

Corticocancellous Bone Graft, Demineralized Bone Matrix and Synthetic Ceramic Bioglass for Defective Bone Fracture in Rats

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Introduction: Trauma, bone infections, and bone defects that emerge during the attempts as musculoskeletal tumor surgery can be treated with bone grafts. The aim of this study is to investigate the level of bone fracture healing that was treated using different kinds of grafts.

Materials and Methods: 40 male Wistar-Albino rats were used as 4 different groups with 10 rats each. The groups included the sham, the corticocancellous analog bone graft, the demineralized bone matrix and the synthetic ceramic bioglass. All rat groups were evaluated biomechanically and histopathologically.

Results: The average scores of the histological evaluations were as follows: 5.5 ± 1.64 in the sham, 5.62 ± 2.38 in the group-1, 6.9 ± 1.19 in the group-2, and 7.4 ± 1.17 in the group-3. There was a significant difference among all groups in terms of the histopathological findings ($p=0.041$). The p-values for the group pairs were as follows: the sham and group-2, $p=0.857$; the sham and group-3, $p=0.049$; the sham and group-4, $p=0.011$; groups 2 and 3, $p=0.22$; groups 2 and 4, $p=0.087$; groups 3 and 4, $p=0.37$.

Conclusion: Histopathologic findings showed the presence of a supporting effect of allograft on healing of defective bone fractures. However, biomechanical positive effects of allograft were seen by the demineralized bone matrix and bioactive glass ceramic.

Keywords: Ceramic, bioglass, corticocancellous, bone matrix, fracture, graft

Introduction

The skeletal system is a vital structure that provides the necessary support for the proper functioning of the organs and systems of the human body. This system is subjected to frequent injury with environmental factors and human factors (1). The skeletal system injuries can vary from a simple soft tissue injury to a severe condition, such as a fracture, depending

on the severity of the trauma (2). The pathology that results from a fracture is not limited to bone tissue. The surrounding soft tissues and organ systems will be negatively affected (3, 4).

The two major factors that influence the healing process of the fracture are the blood supply and the stability of the fracture (5). It is well known that several other factors also influence the healing process of a bone

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fracture, such as condition and configuration of the bone tissue, the periosteum, and the surrounding muscles/soft tissue. These factors' healthy function and their relationships with each other will affect the healing of the fracture (6). The studies on fracture healing focus on the fixation of the fracture, the systemic and local factors that influence the fracture, electrical and audio stimulation of the fracture, and hyperbaric oxygen applications. Many scientists are trying to develop new approaches to better understand the healing process of the bone, and ensure healthy healing of the skeletal system (5, 7).

The bone tissue has an extensive regeneration capacity; it can completely restore its original structure and function (8). However, in some situations such as trauma, infection, cysts, and tumors, the bone defects may not completely heal. In these situations, the bone defects may need to be replaced with bone grafts to initiate, accelerate or facilitate the healing process (9).

