

## Investigation of the effect of combined use of alloplastic-based tricalcium phosphate bone graft and antihemorrhagic plant extract (ABS) on bone regeneration in surgically induced bone defects in nondiabetic rats: an experimental animal study

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**Background/aim:** In this experimental study, we aimed to evaluate the late period effects of the combination of Ankaferd Blood Stopper (ABS), which has bone wound healing effects, and  $\beta$ -tricalcium phosphate (TCP) on the regeneration of bone tissue through histopathological, immunohistochemical, and radiological (dual energy X-ray absorptiometry - DEXA) methods in nondiabetic rats.

**Materials and methods:** Sixty-four Wistar albino male rats were used. In the calvaria of the rats, a bone defect 7.0 mm in diameter was created. These rats were divided into 4 different groups. Group 1 was the control group without any treatment, a 0.125 mL  $\beta$ -TCP graft was applied to Group 2, a 0.125 mL ABS was applied to Group 3, and a 0.125 mL ( $\beta$ -TCP + ABS) mixture was applied to group 4. Half of the rats were sacrificed on day 28 and the other half on day 56. Histopathological, immunohistochemical, and DEXA analyses of the specimens were performed after the experiment.

**Results:** As a result of the histopathological analysis, osteoblastic activity and new bone formation were found to be significantly higher in Group 2, Group 3, and Group 4 than the control group on day 28 ( $P < 0.05$ ). However, inflammatory cell infiltration and vascular dilatation and hemorrhage decreased significantly compared to the control group ( $P < 0.05$ ). The histopathological analysis in rats on day 56 showed that osteoblastic activity in Group 2 and Group 4 was significantly higher than in the control group, but there was a statistically significant decrease in inflammatory cell infiltration and vascular dilatation and hemorrhage compared to the control group ( $P < 0.05$ ). New bone formation in Group 2, Group 3, and Group 4 was significantly higher than in the control group. Western blotting findings revealed that the osteonectin and osteopontin expression on day 28 was increased significantly in Group 2 and Group 4. DEXA analyses revealed that BMC values in Group 2 and Group 4 on day 28 were significantly higher than in the control group ( $P < 0.05$ ). There was no significant difference in bone mineral density values on the 28th and 56th days ( $P > 0.05$ ).

**Conclusion:** The use of both  $\beta$ -TCP + ABS and only ABS had positive effects on wound healing and bone formation in nondiabetic rats.

**Key words:** Ankaferd Blood Stopper, bone regeneration, alloplastic graft, dual energy X-ray absorptiometry

### 1. Introduction

Periodontal disease is an infectious disease with a complex pathogenesis characterized by periodontal tissue and bone loss, in which local factors, host response, environmental factors, and genetic predisposition play important roles in etiology (1). The inflammation occurring during the development of a periodontal disease may result in degeneration of bone tissue causing changes in bone morphology (2). However, in periodontal defects,

various graft materials are used to remove deformities caused by tooth extraction and trauma. The aim of the bone regeneration process is to increase the volume of bone (3). In order to achieve the desired success in these applications, experimental and clinical studies are still being carried out. However, despite these studies, there is not yet an ideal graft material that will allow the lost tissues to be recovered and the bone defects to be filled completely. Studies have focused on bone structure, types of bone

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graft materials, and factors affecting bone metabolism and aimed at accelerating bone regeneration (3,4).

Several biomaterial graft materials are needed for bone regeneration. Today, autogenic, allogenic, and alloplastic bone grafts are used for this purpose. The rapid and predictable regeneration capacity of bone grafts in large bone defects is limited. This is due to the fact that biologically active molecules, bone morphogenesis proteins, and other growth factors are not sufficiently present to replace the bone and provide bone formation in bone grafts (5,6). Autogenous graft materials require a second operation at the donor site, allowing long-term pain, limitation of movement, and a long maintenance period, thus limiting the use of this graft type (7). Beta-tricalcium phosphate ( $\beta$ -TCP), one of the most popular synthetic grafts in recent years, is a safe biocomposite graft material with osteoconductive bioactive properties.  $\beta$ -TCP is partially resorbed, holding the bone mechanically and allowing the replacement of the bone with graft as a biological filler. However, it is a weak bone graft due to improper porosity, having a small grain and dissolving in a period of 6 weeks (4,8).

Ankaferd Blood Stopper (ABS; Ankaferd Medication Cosmetics A.S., İstanbul, Turkey) is an important plant extract in Turkish medicine used as an antihemorrhagic agent, consisting of *Glycyrrhiza glabra*, *Vitis vinifera*, and *Alpinia officinarum* dried leaf extracts and dried root extracts of *Urtica dioica* and *Thymus vulgaris* (9). ABS creates a protein network by forming the aggregation of other protein molecules, especially fibrinogen, in areas where tissue integrity is disrupted. In the formation of this protein network, erythrocytes, thrombocytes, and red blood cells are effective and in particular erythrocyte aggregation occurs in this network. This occurs as a purely physiological process in which no coagulation factor is affected in the hemostatic process (10). In addition, it has been reported that ABS has a beneficial effect on early bone healing (11). As a second feature, ABS has been reported to have antiinflammatory and antioxidant effects. Its late effects on bone have not been determined conclusively (12).

We did not find any study in the literature suggesting the effect of ABS on the late period recovery of bone tissue. The aim of this study was to investigate the effects of combined use of  $\beta$ -TCP, a graft material, and ABS, known as an antihemorrhagic, on bone regeneration by using histological, immunohistochemical, western blot, and radiological methods.

## 2. Materials and methods

The experimental animals local ethics committee of Dicle University approved this study with a protocol number of 2015/13. Operations on experimental animals were

conducted in the operating room of the Dicle University Medical Sciences Research and Application Center, the histological and immunohistochemical experiments in the laboratories of the Dicle University Medical Faculty Department of Histology and Embryology, and the radiological examinations in the laboratories of the Dicle University Medical Faculty Department of Biophysics. Sixty-four 4-month-old Wistar male albino rats with an average weight of 300 g were used. Experimental animals were maintained in a cycle of 12 h of light and 12 h of dark. The rats were provided free access to water and food (*ad libitum*) and the room temperature was kept constant at  $22 \pm 2$  °C.

### 2.1. Surgical method

Experimental animals were fasted 12 h before surgery. Injection anesthesia was applied to the rats with ketamine HCl 10% (Ketasol, Richter Pharma, Welsh, Austria) and 3 mg/kg xylazine HCl 2% (Xylazinbio, Bioveta, Intermed Medicine Depot, Ankara, Turkey). The eyelid reflex was expected to disappear for proper anesthesia depth, and the operation area from the neck to the forehead was shaved and painted with 10% povidone-iodine antiseptic solution (Betakon, Aroma, Tekirdağ, Turkey). An incision was made in the coronal midline of the head's skin with a No. 15 stainless steel surgical scalpel, the heads were in a free position, and then the frontal bones were revealed with periosteal elevator. After that operation, with the aid of a 7.0-mm trephine mill (Trephine, SC Medical Products Industry and Trade Co., İzmir, Turkey), a full layer of bone incision was performed in the midline in a circular pattern. Care was taken to protect the dura mater and superior sagittal sinus during the operation. Separate applications were performed for 7.0-mm-diameter defects of each experimental group. The postoperative skin incision was primarily closed with 3/0 silk suture (Silk, Jinhuan Medical Products Ltd., China). For purposes of prophylaxis, immediately after the operation, a single dose of 50 mg/kg antibiotic (Betamoxla, Active, İstanbul, Turkey) was injected into the right gluteal muscle of each rat one time.

