Clinical and Experimental Health Sciences

Evaluating the Effect of Jointly Administering Synthetic Graft and Ankaferd Blood Stopper for Bone Recovery in Created Bone Deformities in Diabetic Rats

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ABSTRACT

Objective: In the present paper, it was purposed to examine the late impacts of bone tissue recoveries in combination with synthetic grafts and Ankaferd Blood Stopper (ABS), which are effective in bone wound healing in diabetic rats.

Methods: A total of 64 Wistar albino male rats with diabetes were studied. A bone deformity was generated in the calvarium of diabetic rats. These diabetic rats are divided into 4 different groups. Only saline was applied to the bone defect in group 1, Beta-tricalcium phosphate (β-TCP) graft was administered to Group 2, ABS solution to Group 3, (β-TCP+ABS) were administered to group 4. Eight rats from each group were sacrificed on the 28th day and another 8 rats on the 56th day. Immunohistochemical, histopathological, and Dual Energy X-ray Absorptiometry (DEXA) analyzes of the obtained samples were made.

Results: In histopathological measurements, osteoblastic activity and bone regeneration were considerably higher in the group treated with group 2 and group 4 when compared to the control group on the 28th and 56th day ($p< .05$). Western blotting findings showed that the osteopontin (OP) and osteonectin (ON) expression at 28th day increased dramatically in the treated with group 4. DEXA analyzes revealed that BMC values in groups treated with group 2, group 3 and group 4 on 28th day were considerably higher than the control group ($p< .05$).

Conclusion: We can believe and conclude that ABS in combination with a β-TCP bone graft will produce more successful outcomes on wound healing and formation of new bone in diabetic rats.

Keywords: Bone Regeneration, Beta-tricalcium phosphate, Dual-Energy X-Ray Absorptiometry

1. INTRODUCTION

The periodontal disorder is an infectious disease characterized by periodontal tissue and bone loss that develops with bacterial chronic inflammation of tissues that make up the periodontium (1). The etiologic factor of periodontal disease is the pathogen bacteria found in microbial dental plaque and oral cavity. Bacteria are not alone responsible for the formation of periodontal disease. In the development of periodontal disease, the inflammation in the tissue may result in changes in bone morphology and deterioration of bone tissue (1,2). However, a lot of graft materials are used in periodontal defects. The purpose of bone grafting applications is to increase bone volume (3). Studies are still being carried out to achieve the desired effect in these applications. However, in spite of these studies, an ideal graft material has not yet been found to allow the lost tissues to return to their original state and fill the bone defects completely (3,4). β-TCP being a synthetic alloplast is a reliable and biocompatible graft material with osteoconductive and bioactive. It plays a role as a biological filler that is partially resorbed, mechanically clinging to the bone and enables the graft and bone to replace. However, β-TCP is a poor graft due to its inappropriate porosity, small grain and dissolution in a 6-weeks period (4,6).

Although the primary cause of periodontal diseases is a microbial dental plaque, it is accepted that existing systemic diseases increase the risk of periodontal disease by affecting the severity and prognosis of the disease. Diabetes mellitus (DM) is one of the most common systemic diseases (7,8). It has been reported that DM changes bone characteristics and has negative effects on fracture healing in both human and animal studies (9). Diabetes reduces osteoblastic activity and bone mineralization. In addition, microvascular complications of diabetes and therefore decreased blood flow increases bone fragility (10).

ABS (Ankaferd Pharmaceuticals Cosmetic Co., Ltd., Istanbul, Turkey) is the first herbal content used in Turkish medical sector as a haemostatic material containing Glycrhiza Glabra, Vitis Vinifera, Alphina Officinarum's dry leaf extracts, Urtica dioica's dry root extract, Thymus vulgaris' (thyme) dried herb extracts (11). ABS produces a protein network that supports erythrocyte aggregation. In addition, ABS is revealed to have an impact on bone healing at early periods (12). As a second

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characteristic, anti-inflammatory and antioxidant effect of ABS has been reported. The effect on the bone in the late period is not definitively determined (13).

There is limited number of studies evaluating effects of ABS on late-term diabetic bone tissue healing. Thus, the purpose of this research was to evaluate the impacts of jointly applying β-TCP and ABS as anti-bleeding agents on the recovery of diabetic bone tissue by histopathological, immunohistochemical and radiological methods.