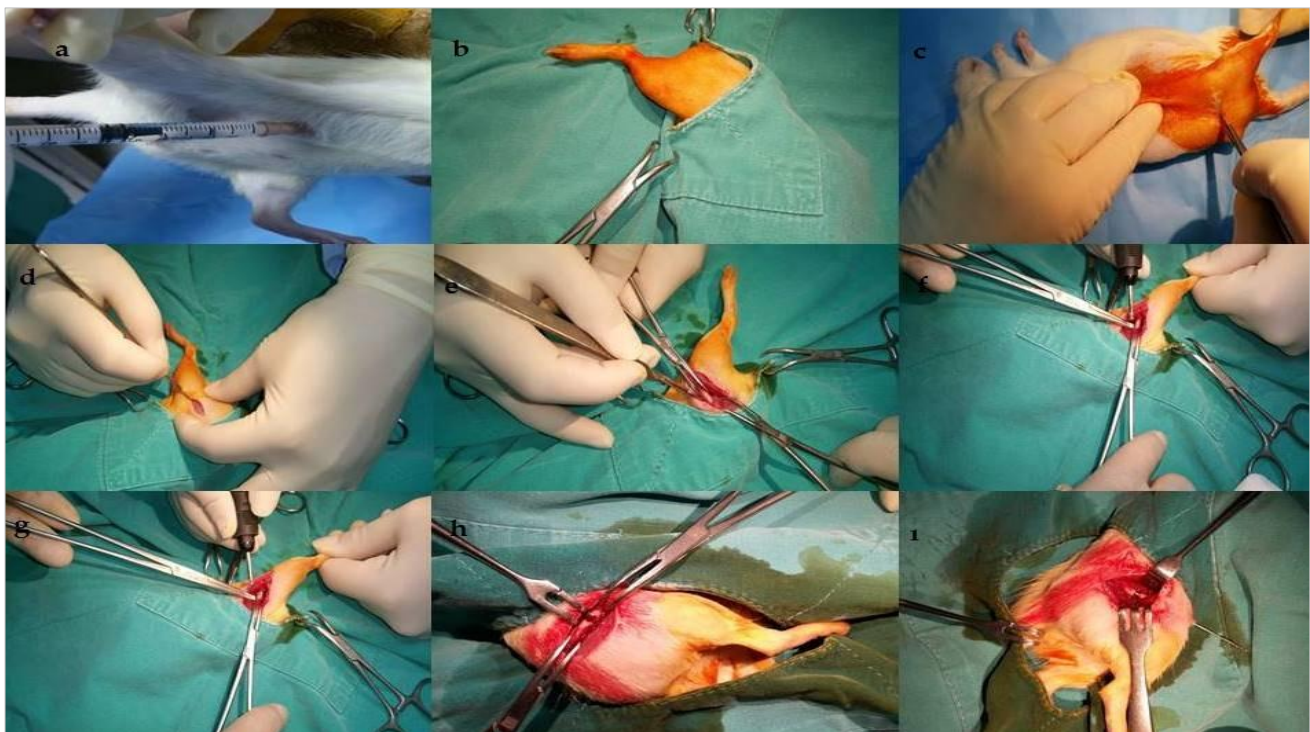
In this study, the rats' right femurs were fractured to form a bone defect, followed by intramedullary fixation. In the sixth week, the fractures were treated with different grafts. The histopathological and biomechanical aspects of the different graft treatment were investigated.

Materials and Methods

Study Design

In this experimental study, we investigated the effects of different graft materials on the healing process of the defective rat femur fractures. The study was carried out at our Experimental Animal Research Laboratory with a total of 80 male Wistar-Albino rats, obtained from the Laboratory Animals and Research Laboratory of Institute. The study was approved by the Research and Examination Committee of the Mustafa Kemal University Medical Faculty Medicine regarding the use of experimental animals (25.12.2016 - 2014/11/1). Wistar Albino rats were all males and older than 12 weeks, and weighed between 280-320 grams (Figure-1).

Figure-1. Preparation of Wistar Albino Rats



Experimental Groups

The rats were randomly divided into 4 groups. The group-1 was treated with the cancellous allograft (TranZgraft®, TBI/Tissue International, USA), group-2 was treated with demineralized bone matrix (DBM) (SureFuse®, Hans Biomed Corp., Seoul, Korea), and the group-3 was treated with bioactive glass-ceramic (BonAlive putty®, BonAlive Biomaterials Ltd., Turku, Finland). The control (*sham group*) was not treated with any graft material. There were no limitations on the diets of the rats. The subjects were kept in the optimal rat shelter, at an average of 22°C temperature and a 12 hours light/12 hours dark cycle. The subjects were fed with dry rat food.

