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Research of the effects of autologous cancellous bone graft and hyaluronic acid on the healing of bone defects experimentally induced in rabbits

Aydın SAĞLIYAN[1,*], Mehmet Cengiz HAN[1], Enis KARABULUT[1], Mustafa ÖZKARACA[2]

[1]Department of Surgery, Veterinary Faculty, Fırat University, Elazığ, Turkey

[2]Department of Pathology, Veterinary Faculty, Atatürk University, Erzurum, Turkey

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Abstract: The present study investigated the effect of autograft and hyaluronic acid (HA) in terms of clinical, radiological, and histopathological aspects on the healing of experimentally induced bone defects. The animals (n = 42) were assorted into 3 groups. After general anesthesia, a 3-mm standard defect was formed from the tibia of the experimental animals. The defects in Group I (n = 14) were left blank. Cancellous grafts in the proximal tibia were collected using a small curette to fill the defects in Group II (n = 14). The defects in Group III (n = 14) were filled by mixing with HA and cancellous grafts taken from the same region of the tibia. To determine the course of healing in the 3 groups in terms of clinical, radiological, and histopathological aspects, 7 rabbits from each group were chosen randomly on days 35 and 70 postsurgery and euthanized under anesthesia. Consequently, higher scores in terms of bone healing criteria were obtained in Group II, in which the graft was applied, compared with Group I. In Group III, in which the graft material was combined with HA, more healing was achieved compared with both Group I and Group II.

Key words: Bone defect, hyaluronic acid, autograft, rabbits

1. Introduction

Orthopedic trauma, oncologic surgery, and congenital disorders often leave patients with large bony defects. Bone defect is a clinically common factor leading to disability. Complete bone regeneration cannot be obtained in critical-sized osseous defects in the absence of an application of osteogenic or osteoinductive bone material. The best experimental results for most bone deficiencies are provided by osteoinductive molecules, such as bone morphogenetic proteins, which are powerful enhancers of bone healing (1,2).

Other molecules may also be used to heal bone defects: transforming growth factor beta 1, heparan sulfates, carboxymethyl benzylamide sulfonated dextran, heparin-binding growth factors, or heparin sulfate proteoglycans are major components of the extracellular matrix (3).

One of the major glycosaminoglycans, hyaluronic acid (HA), has been recently reported to increase osteoblastic bone formation in vitro through increased mesenchymal cell differentiation and migration (4–7). Locally applied high molecular weight HA also has been shown to stimulate differentiation and migration of mesenchymal and muscular cells in vivo (8). The therapeutic use of highly purified HA in human and veterinary medicine now is being advocated for applications such as idiopathic or experimentally induced osteoarthritis, viscosurgery, and viscosupplementation (7,9–11).

HA is a linear polymer of repeating disaccharide units with the structure D-glucuronic acid (1-β-3) N-acetyl-D-glucosamine (1-β-4), and is found in the extracellular matrix, especially in soft connective tissues. It is a negatively charged natural polymer that forms strikingly viscous solutions. HA plays an important role during fetal development and differentiation, facilitating cell migration and tissue morphogenesis. In addition, exogenous HA has been shown to have beneficial effects on healing (12,13).

This study aimed to detect the effect of autograft and HA in terms of clinical, radiological, and histopathological aspects on the healing of experimentally induced bone defects.

2. Materials and methods

2.1. Animals and experimental design

The study included 42 healthy, adult male New Zealand rabbits whose body weights ranged between 2.5 and 3.0 kg. The rabbits were kept under standard laboratory conditions (24 ± 3 °C, 40%–60% humidity, 12 h of darkness, and 12 h of light) and given standard pellet feed (Elazığ Feed

* Correspondence: asaglayan@yahoo.com.tr

Factory, Turkey) and water ad libitum. The protocol for the use of animals was approved by the National Institute of Health and the Local Committee on Animal Research.

Before starting the study, maintenance and feeding of all rabbits were carried out under the same conditions for 10 days. One week before the operation, the animals were randomly separated into 3 groups: Group I (control group, n = 14), Group II (n = 14), and Group III (n = 14).

2.2. Anesthesia technique

The rabbits were anesthetized first with intramuscular injection and premedication of 5 mg/kg of xylazine hydrochloride (Rompun, 23.32 mg/mL, Bayer, Turkey), and then with the intramuscular application of 50 mg/kg of ketamine hydrochloride (Ketalar, 50 mg/mL, Eczacıbaşı, Turkey). After anesthesia, the right back legs of the rabbits were shaved and disinfected.