2. METHODS

This study received an approval from Dicle University Experimental Animals Ethics Committee (No: 2015/13 – Date:18.02.2015). A total of 64 4-month-old Wistar male Albino diabetic rats, weighted around 300 g, were utilized.

2.1. Inducing Diabetes in Rats

0.294 g sodium citrate dihydrate was taken, and its volume was completed to 100 ml with pure water and, its pH was stabilized to 4.5 by hydrochloric acid (HCl). STZ (Streptococin®, Sigma-Aldrich, China) was prepared by sodium citrate buffer. A one shot of 50 mg/kg STZ solution was given to 64 rats to initiate diabetes by intraperitoneal injection. 3 days after STZ injection, glucose measurements were performed in the glucometer device (Optima®, Hsinchu, Tayvan) by taking blood samples from the tail vein of the rats by fasting for 12 h, if the fasting blood glucose was 140 mg/dl or higher, it was considered diabetic.

2.2. Surgical Method and Experiment Grouping

Experimental diabetic animals were left hungry 12 hours before surgery. The rats were applied 10% ketamine HCl and 2% xylazine HCl anesthesia. They were incised in the coronal midline of head skin via a no-15 surgical lancet, the heads were freely positioned. Later, the frontal bones were unveiled via a periost elevator. Following this procedure, a full-thickness bone incision was made in the midline circularly with a 7 mm diameter trephen burs (Trephine®, Turkey). After the operation, the skin incision was mainly covered with 3/0 silk suture for prophylactic purposes. Right after the surgery, one shot of 50 mg/kg antibiotic (Betamoxla®, Turkey) was administered into the muscles of each rat.

Diabetic rats used in our study were planned for 28th day and 56th day. Group 1**:** Saline was performed on bone defects of 16 diabetic rats. Group 2: 0.125 cc β-TCP (KeraOS®, Spain) graft with 0.25-1 mm particle was put into the deformities of 16 rats. Group 3: 0.125 cc ABS was administered to the deformities of 16 rats Group 4: 0.125 cc (β-TCP graft + ABS) was implanted into the bone deformities of 16 diabetic rats. In experimental diabetic animals, euthanasia was performed on 64 rats. Rats were anesthetized through sodium thiopental (Pentalyn Sodyum®, Turkey) whose mortal dose is injected as 60 mg/kg.

2.3. Preparing Sections for Histological and Immunohistochemical Examination

The calvarium bone was taken in an annular form with round burs to coat the deformed area. The samples were put in 10% formaldehyde solution. After completely fixing the samples, they were washed for 12 hours under water. Later, they were kept at the gradually concentration alcohol for dehydration during 12 hours. After that, they were embedded in the paraffin blocks. After making 5 µm sections via a microtome (Rotatory Microtome, Germany) from paraffin blocks, it was stained through Hematoxylin-Eosin (H-E).

Paraffin Sections were put on poly-L-lysine covered slides after the sections were kept in the xylenes for 2 x 5 minutes. Then, they were placed the EDTA (Ethylene-diamine-tetraacetic acid) solution to dissolve the bone tissue. All following processes were performed in this incubation vessel. Then, the Osteonectin and osteopontin antibodies (mouse monoclonal, 1/200, Santa Cruz, CA) were administered to the sections. Main antibody was dropped and kept for 1 hour. After biotinized dropping, secondary antibody (Histostain-Plus Kit, Carlsbad, CA) became consistent with the primary antibody. AEC (Aminoetil Carbazole) was dropped as chromogen. Antigen-antibody reaction was prevented by washing with distilled water. At the last stage, sections for blind evaluation were evaluated by photomicroscope (Nikon Eclipse i50, Japan) immunohistopathologically.

2.4. Western Blotting

The calvarium tissues frosted in liquid nitrogen were pulverized in porcelain mortar. 50 mg of powdered placenta tissue was maintained in 250 µl RIPA lysis solution including a blend of protease inhibitor in frost for 1 hour. Total cellular protein concentration was computed by the BCA Kit (Pierce, Thermo scientific). Protein samples were constituted in 1xSDS loading solution (2% SDS, 5% glycerol, 0.01% bromophenol blue, 8% DL-ditiyotretol) and electrophoresis was conducted in SDS running solution (2.4 mM Tris, 19.2 mM Glycin, 0.01% SDS) for 1 hour at 200 V. Removed proteins were moved from SDS-PAGE to PVDF membrane within transfer solution at 100 V for 1 hour. Following washing, the membrane was applied with horseradish peroxidase conjugated secondary antibodies at a dilution rate of 1/ 10000 for 1 hour at room temperature. Protein bands were visualized using ECL (increased chemiluminescent reagent) chemical (BioRad). Photos were taken using BioRad ChemiDoc™ MP tool.