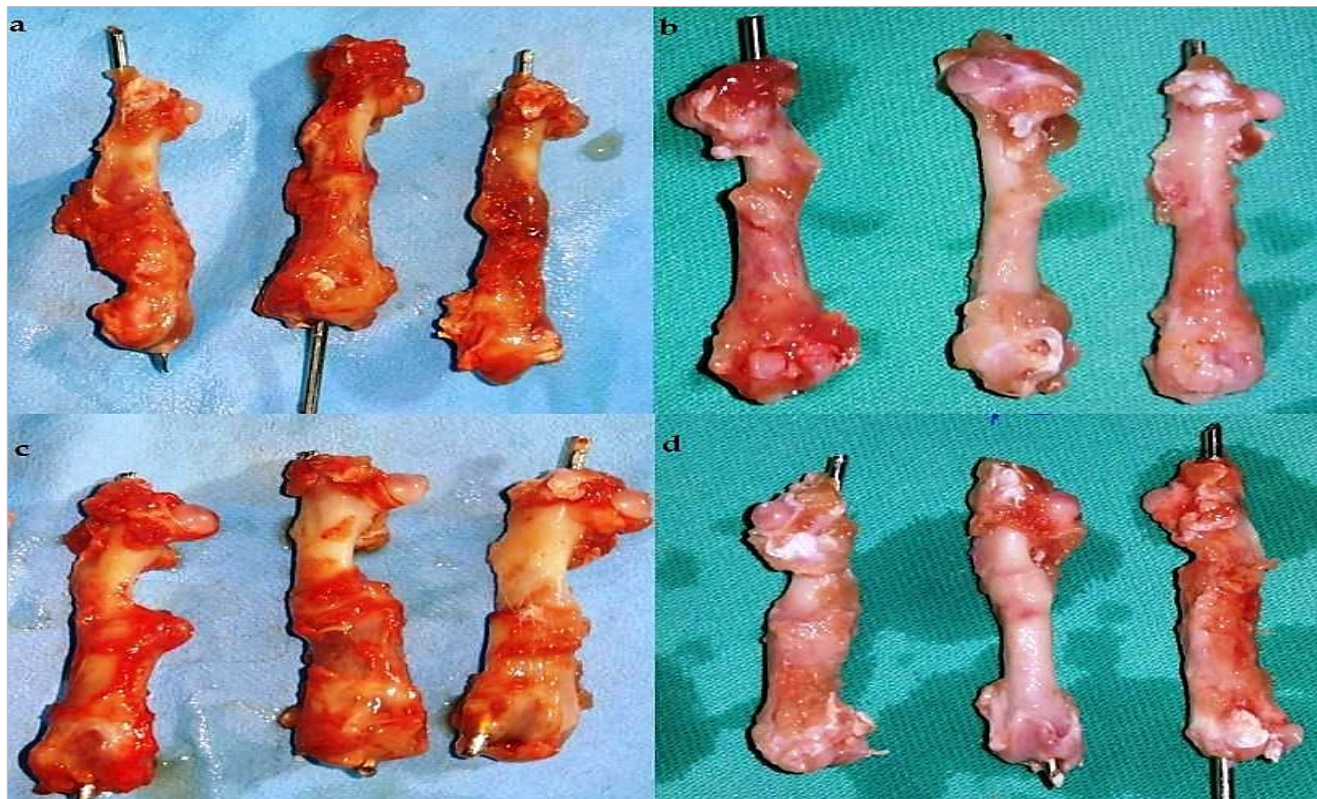
The care and nutrition of the subjects were provided by the designated personnel and was monitored by a veterinarian. After 6 weeks, all

rats were sacrificed with overdose of ketamine (100mg/kg/ketamine) and cervical dislocation. The sacrificed subjects' femurs were removed from the previous incision site and excised (Figure-2). The femurs were kept in a 10% buffer formalin solution until the histopathological and the biomechanical examination.

Surgical Technique

The dose of the drug was individually determined through the weight of each subject. The anesthetic drug [combination of 50 mg/kg Ketamine HCl (Ketalar®, Pfizer, USA) and 10 mg/kg Xylazine (Rompun®, Bayer, Turkey)] was applied from right groin intraperitoneally. After the administration of the anesthetic drug, the local area was sterilized with a Betadine scrub (10% povidoneiod, Merkez-Turkey). An antibiotherapy was applied before and after the operation (IV 50 mg/kg Cefazolin sodium).

Figure-2. Samples of femurs expelled from different working groups



a. Control; b. Allograft; c. Demineralized bone matrix; d. Bioactive ceramic glass

The skin was dissected from the lateral of the shaft with a 3-cm longitudinal incision. The muscles were bluntly dissected to reveal the femoral shaft. The defect was created (2 times the size of the shaft) by cutting the femoral shaft from the middle with a powered saw. The femur was defectively fixated with a 2 mm Kirschner wire (retrograde intramedullary). The part of the wire that remained outside was cut off with a chisel. In the control group, the formed defective region was not filled with a graft. In Group-1, the defective fracture was treated with a cancellous (freeze-dried) allograft; in group-2, with demineralized bone matrix; and in group-3, with bioactive glass-ceramic. The incision area was washed with 10cc of the sterile saline solution and sutured with a basic suture with a 2/0 silk thread. The wound was wiped with povidone-iodine.