2.3. Operation technique

A skin incision, beginning from the proximal end at the edge of the medial cranial part of tibia to the distal end, was made. To create a standard bone defect in the middle of the tibia, the acrylic bilateral external fixator technique was used. For this purpose, 4 pins, 2 of them on the proximal fragment and 2 on the distal fragment, were placed. The pins were located 1 cm far away from the defect edges. The distance between the 2 pins was 1.5 cm, and the pins were connected to each other by an acrylic fixator. Then the muscles were protected and a 3-mm standard defect was formed in the tibia of the experimental animals (Figure 1).

The defects in Group I were left blank. Cancellous grafts in the proximal tibia were collected using a small curette to fill the defects in Group II (Figure 2). The defects in Group III were filled by mixing with HA (Orthovisc, Anika Therapeutics Inc., Bedford, MA, USA, sodium hyaluronate, 15 mg/mL) and cancellous grafts were taken from the same region of the tibia.

The muscle and skin were closed with 3-0 vicryl. After the operation, the wound was protected by dressing for a week, and all the animals were administered 0.25 mL of 800,000 IU penicillin G procaine (Iecilline, I.E. Ulagay İlaç San Turk A.Ş., Topkapı, İstanbul, Turkey) intramuscularly as an antibiotic.

To determine the course of healing in the 3 groups in terms of clinical, radiological, and histopathological aspects, 7 rabbits from each group were chosen randomly on days 35 and 70 postsurgery and euthanized under anesthesia.

2.4. Clinical examination

After the radiographs of relevant tibias were taken, the surroundings were removed from the soft tissues without harming the callus tissue. Knitting tissue in the tibias was assessed by 3 people who participated in the study independently from each other. The inspection was done macroscopically in 2 planes and subjectively according to the scoring system (14,15) shown in Table 1.

2.5. Radiological examination

Radiological assessments were performed numerically with Lane and Sandhu's (16) radiological scoring system (Table 2). The groups were compared using one-way variance analysis and post-hoc Tukey's test, and the differences between intergroup time frames were compared by t test. A P value less than 0.05 was considered statistically significant. Statistical comparisons were made using SPSS 21.0.

2.6. Histopathological examination

After radiological investigations, the tibia samples fixed in 10% buffered formalin solution were decalcified with 10% nitric acid for histopathological examinations. The tissues were passed through a routine alcohol/xylene series and were stained with hematoxylin–eosin (HE) and Masson's trichrome. Histopathological assessment was performed

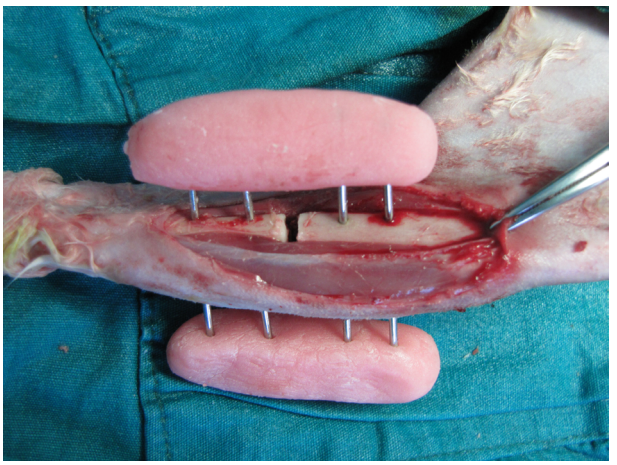

Figure 1. Establishing a standard 3 mm defect in the tibia of experimental animals (black arrow).

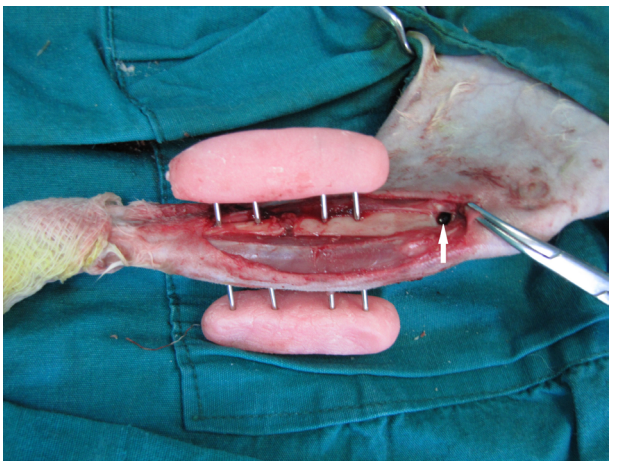

Figure 2. The region receiving autograft (white arrow).