### 2.2. Groupings of experimental animals in the study

Nondiabetic rats used in our study were used for 2 different periods, as the 28th day and 56th day. In these two periods, 4 different subgroups were created:

**Group 1:** No procedure was performed to the bone defects of 16 rats; the defects were left empty and the skin incisions were closed. Eight of the rats were sacrificed on the 28th day and the remaining 8 on the 56th day.

**Group 2:** A 0.125 mL  $\beta$ -TCP graft with 0.25–1 mm particles was inserted into the defects of 16 rats. Eight of the rats were sacrificed on day 28 and the remaining 8 on day 56.

**Group 3:** 0.125 mL of ABS was applied to the defects of 16 rats. Eight of the rats were sacrificed on day 28 and the remaining 8 on day 56.

**Group 4:** 0.125 mL of  $\beta$ -TCP graft + ABS was applied to the bone defects of 16 rats. Eight of the rats were sacrificed on day 28 and the remaining 8 on day 56.

In experimental animals, euthanasia was performed on 32 rats on day 28 and the other 32 rats on day 56. Rats were anesthetized with 45 mg/kg ketamine HCl 10%, then sodium thiopental (Pentalyn Sodyum, Ulagay, İstanbul, Turkey), for which the lethal dose of 60 mg/kg was injected.

### 2.3. Preparation of sections for histological evaluation

After the rats were euthanized, the calvarial bone was removed in a circular way with round dental bur to cover the defect area. The samples were placed in 10% formaldehyde solution, then directly fixed in a neutral buffered formalin solution in the histology laboratory. After the complete fixation of the samples, they were washed for 12 h under water, then were suspended in gradually concentrated alcohol for dehydration (30%, 50%, 70%, 80%, 90%, 96%, and 100%) for 12 h. After a transparency process in xylol, infiltration of the tissues was immediately done and they were buried in paraffin blocks. After sectioning with a microtome (RM 2265 Rotatory Microtome, Leica, Germany), paraffin blocks with a thickness of 5  $\mu$ m were stained with hematoxylin and eosin (H&E) for routine staining. In this study, the osteoblastic activity, inflammatory cell infiltration, vessel dilatation and hemorrhage, and new bone formation parameters were histopathologically evaluated.

### 2.4. Preparation of sections for immunohistochemical evaluation

Sections of paraffin blocks of 5  $\mu$ m were placed on poly-L-lysine-coated slides and then were held at room temperature. They were kept in a 60 °C incubator for 1 night. After the sections cooled, they were kept in the xylenes for 2 to 5 min, followed by a 96%, 80%, 70%, and 60% ethyl alcohol for 5 min. After passing through alcohol concentrations, they were kept in distilled water for 5 min. Then they were put into EDTA solution to dissolve the bone tissue. The samples taken on slides were surrounded by Dako Pen (Huiyou, China). In a 700 W microwave oven, they were kept in citric acid (pH 6.0) for 7 + 5 min to remove the antigen masking. They were then cooled for 20 min at room temperature, washed with phosphate-buffered saline (PBS) solution for 3  $\times$  5 min, and kept in 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to provide endogenous peroxide blockade for 20 min. The sections were again kept in PBS for 3  $\times$  5 min and taken into the incubation container. All subsequent operations were carried out in this incubation container. Block solution (Histostain-Plus Kit, Invitrogen, Carlsbad, CA, USA) was dripped and kept for 1 h, and then osteonectin (mouse monoclonal,

1/2200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and osteopontin (mouse monoclonal, 1/200, Santa Cruz Biotechnology) antibodies were applied to the sections. The primary antibody was dropped and kept for 1 h, then washed with PBS solution for 3  $\times$  5 min. After dropping the biotinized secondary antibody (Histostain-Plus Kit, Invitrogen) compatible with the primary antibody, it was kept in a closed moist box at room temperature for 30 min. Immediately after washing with PBS solution for 3  $\times$  5 min, the secondary antibody (Zymed, Histostain-Plus Kit, Invitrogen) marked with streptavidin was dripped and waited for 30 min at room temperature in the closed moist box, then washed with PBS solution for 3  $\times$  5 min. Aminoethyl carbazole was dripped as a chromogen, then washed with distilled water to prevent the reaction of antigen/antibody. Contrast staining with Mayer's hematoxylin was done, and the sample was again distilled with water and closed with a cover slide. In the final stage, the sections were evaluated and viewed immunohistopathologically with a photomicroscope (Nikon Eclipse i50, Tokyo, Japan) for blind evaluation.

### 2.5. Western blotting method

#### 2.5.1. Cell lysis and protein quantitation

The calvarial tissues frozen in liquid nitrogen were powdered in a porcelain mortar, while 50 mg of powdered placenta tissue was kept in 250  $\mu$ L of RIPA lysis solution containing a mixture of protease inhibitor in ice for 1 h. Lysed placenta samples were stored at -86 °C. All the steps were taken on ice to prevent protein degradation. Total cellular protein concentration was calculated using the BCA Kit (Pierce, Thermo Scientific, Waltham, MA, USA). The BCA measurement was made in 96-well plates with a microplate reader (Multiscan Go, Thermo Scientific).

#### 2.5.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were prepared in 1X SDS loading solution (2% SDS, 5% glycerol, 0.01% bromophenol blue, 8% DL-dithiothreitol (DTT), then boiled at 95 °C for 5 min. Next, 20  $\mu$ g of protein samples were loaded onto 10% polyacrylamide gel, and electrophoresis was performed in SDS running solution (2.4 mm Tris, 19.2 mm glycine, 0.01% SDS) for 1 h at 200 V.

#### 2.5.3. Membrane transfer and antibody staining of proteins

Separated proteins were transferred from SDS-PAGE to PVDF membrane in transfer solution at 100 V for 1 h. After transfer, the membrane was blocked at room temperature for 1 h in 5% milk powder prepared in PBS-T solution. After the blockage process, the membrane was treated at room temperature for 2 h with primary antibodies and the membrane was washed 4 times with PBS-T for 30 min. After washing, the membrane were treated with

horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h at 1:10,000 dilution rate. The membrane was washed again 4 times with PBS-T for 30 min. Protein bands were visualized using the enhanced chemiluminescent reagent chemical (Bio-Rad, Hercules, CA, USA). Photos were taken using the ChemiDoc MP (Bio-Rad) device.

## 2.6. Radiological evaluation

Bone defect measurements of rats were taken by dual energy X-ray absorptiometry (DEXA). Bone mineral density (BMD) measurements of all rats were made with the QD 450 ACCLAIM SERIES ELIENTE HOLOGIC DEXA (Hologic, Bedford, MA, USA) device, but only the BMD of the head was evaluated. After all rats were anesthetized with injections, the DEXA tool was withdrawn before starting and being stored. Measurements were obtained as bone mineral content (BMC) in grams and bone mineral density (BMD) values in  $\text{g}/\text{cm}^2$ .

