Histopathological and biomechanical evaluation of bone healing properties of DBM and DBM-G90 in a rabbit model

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Objective: The present study was designed to investigate the effects of DBM and DBM-G90 on bone healing in a rabbit model.

Methods: Thirty male white albino rabbits were used in this study. An incision was made in all rabbits under general anesthesia directly over the radius in order to expose it. A 10-mm segmental defect was created on the middle portion of each radius. The defects of 10 rabbits (Group I) were filled with DBM Block and Strip (Zimmer, Inc., Warsaw, IN, USA), the defects of 10 rabbits (Group II) were filled with DBM soaked in G90, and the defects of 10 rabbits (Group III/control) were left empty. The rabbits were euthanized at 60 days postoperatively for histopathological and biomechanical evaluation.

Results: At the histopathologic level, the defects of the animals in the DBM and DBM-G90 groups showed more advanced healing criteria than those of the control group. In biomechanical findings, there was a statistically significant difference between the injured bones and contralateral normal bones of the control group in terms of measured strength. There was not a statistically significant difference between the treated bones of the DBM and DBM-G90 groups with contralateral normal bones, nor was there a statistically significant difference between the treated bones of the DBM and DBM-G90 groups with the treated bones of the control group, in terms of other biomechanical tests.

Conclusion: Based on the histopathological and biomechanical findings, the DBM and DBM-G90 groups demonstrated superior osteogenic potential; however, G90 shows no superiority over DBM on bone healing.

Keywords: Bone healing; DBM; DBM-G90; rabbit model.

It remains challenging for orthopedic surgeons and researchers to find an ideal biomaterial for treatment of large bone defects, delayed unions, and nonunions. In the treatment of bone defects and nonunions, autograft is the gold standard for bone repair. However, there are some disadvantages associated with autografts, such as limited supply, new nerve damage, persistent pain, and new fractures.[1–3] Allografts have been used successfully in orthopedic operations, owing to their excellent osteoconductivity and abundant supply. However, allografts

carry the potential risk of infection, disease transmission, and immune response.[1,4,5] Studies have been conducted to promote bone regeneration, focusing on the application of bone marrow with static magnetic field,[6] coral with human platelet-rich plasma (PRP),[7] hydroxyapatite with human PRP,[8] omentum with adipose tissue stem cells,[9] demineralized bone matrix (DBM),[3,10] nano-hydroxyapatite/collagen, synthetic poly (glycolic-co-lactic) acid polymer,[11] and true bone ceramics or sintered bovine bone.[12,13] Each method presents its own advantages and disadvantages. For example, ceramic and polymer-based bone graft substitutes are mostly osteoconductive but are not potentially osteoinductive. Other problems may include unsuitable degradation rates and inferior mechanical properties. In addition, protein- or growth factor-based bone graft substitutes usually require the addition of an osteoconductive scaffold for structural support.[14,15]

Allogenic DBM has been used for several decades in human orthopedic surgery.[16] The process of demineralization with hydrochloric acid destroys antigenicity, decreases antigenic stimulation, and may enhance the release of bone morphogenic protein (BMP).[17] DBM has osteoinductive and osteoconductive properties. Research continues to identify BMPs that might be osteoinductive, which are used for clinical application.[18–21] Beyond their role in osteoinduction, DBM and certain BMPs have shown promise in aiding the repair of osteochondral defects.[22,23] Unlike tricalcium phosphate and hydroxyapatite, DBM is advantageous over other substitutes in that it is inherently osteoinductive and available in large quantities.

G90 was obtained from the tissue homogenate of the earthworm Eisenia foetida (phylum Annelida, family Lumbricidae). The earthworms, which possess antibacterial activity, have been widely used in traditional Chinese medicine.[24] It has been shown that G90 is neither an allergen nor toxin, and it possesses antibacterial activity which aids wound healing.[25,26] G90 mixture contains the growth factors of the insulin superfamily, adhesins of the immunoglobulin superfamily, and proteolytic enzymes of the trypsin family.[25,27–29] Additionally, it contains antitumor, antipyretic and antioxidative activities.[30] Therefore, the present study was designed to investigate the effects of DBM-G90 on experimental critical size bone defects in a rabbit model.