Table 1. Clinical assessment of the callus tissue.

Score	Clinical findings in the broken part
0	No knitting (movement in both the planes)
1	Medium-level fusion (movement in a single plane)
2	Full fusion (no movement)

Table 2. Radiographic scoring system.

Score	Radiological findings
0	No healing
1	Callus formation
2	Onset of osseous knitting
3	Broken line starts disappearing
4	Full osseous knitting

according to the criteria used by Emery et al. (17) and analyzed using the Kruskal–Wallis statistical analysis (Table 3).

3. Results

3.1. Clinical findings

Clinical examination revealed a statistically important difference between Group I, and Groups II and III ($P < 0.05$). However, no difference was found between Group II and Group III (Table 4).

3.2. Radiological findings

Assessment of the radiographs taken on days 35 and 70 showed a statistically significant difference between Group I, and Groups II and III ($P < 0.05$). However, no difference was found between Group II and Group III (Table 5; Figure 3).

When the groups' results with regards to their own time frames were compared, there was no statistically significant differences found in either clinical or radiographic assessments.

Table 3. The scoring system used to assess histological healing of fractures.

Score	Tissue present
0	Empty cavity
1	Fibrous tissue only
2	More fibrous tissue than fibrocartilage
3	More fibrocartilage than fibrous tissue
4	Fibrocartilage only
5	More fibrocartilage than bone
6	More bone than fibrocartilage
7	Bone only

Table 4. Assessment of clinical findings on days 35 and 70.

Time	Group I	Group II	Group III
Day 35	0.28 ± 0.21^{aA}	1.28 ± 0.31^{bA}	1.62 ± 0.30^{bA}
Day 70	0.90 ± 0.33^{aA}	1.57 ± 0.30^{bA}	1.90 ± 0.25^{bA}

Different superscripts (a, b) in the same row indicate significant differences ($P < 0.05$) between the 3 groups (Groups I, II, and III). Different superscripts (A, B) in the same column indicate significant differences ($P < 0.05$) according to time within the same group.