2.5. Radiological Examination

Bone deformities of rats were measured with DEXA. Bone Mineral Amount (BMC) (gr) and Bone Mineral Density (BMD) (gr/cm2) calculations of all rats were carried out via the DEXA (Hologic®, USA) tool. But, solely, the measurements of the head was examined.

2.6. Statistical Analysis

The effect size was 2.56 and the α value was set at .05. The power of this experimental animal study was calculated to be 95%. The sample size was calculated in a computer program (G*Power, ver; 3.1.9.2, Kiel, Germany). In our study, statistical analyses of data were done with SPSS (IBM® Ver: 21.0, USA) statistical program. Mann Whitney U test was employed to make a comparison for the data with non-normal distributions for two groups, and the Kruskal Wallis test was employed for the two groups. Mann Whitney U test, as corrected by Bonferroni, was also employed to compare the groups. Wilcoxon test was employed for intra group comparison. p< .05 was considered as statistically important in all the tests.

3. RESULTS

3.1. Histological Findings

On the 28th day of group 1, intense vascularization and increased fibrous tissue were observed in the defect area. Vacuolar structures and few osteoblastic cells were witnessed in areas where bone trabeculae developed (Fig. 1A). On day 56 of group 1, dilatation and hemorrhage in blood vessels, inflammatory cell infiltration and an increase in fibrous tissue were observed (Fig. 1E). On the 28th day of group 2, reduced vascularity and diffused mononuclear cells in the outside area of the defect site were observable. We saw that the bone trabeculae where the defect rings are located was prominent. In addition, the osteocyte cells came to the forefront with the increase of osteoblastic activity (Fig. 1B). On the 56th day of group 2, an increase in the osteoblastic activity around the graft and prominent bone trabeculae with enhanced osteocyte formation was clearly observed. Hemorrhage was seen in blood vessels with inflammatory cells in the form of clusters (Fig. 1F). On the 28th day of group 3, small increases in vascularization and inflammatory cells were observed, and osteoblastic cells began to stand out in small trabecular parts where islet-like bone fragments were observed (Fig. 1C). On the 56th day of group 3, dilatation in blood vessels was seen, inflammatory cells were placed in clusters, and osteoblastic activity began in small bone trabeculae as wrapped around the graft (Fig. 1G). On the 28th day of group 4, a rise in collagen fibers and a decline in inflammatory cells in the deformed zone were seen, and the development of osteoblastic activity and osteocytes showed some increase. The bone trabeculae expanded and the osteocytes within the lacunae became evident (Fig. 1D). On the 56th day of group 4, there was a parallel distribution of collagen fibers along with hemorrhage and inflammatory cell density. Osteoblastic activity and osteocyte organization were clearly observed in the expanding bone trabeculae. New bone formation began to take shape (Fig. 1H).

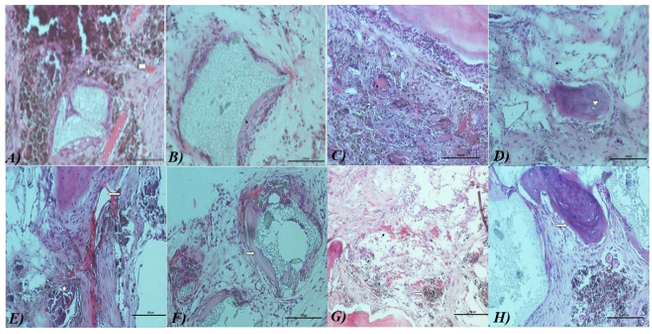

Figure 1. *Hematoxylin-eosin stained sections of the groups. A) Vascularization (stellate) and osteoblastic cells (white arrow) are shown on a histopathologic section taken at 28th day. B) Osteocyte cells (thin black arrow) at 28th day. C) A histopathologic section taken at 28th day showed vascularization and inflammatory cells (white arrow) and islet-shaped bone fragments (black thin arrow). D) The histopathologic section taken at 28th day showed collagen fibers (thin black arrow) and osteocytes within lacunae (triangle). E) On the histopathologic section taken at day 56, dilated blood vessels (thick white arrow) and inflammatory cells (stellate) are shown. F) A histopathologic section taken at 56th day demonstrates enhanced bone trabeculae (thick white arrow) developing osteocytes. G) Histopathological examination taken at 56th day revealed inflammatory cells (thick white arrow) and the onset of osteoblastic activity (thin black arrow). H) On the histopathologic section taken at day 56, inflammatory cells (stellate) and prominent osteocytes are shown (thick white arrow). Hematoxylin-Eosin staining Bar 100μm.*