Materials and methods

Thirty male white albino rabbits, 10–12 months in age and weighing 2.0±0.2 kg, were used in this study. Before the experiment, the animals were kept in their new location for 10 days in order to be properly adapted to the experimental environment. Each rabbit was kept separately in an individual standard rabbit cage and maintained on a standard rabbit diet, with no limitation of access to food or water. The experimental protocol was approved by the Animal Care and Experiment Committee of the University, in accordance with the ethics standards of the Principles of Laboratory Animal Care.

Hrzenjak et al.'s method[25] was used to prepare G90. Two hundred earthworms were cleansed by washing with warm water several times and were then immersed in 10% sodium chloride solution for one hour at room temperature until they expired. The worms were washed again, cut into pieces with scissors, and homogenized with a homogenizer machine. The mixture was transferred to a beaker, ethanol and chloroform in a 1:1 ratio were added to the solution and left at 4°C overnight, after which distilled water was added to produce a final volume of 200 cc of mixture. After stirring, it was filtered several times, until the mixture was light brown in appearance. The mixture was centrifuged at 4000 rpm for at least 10 minutes in 50 mL Falcon® tubes (Corning Life Sciences, Corning, NY, USA). After centrifugation, 3 layers developed in each tube: The top layer was a clear light brown-colored liquid, the middle layer was a brown-colored solid, and the bottom layer was a straw-colored liquid. The solid middle layer was placed on a filter paper until the remaining liquid slowly evaporated, the pellet dried, and the brown color was clearly visible. The discs were transferred into 1000 cc balloons and freeze dried at −50 °C. The resultant powder (G90) was placed under UV light for 30 minutes.

DBM was prepared from the midshafts of the long bones of a normal 2-year-old Holstein cow slaughtered in a local slaughterhouse. The bones were collected aseptically, and the soft tissues were removed before storage at −70°C. Fascia was cleaned from the bones, which were cut into 1-cm pieces with a Stryker saw under saline (0.9% NaCl) solution lavage. The bone pieces were stored at −70°C until further use. The pieces were thawed in ethanol and air-dried. All bones were milled (Universal Mill A-20, Tekmer Co, Cincinnati, OH, USA) and sieved to collect the 2- to 4-mm pieces. These bone pieces were then decalcified in 0.6 mol/L HCL at 4°C for 8 days under constant agitation.

Demineralization was evaluated by radiography and calcium analysis.[31] Loss of density radiographically was used to subjectively evaluate demineralization. In addition, random samples of DBM were dried at 95°C, weighed, and reduced to ash at 600°C for 24 hours. These samples were then dissolved in 0.6 mol/L

nitric acid and analyzed by atomic absorption spectrophotometry to determine the calcium concentration per gram dry weight (% Ca:DW).[32,33] Demineralization was considered adequate when the samples were no longer visible radiographically and when the calcium content was <1%.[34] After demineralization, all bone pieces were rinsed in sterile water and placed in phosphate buffer solution overnight. The bone pieces were then rinsed in distilled water, and the pH was adjusted to 7.3. They were immersed in ethanol, the ethanol was allowed to evaporate overnight, and the pieces were packaged aseptically and stored at 4°C.

The animals were anesthetized with ketamine (40 mg/kg, intramuscular [IM]) and xylazine (5 mg/kg, IM). The left forelimb was shaved and prepared aseptically with povidone iodine, and the limb was covered with sterile drapes. An incision was made directly over the radius to expose it by dissecting the surrounding muscles. An osteoperiosteal 10-mm segmental defect was created at mid-diaphysis with an electrical bone cutting saw.[35] Radial bones of rabbits have been reported previously to be suitable because there is no need for internal or external fixation, which influences the healing process.[2,36–38]

The defects in 10 rabbits (Group I) were filled with DBM block of the same size as the defect. The defects in 10 rabbits (Group II) were filled with soaked DBM in G90 (10 nanograms soaked in DBM Block and Strip [Zimmer, Inc., Warsaw, IN, USA]), and the defect area of the control group (Group III) was left empty.