## 2.7. Statistical analysis

Statistical analysis of the data obtained in our study was realized using SPSS 21.0 Windows (IBM Corp., Armonk, NY, USA). Radiological and histological data are shown as numerical values, mean (M), and standard deviation (SD). The Mann–Whitney U test was used to compare the data with nonnormal distributions for binary comparisons, and the Kruskal–Wallis test was used to compare multiple comparisons. The Bonferroni corrected Mann–Whitney U test was performed in comparisons of multiple groups. The Wilcoxon test was used for in-group comparisons.  $P < 0.05$  was accepted as significant in all statistical tests.

## 3. Results

Experimental animals were in cages provided with appropriate environmental conditions after surgery until the 28th and 56th days of healing periods.

### 3.1. Histological results

As a result of the H&E staining procedure (Figures 1A–1H), the following results were obtained. In Group 1, histological analysis on day 28 revealed that inflammatory cells around the vessel in the defect region were observed clearly, fibrous tissue was increased, and bone trabeculae and osteoblastic cells became apparent (Figure 1A). In the histopathological analysis of Group 1 performed on day 56, bone trabeculae in the form of small islets and collagen fiber increase with fibrous tissue began to appear (Figure 1E).

On histopathological evaluation in Group 2 on day 28, we observed that cellular infiltration intensified and vascular hemorrhages decreased. The osteoclastic activity also decreased and osteoblast cells were activated, and osteocytes became apparent in lacunar structures and bone trabeculae. Small islet bone marrow fragments were observed in the middle parts of the enlarged bone

trabeculae (Figure 1B). On histopathological evaluation at day 56 in group 2, a significant increase in collagen fibers and connective tissue cells and an increase in osteoblast activity were found, and osteocytes were seen in the lacunae. Bone trabeculae were observed to enlarge for new bone formation (Figure 1F).

In Group 3, histopathological evaluation on day 28 showed that bone trabeculae became apparent, and osteoblastic activity and osteocyte formation developed. Cellular activity of collagen fiber development in connective tissue increased (Figure 1C). On histopathological evaluation in Group 3 on day 56, dilatation and free-spreading erythrocytes in blood vessels, a partial reduction in inflammatory cells, increased osteoblastic activity, and osteocytes beginning to form new bone precursors and bone trabeculae as small pieces were all observed (Figure 1G).

On histopathological evaluation in Group 4 on day 28, bone trabeculae had matured and bone marrow became apparent. The formation of new bone was observed. Collagen fibers and connective tissue cellular increase occurred (Figure 1D). On histopathological evaluation in Group 4 on day 56, bone trabeculae displaying the formation of new bone began to enlarge, enabling the creation of bone cells among them. Osteoblastic activity and osteocyte formation enlarged by spreading the graft area (Figure 1H).

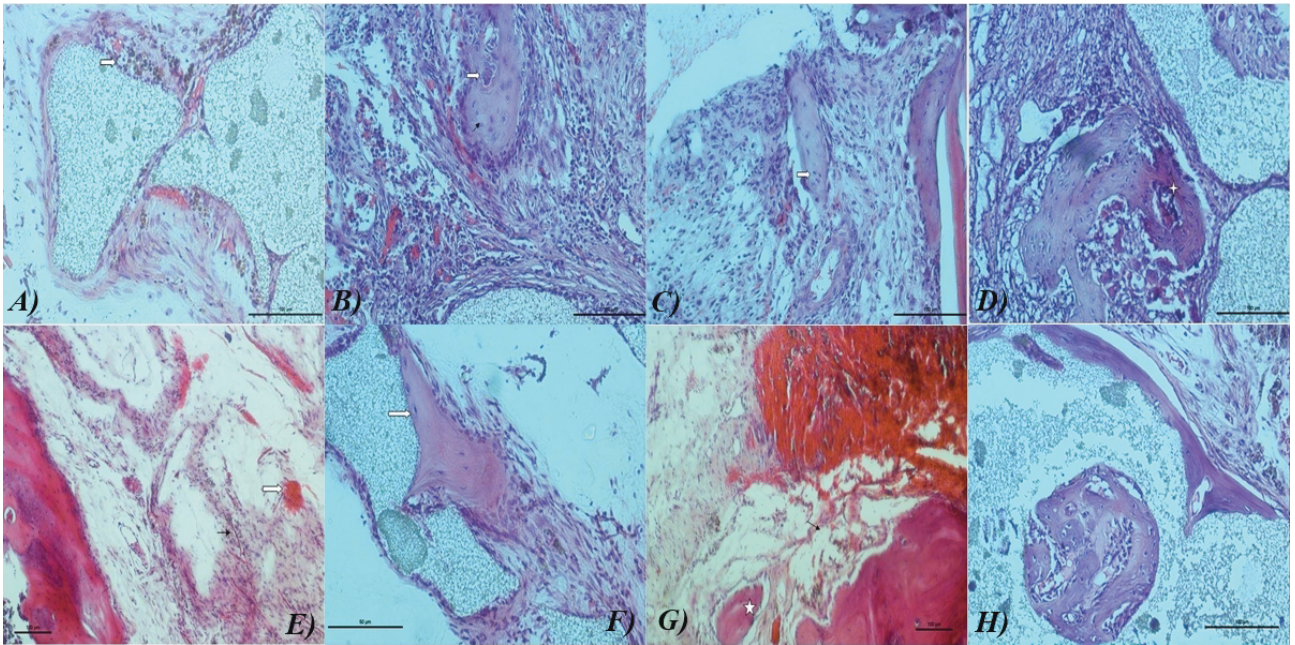
### 3.2. Immunohistochemical results

As a result of the osteopontin immune staining procedure (Figures 2A–2H), the following results were obtained. In Group 1, osteopontin staining at day 28 showed positive osteopontin expression in osteoblasts present in small bone fragments (Figure 2A). Immunohistochemical analysis of osteopontin staining in Group 1 on day 56 revealed positive osteopontin expression in osteoblastic cells around bone trabeculae together with an increase in connective tissue outside the graft site (Figure 2E).

In the immunohistochemical analysis performed in Group 2 on day 28, osteoblast expression was positive (Figure 2B) in osteoblast cells in the enlarged bone trabeculae between the grafts. In group 2, immunohistochemical analysis at day 56 showed that the number of bone trabeculae increased, and osteoblast cells showed obvious osteopontin expression and osteocyte cells occurred (Figure 2F).

In Group 3, we found that an increase in osteoblastic activity and osteopontin-positive reaction were determined in osteoblast cells in the immunohistochemical analysis performed at day 28 (Figure 2C). In Group 3, the immunohistochemical analysis performed at day 56 showed positive osteopontin expression in osteoblast cells other than bone trabeculae in different shapes within the graft area (Figure 2G).





**Figure 1.** A) The inflammatory cells in the histopathological section taken from Group 1 on day 28 are shown with a thick white arrow. B) In the histopathological section taken from Group 2 on day 28, the lacunar structures of the osteocyte cells are shown with a thin black arrow, and the bone marrow sections are shown with a thick white arrow. C) Osteoblastic activity and osteocyte formation in histopathological section taken from Group 3 on day 28 are shown with a thick white arrow. D) Bone marrow in the histopathologic section taken from Group 4 on day 28 shown with a star. E) A histopathologic section taken from Group 1 on day 56; collagen fibers and fibrous tissue are shown with a thin black arrow, and islet-shaped bone trabeculae are shown with a thick white arrow. F) A histopathologic section taken from Group 2 on day 56; osteocytes are shown with a thick white arrow. G) A histopathologic section taken from group 3 on day 56; erythrocyte cells are shown with thin black arrow and bone trabeculae pieces with stars. H) Histopathologic section taken from Group 4 on day 56; bone trabeculae enlarge to form bone marrow, and osteocytes spread to the graft site. Hematoxylin and eosin staining; bar = 100 µm.