3.2. Immunohistochemical Findings

On day 28 of group 1, negative osteopontin expression was observed in osteoblasts (Fig. 2A). On the 56th day of group 1, osteopontin expression was demonstrated in osteoblast cells between inflammatory cells outside the graft sites (Fig. 2E). On day 28 of group 2, positive osteopontin expression was observed in osteoblast cells in the periphery of bone trabeculae (Fig. 2B). On the 56th day of group 2, osteoblast cells in the bone grafts demonstrated positive osteopontin expression in bone trabeculae in the graft region, and osteocyte cells initiated to form new bone structure (Fig. 2F). On day 28 of group 3, osteopontin positive expression was seen in tiny bone trabeculae between inflammatory cells (Fig. 2C). On day 56 of group 3, weak osteopontin expression in osteoblast cells outside the bone trabeculae in the graft site and positive osteopontin expression among inflammatory cells were observed (Fig. 2G). Positive osteopontin expression in osteoblast cells in flat bone fragments at the graft site was demonstrated on the 28th day of group 4 (Fig. 2D). On the 56th day of group 4, osteopontin positive expression was seen in osteoblasts outside the bone trabeculae of different sizes except for graft sites, and osteopontin positive cells were observed in connective tissue cells (Fig. 2H).

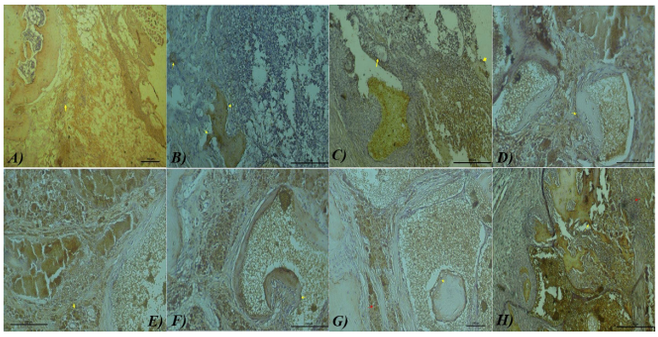

Figure 2. *Photomicroscope view of sections prepared with osteopontin antibody. A) Negative osteopontin expression in osteoblasts (yellow arrow) on the immunohistochemical section on day 28. B) The osteoblast cells in the periphery of the immunohistochemical section on day 28 were positive for osteopontin expression (yellow arrow). C) Osteopontin expression in bone trabeculae positive (yellow arrow) on the immunohistochemical cut at day 28. D) Positive osteopontin expression in osteoblast cells (yellow arrow) in the immunohistochemical section taken on 28th day. E) Osteopontin expression of osteoblast cells (yellow arrow) in the immunohistochemical section taken on day 56. F) On the 56th day, osteoblast cells marked positive osteopontin expression (yellow arrow). G) An immunohistochemical cross-section taken on day 56, osteoblast cells showed weak osteopontin expression (yellow arrow), and inflammatory cells showed positive osteopontin expression (red arrow). H) At day 56, positive osteopontin expression in bone osteoblast cells (yellow arrow) and positive osteopontin expression in osteoblast cells of connective tissue (red arrow). Osteopontin immunostaining Bar 100µm.*