Biomechanical Assessments

In the first step, the muscle tissue was completely removed from the femurs, as this is necessary for an accurate measurement. The soft tissue can alter the refracture of the bone. The bones were tried and fractured from the initial fracture line. The values that resulted from the fracture were digitally recorded. Two rats were excluded from the study. One rat from the allograft group had osteomyelitis, and another one rat from the glass-ceramic group did not heal and developed an abscess. The bones that were obtained from the sacrificed rats were at once tested with the three-point bending method, at room temperature (23 °C). This test was conducted by adding two attachments to the ZWICK/Z100 drawbench (Zwick Roell AG, Ulm, Germany). The femurs were placed to the lower attachment to provide an anteroposterior bending force. The effective span was 20 mm. The pull force was applied symmetrically to the

center of the rat femur, at the callus line. The initial pull force was 0 Newton and was increased at 20 mm/min until the femur was fractured. The ultimate load at the point of fracture was digitally recorded by a computer.

Histopathological Evaluation

There were a total of 40 rats that were divided into four groups of 10 rats. The surrounding soft tissue was removed from each femur, and the femurs were kept in a 10% neutral formalin solution for 48 h. Subsequently, the specimens were decalcified with a 10% formic acid solution. After one week of decalcification, an auto-technicon was used to dehydrate, clear and embed the femurs in paraffin. The longitudinal sections (5-6 µm) were perpendicular to the fracture line and in close proximity to the callus. They were later stained with hematoxylin and eosin (HE). The prepared slides' micrographs were evaluated by a pathology specialist with a binocular research microscope. The slides were evaluated regarding fibrous tissue, cartilage, new bone, and mature bone content, according to the scale reported by Huo et al. (10)(Table-1).

Table-1. Scoring system for histopathological evaluation

Grade	Histological findings
1	Fibrous tissue
2	Weighted fibrous tissue, a small amount of cartilage
3	Congenital fibrous and cartilaginous tissue
4	Weighted cartilage, a minimal amount of fibrous tissue
5	Cartilage tissue
6	Massive cartilage, a small amount of immature bone
7	Equilibrium cartilage and immature bone tissue
8	Weighted immature bone, a small amount of cartilage tissue
9	Fracture healing with immature bone
10	Fracture healing with mature bone

Statistical Analysis

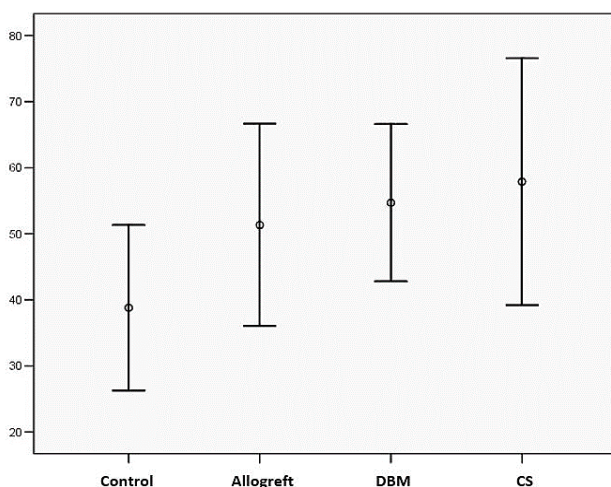
Means and standard deviation are used to report normally distributed data. It was agreed at the start of analysis that any variable with 5% or more missing data would be excluded. The obtained data were analyzed with the SPSS v21 Statistical Package (IBM Inc., Chicago, Illinois, USA), through the Kruskal Wallis analysis. The groups were compared in pairs with the Mann-Whitney U-test. In all the comparisons, a p-value of <0.05 was considered significant.

Results

Biomechanical Findings

The femurs obtained from the rats that were sacrificed on week six were tested with the three-point bending test (11). After the removal of the intramedullary material, the number of samples with sufficient integrity for the test were as follows: 10 femurs from the control group, 9 femurs from group 1, 10 femurs from group 2 and 8 femurs from group 3. The data from the biomechanical test are presented in Figure-3.