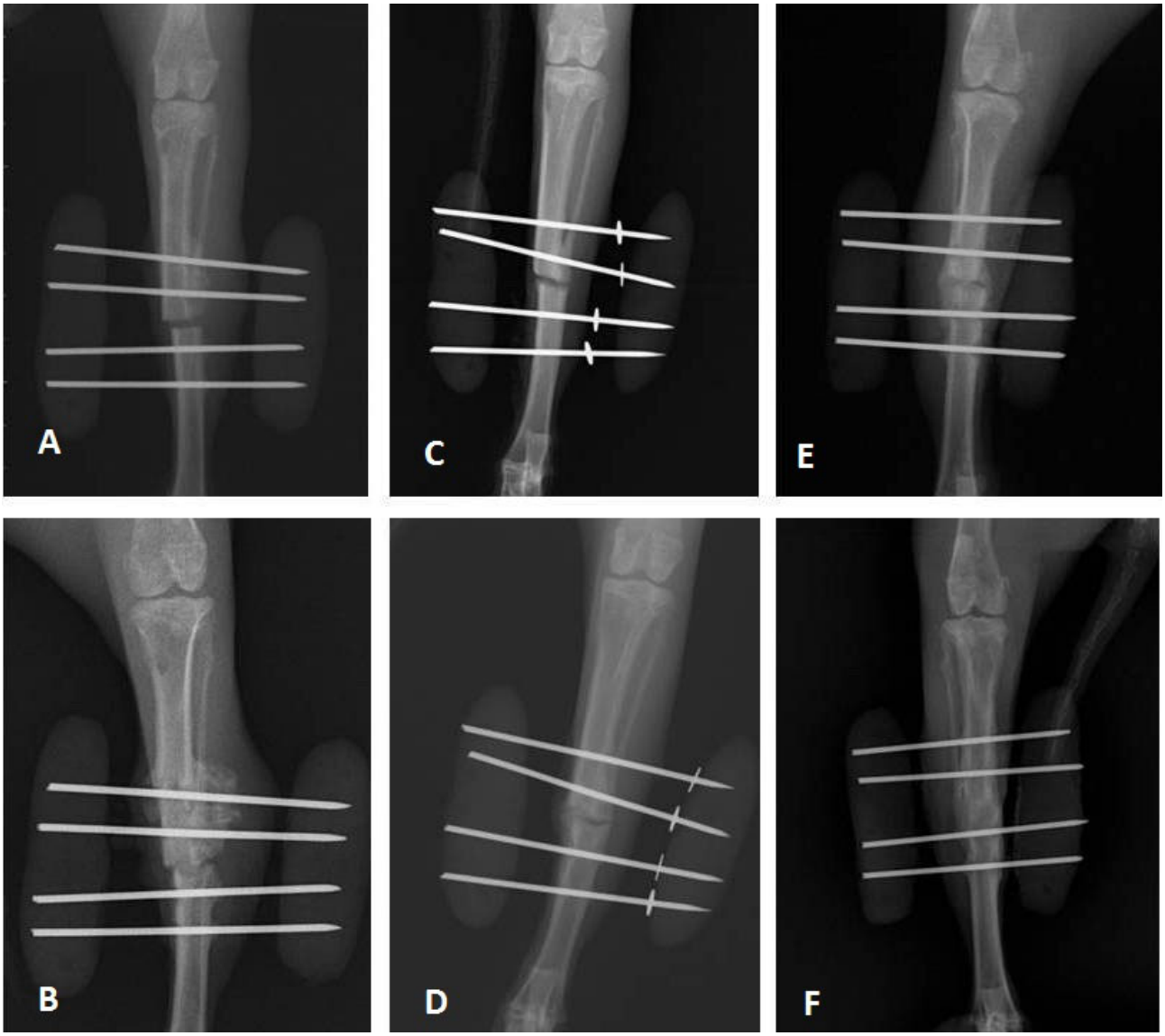

Figure 3. Radiographic results. (A) Group I, day 35; (B) Group I, day 70; (C) Group II, day 35; (D) Group II, day 70; (E) Group III, day 35; (F) Group III, day 70.

Table 5. Assessment of the radiographs taken on days 35 and 70.

Time	Group I	Group II	Group III
Day 35	0.95 ± 0.40^{aA}	2.89 ± 0.61^{bA}	3.17 ± 0.51^{bA}
Day 70	1.21 ± 0.42^{aA}	3.56 ± 0.19^{bA}	3.76 ± 0.19^{bA}

Different superscripts (a, b) in the same row indicate significant differences ($P < 0.05$) between the 3 groups (Groups I, II, and III). Different superscripts (A, B) in the same column indicate significant differences ($P < 0.05$) according to time within the same group.

3.3. Histopathological findings

On day 35, when the cavities of the rabbits in Group I were examined, 3 of them were found to have only fibrous tissue while 4 of them had a fibrous tissue also containing fibrocartilage (Figures 4A and 5A). In Groups II and III, the situation seemed to be more advanced compared with Group I. Four rabbits in Group II had fibrocartilage containing fibrous tissue and 3 rabbits had a structure