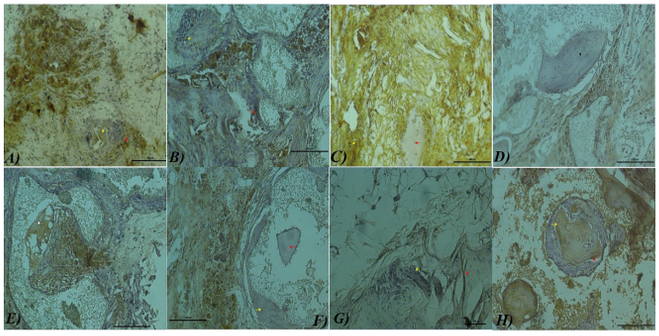

Figure 3. *Photomicroscope view of sections prepared with osteonectin antibody. A) The osteonectin expression in the connective tissue cells is positive (red arrow) and the osteocyte expression in osteocyte cells of bone is negative (yellow arrow) in the immunohistochemical section on day 28. B) Positive osteonectin expression in the connecting tissue cells (red arrow) and positive osteonectin expression in the osteocytes in bone (yellow arrow) are shown in the immunohistochemical section on day 28. C) Positive osteonectin expression (yellow arrow) in osteocytes and negative osteonectin expression in bone trabeculae (red arrow) are shown through immunohistochemical section taken on the 28th day. D) Osteonectin expression in osteocytes in the immunohistochemical section on day 28 (thin black arrow). E) Negative osteonectin expression (stellate) in osteocytes in the immunohistochemical section on day 56. F) Positive osteonectin expression (yellow arrow) in osteocytes and negative osteonectin expression (red arrow) in osteocyte cells in bone within the graft in the immunohistochemical section at day 56. G) Weak osteonectin expression in osteocytes (yellow arrow) and positive osteonectin expression in collagen bands (red arrow) immunohistochemical section taken on day 56. H) Positive osteonectin expression in osteocytes in bone (yellow arrow) and positive osteonectin expression in bone matrix (red arrow) at the immunohistochemical section on day 56. Osteonectin immunostaining Bar 100µm.*

On day 28 of group 1, positive expression was seen in the connective tissue cells in the collagenous fiber spaces among the inflammatory cells, while osteocytes in the small bone fragments showed an osteonectin-negative reaction (Fig. 3A). On day 56 of group 1, osteocyte cells did not develop in bone trabeculae within the graft site, and osteonectin expression was negative (Fig. 3E). On the 28th day of group 2, osteonectin-positive expression was less in certain connective tissue cells between the collagen fibers around the graft sites, while there was the osteonectin-positive reaction in a small number of osteocytes in bone trabeculae (Fig. 3B). On the 56th day of group 2, bone trabeculae began to mature within the connective tissue outside the graft site and positive osteonectin expression in osteocytes was observed. Negative expression was also detected in osteocytes in trabeculae within the graft site (Fig. 3F). On day 28 of group 3, positive osteonectin expression in osteocytes in some bone trabeculae was observed, while negative expression was also determined in certain trabeculae (Fig. 3C). On the 56th day of group 3, weak osteonectin expression in the osteocytes of the small bone trabeculae and positive osteonectin expression in the thickened collagen bands were seen (Fig. 3G). Osteonectin expression was demonstrated in osteocytes in bone trabeculae on the 28th day of group 4 (Fig. 3D). On day 56 of group 4, osteonectin expression was positive in a few osteocytes in the bone trabeculae placed between connective tissue cells in the graft site, while osteonectin expression in the bone matrix was positive (Fig. 3H).

3.3. Western Blotting Findings

The expression level of OPN and ON in the calvarium tissue rose drastically in β – TCP and ABS+ β – TCP groups on day 28. Twenty µg total protein was run on a gel. Anti-osteonectin and anti-β-actin antibodies were employed in Western Blotting method, and β-actin was utilized for load control (Fig. 4).

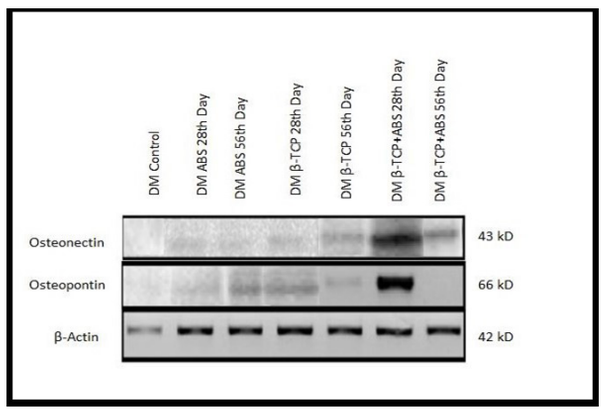

Figure 4. *Osteopontin and osteonectin expression in calvarium tissue rose dramatically in β-TCP and ABS+ β-TCP groups on day 28. DM: diabetes mellitus, β-TCP: beta-tricalcium phosphate, ABS: ankaferd blood stopper, kD: kilodalton.*