Immunohistochemical analysis in Group 4 at day 28 showed an increase in osteopontin expression in osteoblast cells in enlarged bone trabeculae between the graft areas (Figure 2D). On day 56, the new bone structure was fully formed, and positive osteopontin expression occurred in the osteoblast cells, matrix area, graft area, and outside the large bone trabeculae (Figure 2H).

As a result of the osteonectin immunostaining procedure (Figures 3A–3H), the following results were obtained. According to the results of osteonectin staining in Group 1 on day 28, its expressions were observed positive in some precursor connective tissue cells other than graft sites (Figure 3A). The analysis of osteonectin staining in Group 1 on day 56 revealed negative osteonectin expression in small bone trabeculae outside the graft sites (Figure 3E).

Immunohistochemical analysis at day 28 in Group 2 revealed osteonectin-positive expressions in osteocytes in the trabeculae enlarging between the graft fragments (Figure 3B). In Group 2, analysis at day 56 showed that bone trabeculae began to develop within the graft site,

and the expression was positive in osteocytes of bone trabeculae (Figure 3F).

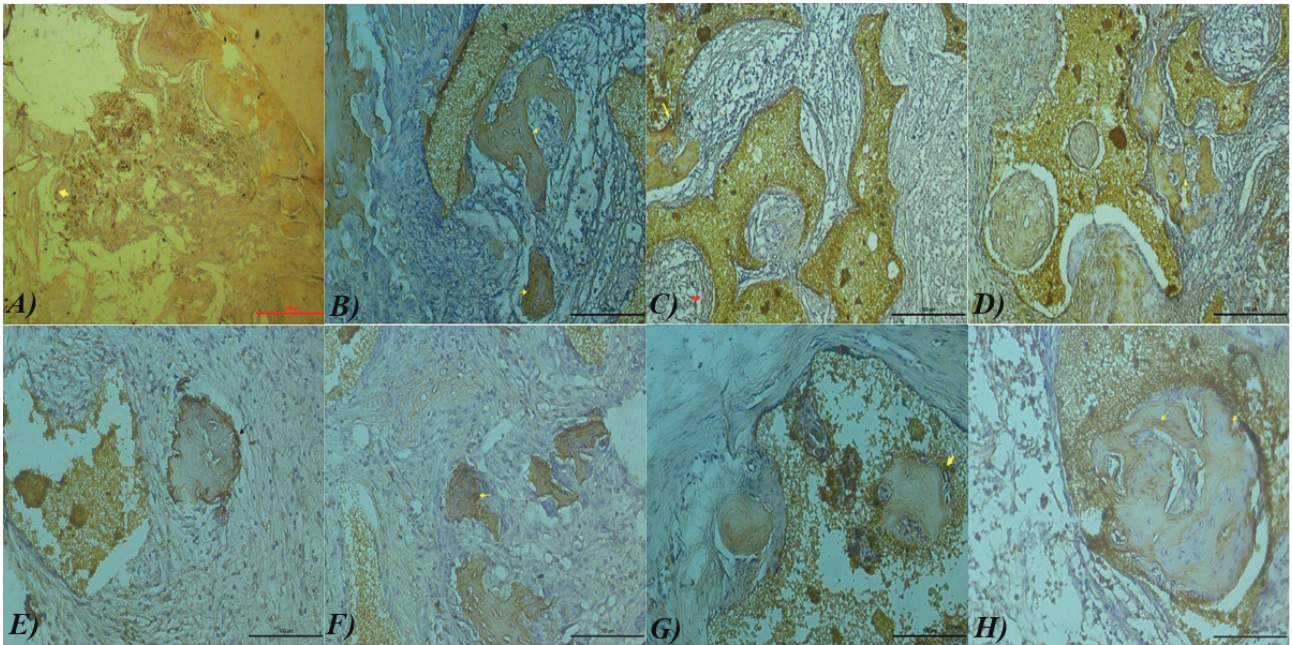
In Group 3 at day 28, osteonectin expression was observed in a small number of osteocyte cells among small bone trabeculae formed within the graft area (Figure 3c). In Group 3, the immunohistochemical analysis performed on day 56 showed osteonectin-positive expression in a small number of osteocyte cells and the prominence of bone matrix in long bone trabecula (Figure 3G).

In the analysis performed on the 28th day in Group 4, osteocyte cells showed positive osteonectin expression in bone trabeculae that tended to be unified with graft fragments (Figure 3D). Immunohistochemical analysis at day 56 of Group 4 revealed that bone trabeculae were enlarged in the graft site and osteonectin expression was positive in osteocytes (Figure 3H).

### 3. 3. Western blotting results for osteonectin and osteopontin expression

The expression level of osteonectin in the calvarial tissue increased dramatically in the  $\beta$ -TCP and ABS +  $\beta$ -TCP groups on day 28 (Figure 4). Total protein (20 µg) was





**Figure 2.** A) Positive osteopontin expression in osteoblasts in immunohistochemical section taken from Group 1 on day 28 (yellow arrow). B) The osteopontin expression was positive in bone trabecula in the immunohistochemical section taken from Group 2 on day 28 (yellow arrow). C) There was an osteoblastic increase (red arrow) and positive expression of osteopontin (yellow arrow) in immunohistochemical section taken from Group 3 on day 28. D) The immunohistochemical section from Group 4 on day 28; there was an increase of osteopontin expression in osteoblast cells (yellow arrow). E) An immunohistochemical section taken from Group 1 on day 56; there was an increase of osteopontin expression in osteoblast cells (thin black arrow). F) An immunohistochemical section taken at 56th day from Group 2; there was a marked increase of osteopontin expression in osteoblast cells (yellow arrow). G) Immunohistochemical section taken at 56th day from Group 3; there was a positive increase in osteopontin expression in osteoblast cells (yellow arrow). H) Immunohistochemical section taken at 56th day from group 4; there was a positive increase in osteopontin expression in osteoblast cells (yellow arrow). Osteopontin immunostaining; bar = 100  $\mu$ m.

run on a gel. Antiosteonectin and anti- $\beta$ -actin antibodies were used in western blotting.  $\beta$ -Actin was used for load control.

The level of osteopontin expression in calvarial tissue had increased dramatically in the  $\beta$ -TCP and ABS +  $\beta$ -TCP groups on day 28 (Figure 5). Total protein (20  $\mu$ g) was run on a gel. Antiosteonectin and anti- $\beta$ -actin antibodies were used in western blotting.  $\beta$ -Actin was used for load control.