Figure-3. Graph showing force values of biomechanical results

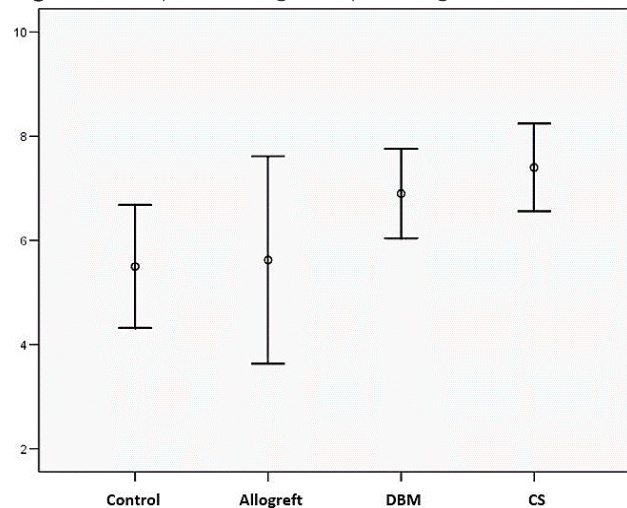


Histopathological Findings

The callus formation in the control group mostly had a regular appearance. The samples of study groups had fibrous tissue formation,

but in some of the samples, this appearance was replaced with cartilage and immature bone formation. The average scores of histological evaluations were as follows: 5.5 ± 1.64 in control, 5.62 ± 2.38 in the group-1, 6.9 ± 1.19 in the group-2, and 7.4 ± 1.17 in the group-3. The distribution of the histopathological scores is presented in Figure-4.

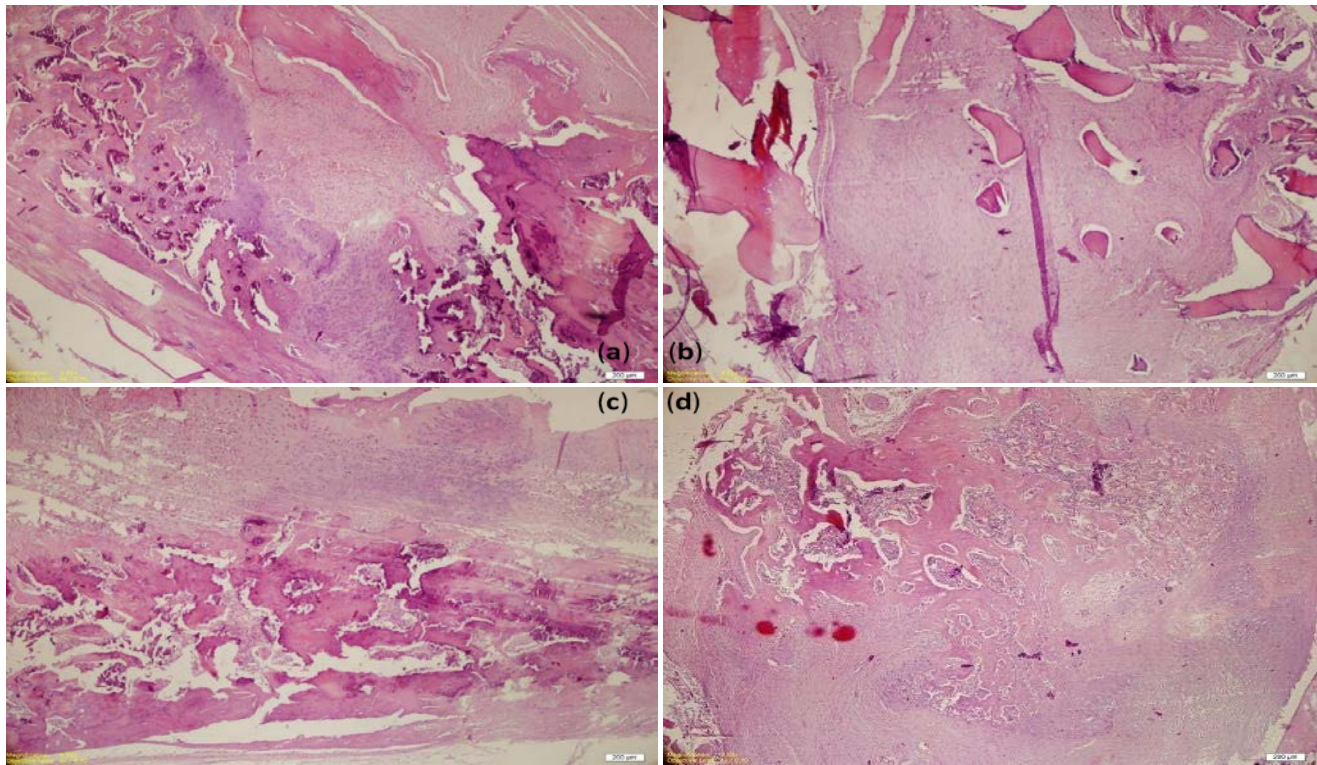
Figure-4. Graph showing histopathologic evaluations