3.4. Histopathological Findings

The bone specimens taken on the 28th and 56th days from calvariums of diabetic rats were histopathologically examined, and significant difference was identified when the values of osteoblastic activity, inflammatory cell infiltration, vascular dilatation and hemorrhage and new bone formation were compared between groups (p< .001). Post hoc test was carried out on the 28th day of diabetic rats. There was statistically significant difference in terms of osteoblastic activity and new bone formation scores between group 1 and group 2, group 1 and group 4, group 2 and group 3, and group 3 and group 4. There was also significant difference in terms of inflammatory cell infiltration, vascular dilatation and hemorrhage scores between group 1 and group 2, group 1 and group 3, and group 1 and group 4. Post hoc test was applied to diabetic groups on the 56th day. There was significant difference in terms of osteoblastic activity and new bone formation scores between group 1 and group 2, group 1 and group 3, and group 1 and group 4 (Table 1).

Table 1. *Comparing histopathological values between groups on days 28 and 56 of rats with diabetes*

Groups		Group 1 (mean±S)	Group 2 (mean±SD)	Group 3 (mean±SD)	Group 4 (mean±SD)	p	p_{1-2}	p_{1-3}	p_{1-4}	p_{2-3}	p_{2-4}	p_{3-4}
Osteoblastic activity	28. Day	1.00±0.53	2.63±0.52	1.18±0.54	2.63±0.52	< .001**	.001**	.078	.001**	.001**	1.000	.001**
	56. Day	1.00±0.54	2.75±0.46	2.13±0.35	2.75±0.46	< .001**	.001**	.001**	.001**	.015*	1.000	.015*
Inflammatory cell infiltration	28. Day	3.63±0.52	1.38±0.52	2.88±0.64	1.25±0.46	< .001**	< .001**	.028*	< .001**	.001**	.602	.001**
	56. Day	2.88±0.64	1.13±0.36	200±0.54	1.13±0.64	< .001**	.001**	.013*	.001**	.004*	.945	.013*
Vessel dilatation and hemorrhage	28. Day	3.13±0.64	2.00±0.54	1.50±0.54	1.38±0.52	< .001**	.004*	.001**	.001**	.085	.036*	.626
	56. Day	3.38±0.52	1.00±0.54	1,.75±0.46	1.25±0.46	< .001**	< .001**	< .001**	< .001**	.013*	.332	.053
New bone formation	28. Day	1.13±0.36	2,.50±0.54	1.38±0.52	3.38±0.52	< .001**	.001**	.264	< .001**	.003*	.010*	< .001**
	56. Day	0.75±0.46	2.75±0.46	2.25±0.46	2.75±0.46	< .001**	< .001**	< .001**	.001**	.053	.880	.082

*SD: standard deviation, *p≤ .005: significant, ** p≤ .001: very significant, p: Kruskal Wallis Test; p1-2, p1-3, p1-4, p2-3, p2-4 and p3-4:Mann Whitney U Test*

Table 2. *Comparing BMC and BMD values between groups of diabetic rats*

		Group 1 (mean±SD)	Group 2 (mean±SD)	Group 3 (mean±SD)	Group 4 (mean±SD)	p	p_{1-2}	p_{1-3}	p_{1-4}	p_{2-3}	p_{2-4}	p_{3-4}
BMC	Beginning	2.25±0.09	2.28±0.10T	2.26±0.08	2.30±0.07	.800	-	-	-	-	-	-
	28. Day	1.97±0.20	2.65±0.17	2.75±0.12	2.89±033	.021*	.021*	.021*	.020*	.663	.146	.375
	56. Day	2.61±0.21	2.78±0.26	2.81±0.13	2.76±0.15	.442	-	-	-	-	-	-
BMD	Beginning	0.30±0.01	0.31±0.01	0.29±0.03	0.30±0.03	.708	-	-	-	-	-	-
	28. Day	0.30±0.04	0.31±0.01	0.31±0.01	0.33±0.03	.301	-	-	-	-	-	-
	56. Day	0.31±0.03	0.32±0.01	0.32±0.01	0.33±0.01	.560	-	-	-	-	-	-

*BMC: bone mineral amount, BMD: bone mineral density, SD: standard deviation, *p< .05: significant, p: Kruskal Wallis Test, p1-2, p1-3, p1-4, p2-3, p2-4 and p3-4: Mann Whitney U Test..*

3.5. DEXA Findings

The BMC and BMD values were measured in the bone defect in the calvarium of diabetic rats on the 28th day. Comparison of the mean BMC and BMD values between groups showed that 28th day BMC values were statistically significant (p= .021)**.** Post hoc test was performed on diabetic rats on the 28th day. Significant difference was seen in the BMC values between group 1 and group 2, group 1 and group 3, and group 1 and group 4 (Table 2).

