



Effects of demineralized bone matrix on tendon-bone healing: an *in vivo*, experimental study on rabbits

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Objective: The aim of this study was to investigate the effects of demineralized bone matrix (DBM) on tendon-bone healing.

Methods: The extensor digitorum longus tendon was fixed with pegged suture technique in a tunnel at the proximal tibia in both legs of 12 New Zealand rabbits. Rabbit DBM was applied in the tunnel on the right limbs before fixation (study group), while the fixation was performed without DBM in the left legs (control group). Randomly, four rabbits were sacrificed at the 3rd, four rabbits at the 6th and the remaining four rabbits at the 9th week with an intravenous high dose (200 mg/kg) pentothal and both legs were collected for histological analysis. Each specimen was blindly and independently examined to assess fibrocartilage formation, new bone formation, tendon graft bonding to adjacent tissue and Sharpey's fiber formation. A scoring system was used for quantification of histopathological analysis.

Results: The DBM group showed higher number of Sharpey's fibers, slightly increased fibrocartilage formation and new bone formation scores than the control group in the 3rd week. All histological scores were similar in both groups in the 6th and 9th weeks ($p>0.05$).

Conclusion: DBM increased new bone formation and the number of Sharpey's fibers in a bone tunnel animal model within the first three weeks of tendon-bone healing process.

Key words: Tendon-bone healing; demineralized bone matrix; bone tunnel.

The healing of a tendon graft within the bone tunnel is a basic biological process necessary for successful results in many reconstruction techniques. The quality and speed of tendon-bone healing within a tunnel are essential for early rehabilitation and return to sport.^[1]

Extended immobilization after reconstruction weakens the tendon, causes joint stiffness and may impair the results.^[2] On the other hand, it is also argued that avoidance of early activity during the post-reconstruction period is useful for a healthy and continuous

biological healing process at the tendon-bone interface. Most studies, however, advocate techniques to increase the strength of the initial fixation and the initiation of early controlled motion.^[3-5]

Gallie and Le Mesurier^[6] examined the behavior of tendon within bone tissue in 1922. Many animal studies showing the bonding of tendons on or within the bone in an ossified way during the healing period have since been conducted. The fibers newly produced during the healing process between the tendon and bone

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are called Sharpey's fibers. They lie vertically to the long axis of the tendon and appear to perforate the bone. These fibers are the primary structures enabling the tendon to attach to the bone firmly in an indirect way.^[7,8] Many biological and non-biological (with different fixation techniques) studies have been carried out on earlier and stronger fixation since the description of Sharpey's fibers. The addition of growth factors within the bone tunnel (such as bone morphological proteins, transforming growth factor- β), mesenchymal stem cell implementations and the application of agents that inhibit collagen degrading matrix metalloproteinases have been studied. These studies have also reported the positive effects of biological adjuvants on tendon-bone healing.^[9-12] In a different study, hyperbaric oxygen treatment in rabbits had positive biologic and mechanic impacts on tendon-bone healing.^[13] Hybridizing the tendon with calcium phosphate during surgery before placing it into the tunnel and wrapping the tendon with periosteum before placing it into the tunnel also improved tendon-bone healing.^[14,15] Although these studies reported successful outcomes in experimental healing processes, there is currently no biological agent for healing process in clinical routine.

Demineralized bone matrix (DBM) is obtained by extracting the mineralized component of the bone with acid.^[16] DBM contains collagen and non-collagen proteins, bone morphologic proteins (BMP), transforming growth factors (TGF- β 1, 2 and 3) and growth differentiation factors.^[17] DBM enhances enchondral ossification and so is osteoinductive.^[18,19] Currently, DBM is used as an adjunctive agent in non-unions, spinal fusion and the filling of bone defects.^[20-22] Tendon-bone attachment is achieved with progressive mineralization of the tendon within the bone tunnel surface and the remodeling of this tissue under mechanical loads.^[23] Possible effects of DBM on tendon-bone healing may be due to the growth factors which enable enchondral ossification and may form a framing substructure for many cells which will serve for healing in the tendon-bone interface.

The aim of the present study was to determine the effect of DBM on the healing of tendons within the bone tunnel and to histologically evaluate possible abnormal bone formation around the bone tunnel.