The rabbits were fed a standard diet and allowed to move freely during the experimental period. Postoperatively, the animals were kept in separate cages, fed a standard diet, and allowed to move freely with weight-bearing during the experimental period. Flunixin meglumine (1.0 mg/kg, subcutaneous) was administered every 6 hours postoperatively for 24 hours to control pain and discomfort.[39]

Sixty days postoperatively, the rabbits were euthanized for histopathological and biomechanical evaluation. Histopathological evaluation was performed randomly on 5 rabbits from each group. The left forelimb in each animal was harvested and dissected free of soft tissues. Routine hematoxylin and eosin (H&E) stain-

ing was used for histopathological evaluation and scoring the bone growth in the defected area, as well as for evaluation of inflammatory response in the implanted area.[2] The sections were blindly evaluated and scored by 2 pathologists according to Emery's scoring system.[40] Based on this scoring system, the defects were evaluated as follows: 0=gap empty; 1=gap filled with fibrous connective tissue only; 2= gap filled with more fibrous tissue than fibrocartilage; 3=gap filled with more fibrocartilage than fibrous tissue; 4=gap filled with fibrocartilage only; 5=gap filled with more fibrocartilage than bone; 6=gap filled with more bone than fibrocartilage; 7=gap filled only with bone.

Biomechanical performance was evaluated on the injured and normal contralateral bones of each rabbit. The test was performed using a universal tensile testing machine (Instron, London, UK).[41–43] Three-point bending test was performed to determine the mechanical properties of bones. The data derived from the load deformation curves were expressed as Mean±SEM for each group, and maximum load, stiffness, stress, and load at yield point were measured and recorded.

The histopathological data were compared by Kruskal-Wallis and non-parametric analysis of variance (ANOVA) tests; when p values were found to be <0.05, then pairwise group comparisons were performed by Mann-Whitney U test. Student's t-test was used for comparison of the treated and normal limb biomechanical data. One-way ANOVA test was used for biomechanical analysis of the treated bones of all groups (SPSS v17.0 for Windows, SPSS Inc., Chicago, IL, USA).

Results

All animals were alive until the end of the study. Ulna bone fracture was not observed at the radial bone defect of any rabbit.

As has been shown in Table 1, the defects of the animals in the DBM and DBM-G90 groups showed more advanced healing criteria than those of the control group. There were no significant differences between the DBM and DBM-G90 groups in histopathological evaluation. Fibrous nonunions or fibrocartilages in the defects of the animals of the control group were dominant, and the

Table 1. Bone measurements at microscopic level.

#of animals/group	Median (min–max)			p[a]
	Control group (n=5)	DBM group (n=5)	DBM-G90 group (n=5)	
Microscopic scores	3 (2–3)[b]	6 (6–7)	6 (6–7)	**0.05**

Significant p values are presented in bold face. [a]Kruskal-Wallis non-parametric ANOVA; [b]Compared with DBM group (p=0.04) and DBM-G90 group (p=0.04) by Mann-Whitney U-test. DBM and DBM-G90 groups were significantly superior (p<0.05) to the control group.
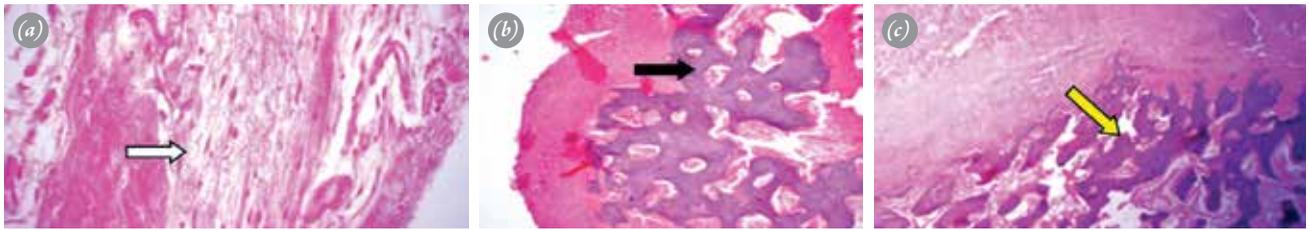
Fig. 1. Photomicrograph of the defect in the control group (**a:** H&E, ×10). Extensive fibrous connective tissue is seen in the defected area of an animal of the control group (white arrow). The bone marrow has not yet developed. Note the woven and trabecular bone formation (black arrow) in the defect of an animal in the DBM group (**b:** H&E, ×10). The woven bone, fibrocartilaginous tissue, and bone marrow have formed perfectly in the animals treated with DBM-G90 at this stage (yellow arrow) (**c:** H&E, ×10). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