### 3.4. Statistical results

#### 3.4.1. Histopathological statistics

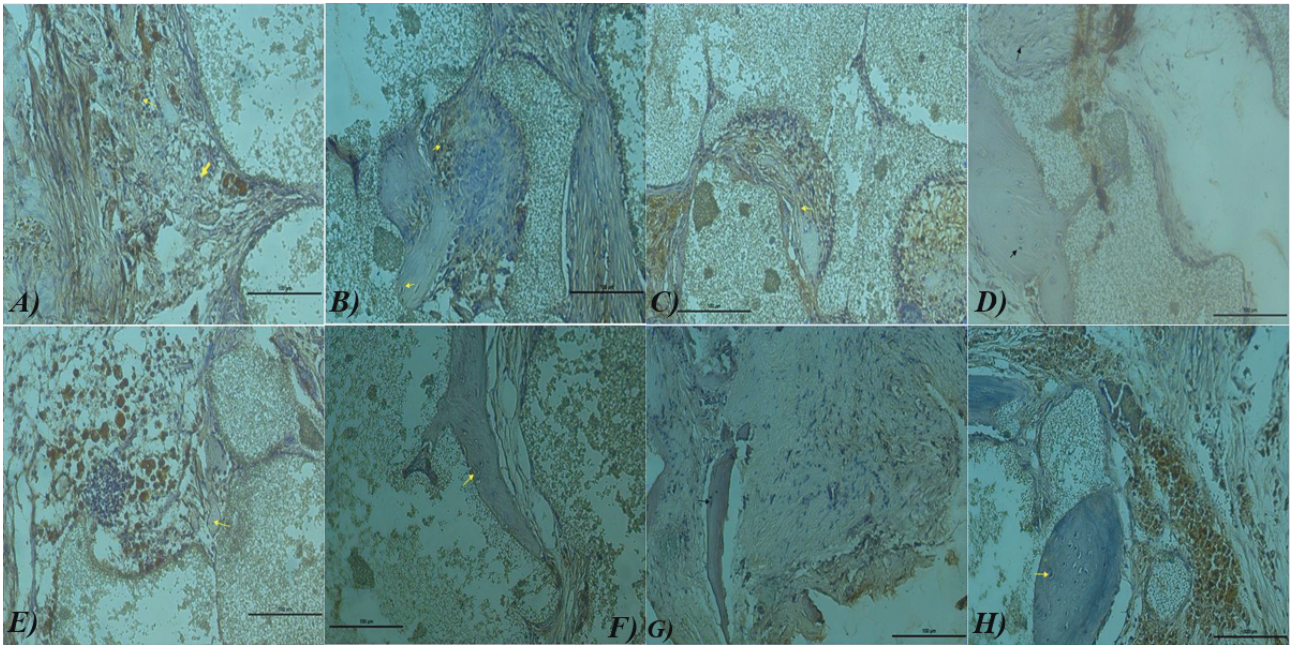
For the results of the histopathological examination of the bone samples taken the calvaria of the nondiabetic rats on the 28th and 56th days, the values of osteoblastic activity, inflammatory cell infiltration, vascular dilation and hemorrhage, and new bone formation were compared between all groups by Kruskal–Wallis test and a statistically significant difference was found between all groups (Table 1) ( $P < 0.001$ ).

The Mann–Whitney U test with Bonferroni correction was performed on the 28th day for nondiabetic rats' data that were also significant by Kruskal–Wallis test.

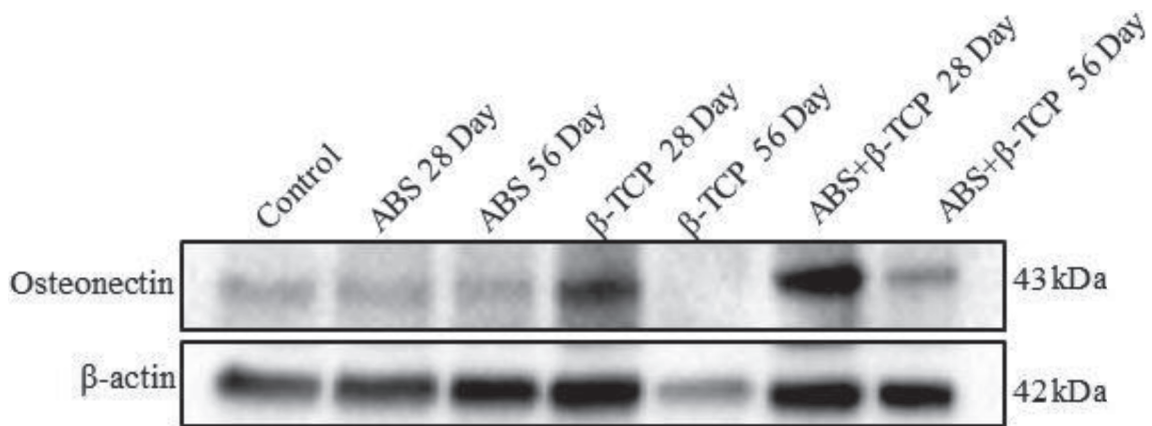
Osteoblastic activity, inflammatory cell infiltration, and new bone formation scores were compared between groups; there was a significant difference between the control group and the other groups. In comparison of vascular dilatation and hemorrhage scores between groups, significant differences were found between Groups 1 and 2, 1 and 3, 1 and 4, 2 and 3, and 3 and 4 (Table 1).

The Mann–Whitney U test with Bonferroni correction was also performed for nondiabetic rats' 56th day data that were also significant by Kruskal–Wallis test. Comparison of osteoblastic activity scores between groups was conducted. Significant differences were found between Groups 1 and 2, 1 and 4, 2 and 3, 2 and 4, and 3 and 4. There was a significant difference between Group 1 and Group 2, Group 1 and Group 3, and Group 3 and Group 4 in the comparison of inflammatory cell infiltration and vessel dilatation and hemorrhagic scores between groups. According to the comparison of new bone formation scores between groups, there was a significant difference between Groups 1 and 2, 1 and 3, 1 and 4, 2 and 3, and 3 and 4 (Table 1).





**Figure 3.** A) Immunohistochemical sections taken from Group 1 on day 28; osteonectin expression in connective tissue cells was positive (yellow arrow). B) Immunohistochemical section from Group 2 on day 28; osteonectin expression in osteocytes was positive (yellow arrow). C) Immunohistochemical section taken from Group 3 on day 28; osteonectin expression in osteocytes was positive (yellow arrow). D) Immunohistochemical section taken from Group 4 on day 28 shows positive osteonectin expression in osteocytes (thin black arrow). E) Immunohistochemical section taken from Group 1 on day 56 shows negative osteonectin expression in osteocytes (yellow arrow). F) Immunohistochemical section taken at 56th day from Group 2 shows positive osteonectin expression in osteocytes (yellow arrow). G) Immunohistochemical section taken at 56th day from Group 3 shows positive osteonectin expression in osteocytes (black arrow). H) Immunohistochemical section taken at 56th day from Group 4 shows positive osteonectin expression in osteocytes (yellow arrow). Osteonectin immunostaining; bar = 100  $\mu$ m.