Materials and methods

Twelve adult male New Zealand albino type rabbits from İstanbul University, İstanbul Faculty of Medicine, Laboratory of Animal Subjects (age: 8 to 12 months; weight: 2.7 to 4.0 kg) were included in this study. Ethics

committee approval for the study was obtained from the Veterinary Faculty of İstanbul University. Animal model for tendon-bone healing was recreated in both lower limbs. The extensor digitorum longus (EDL) tendon was cut from the lateral femur condyle in both knees of each rabbit and fixed to the extra-articular bone tunnel in the proximal tibia.^[24] The study group was formed by the application of 1.5 ml DBM, obtained from the long bones of the rabbits (Musculoskeletal Transplant Foundation; Edison, NJ, USA) to the tunnels opened in the right tibia of each rabbit.^[25] In the control group, the EDL tendons were fixed within the tunnel of the left tibias of the same animals.

Intramuscular anesthesia was applied to the rabbits in the form of 100 mg/kg ketamine (Ketalar; Sigma, St Louis, MO, USA) and 8 mg/kg xylazine (Rompun; Sigma, St Louis, MO, USA). Preoperative infection prophylaxis was provided with 50 mg/kg cefazolin sodium. Operations were performed between 9 and 12 o'clock in the morning in order to standardize rabbits' daily diurnal changes. Both knees were shaven, the surgical field was cleaned with povidone-iodine solution and a sterile cover applied. A lateral parapatellar incision extending to the distal tibia was used for entering the knee joints of both knees.^[24] The long extensor tendon was freed from the lateral femur condyle (Fig. 1a). A 2.5-mm-wide bone tunnel in the tibia proximal metaphysis was created at 30° to the longitudinal axis of the tibia (Fig. 1b). Tunnel lengths were measured and care was taken for the maximum contact of the freed tendon with the bone all along the tunnel. The tendon was fixed within the tunnel by a suspension suture using 3/0 non-absorbable suture material (Ethibond). Rabbit-originated DBM was placed into the tunnel with an injector in the right tibia of each rabbit before the tendon was fixed (Fig. 1c). The joint capsule, subcutaneous tissues and skin were closed using 3/0 silk. Rabbits were placed into individual cages and no postoperative immobilization was applied. Randomly, four rabbits were sacrificed at the 3rd, four rabbits at the 6th and the remaining four rabbits at the 9th week with an intravenous high dose (200 mg/kg) pentothal. The right and left tibias of each rabbit were detached from the tibiofemoral joint surface and prepared for histological examination.

The soft tissues surrounding the tibias were immediately cleaned after sacrifice and tibias were fixed within a buffered 10% formalin solution. Samples were then subjected to decalcification with nitric acid for 7 days. Coronal plane cut pieces were made and embedded into paraffin blocks. Three different sections were sampled from each paraffin block (Fig. 2). After the deparaf-