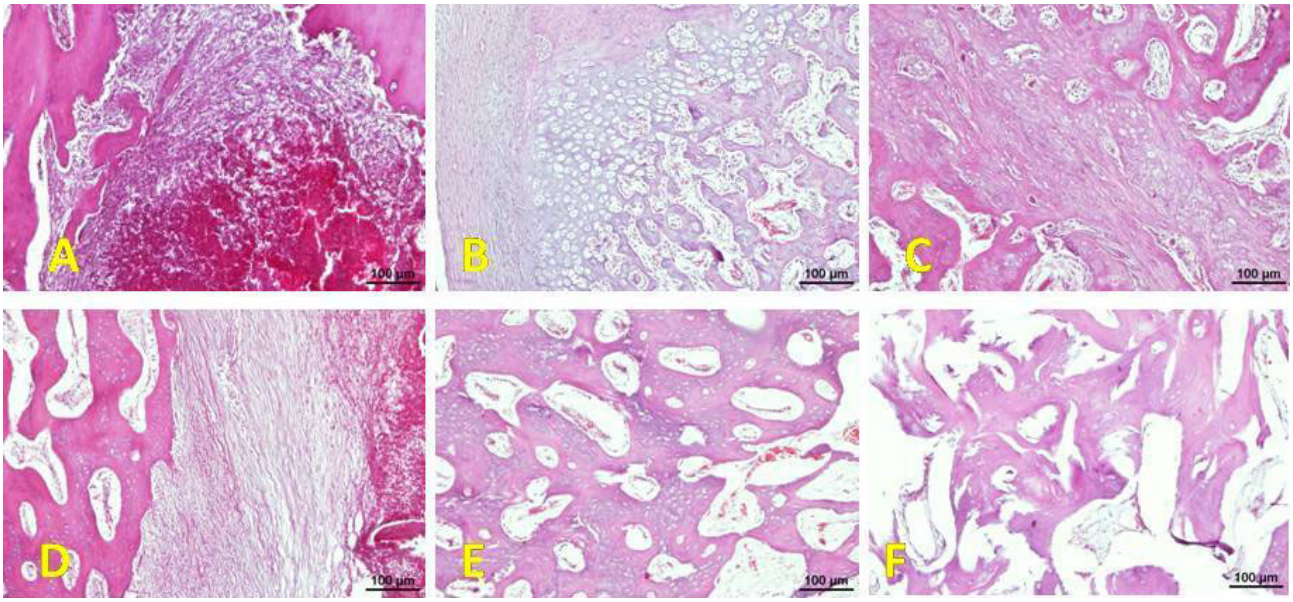

Figure 4. (A) Group I, day 35 (hematoxylin–eosin); (B) Group II, day 35 (hematoxylin–eosin); (C) Group III, day 35 (hematoxylin–eosin); (D) Group I, day 70 (hematoxylin–eosin); (E) Group II, day 70 (hematoxylin–eosin); (F) Group III, day 70 (hematoxylin–eosin).

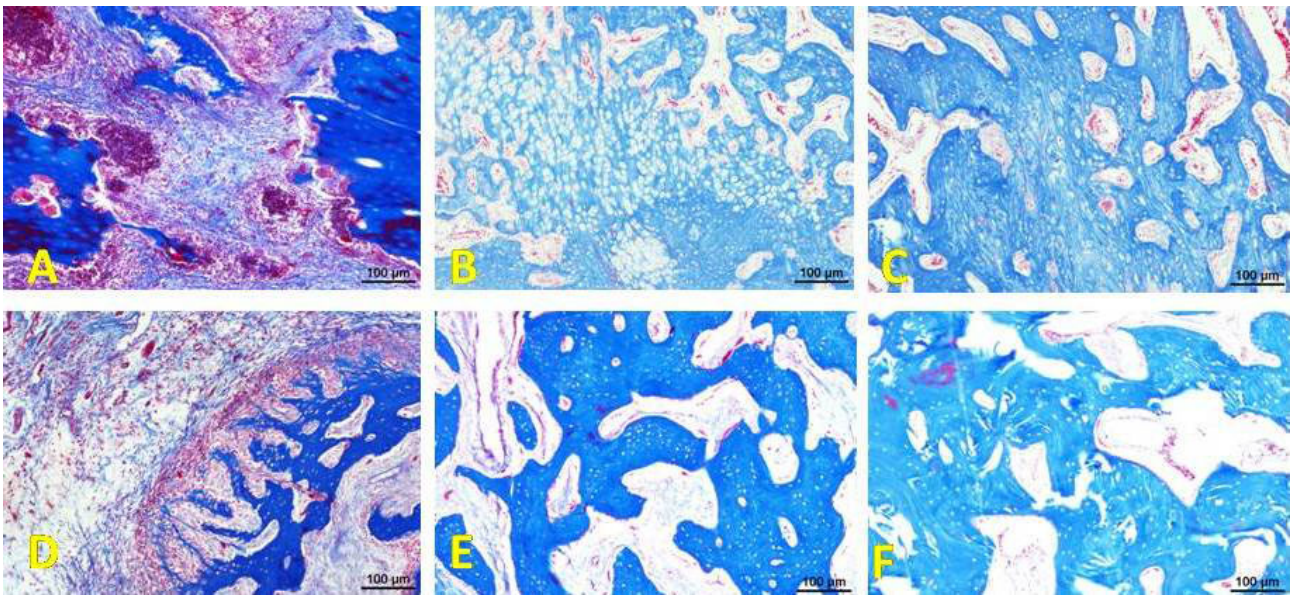

Figure 5. (A) Group I, day 35 (Masson's trichrome stain); (B) Group II, day 35 (Masson's trichrome stain); (C) Group III, day 35 (Masson's trichrome stain); (D) Group I, day 70 (Masson's trichrome stain); (E) Group II, day 70 (Masson's trichrome stain); (F) Group III, day 70 (Masson's trichrome stain).

containing only fibrocartilage (Figures 4B and 5B). In Group III, the healing was at an advanced level. Six rabbits had reshaping of a tissue containing only fibrocartilage and 1 rabbit had reshaping of a fibrocartilage tissue containing a little bone in it (Figures 4C and 5C; Table 6).