We conducted an intragroup comparison of diabetic rats. No significant difference was found in the BMC and BMD values between the baseline days (28th day) and between days 28 and 56 of the rats. In Group 1, significant difference was found in the BMD values between 28th and 56th days (p=.021) (Table 3).

Table 3. *Comparing intragroup BMC and BMD values of diabetic rats*

		Beginning (mean±SD)	28. Day (mean±SD)	56. Day (mean±SD)	p_{0-28}	p_{0-56}	p_{28-56}
Group 1	BMC	2.25±0.09	1.97±0.20	2.61±0.21	.144	.068	.021*
	BMD	0.30±0.01	0.30±0.04	0.31±0.03	.713	.414	.375
Group 2	BMC	2.28±0.10	2.76±0.20	2.78±0.26	.068	.068	.189
	BMD	0.31±0.01	0.32±0.01	0.32±0.01	1.000	.180	.129
Group 3	BMC	2.26±0.08	2.75±0.12	2.81±0.13	.068	.068	.772
	BMD	0.29±0.03	0.31±0.01	0.32±0.01	.144	.144	.369
Group 4	BMC	2.30±0.07	2.89±0.33	2.76±0.15	.068	.068	.561
	BMD	0.30±0.03	0.33±0.03	0.33±0.01	.144	.144	1.000

*BMC: bone mineral amount, BMD: bone mineral density, SD: standard deviation, *p< .05: significant, p0-28 and p0-56: Wilcoxon Test, p28-56: Mann Whitney U Test.*

4. DISCUSSION

The main purpose of using these materials is to accelerate the healing process in bone tissue, restoring alveolar bone, gaining clinical attachment, decreasing pocket depth, and achieving desired bone healing in the regeneration zone (14).

Synthetic TCP and HA grafts are biocompatible, therefore, are widely used instead of autogenous bone grafts due to their osteoconductive properties. β-TCP is resorbed at the earliest 4 weeks on radiographs and SEM. After using β-TCP; new bone formation differs depending on the material combination, pore size, geometry and particle structure (15).

Diabetes is known to be a high-risk group for bacterial infections such as periodontitis, and the relationship between periodontitis and diabetes has been a topic of many research for years. Even periodontal diseases have been recognized as complications of diabetes (7,16). Studies show that diabetes affects the wound healing during the formation of the bone. It has been reported that diabetes reduces the production of osteoblasts, osteoids, and osteocalcin in bone tissue and destabilizes bone formation and destruction (17).

Experimental diabetes animal models can be created by chemical, viral and surgical methods. By administering a single dose of STZ chemically, diabetes can be formed by creating partial damage to pancreatic β cells. Junod et al. (18) reported that their STZ administration dose is 25-100 mg/kg. According to the literature, there is no difference in the diabetes effect between 55-65 mg/kg STZ doses. In addition, the general metabolism of diabetes in STZ-induced diabetic rats is similar to that of DM in humans (19). In the present study, diabetic rats were formed with a single dose of 50 mg/kg STZ.

There are some studies evaluating antibacterial, antimicrobial and antifungal efficacy besides the hemostatic effect of ABS (20). Studies have also been performed to examine the impact of healing bone and soft tissue scars (21,22). Bulut et al. (23) found that in studies evaluating the effect of ABS on bone healing in diabetic rats, forming new bone in the diabetic group was considerably reduced on both 7th and 14th day when compared to the control group. It was observed that the amount of forming new bone in the diabetic group treated with ABS was greater than in the control group.

In our study, the difference between ß-TCP (ß-TCP +ABS) group and the control group was statistically important when comparing the histological scores between the groups in diabetic rats on the 28th day. The control group was compatible with the findings of Alpan et al. (24). The fact that there is no statistically significant difference in new bone formation between ABS and the control group is compatible with the researches of Bulut et al. (23). Although ABS does not increase osteoblastic activity and new bone formation on day 28th there is a positive effect on the reduction of inflammation, vascular dilatation, and hemorrhage during bone healing. ABS was found to be highly effective in combination with ß-TCP, although it was not effective by itself in bone formation.