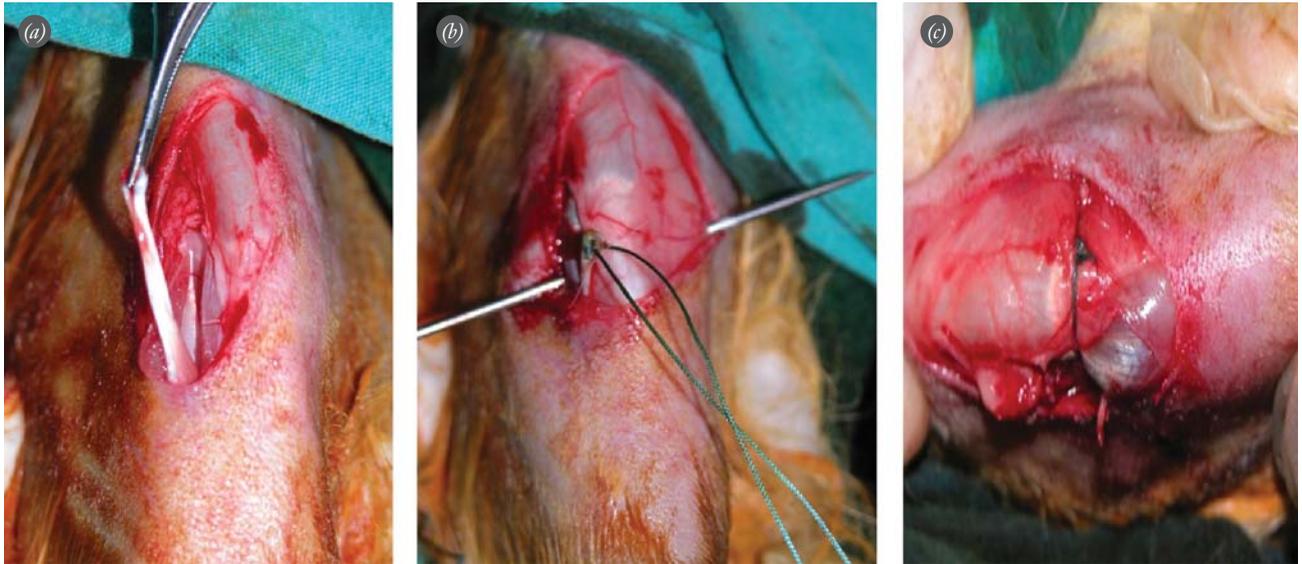


Fig. 1. (a) Long extensor was disjoined from starting point of lateral femur condyle after extensor digitorum tendon was found. (b) In tibia proximal metaphysis, a bone tunnel in width of 2.5 mm was opened at 30° to long bone axis of tibia. (c) The tendon was fixed within the tunnel by means of suspension suture fixing method using 3/0 non-absorbable suture material. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

finization process, the sections were stained with hematoxylin-eosin (H&E) and Masson's trichrome. Each stained section was rated using a scoring system based on new bone formation between the bone and tendon, fibrocartilage formation and the holding of the tendon graft within the bone tunnel (Table 1).^[13] Furthermore, the number of Sharpey's fibers in the target field was determined by number of units covered in a scaled ocular micrometer. All histological evaluations and scorings were performed by two different pathology specialists independently who were blinded the study groups.

For statistical analysis, the Mann-Whitney U test was used for histological scores and number of Sharpey's fibers. Threshold value of $p < 0.05$ was accepted as the limit of significance.

Results

No surgical site infection or DBM-originated skin reaction was observed in any animal during the trial. Total mean score was 6.5 ± 0.6 for the DBM group and 5.25 ± 0.5 for the control group at the 3rd week, and 5.75 ± 0.25 and 6 ± 0 for the DBM and control groups, respectively, at the 6th week. In both groups, the total mean score was 5.75 ± 0.25 at the 9th week (Table 2). However, more Sharpey's fibers extending to the tendon graft (number of units observed in scaled ocular micrometer in target field) were observed in the DBM group (mean $n=19$) within the first 3 weeks. A mean of 14 and 13 Sharpey's fibers were detected in the target field at the 6th and 9th weeks, respectively.

In the first 3 weeks, new bone formation, fibrocartilage formation and holding of the tendon graft within the tunnel were higher in the study group than the control group; however, no statistically significant dif-

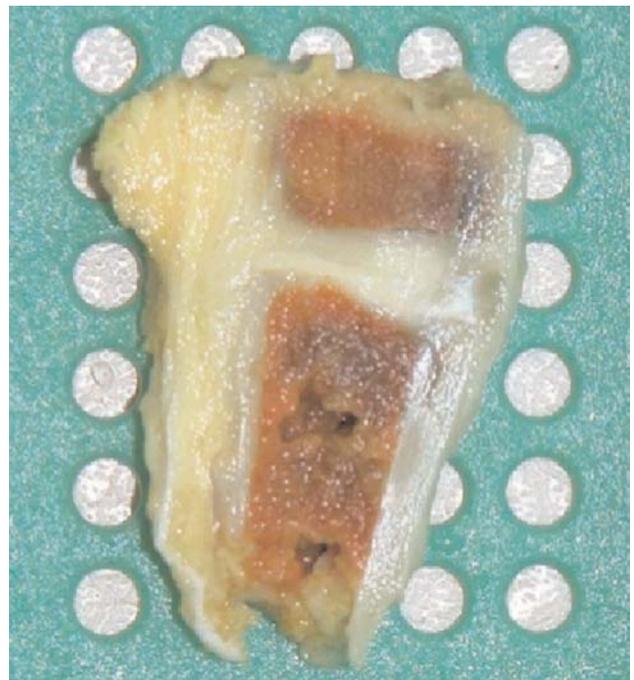


Fig. 2. After decalcification, relation between the EDL tendon and bone tunnel were seen macroscopically in coronal plan cutting at the proximal tibia. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

Table 1. Scoring system for histological evaluation.

Characteristic features	Points
Fibrocartilage formation	
Very	3
Medium	2
Little	1
None	0
New bone formation	
Very	3
Medium	2
Little	1
None	0
Holding of tendon graft within tunnel	
Very	3
Medium	2
Little	1
None	0
Perfect Point	9

ference was detected ($p>0.05$). After the 6th week, no difference was detected between the groups in the histological evaluation criteria. Although the number of Sharpey's fibers was higher in the DBM group in the first 3 weeks, no statistically significant difference was observed between both groups (Fig. 3). No statistically significant difference was detected for histological evaluation parameters of tendon-bone healing between the 3rd, 6th and 9th weeks. The decrease in 6th and 9th week scores when compared to the 3rd week is

Table 2. Results of histological scoring.