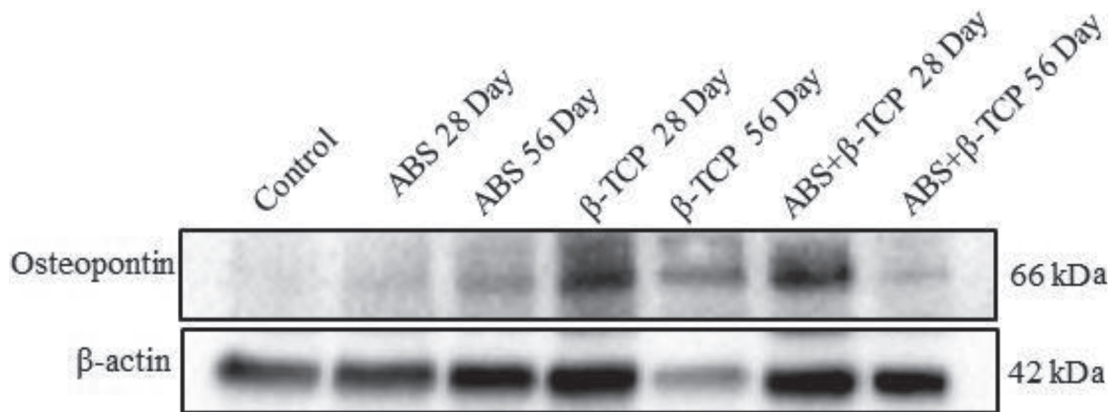


**Figure 4.** Osteonectin expression in calvarial tissue increased significantly in  $\beta$ -TCP and ABS +  $\beta$ -TCP groups at day 28.

### 3.4.2. DEXA results

The Kruskal–Wallis test was used to compare mean BMC and BMD data in the calvarial bone defects of diabetic rats. According to this test, BMC and BMD were found to be statistically insignificant between the groups, and the BMC values of days 28 and 56 were found to be statistically significant (Table 2).

The Mann–Whitney U test with Bonferroni correction was applied to nondiabetic rats' 28th day data that were significant by Kruskal–Wallis test. According to the comparison of BMC values between groups, a significant difference was found between Groups 1 and 2, 1 and 4, 2 and 3, and 3 and 4. According to the comparison of BMC values between groups of 58-day-old rats, a significant



**Figure 5.** Osteopontin expression in calvarial tissue increased significantly in  $\beta$ -TCP and ABS +  $\beta$ -TCP groups on day 28.

**Table 1.** Comparison of histopathological values of nondiabetic rats at 28th and 56th days.

Groups		Group 1 (mean $\pm$ SD)	Group 2 (mean $\pm$ SD)	Group 3 (mean $\pm$ SD)	Group 4 (mean $\pm$ SD)	P	P <sub>1-2</sub>	P <sub>1-3</sub>	P <sub>1-4</sub>	P <sub>2-3</sub>	P <sub>2-4</sub>	P <sub>3-4</sub>
Osteoblastic activity	28th day	1.38 $\pm$ 0.52	2.38 $\pm$ 0.74	2.50 $\pm$ 0.54	3.00 $\pm$ 0.54	<0.001**	0.014*	0.003*	0.001**	0.814	0.077	0.085
	56th day	1.75 $\pm$ 0.46	2.87 $\pm$ 0.35	2.00 $\pm$ 0.54	3.62 $\pm$ 0.52	<0.001**	0.001**	0.332	<0.001**	0.004*	0.008*	0.001**
Inflammatory cell infiltration	28th day	3.00 $\pm$ 0.54	1.50 $\pm$ 0.53	2.00 $\pm$ 0.54	1.00 $\pm$ 0.54	<0.001**	0.001**	0.004*	<0.001**	0.085	0.085	0.004*
	56th day	2.50 $\pm$ 0.54	1.25 $\pm$ 0.46	2.50 $\pm$ 0.54	0.75 $\pm$ 0.46	<0.001**	0.002*	0.694	<0.001**	0.002*	0.053	<0.001**
Vessel dilatation and hemorrhage	28th day	2.50 $\pm$ 0.54	1.00 $\pm$ 0.54	1.63 $\pm$ 0.52	1.00 $\pm$ 0.54	<0.001**	0.001**	0.010**	0.001**	0.036*	0.525	0.036*
	56th day	2.50 $\pm$ 0.54	1.38 $\pm$ 0.52	2.87 $\pm$ 0.35	1.00 $\pm$ 0.54	<0.001**	0.003*	0.117	0.001**	0.001**	0.175	<0.001**
New bone formation	28th day	1.38 $\pm$ 0.52	3.00 $\pm$ 0.54	2.63 $\pm$ 0.52	3.00 $\pm$ 0.54	<0.001**	0.001**	0.002*	0.001**	0.175	0.248	0.175
	56th day	1.25 $\pm$ 0.46	3.25 $\pm$ 0.46	1.88 $\pm$ 0.35	3.50 $\pm$ 0.54	<0.001**	<0.001**	0.015*	<0.001**	<0.001**	0.317	<0.001**

\*: Significant difference between groups at  $P \leq 0.005$ .

\*\* : Significant difference between groups at  $P \leq 0.001$ .

P, Kruskal–Wallis test; P<sub>1-2</sub>, P<sub>1-3</sub>, P<sub>1-4</sub>, P<sub>2-3</sub>, P<sub>2-4</sub> and P<sub>3-4</sub>, Mann–Whitney U test.

difference was found between Group 2 and 3, and Groups 3 and 4 (Table 2).

In-group comparisons of bone defects in calvaria of nondiabetic rats revealed no significant difference in the

comparison of BMC and BMD values up to the 28th day and 56th day. In the comparison of the groups for bone defects in the calvaria of nondiabetic rats, a significant difference was found in the comparison of BMC values of



the 28th and 56th days in all groups. There was a significant difference in BMD values between groups at the 28th day and the 56th day in Groups 2 and 4 (Table 3).

**4. Discussion**

The strength and continuity of bone tissue can be impaired for different reasons. Bone loss can occur in patients with

periodontal diseases, acute and chronic pathological processes, cysts, and trauma. Small bone defects can heal spontaneously without any surgical intervention, but large bone defects require reconstructive surgery. The success of bone regeneration depends on defect morphology and graft material. The applicability of regenerative procedures is limited and it is still difficult to obtain the desired new

**Table 2.** Comparison of BMC and BMD values between groups of nondiabetic rats.

		Group 1 (mean ± SD)	Group 2 (mean ± SD)	Group 3 (mean ± SD)	Group 4 (mean ± SD)	P	P <sub>1-2</sub>	P <sub>1-3</sub>	P <sub>1-4</sub>	P <sub>2-3</sub>	P <sub>2-4</sub>	P <sub>3-4</sub>
<b>BMC</b>	Beginning	2.34 ± 0.21	2.25 ± 0.10	2.25 ± 0.11	2.26 ± 0.11	0.904	-	-	-	-	-	-
	28th day	2.44 ± 0.11	2.76 ± 0.20	2.55 ± 0.03	2.73 ± 0.09	0.005*	0.014*	0.110	0.014*	0.020*	0.769	0.021*
	56th day	2.88 ± 0.12	3.03 ± 0.07	2.81 ± 0.17	3.10 ± 0.16	0.040*	0.050	0.539	0.065	0.043*	0.663	0.043*
<b>BMD</b>	Beginning	0.31 ± 0.01	0.30 ± 0.01	0.31 ± 0.01	0.30 ± 0.02	0.669	-	-	-	-	-	-
	28th day	0.31 ± 0.11	0.32 ± 0.01	0.31 ± 0.01	0.31 ± 0.01	0.074	-	-	-	-	-	-
	56th day	0.33 ± 0.01	0.34 ± 0.01	0.33 ± 0.02	0.35 ± 0.02	0.076	-	-	-	-	-	-

\*: P < 0.05, significant difference between groups.  
 P, Kruskal–Wallis test; P<sub>1-2</sub>, P<sub>1-3</sub>, P<sub>1-4</sub>, P<sub>2-3</sub>, P<sub>2-4</sub>, and P<sub>3-4</sub>, Mann–Whitney U test.