In our study, the difference between ß-TCP (ß-TCP +ABS) group and the control group was statistically important when comparing the histological scores between the groups in diabetic rats on the 56th day. But, the control group was contrary to Alpan et al. (24). ß-TCP and ABS have been found to have a favorable impact on bone healing in the late period, but the effect of ß-TCP on the bone was found to be higher than that of ABS. It may be due to the short-term resorption of the clot formed by the ABS in the defect area.

In our study, there was an increase in new bone formation on the 28th and 56th days when ß-TCP and ABS were applied separately compared to the control group. However, it was observed that this increase was higher in the ß-TCP group than in the ABS group. However, using ß-TCP and ABS together had a synergistic effect in forming new bone. The results of our study by Tanik et al. (22) are consistent with the findings of his study in rats nondiabetic. The reason for this can be explained by the fact that the ß-TCP graft remains in the defect area for a long time without resorption, and ABS is resorbed in the defect area quickly.

OPN enables the production and differentiation of different levels of osteoblastic cells in the bone and is secreted from many tissue cells such as wound healing areas (25). ON is

used to show the deep layers of osteoid and is thought to be effective in the mineralization of the bone matrix (26). In our study, a rise was found in OPN and ON expressions of diabetic rats on day 28th. Expression of OPN and ON were found considerably higher in β-TCP +ABS groups at day 28th. This may be due to high osteoblastic activity or the positive effect of ABS on bone wound healing with bone graft.

DEXA is frequently used in the diagnosis of osteoporotic patients. This method measures bone mineral density and quantity by the amount of absorption of photons at different energy levels[27]. Barou et al. [28] studied bone loss and changes in osteoporotic rats and utilized high-resolution three-dimensional microcomputer tomography (3D-µCT), DEXA and histomorphometric analysis. DEXA and 3D-µCT detected the bone loss earlier than histomorphometry on day 13. In another study where the density of bone trabeculae in rats was measured by DEXA, histomorphometry and computed tomography (CT), the DEXA and CT findings were found significantly different from the histometry findings; that's why DEXA and CT make cortical and cancellous bone measurements, histomorphometry only makes cancellous bone measurements [27]. For this reason, in the present study, DEXA was used to evaluate both early cortical and cancellous bone healing. In the study of AboElAsrar et al. (29) investigating the insulin-dependent impacts of cross-talk and growth factor-1 cytokines in Type 1 DM patients, DEXA analysis was performed using follow-up records of 10 months, so no significance was found between the BMC and BMD values of whole body and waist region among well controlled and uncontrolled diabetic patients. In a study by Duartae et al. (30) evaluating DM-related bone diseases, the DEXA analysis at day 120 did not show a significant in BMD values in the femur and metadiaphyseal region.

Studies that are similar to our study were taken as reference because of the lack of similar work. Only BMC values at 28th day were found significantly higher in comparison of BMC and BMD values. BMD findings of the present study support the results of AboElAsrar et al. (29) and Duartae et al. (30). In addition, it is compatible with the study of AboElAsrar et al. (29) in terms of BMC value at day 56th. Findings of BMC on the 28th day of the present study are inconsistent with AboElAsrar et al. (29). It may result from the shorter duration of 4-week of the present study than AboElAsrar et al. (29).

In our study, the diabetic rats of all experimental groups on the 28th day were significantly higher in BMC values than control groups. This is because the graft and ABS used in the early period of diabetes may have a slight effect. The BMD values on the 28th and 56th day is consistent with the literature. This may be because DM decreased bone density due to decreased bone trabeculae.

No statistically important divergence was found for BMC and BMD values between the baseline to 28th and the baseline to 56th day. The reason for not having a significant difference may be that the adverse effects of diabetes on bone delayed wound healing, the graft material was late resorbed or the amount of ABS was inadequate. There was a high degree of difference in BMC values only in the control group on the 28th and 56th day of the diabetic rats. These results are important to us in showing that diabetes has an adverse effect on bone regeneration.

As a limitation of our study, it is stated in the literature that the results of studies with a longer term of 6 months on bone regeneration treatments will be more successful. However, since it would be difficult for many rats to survive due to the side effects of diabetes in long-term bone regeneration studies in diabetic rats, we had to make plans to finish our study earlier.

5. CONCLUSION

ABS has an effect on wound healing and new bone formation in diabetic groups. However, we think that the use of ABS with a bone graft, especially in bone recovery, will yield more positive outcomes. Thus, further studies should be done in this field.

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