On day 70, fibrous tissue containing fibrocartilage was observed in 6 of the rabbits in Group I (Figures 4D and 5D). In Groups II and III, the healing was at a more advanced level compared with Group I. In 5 rabbits in Group II, fibrocartilage containing a little bone and, in 2 rabbits, bone tissue containing a little fibrocartilage emerged (Figures 4E and 5E). In 1 rabbit in Group III, the emergence of new bone was observed. In another 2 rabbits, the emergence of bone tissue containing a little fibrocartilage was detected (Figures 4E and 5E; Table 6).

Table 6. Assessment of clinical findings on days 35 and 70.

Time	Group I	Group II	Group III
Day 35	1.57 ± 0.20^{a}	3.42 ± 0.20^{b}	4.14 ± 0.14^{c}
Day 70	1.85 ± 0.14^{a}	5.14 ± 0.14^{b}	5.57 ± 0.12^{c}

Different superscripts (a, b, and c) in the same row indicate significant differences ($P < 0.01$ or more) between the 3 groups (Groups I, II, and III).

4. Discussion

Fracture healing is one of the issues in orthopedics not completely resolved yet. Researchers are investigating the factors that affect fracture healing and that can accelerate the process (18). In addition, the effects of frequently used drugs and the negative influence of nonsteroidal antiinflammatory drugs on fracture healing have a significance and are being explored (14,19,20).

In the present study, standard bone defects were created in the tibia of rabbits. The experimental animals were dividing into 3 groups (n = 42). The defects formed in the rabbits in Group I (n = 14) were left blank. The defects formed in the rabbits in Group II (n = 14) were filled with autogenous cancellous graft taken as fresh, while the defects in Group III (n = 14) were filled with autogenous cancellous graft mixed with HA.

Congenitally acquired bone defects frequently need a bone graft. In spontaneous healing, a mature fibrous tissue begins to form in the defect regions. With fibrotic healing, some clinical complications, such as nonunion and encapsulation, can emerge. To avoid such complications, and to provide regeneration of bone tissue in the region, reconstruction of defects with graft materials is required (21,22). In the defects that are not reconstructed with graft material, the cells of fibrous tissue migrate to the defect region and activate fibrotic healing. The defects are filled with collagen tissue until osteoblasts are formed, which negatively affects osteogenesis. The biggest disadvantage of fibrotic healing is that the fibrotic tissue formed does not function like bone tissue and is not shear resistant. Since the fibrotic tissue is not firm like bone tissue structurally, it cannot heal the defects and leads to problems such as pseudarthrosis, encapsulation, and nonunion (23,24).

Studies on allogeneic bone grafts showed that one of the biggest problems in using these materials was the risk of carrying illness from the donor. However, this problem could easily be solved by a freeze-drying method and adhering to tissue-banking standards. Although fresh autogenous bone graft provides the most ideal healing among graft materials it has some disadvantages, such as limited availability, an increase in postoperative morbidity, and formation of a new wound (25,26). In this study, cancellous grafts in the proximal tibia were collected using a small curette and the defects were filled. Sufficient amounts of bone graft were collected from all the rabbits in Groups II and III. The region where the graft was collected postoperatively did not encounter any complications. Furthermore, it was realized that the systematic application of antibiotics to all experimental animals for 7 days could be effective in preventing complications.

This type of healing has been used in ocular and joint surgery, orthopedic applications, burn treatment, closure of tympanic membrane perforations, prevention of intraabdominal adhesions, and early treatment of osteoarthritis. Sasaki et al. (11) followed the healing for 14 days after applying HA in bone defects in rat femurs and reported the formation of new bone from day 4 on the bottom and walls of the cavities in the HA group; at the end of week 1, the cavities were filled completely with newly formed trabecular bone. Furthermore, they observed newly formed bone tissue in the control group at the end of day 7; the cavities were filled completely with newly formed trabecular bone at the end of day 14. Moreover, they found that the granulation tissue formed in the cavities to which HA was applied in the first period left its place and moved rapidly to the newly formed bone marrow tissue.

