regeneration. **Materials and Methods:** Three bone defects were created via an extraoral approach on the mandibles of nine domestic pigs. The first defects were filled with ABS solution (0.3 mL/defect) in a transporting agent of ABB (0.3 cc/defect), whereas the second defects were filled with ABS (0.3 mL/defect) in microcapsules for controlled drug release, combined with ABB (0.3 cc/defect) again. The third defects were left empty. After a 10-week healing period and the sacrifice, undecalcified sections were prepared for histomorphometric analysis. **Results:** The mean total area of hard tissue was 29.54% ± 3.2% in the control group, 59.78% ± 5.4% in the conventional group, and 63.67% ± 4.2% in the microsphere group ($P < .001$). The mean area of newly formed bone was 29.54% ± 3.2% in the control group, 34.79% ± 3.9% in the conventional group, and 37.95% ± 5.3% in the microsphere group ($P = .003$). The mean residual graft area was 24.99% ± 2.4% in the conventional group and 25.71% ± 4.4% in the microsphere group ($P = .730$). **Conclusion:** The combined usage of ABS and ABB in both ways increased bone regeneration statistically. However, there was no significant difference between the two methods for ABS delivery systems in terms of new bone regeneration. **Keywords:** bovine-derived anorganic bone graft, controlled release, hemostatic plant extract, histomorphometry

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a positive effect of ABS on early bone tissue healing in their preliminary experimental study.\textsuperscript{10}

To replace the lost bone tissue, materials such as hormones, growth factors, and proteins are placed in various carrier matrices, and recently, the distribution of those materials into the bone tissue has been possible as a result of developments in tissue engineering.\textsuperscript{11} In vivo studies have demonstrated that poly lactic-co-glycolic acid (PLGA) microparticles prolonged retention and release of rhBMP-2 and rhBMP-7 to sufficiently buttress rat and bovine critical-size cranial defects, respectively.\textsuperscript{12} The aim of the controlled slow release method that arises at this stage is to ensure that the active substance is present in the environment for as long as possible and to increase its therapeutic effect. For this purpose, the method involves creating a physical and chemical barrier to delay the release of the active substance into the environment.\textsuperscript{13,14} This method prevents short serum half-time due to catabolism and liver involvement.\textsuperscript{13} PLGA is a commonly used material for controlled drug release. It is biocompatible, biodegradable, shows rapid degradation, and can be produced in various forms.\textsuperscript{14}

The aim of this experimental study was to evaluate the bone regeneration effect of the combined use of anorganic bovine bone graft (ABB), a hard tissue grafting material, and ABS, which is mainly known to provide hemostasis. The hypothesis of the study was that grafting and controlled release methods can stimulate significant improvement in bone regeneration.

MATERIALS AND METHODS

Preparation of Gelatin Microspheres Loaded with ABS

In order to prepare the microspheres in the laboratory, the double emulsion method was used in which the solvent was evaporated.\textsuperscript{15,16} ABS used in the present study was in the 2-mL ampule form, of which 0.12 mg were \textit{Urtica dioica}, 0.16 mg \textit{Vitis vinifera}, 0.18 mg \textit{Glycyrrhiza glabra}, 0.14 mg \textit{Alpinia officinarum}, and 0.1 mg \textit{Thymus vulgaris}. First, 0.4 mL of ABS was added as a bioactive agent in a polymer solution (10%, w/v) containing 0.6 mL of dichloromethane (Sigma-Aldrich Chemie) and 60 mg of PLGA (50:50; Sigma-Aldrich Chemie), and the mixture was ultrasonicated at 50 W for 15 seconds. This first emulsion was added to 2 mL of polyvinyl alcohol (Sigma-Aldrich Chemie; 4%, w/v), and sonication was repeated. The resulting double emulsion was then added to the low concentration of polyvinyl alcohol (50 mL, 0.33% [w/v]), and the organic solvent was removed by continuous stirring overnight. The resulting spheres were washed twice with Tris-HCl (10 mM, pH: 7.4) solution and lyophilized.

Study Sample

Nine male four-and-a-half-month-old domestic pigs (\textit{Sus domesticus} bred at Cukurova University (CU) Medical Experimental Research Center (TIBDAM) were included in the study. Their body weight was 20 ± 5 kg at the time of surgery. The study was approved by CU Medical Ethics and Scientific Research Center (DHF2011D2).