**Table 3.** In-group comparisons of BMC and BMD values of nondiabetic rats.

		Beginning	28th day	56th day	P <sub>0-28</sub>	P <sub>0-56</sub>	P <sub>28-56</sub>
<b>Group 1 (mean ± SD)</b>	BMC	2.34 ± 0.21	2.44 ± 0.11	2.88 ± 0.12	0.345	0.068	0.009*
	BMD	0.31 ± 0.01	0.31 ± 0.11	0.33 ± 0.01	0.655	0.059	0.166
<b>Group 2 (mean ± SD)</b>	BMC	2.25 ± 0.10	2.76 ± 0.20	3.03 ± 0.07	0.066	0.068	0.042*
	BMD	0.30 ± 0.01	0.32 ± 0.01	0.34 ± 0.01	0.066	0.066	0.015*
<b>Group 3 (mean ± SD)</b>	BMC	2.25 ± 0.11	2.55 ± 0.03	2.81 ± 0.17	0.068	0.068	0.043*
	BMD	0.31 ± 0.01	0.31 ± 0.01	0.33 ± 0.02	0.655	0.102	0.089
<b>Group 4 (mean ± SD)</b>	BMC	2.26 ± 0.11	2.73 ± 0.09	3.10 ± 0.16	0.068	0.068	0.021*
	BMD	0.30 ± 0.02	0.31 ± 0.01	0.35 ± 0.02	0.180	0.068	0.017*

\*P < 0.05, significant difference between two groups.  
 P<sub>0-28</sub> and P<sub>0-56</sub>, Wilcoxon test; P<sub>28-56</sub>, Mann–Whitney U test.

bone formation. For this purpose, new methods aimed at bone engineering and tissue regeneration are investigated. Tissue engineering applications are used to provide bone regeneration and cell proliferation in regions of bone loss. Tissue engineering, unlike traditional biomaterial applications, aims not only at the filling of the defect regions of the lost tissues but also the formation of the tissue having regeneration capacity (3,4).

Animal experiments are carried out mostly in dogs, rabbits, mice, pigs, and hamsters because the tissue structure of these animals is similar to that of humans. Rats are used in research because of easy availability, low cost, and the ease of obtaining tissue samples (13). For these reasons, we did our work on rats. There is no complete consensus on the size of the critical bone defect. While many authors accepted the critical bone defect size as 8 mm, others defined 4, 5, and 6 mm (14,15). In our study, a 7-mm critically sized bone defect was created.

The second phase of bone healing, the granulation phase, takes 7–12 days structurally. Granulation tissue formed after this phase slowly transforms into soft fibrous tissue. The soft callus formed during this process forms a harder callus by the precipitation of Ca and P salts. Callus formation occurs between 4 and 16 weeks (16,17). In our study, the euthanasia days of the experimental animals were determined by considering the bone healing rate and by reference to similar studies in the literature. Many organic, semisynthetic, fully synthetic, and alloplastic graft materials were used in order to improve the bone tissue with various losses of material due to different reasons (18). The duration and rate of resorption are important in bone graft materials. Early resorption may result in failure to achieve the desired bone regeneration in bone tissue. In recent years, research has focused on alloplastic grafts. Among these grafts,  $\beta$ -TCP is a type of graft with late resorption features that preserves its physical structure for a long time in the defect area. Sometimes this resorption period lasts for 1–1.5 years (19).

$\beta$ -TCP provides a very strong osteoconductive effect by providing intercellular communication between the osteogenic cells that remain in the lacunas, whereas  $\beta$ -TCP itself is very fragile. The skeletal structure is not resistant to strong mechanical forces (8). When condensing this graft at the defect site, excessive force should be avoided because changes in the physical geometry of  $\beta$ -TCP and structural breaks can occur. Although the pore size of  $\beta$ -TCP varies between 1 and 1000  $\mu\text{m}$ , the ideal pore size is between 50 and 150  $\mu\text{m}$  (20). The size of the  $\beta$ -TCP graft pore used in our study is between 25 and 100  $\mu\text{m}$ .

Goshima et al. (21) formed a segmental bone defect in rabbit tibia, and at week 4, they observed that the granulation tissue began to mineralize and that osteoclasts appeared. In the 6th and 8th week of the study, they found

that some of the graft particles began to be resorbed and new bone islets were formed. In our study, inflammatory cell infiltration and vascular dilatation and hemorrhage in the  $\beta$ -TCP group of the non-diabetic rats on day 28 were significantly lower than in the control group.

Ağacayak et al. (19) studied the effect of mesenchymal stem cells in critically sized bone defects and reported that very little osteoblastic activity occurred in the second week compared to that rate in the 8th week. Similar to this study, we found osteoblastic activity to be significantly higher in the  $\beta$ -TCP group than in the control group when comparing the histologic scores of the 56th day of nondiabetic rats. According to these results, existing osteoblastic activity on both the 28th day and the 56th day in  $\beta$ -TCP groups indicates that bone healing is continuing and defect filling occurs.

Chawla et al. (22) evaluated the  $\beta$ -TCP effect in 12 patients with a periodontal bilateral bone defect and reported that the defect filling was significantly higher in the  $\beta$ -TCP group compared to the control group.

Rojbani et al. (23) evaluated the osteoconductive effect of the combination of simvastatin, HA,  $\beta$ -TCP, and  $\alpha$ -TCP in calvarial defects of rats; after 6 and 8 weeks, the new bone formation with  $\beta$ -TCP and  $\alpha$ -TCP was higher in the samples.

Similar to the above studies, new bone formation was found to be significantly higher in the  $\beta$ -TCP group than in the control group when histological scores for days 28 and 56 of nondiabetic rats were compared. Thus, the evidence of significant increases in new bone formation after the 4th week in most of the studies performed with  $\beta$ -TCP grafts are consistent with the study of Rojbani et al. (23).

ABS is a plant-based hemostatic agent derived from *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum*, and *Urtica dioica*, approved by the Ministry of Health of the Republic of Turkey on 26 October 2007. All of these plants show their effects on the endothelium, blood cells, angiogenesis, cellular proliferation, vascular dynamics, and mediators. There are many studies reporting that ABS can be used as a hemostatic agent to control hemorrhage after dental extraction, surgical operation, subgingival curettage, and periodontal plastic surgery (24–26). Studies have also been conducted on the effect of ABS on bone and soft tissue wound healing (26). ABS has been reported to have a positive effect on new bone formation in the wound healing process (25).

İşler et al. (12) investigated the effects of ABS on early bone healing and fewer infections, less inflammation, and less necrosis was detected in the defect group with ABS while more new bone formation was reported. In our study, rates of inflammatory cell infiltration, vascular dilatation, and hemorrhage of nondiabetic rats were examined on day 28, when the histological scores of the groups

were compared. There was a significant decrease in the ABS group compared to the control group. A significant difference between the ABS group and the control group is consistent with the results of this study.