The present study found a significant difference between Group I and the experimental groups (Groups II and III) on days 35 and 70 (Table 6). Although no significant difference was noted between Group II and Group III, Group III was better in terms of clinical, radiological, and histopathological aspects. These results indicate that HA has a positive effect on the healing on bone defects.

The researchers suggest that HA plays a role in maintaining some growth factors released locally and accelerates new bone formation during bone wound healing by stimulating osteogenic differentiation (11,27). In a study on the healing of experimentally induced defects in femurs in rats, HA was applied alone and combined with demineralized bone matrix (DBM). The results were monitored for 16 weeks and, during histopathological evaluation, the most common endochondral ossification was observed in cavities treated with DBM combined with HA. Oakes et al. (28), who suggested that HA had osteoinductive potential, also reported that HA might

have clinical applications with and without bone graft material. Hunt et al. (29) examined the effects of HA and bone morphogenetic protein (BMP) on bone healing in bone cavities. They demonstrated that HA stimulated bone healing but did not have any osteoconductive effect. They found that the healing rate in bone cavities where a combination of BMP and HA was applied was higher than the healing in cavities where BMP and HA were applied individually. Furthermore, they stated that HA could be resorbed in 12 weeks and used as a suitable carrier material.

In this study, 3 mm of the defects formed from the tibia of the rabbits as standardized were left blank in Group I, filled with autogenous cancellous grafts in Group II, and filled with grafts combined with HA in Group III. On days 35 and 70, fraction healing was assessed in terms of clinical, radiological, and histopathological aspects. Fraction healing was found to be the best in Group III. These results were consistent with the findings of previous studies.

Solchaga et al. (27) compared the use of HA gel and calcium phosphate-based graft material with rabbit mesenchymal progenitor cells as osteogenic or chondrogenic carrier. Three weeks after implantation cartilage tissue was observed in HA gel samples, and at the end of week 6 bone cells were located more commonly and cartilage tissue developed. At the end of weeks 3 and 6, only loose collagen tissue emerged in the control group. At the end of week 3, bone and cartilage tissue scores were approximately 2 times more than graft scores in the HA gel group. The researchers suggested that this difference between histopathological scores resulted from the fact that the surface of the HA gel had a porous structure and was able to connect to the tissues.

In another study conducted on rabbits, bone grafts treated with HA were applied to bone defects that were formed, and their knitting status as histological was investigated. On days 30 and 40, the knitting in the experimental group, to which grafts treated with hyaluronic acid was applied, was found to be significantly more compared with the control group (30).

In this study, on day 35, 3 of the rabbits in Group I were found to have fibrous tissue while 4 had fibrous tissue containing fibrocartilage (Figures 4A and 5A). In Groups II and III, healing was at a more advanced level compared with Group I. In Group II fibrocartilage tissue containing a fibrous structure was detected in 4 rabbits and a structure containing only fibrocartilage in 3 rabbits (Figures 4B and 5B). In Group III, the healing was at the most advanced level. A tissue containing only fibrocartilage was formed in 6 rabbits and a fibrocartilage tissue containing a little bone developed in 1 rabbit in this group (Figures 4C and 5C; Table 6).

On day 70, fibrous tissue containing fibrocartilage developed in 6 of the rabbits in Group I (Figures 4D and 5D). In Groups II and III, healing was at a more advanced level compared with Group I. It was detected that in 5 rabbits in the graft group, a fibrocartilage containing a little bone developed and, in 2 rabbits, bone tissue containing a little fibrocartilage was formed (Figures 4E and 5E). In Group III, the healing was at the most advanced level. In 1 rabbit in this group, new bone formation was observed. In the other 6 rabbits, the development of a bone tissue containing a little fibrocartilage was detected (Figures 4F and 5F; Table 6). In the present study, one of the reasons why better healing values were obtained in Group III may be the suitable environment provided by the material combined with HA for the osteoinductive effect.

The present study demonstrated higher scores in terms of bone healing criteria when graft material combined with HA was used. Hence, the combination can be considered an alternative choice for healing bone defects.

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