There was a significant difference between the histopathological findings ($p=0.041$). The p-values for the group pairs were as follows: the control group and group 1, 0.857; the control group and group 2, 0.049; the control group and group 3, 0.011; groups 1 and 2, 0.22; groups 1 and 3, 0.087; groups 2 and 3, 0.37. The analyses revealed that the difference was significant only between the control group and groups 2 and 3 (Figure-5).

Discussion

Various methods have been developed to improve the orthopedic healing process of bone fractures. The applications that were found to improve bone healing started to be commonly used in clinical practice (12). There are also graft applications that are developed to biologically improve the healing

Figure-5. Histopathological images of 6th week**a.** Control group; **b.** Allograft group; **c.** Demineralized bone matrix; **d.** Bioglass ceramic group

process (13). The applications serve 2 important purposes: the replacement of a lost bone, and the correction of bone deformities to restore integrity and function. In our study, we created a defective fracture in the femur and stabilized it with intramedullary fixation. The purpose was to compare the biomechanical and histopathological results of the treatments including different kinds of grafts. We believe that the findings will contribute to the routine graft practices performing by surgeons.

Until recently, orthopedic surgeons could only use autologous bone grafts and allografts to replace bones during treatment. The bone autograft is regarded as the gold standard for the treatment of bone defects due to the low immune response (14). However, developments in tissue engineering applications, it is now possible to choose among many different options; including ceramics, demineralized

bone matrix, bone marrow, autologous growth factors, and composite grafts. The basis of bone tissue engineering is to create a response that will lead to healing in the desired anatomical area. The treatment aims to regenerate and repair the bone tissue through the stimulation of a combination of cells and inducing factors. Tissue engineering tries to imitate the body's natural tissue formation process to create functional living tissues (15). There are different factors that influence this process, such as the variety of the cell at the injury site, and the levels of different factors (16, 17). Closed fractures are regarded to be easier to heal than open fractures, as the hematoma has not drained, and there is less damage to the surrounding soft tissue. The experimental studies have also used closed fracture methods. In the open fracture model, the healing process is longer, and thus, it is easier to determine the rate of

healing between different groups (16). In our study, we have chosen to experiment with osteotomy in order to be able to form a bone defect and apply the grafts to the fracture line.

The experimental studies concerning bone healing use different animal models, such as dogs, rats, rabbits, sheep, and pigs. In general, it can be said that the large animal models are used to develop new devices, and the small animal models are used to better understand the molecular mechanisms and genetics of healing. The fact that most of the studies on fracture healing were performed on rats led us to make this decision (18). We decided to use isogenic mice that we obtained from the experimental animal research laboratory of our university. The studies on bone fracture healing that use rats mostly experiment on the tibia or the femur, due to the ease of manipulation, exposure, and preparation of the slides (19). Also, the morphology of the Wistar-type rats' femur is similar to that of the human (20). Therefore, Wistar type rats were preferred in fracture healing studies.

In the defective fracture studies, the purpose is to create a gap in the site of the fracture in order to investigate the activities of different modalities, such as grafts, growth factors, and hormones; and to subsequently fill this gap. The most commonly used model for the experimental studies is the rabbit and rat skull defects (21, 22). In our clinic, we mostly encounter defective fractures in the long bones, which are internally or externally fixated. In light of the literature, we chose to work with the femur and used the intramedullary nailing method which is accepted as the gold standard for femur fractures. After the formation of the defective fracture, the bone was fixated with the intramedullary application of the 2 mm K-wire.

It was easy to apply the technique and to obtain the material, however, there were structures that were not rigid. We accepted this drawback to be an advantage, as it would provide more reliable data regarding the observation of secondary bone healing, and that there is a more prominent callus formation in non-rigid fixations. Due to the small size of the rat femur, most of the studies choose intramedullary fixation. After the intramedullary fixation, the femurs were not further fixated. The studies mostly examine the healing in the sixth week (23, 24). This is because, at six weeks, the callus is now entirely replaced by the bone tissue; and the mineralization can be observed. Thus, we have chosen to examine the fractures in the sixth week.