In a study of the late effects of ABS on bone surfaces, histopathological examination revealed that ABS accelerated early bone healing and did not cause any foreign body reactions (24).

In one study, the effect of ABS and heterologous bone graft on bone healing was evaluated with sinus floor augmentation applications of bone graft; there was no evidence that a new bone formation occurred in any group at week 1, but there was an increase in the formation of new bone in all groups during the other weeks and the maximum increase was observed in the ABS + heterograft group, with the results reported as similar in all groups at week 8 (27). In our study, the histological scores of the nondiabetic rats on the 28th day were compared, and although no statistically significant difference was found between the ABS and  $\beta$ -TCP + ABS groups, new bone formation was significantly higher in the  $\beta$ -TCP + ABS group than in the control group. This study is also consistent with the effect of  $\beta$ -TCP on the 56th day. A significant difference between the  $\beta$ -TCP + ABS group and the control group is consistent with the results of this study.

In our study, we found a significant difference between the ABS and control group when the histological scores of nondiabetic rats on day 56 were compared. A statistically significant difference between the ABS and control groups indicates that ABS is effective both in early and late bone healing. This is not consistent with the studies of Şimşek et al. (24) and Günay et al. (28). In our study, histologic scores of nondiabetic rats on day 56 were compared. The new bone formation was found to be significantly higher in the ABS and  $\beta$ -TCP + ABS groups than in the control group. The ABS and  $\beta$ -TCP + ABS groups were found to be significantly higher among themselves. Based on these results, it was found that the  $\beta$ -TCP grafts had a positive effect on bone regeneration on the 56th day alone and in combination with ABS, and the highest efficacy was with ABS.

Osteopontin is a major sialoprotein of the extracellular bone matrix obtained from animal bone for the first time. It has high affinity for hydroxyapatite, found in the bone structure, and is a bone protein that acts as a bridge between hydroxyapatite and cells (29). It is released from many tissue cells, such as bone, dentin, semen, kidney, fibroblasts in embryonic stroma, and wound healing areas (30). Osteonectin, an extracellular matrix glycoprotein with noncollagenous acidic properties, is synthesized by osteoblasts in the bone matrix. It is found in periodontal ligament, semen, bone, fibroblast, and osteoblast cells (31).

In mineralization, proteoglycans and osteonectin provide the release of calcium salts on collagen fibers because osteonectin shows high affinity in binding calcium salts (32).

We used the western blot method for serologic methods, which showed more reliable results to eliminate positively or negatively subjective evaluation of osteonectin and osteopontin expressions that resulted from our immunohistochemical staining method. More specific protein bands are formed by the western blot method. Thus, it enabled us to detect only certain proteins precisely among a large number of proteins. In our study, we found an increase in osteopontin and osteonectin expressions of nondiabetic rats at day 28. The increase in osteopontin expression is compatible with the findings of Nagata et al. (33) and Thorwarth et al. (34). In our study, the western blot method was used to determine which groups had a significant increase in osteopontin and osteonectin expressions. Osteopontin and osteonectin expressions were found to be significantly higher in the  $\beta$ -TCP and  $\beta$ -TCP + ABS groups on day 28. The expression of osteopontin and osteonectin expression were significantly higher in these two groups, indicating that osteoblast cells and osteocytes were observed intensively. The presence of cells that are effective in making bones in these groups indicates that there is bone formation in these defects.

In our study, there was an increase in osteopontin and osteonectin expressions in nondiabetic rats on day 56. The present study is compatible with that of Agacayak et al. (19). However, osteopontin and osteonectin expressions were found to be not significant in any group by the western blotting carried out to determine which groups had increased expression. The osteopontin and osteonectin expressions were higher in the early period, and there was no increase in the late period, which may be due to the osteoblastic activity being more intense in the early period.

DEXA measures the bone mineral density and amount through the absorption of photons at different energy levels (35). Today, it is still used as a gold standard. Scans are two-dimensional and are used to determine the area density. Barou et al. (36) examined bone loss and changes in rats with osteoporosis using high-resolution three-dimensional microcomputer tomography (3D- $\mu$ CT), DEXA, and histomorphometry. DEXA and 3D- $\mu$ CT detected bone loss changes on day 13 earlier than histomorphometry. In another study where the density of bone trabeculae in rats was measured by DEXA, histomorphometry, and computed tomography (CT), DEXA and CT were found significantly different from histomorphometry because DEXA and CT measure cortical and cancellous bone, whereas histomorphometry measures only cancellous bone (35). For this reason, in our study, DEXA was used to evaluate both early cortical and cancellous bone healing.



Findik et al. (35) investigated the effect of low-intensity ultrasound waves on autogenous bone graft healing; through DEXA analysis in 18 days, there was a significant difference between groups for BMC and BMD values. Reikeras et al. (37) investigated the effect of soft tissue on the healing of tibial fractures in rats and DEXA analysis on day 56 showed that there was no significant difference between BMC and BMD values between groups in fracture healing. BMD values of nondiabetic rats in our study are in agreement with the findings of Reikeras et al. (37) and are incompatible with the findings of Findik et al. (35). The fact that the findings of our study are inconsistent with the findings of Findik et al. (35) suggests that there may be an increase in bone density due to the early resorption of the autogenous bone used by Findik et al. (35). In the present study, BMD values were found to be low due to the long resorption time of the alloplastic graft material.

BMC values of the nondiabetic rats on day 28 were found significantly different between the control group and the  $\beta$ -TCP and  $\beta$ -TCP + ABS groups, and also between the  $\beta$ -TCP group and ABS and the ABS group and  $\beta$ -TCP + ABS. According to the findings of this study, the  $\beta$ -TCP graft and ABS increase the amount of bone on day 28 radiologically; however, the  $\beta$ -TCP graft is more effective in bone regeneration. The effects of  $\beta$ -TCP + ABS and  $\beta$ -TCP graft on bone regeneration were found to be similar. When BMC values of the nondiabetic group on day 56 were evaluated, the  $\beta$ -TCP group's value was significantly

higher than that of the ABS group. In the same way, there was a significant difference between the ABS group and the  $\beta$ -TCP + ABS group. In this study, it was found that  $\beta$ -TCP grafts on day 56 radiologically increased the amount of bone more than ABS but in similar amounts with  $\beta$ -TCP + ABS. There was no significant difference in BMD values between groups on the 28th and 56th days. Yuan et al. (8) reported that there was a difference between the control group and the  $\beta$ -TCP graft group when comparing the BMD values between groups at week 32. In our study, the planned 8-week duration was less in terms of BMD change, so no significant results were found. In general, there is no significant difference between BMD values in short-term studies in the literature.

There was no significant difference in the nondiabetic rats' BMC and BMD values up to the 28th day and up to the 56th day. This may be due to the fact that the period of the study was not long enough and due to the late resorption of the synthetic graft material we used. It may also be due to the material composition of  $\beta$ -TCP, pore size, geometry, and particle structure.

In conclusion, this study presents research on the combined use of a synthetic bone graft and ABS in bone regeneration in nondiabetic rats. We believe that although ABS suggests favorable wound healing and new bone formation in nondiabetic rats, using it together with a bone graft in cases where full bone regeneration is desired will lead to more successful results.

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