Anesthesia and Medication

Premedication was administered with intramuscular (i.m.) injections of 20 mg/kg ketamine (Alfamyn, Egevet) and 2 mg/kg xylazine (Alfazyne, Egevet). After sedation was achieved, general anesthesia was induced by intravenous (iv) administration of 5 mg/kg thiopental sodium (Pental Sodium, Ibrahim Etem) with controlled infusion. As a prophylactic antibiotic and analgesic, 500 mg cephalosporin (lespor, I.E. Ulagay) and 500 mg metamizole sodium (Novalgine, Aventis) was administered i.m. perioperatively to all animals.

Surgical Procedure

All surgical procedures were performed with attention to aseptic precautions. A 4-cm-long horizontal incision was made from 2 cm medial to the lower border of the mandibular bone. The mucoperiosteal flap was elevated. The corpus was approached laterally, and cylindrical defects of 5 mm in depth and 10 mm in diameter were created, with three pieces in each animal. All equal-sized defects were created using a single-sized trephine bur. One of the defects was left blank in each of the animals (control group). Liquid ABS (0.3 mL/defect) and ABB (Integros Boneplus-xs, Integros Health Products Ar-Ge Manufacturing, Import, Export, Industry and Trade; 0.3 cc/defect) were mixed in a surgical mixing well, and second defects were filled with this mixture (conventional group). The remaining third defects were filled with a mixture of microspheres, which were previously loaded with ABS (0.3 mL/defect) and ABB (0.3 cc/defect; microsphere group; Fig 1). The amount of ABS and ABB were the same for the last two groups, but the only difference was that ABS was encapsulated in microspheres in the microsphere group in order to provide slow releasing of ABS. The grafts used were in granular form with a particle size in the range of 0.25 to 0.5 mm. Before the membranes were placed, markings with screws were made near defect areas, in order to avoid problems that may arise during the histomorphometry-section-taking process. A resorbable, porcine-derived collagen membrane of 30 × 40 × 0.5 mm was covered over all three defects (TheraForm, Sewon Cellontech). Flaps were sutured primarily with resorbable 3.0 sutures.

Five hundred milligrams cephalosporin (lespor, I.E. Ulagay) 1 × 1 and 500 mg metamizole sodium (Novalgine, Aventis) 1 × 1 was administered i.m. postoperatively to...
all animals for 3 days. At the end of a 10-week healing period, all animals were sacrificed. Animal care protocols and the policies of the Ministry of Health in Turkey were followed at all stages of the surgery.

**Histomorphometry**

The specimens were removed as a block containing some of the main bone around the defects and then kept in a 4% buffered formalin solution for at least 24 hours. After the fixation, the specimens were gradually dehydrated and infiltrated under vacuum by staying in methyl methacrylate resin (Technovit 7200 VLC, Kulzer & Co) for 24 hours. The molds with one sample in each were polymerized for 8 hours under light at a wavelength of 450 nm at 40°C.

The flat bottom surfaces of the transparent methyl methacrylate resin blocks containing the samples were adhered onto the plexiglass slide under vacuum using Technovit 7210 VLC (Kulzer & Co). Two histologic sections with 100-μm thickness were obtained by using a precise cutting device (Exakt 400 CS, Exakt Apparatabau) from each sample. These sections were stained with toluidine blue. The stained histologic samples were allowed to dry and covered with cover slip using methyl methacrylate. The best sections were selected according to their demonstrative properties, such as lack of watermarks, scratches, and overstaining. A digital camera (Olympus DP70, Olympus) attached to a microscope (Olympus BX50, Olympus) at a magnification of ×4 was used to obtain digital images of the sections. The measurements were made blindly using WinTAS image analysis program (WinTAS Trabecular Analyze System, version 1.2.9), which was calibrated before use according to the manufacturer’s recommendations. The following parameters were evaluated:

1. Total hard tissue (ratio of total hard tissue area of residual graft and newly formed bone to the entire defect area)
2. Newly formed bone (ratio of newly formed bone area to whole defect area)
3. Residual graft (ratio of area of residual graft material relative to total area of the defect)
For statistical analysis of the acquired data, the SPSS 19.0 (Statistical Package for the Social Sciences) software package was used. Data entry and statistical analysis were carried out blindedly. Total hard tissue, newly formed bone, and residual graft material percentage values were assessed. Mean, standard deviation, median, minimum, and maximum values were summarized. The Mann-Whitney U test and Kruskal-Wallis test were used for the general comparison of numerical measurements. The Bonferroni correction was applied for multiple group comparisons, and $P < .05$ was considered statistically significant.