Week	Control group	Study group (subjected to DBM)	P value
3rd week	5,5,6,5	6,6,7,7	$p>0.05$
6th week	6,6,6,6	5,6,6,6	$p>0.05$
9th week	6,6,5,6	6,6,5,6	$p>0.05$

thought to be caused by the replacement of fibrocartilage formation with calcified cartilage and new bone at the 6th and 9th week.

Discussion

The use of DBM for accelerating or facilitating tendon healing within the bone tunnel or for increasing its quality has not been sufficiently researched. In the most comprehensive animal study carried out on this subject Sundar et al.,^[26] cutting the patellar tendon of sheep from the bone and re-fixing by means of suture anchor, reported that augmentation of tendon-bone interface with DBM was biomechanically stronger and morphologically and functionally superior. In addition, it was histologically determined that the mineralized fibrocartilage tissue was significantly different than in the control group at the 12th post-procedure week. However, there was no significant difference between both groups in biomechanical tests performed at the 12th week. In the current histological study, we found no distinctive superiority in DBM application. By con-

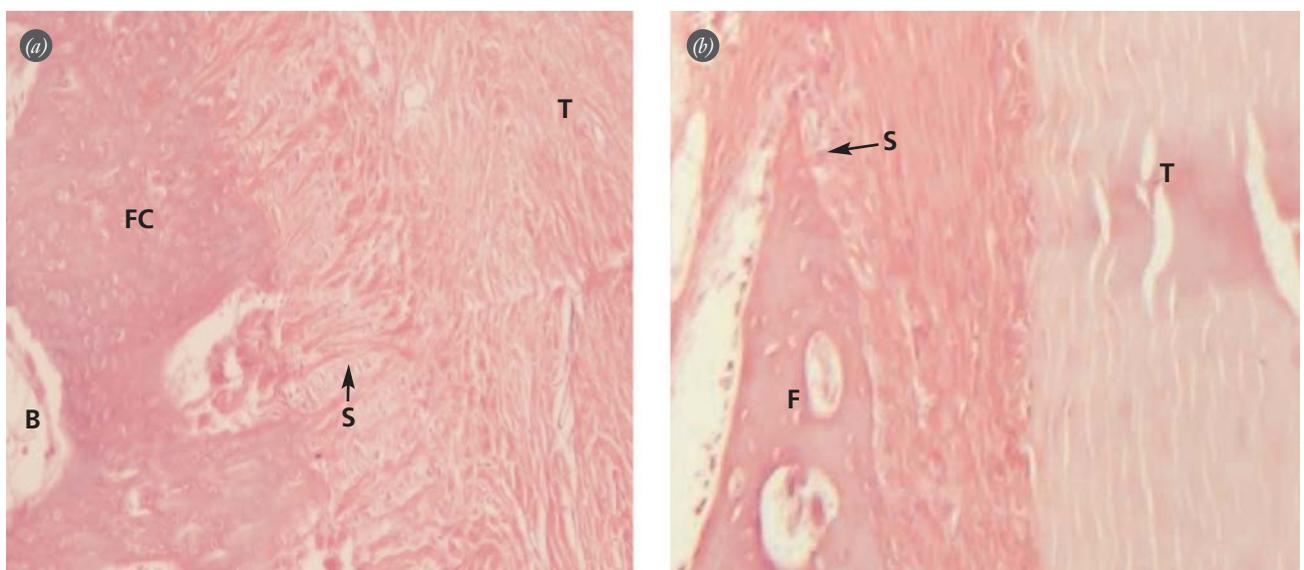


Fig. 3. (a) Number of Sharpey's fibers and new fibrocartilage formation was higher in the group subjected to DBM in the first 3 weeks as seen in the sections painted with H&E (B: bone, FC: fibrocartilage, S: Sharpey's fibers [black arrow], T: tendon). (b) Fibrocartilage tissue and Sharpey's fibers were seen in 3rd week experiments tendon-bone interface at the section painted with H&E, in the control group. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

trast, in sections tested in the 3rd week, Sharpey's fiber formation and all histological evaluation parameter scores were higher in the DBM group. In our time-dependent groups, no significant difference was detected between the DBM and control groups. Our study differed from Sundar et al.'s^[26] in that our study examined the healing of the tendon-bone within the tunnel and applied different fixation techniques.