The literature presents several different methods of histologically examining the tissue. Allen et al. have given the tissues a score from 0 to 4, concerning the healing process of the callus tissue. Huo et al. have given a score from 1 to 10 to determine the cellular changes in the fracture site (10). This scale uses the fibrous, cartilage, mature and immature bone tissues in the healing site to determine the degree of healing. In our study, we have used a histologic evaluation scale developed by Huo et al.

There are several studies concerning the effects of different grafts on healing. Tiedeman et al. have used the demineralized matrix in the nonunion tibia fractures of dogs (25). Their histological examination was performed on the 12th week instead of the 6th week, and they observed lots of cartilage, isolated calcified cartilage regions, and new bone formation through endochondral ossification. Bernabe et al. have investigated the effects of bioactive glass and calcium sulfate on the healing of rat tibias (26). On the 10th postoperative day, there

were no differences between the study groups. On the 30th postop. day, the bone formation was more prominent in the calcium sulfate barrier group. Rabie et al. have implanted endochondral autogenous graft, demineralized bone, and a combination of these two grafts to observe the healing process of the rabbit parietal bones (27). They reported that the bone healing was through endochondral ossification in all groups and that the healing had significantly improved in the group where the two materials were used together: the demineralized bone powder had increased the osteoinduction of both the receiving site and the bone graft.

There are many studies indicating that the demineralized bone matrix has similar results to autologous bone graft in the nonunion of long bones and the bone losses that occur due to fractures. Tiedeman et al. (28) have compared the bone marrow and DBM composite graft treatment (10 subjects) with the autograft from the iliac crest (8 subjects). They reported that after a 12-month radiological follow-up, 9 out of 10 patients with the DBM treatment had fully recovered, whereas only 6 out of 8 patients had a complete recovery. DBM is a mixture of bone morphogenic proteins (BMP) and it is immunogenic. The BMP mixtures are obtained from purified bone extracts. Johnson et al. have used a human BMP-DBM composite to treat 25 patients with resistant nonunion with a partial or complete segmental defect (29). The average follow-up was 21 months (5-82 months). They obtained 14 perfect, five good and five moderate results. Amorosa et al. have compared the physiological load on the callus tissue following the application of allograft, autograft, and scaffolding to the rat femur segmental defective fracture (9). They have

found that the biomechanical results were different between the groups. The rats were divided into four groups, and it was observed that the biomechanical results of the autograft and the allograft groups were better than those of the scaffold. In our study, we compared histopathological and biomechanical results of the different graft materials: freeze-dried cancellous allograft, demineralized bone matrix, and bioactive glass granules.

In the current study, the biomechanical data which were obtained from the three-point bending test were digitally measured and recorded. The control and study groups were not found to be significantly different. However, the callus tissue was macroscopically observed to be larger; the data were higher in the study group, which indicates the positive effect of graft use on the healing process of a defective bone. We believe a study with more extended healing periods may yield significant results. We saw that an immature bone tissue had developed in all groups except the control group. In the control group, cartilaginous and fibrous tissue were present in approximately equal parts. The comparison of the histological findings and scores revealed a significant difference between the control group and the graft groups. The different graft groups' histological scores also differed among themselves. This difference may be due to the different healing rates that arose from the non-rigid fixation. However, the significant difference between the control group and the graft groups indicate that these materials improve the healing process.

The limitation of this study is the lack of radiographic way. Similar studies radiologically evaluated parameters such as the bridging status, the prominence of the fracture line, and

the width of the callus. As we believed that these parameters might lead to errors in measurement, we did not include in our study.

Conclusion

In our study, we have found that the presence of an allograft, demineralized bone matrix and bioactive glass-ceramic did not improve the biomechanical strength of the fractured bone; however, the histopathological findings indicate that these grafts improve the healing process. These findings indicate that the graft materials can replace autografts, when the autograft leads to morbidity in the donor site or where sufficient material cannot be obtained due to the massive bone loss. We hope that the longer-term follow-up results of these materials will provide more meaningful results.

Conflict of Interests

The authors have declared no conflict of interest for the present article.

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