**RESULTS**

During the 10-week follow-up, no wound dehiscence or membrane exposure was observed.

Histologically, after 10 weeks of recovery, residual xenograft particles were detected in the conventional and microsphere groups. At the end of the 10th week, new bone formation was detected in each of the control, conventional, and microsphere groups (Fig 2).

**Histomorphometric Analysis**

After 10 weeks of healing, the mean proportion of the total area of hard tissue was 29.54% ± 3.2% in the control group (CG), 59.78% ± 5.4% in the conventional group (CVG), and 63.67% ± 4.2% in the microsphere group (MG). The differences between the CG and the CVG ($P < .001$) and between the CG and the MG were statistically significant ($P < .001$), whereas there was no statistically significant difference between the CVG and the MG groups in terms of total hard tissue percentage ($P = .217$; Table 1).

The mean percentage area of newly formed bone was 29.54% ± 3.2% in the CG, 34.79% ± 3.9% in the CVG, and 37.95% ± 5.3% in the MG. The difference was statistically significant between the CG and the CVG ($P = .047$) and the CG and the MG ($P = .001$) in terms of newly formed bone percentages (Table 1).

The mean percentage area of residual graft was measured as 24.99% ± 2.4% in the CVG and 25.71% ± 4.4% in the MG. There was no statistically significant difference between these groups ($P = .730$; Table 1).

**DISCUSSION**

The aim of this study was to investigate the bone healing effect of combined usage of bovine-derived anorganic bone graft and hemostatic plant extract placed in polymer-based microspheres in surgically created defects. ABS combined with ABB, regardless of the methodology, significantly increased new bone formation in surgical defects.

The blood flow of the tissue, circulatory system, fracture healing, and especially the rate of new bone formation (1.2 to 1.5 μm/day for pig, 1.0 to 1.5 μm/day for human) of adult domestic pigs are similar to humans.19 For these reasons, they were seen as a suitable animal model for the present study.
In a study using similar methodology, one of the defects was intentionally left blank for control purposes, and ABB graft was placed in the second defect. As a result of this study, the amount of newly formed bone in the group left empty without grafting was consistent with the rate of newly formed bone in the CG of the present study. In the present study, the newly formed bone percentages at the 10th week and the percentages of total hard tissue were both greater than the results of Görmez et al for the CVG and the MG. These results revealed that the use of ABB with ABS increases bone regeneration more than the use of ABB alone. This increase may have been the result of the positive effect of ABS on bone regeneration. Since the same animal model was used in the study by Görmez et al, the same amount of ABB was placed in similar-size defects, and both studies had the same follow-up periods, the size of the defects was similar to those mentioned earlier. Regenerative methods applied to conventional and microsphere groups have significantly contributed to new bone formation compared with the control group.

Zitzmann et al compared the guided bone regeneration characteristics of polytetrafluoroethylene material (Gore-Tex) with resorbable collagen membrane. They indicated significant bone gain for both membrane groups, but no significant difference between two membranes. In the present study, resorbable collagen membranes showed a successful performance and did not cause any complications. It is thought that the isolation of the operation area and creating a barrier will have a positive effect on the accuracy of the results. The present authors have concluded that resorbable membranes can be a better alternative to non-resorbable membranes without the disadvantage of requiring a second surgery.

Different results have been reported in terms of total hard tissue after different healing periods. Van der Pol et al reported 26.9% in the group without graft at the end of fourth month. When the follow-up differences between these studies were considered, the percentage of total bone area measured in the CG in the present study was at similar levels compared with the literature.

The reported newly formed bone amounts in various studies have a range from 42% to 60%. These range differences can be explained by the different

### Table 1 Results of Histomorphometric Analysis of All Parameters

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SD (min/max)</th>
<th>Median</th>
<th>P</th>
<th>P control vs conventional group</th>
<th>P microsphere vs control group</th>
<th>P conventional vs microsphere group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residual graft, %</td>
<td>24.99 ± 2.40 (21.65/28.30)</td>
<td>25.71 ± 4.45 (17.75/32.15)</td>
<td>.730*</td>
<td>*Mann-Whitney U test.</td>
<td>.003**</td>
<td>.047*</td>
</tr>
<tr>
<td>Newly formed bone, %</td>
<td>29.54 ± 3.22 (24.53/33.63)</td>
<td>34.79 ± 3.93 (27.26/40.88)</td>
<td>.003**</td>
<td>.047*</td>
<td>.001*</td>
<td>.389*</td>
</tr>
<tr>
<td>Total hard tissue, %</td>
<td>29.54 ± 3.22 (24.53/33.63)</td>
<td>30.58</td>
<td>62.88 ± 4.20 (58.31/69.64)</td>
<td>.001*</td>
<td>.001*</td>
<td>.217</td>
</tr>
</tbody>
</table>