In our study, fields similar to heterotopic ossification were found in some of histological sections, especially those from the 6th and 9th weeks. This may be resultant of contamination from the DBM to the surrounding tissues (Fig. 4). Beyond the contamination caused during DBM application, BMPs released from the DBM reached higher doses in the surgical field which might lead to this situation. This remarks medium and long-term complications that may result from induction of new bone formation. Possible complications are leakage of DBM into the joint after anterior cruciate ligament reconstruction which may lead to arthrofibrosis and re-ossification of the acromioplasty surface due to the same implementation in rotator cuff surgery. Therefore, the use of DBM has still not found a place for clinical use in tendon reconstruction. New studies to evaluate the medium and long-term biomechanical features of tendons which are subjected to DBM in order to examine the flexibility of tendons are warranted.

In an experimental study using similar animal model and tendon augmentation with BMP-2 and periosteal progenitor cells, a higher amount of Sharpey's fibers and fibrocartilage interface tissue were found in the study group than the control group at the 3rd and 6th weeks.^[24] In another study^[15] that examined the healing of a periosteum covered EDL tendon within a bone tunnel, the amount of fibrocartilage and granulation tissues in the tendon-bone interface and Sharpey's fibers were higher than the control group at the 3rd and 6th weeks. Mean tensile strength of the periosteum covered tendon group was also significantly different at the 3rd and 6th weeks. Our study produced similar findings to these two studies using the same animal model in terms of histological evaluation parameters. Total scores were higher at the 3rd week than the 6th and 9th weeks, which we believe was resultant from the fact that fibrocartilage formation was higher in the first 3 weeks. A high amount of fibrocartilage and fibrovascular tissues may enable easy formation of Sharpey's fibers which are also composed of collagen fibers.^[13,15] In an experimental study by Rodeo et al.,^[27] no uniform healing was observed in every field within the tunnel during the holding of the bone to the tendon. Fibrovascular granulation tissue forming

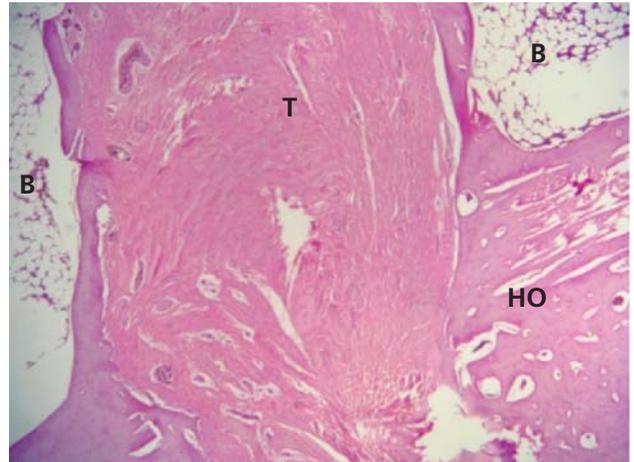


Fig. 4. In this section painted with H&E and belonging to the DBM group from 9th week, heterotopic ossification-like fields can be observed (B: bone, HO: heterotopic ossification center which was found outside of the bone tunnel, T: tendon). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

between the tendon and bone was found to be heterogeneous, with poorly and well organized fields (consisting of parallel array of collagen fibers).^[1,27] As a result, different numbers of Sharpey's fibers may have been observed during histological examination of the sections sampled from the tunnel. The high number of Sharpey's fibers detected in the DBM group within the first 3 weeks should not be regarded as significant for this reason.

A limitation of our study was the insufficient number of subjects for accurate histological and statistical evaluation. Furthermore, an important factor which decreases the power of the study was that biomechanical strengths were not evaluated according to the weeks during the healing process. In our opinion, the lack of difference between the groups in our study resulted from the low number of samples.

DBM may have an effect on tendon-bone healing as it is osteoconductive, provides enchondral ossification, contributes to mineralization process of fibrocartilage tissue through growth factors and increases formation of Sharpey's fibers serving for indirect healing. However, histological evaluation is not sufficient alone to evaluate the effects of DBM upon tendon healing within bone tunnel; its possible effects on this healing should be proven biomechanically.

In conclusion, DBM increased new bone formation and the number of Sharpey's fibers in a bone-tunnel animal model within the first three weeks of tendon-bone healing process.

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Conflicts of Interest: No conflicts declared.

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