lesions of these animals showed poor revascularization. Bridging callus or histological union did not develop in any of these defects. These findings reflect that healing occurred very slowly in the control group (Figure 1a). The defects of rabbits of the DBM group were filled with trabecular bone and fibrocartilage tissues (Figure 1b). Normal trabecular and woven bones were uniformly formed within the defects of the animals that were treated with the DBM-G90 regimen, and the lesions of animals in this group were filled with woven bone and showed proper maturation. The regenerated bone completely spanned the defect and rapidly produced full histologic union. Active endochondral ossification and secondary fracture repair took place in the middle of the defect of the animals of the DBM-G90 group (Figure 1c). No significant inflammatory response was evident in the lesions of any animals at 60 days post-injury.

A significant difference (p=0.05) was found between the injured bone and normal bone of the control group in terms of ultimate strength, and the normal bones had superior ultimate strength compared to corresponding treated contralateral bones. However, the ultimate strength and stiffness of the treated animals of both the DBM and DBM-G90 groups showed more advanced values that were not statistically significant with those of their normal contralaterals. There was no significant difference between the stress and yield strength of the treated bones of the DBM and DBM-G90 groups with

those of their normal contralaterals or those of the normal bones of the control group (Table 2).

Discussion

This study was designed to clarify the efficacy of the concurrent use of DBM with G90 and give more insight into the effect of DBM-G90 on bone regeneration. To the authors' knowledge, this is the first study which presents new data on the bone regenerative properties of DBM-G90 in a rabbit model. Such defect in the radius in the rabbit model has previously been reported suitable because there is no need for internal or external fixation, which influences the healing process.[44] The segmental defect was created on the middle portion of the radius, as long as 10 mm, to prevent spontaneous and rapid healing.[35] In the present study, the DBM and DBM-G90 groups demonstrated superior histopathological and biomechanical osteogenic potential in healing of the radial bone defect in this rabbit model. However, the control group was inferior by these parameters to the DBM and DBM-G90 groups.

The bone inductive activity of DBM has been well established.[2,3,10]

DBM is both osteoconductive and osteoinductive, as well as biodegradable, making it an ideal graft substitute. It is available as an injectable paste or putty, graft, gel, crunch, and flex. It is often conjugated or embedded

Table 2. Biomechanical performance of the treated and untreated defects at 60th postoperative day.

| | Mean±SEM | | | | | |
| | Control (n=5) | | DBM (n=5) | | DBM-G90 (n=5) | |
3-point bending test criteria	Normal limb	Defected limb	Normal limb	Treated limb	Normal limb	Treated limb
Ultimate strength (N)	(82.2±7.5)[a]	(53±12.4)	(63.2±25.7)	(42.5±25)	(92.5±40.9)	(83.7±46.7)
Stress (N/mm²)	(21.6±3.03)	(17.8±2.1)	(16.3±6.8)	(12.6±6.6)	(24.3±9.5)	(27.8±13)
Stiffness (N/mm)	(3.7±1.4)	(2.1±0.8)	(3.3±0.6)	(2.7±0.5)	(3.0±1.0)	(3.6±1.8)
Yield strength (N)	(70.5±7.3)	(54.2±7.6)	(55.5±26.3)	(37±14.8)	(76.7±30.6)	(70.7±47.4)

[a]p= 0.05 (normal limb compared with treated limb in control group by Student's t-test).