* P < .05 is significant.
* *Mann-Whitney U test.
** Kruskal-Wallis test.
collagen contents of the grafts and the follow-up periods of the studies. Nevertheless, these findings were consistent with the results of the present study. The amount of ABB grafts used in each of the defects in the CVG and the MG was equal (0.3 cc/defect). In addition, the amounts of ABS placed in gelatin microspheres and ABS mixed directly with the graft were also equal in these two groups (0.3 mL/defect). According to this, the application of ABS by the controlled release method impregnated into gelatin microspheres did not show a statistically significant contribution to bone regeneration, compared with administration by mixing with ABB directly. Even though there seems to be minimal bone gain for both study groups in comparison with the control group, the differences were statistically significant. These results suggest that the ABB-ABS combination in the present study promoted bone regeneration. A possible reason to have low new bone rates could be that the present study had only one sacrification time, which was at 10 weeks. The relatively small sample size of the present study prevented different sacrification times, which can be considered as a limitation. Further studies that have longer and shorter sacrification times could help in better understanding the direction of bone regeneration.

The xenograft, which was placed in the bone defect, continued its displacement during new bone formation, regardless of the effect of ABS on the tissues. New bone formation and resorption of the graft particle with osteoclastic activity at the outer border of the graft was observed in both the CVG and the MG around the ABB graft used in the present study. In the literature, different residual graft percentages have been reported using similar surgical methods. Takaori et al. reported this value as 27.58% and 25.42%. Although the follow-up period in that study was shorter (8 weeks), the results of the present study were comparable with theirs. In addition, some of these studies are clinical and the rest are experimental studies, and in these experimental studies, various animals such as dogs and pigs were used. Bone turnover on the mandibles of pigs is faster than canine, and the sacrification times in these studies also differ. In order to achieve lower residual graft rates clinically, and thus higher newly formed bone rates, it may be suggested to extend the 10-week follow-up period. Regardless of the study structure, one of the important features of xenografts is the relationship of the graft with the newly formed bone around it. In the present study, it was observed that the residual graft particles were in contact with the newly formed bone after 10 weeks. The integration of residual graft particles with newly formed bone was consistent with the results of previous studies.

In a clinical study using Integros Boneplus-xs for maxillary sinus augmentation, the residual graft was reported to be 31.8% at the end of 6.5 months of follow-up, and graft particles were still present at 8 months. In the present study, histologic sections showed resorption due to osteoclast activity at the outer border of residual graft particles. These results were predictable at 10 weeks of follow-up and were consistent with this study. While xenografts are known to show slow resorption and PLGA 50:50 shows fast degradation rates, a 10-week follow-up was considered an appropriate time period to see resorption of the graft, formation of new bone around it, and release of ABS due to degradation of PLGA.

For controlled release, many organic and inorganic matrix carriers are used. Both nano- and micro-size carrier systems of synthetic materials, natural polymers, and hydroxyapatite-based particles have been reported. Polymers are used for controlled drug release in many ways, such as granules, pellets, waxes, spheres, and liquids. PLGA is known to exhibit rapid degradation in vivo. It has a degradation rate of 99% in 6 months. A 10% polymer concentration of PLGA provides maximum encapsulation efficiency. Yilgor et al. study showed that this concentration has five times more encapsulation efficiency than the same concentration of another polymer, poly (3-hydroxybutyrate-co-3-hydroxyvalerate; PHBV). Thus, the microspheres used in the present study were prepared using 10% PLGA solution.

CONCLUSIONS

At the end of the 10-week recovery period, ABS combined with ABB, applied with two different methods, significantly increased new bone formation in surgical defects. However, there was no significant difference between these two methods in terms of new bone formation. Further studies are required to evaluate the effectiveness of ABS on soft and hard tissue healing with different study models such as different flap designs, extraction sockets, or assessment of in vivo antibacterial properties.

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