with collagen type I, alginate, gelatin, sodium hyaluronate, glycerol, starch, and calcium sulfate. Regardless of the DBM carrier, the material can also be mixed with bone marrow aspirate prior to surgery. The second well-known option is the calcium phosphate group, including mono-, bi- and tricalcium phosphate. These are often available in conjugation with collagen type I and carboxymethylcellulose (CMC), and all are osteoconductive and biodegradable products available as an injectable paste, moldable putty, and various-sized pellets. Bone morphogenetic protein-2 (rhBMP-2) and bone morphogenetic protein-7 (rhBMP-7) proteins on an absorbable collagen sponge are other biodegradable options with osteoinductive characteristics.[45]

Decalcification for DBM preparation from the cortical bone exposes these osteoinductive growth factors buried within the mineralized matrix, thereby enhancing the bone formation process.[46] These proteins promote the chondroblastic differentiation of the mesenchymal cells, followed with new bone synthesis by endochondral osteogenesis.[46,47] In the present study, it was found that the morphological and biomechanical properties of the defected bones of the DBM group at 60 days post-injury were statistically superior in comparison with those of the control group. It appears that the grafted DBM released BMPs which produced osteoinductive activity and led to superior results in terms of enhanced morphogenesis and biomechanical performance in comparison with the control group. Additionally, DBM appears to support new bone formation through osteoconductive mechanisms.[48] No significant differences were found in histopathologic and biomechanic evaluation between the injured bones in animals treated with DBM and DBM-G90, and none of the graft material elicited a significant inflammatory reaction. It has been reported that the demineralization process destroys the antigenic materials in bone, making DBM less immunogenic than the mineralized allograft.[49] The authors did not observe any inflammatory reaction in the DBM and DBM-G90 groups. This finding is in accordance with those of previous studies which have shown that G90 possessed anti-inflammatory and anti-pyretic properties.[50,51]

Histopathological and biomechanical evaluation in our study showed superior osteogenic properties in the defects of the DBM-G90 treated animals in comparison with those of the control group.

In the DBM-G90 group, woven bone was observed in the defected area. Based on the pattern of collagen forming the osteoid, 2 types of bone were identified: woven bone, which is characterized by a haphazard organization of collagen fibers, and lamellar bone, which is char-

acterized by a regular parallel alignment of collagen into sheets (lamellae).[52] Lamellar bone, as a result of the alternating orientations of collagen fibrils, has a significant mechanical strength, similar to plywood. This normal lamellar pattern is absent in woven bone, in which the collagen fibrils are laid down in a disorganized manner. Hence, woven bone is weaker than lamellar bone. Woven bone is produced when osteoblasts produce osteoid rapidly. This occurs rapidly in initial fracture healing with osteoinduction materials, but the resulting woven bone is replaced by the deposition of more resilient lamellar bone in a process called remodeling.[52] In the DBM-G90 group, rapid woven bone formation was primarily related to the presence of DBM and secondarily related to the presence to G90. Earlier investigations have shown that G90 glycolipoprotein mixture is capable of undertaking different activities such as mitogenesis, as well as stimulating the synthesis of transforming growth factor (TGF) and epithelial growth factor, all of which could contribute to the rapidness of wound healing.[28] The mitogenic activity of G90[28,53] could be responsible for the proliferation of fibroblasts cells, contributing in that manner to enhancing the healing process.[54] Furthermore, previous studies have shown that G90 could enhance fibroblast proliferation, collagen production, and angiogenesis by enhancing the concentration of fibroblast growth factor (FGF) in the wound area.[53] Through this mechanism, FGF is effective in inducing angiogenesis and fibroblast growth.[53] Therefore, such enhancement in hierarchical organization in the treated animals of the present study resulted in improved histopathological and biomechanical parameters. However, since we did not use G90 solely in the present study, the differences between the control group and the DBM-G90 group may be largely or entirely due to the DBM Block and Strip.

Radiography could be a technique for further evaluation that was not performed in this study. Nonetheless, this useful tracking method could be applied in such experiments in future.

Based on the histopathological and biomechanical findings of the present study, the DBM and DBM-G90 groups demonstrated superior osteogenic potential in healing of the radial bone defect in a rabbit model. However, there were no significant differences between the DBM and DBM- G90 groups at this stage, demonstrating no positive effect of G90 over DBM on bone healing. Future studies are needed to evaluate the effects of G90 alone on bone healing.

Conflics of Interest: No conflicts declared.

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