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The role of cortical autograft, commercial-demineralized bone matrix, calf fetal demineralized bone matrix and calf fetal growth plate powder on bone healing in rabbits

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ABSTRACT

Trauma, developmental anomalies, infection, and pathological injuries can lead to defects in the bony skeleton and it is a current challenge for surgeons and investigators to restore lost tissues. This study was designed to evaluate the effects of cortical autograft, commercial-demineralized bone matrix (DBM), calf fetal demineralized bone matrix and calf fetal growth plate powder on bone healing in a rabbit model. Five round defects were created on a tibial bone with an electric drill in ten adult white New Zealand rabbits. One of the defects was left empty as a control group but the other defects were filled with different biomaterials. Radiographs of each hind limb were taken postoperatively on the 1st day and at the 2nd, 4th and 6th weeks post injury, to evaluate the bone healing criteria of the defect. The operated tibial bones were removed on the 42nd postoperative day and evaluated for histopathology criteria. Based on the radiology and histopathology findings of the present study, autografts, commercial-demineralized bone matrix, calf fetal demineralized bone matrix and calf fetal growth plate groups demonstrated superior osteogenic potential in healing of the tibial bone defects in the rabbit model. However, the control group was inferior to the autograft, commercial-DBM, calf fetal DBM and calf fetal growth plate powder groups at this stage.

Key words: cortical autograft, demineralized bone matrix, calf fetal growth plate powder, bone healing, rabbit

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Introduction

Large bone defects may be caused by trauma, tumors and osteitis. It has been stated that reconstructive surgeons perform more than 250,000 bone graft procedures each year to restore lost tissues (MOGHADAM et al., 2004). An ideal bone graft substitute should have osteoconductive, osteoinductive and osteogenic properties. An autogenous bone graft is the gold standard among graft materials, because it provides all these properties (FINKEMEIER, 2002; GREENWALD et al., 2001; PARIKH, 2002). An autogenous bone graft has been implicated as the implant of choice for most orthopaedic procedures (BAUER and MUSCHLER, 2000). However, autogenous and allogeneic bone grafts have several limitations, such as donor-site infection, pain, and disease transfer (BAUER and MUSCHLER, 2000; BURCHARDT, 1983; FLEMING et al., 2000). As a result of these limitations, various bone graft substitutes, including autografts, allografts, xenografts, biosynthetic bone graft substitutes, polymers, ceramics and some metals have been employed to promote bone reunion (FRIEDLAENDER, 1987; INOUE et al., 1997; KOS et al., 2006; PARIZI et al., 2010). Numerous studies have previously compared the biopotency of xenografts, such as Coralline and bovine grafts, with the available autografts (KARAISMAILOGLU et al., 2002; KUBOKI et al., 1998). An allogeneic, demineralized bone matrix (DBM) has been used for several decades in human surgery for the treatment of nonunions, osteomyelitis, and large defects resulting from removal of benign tumors (JIN, 1991). The process of demineralization with hydrochloric acid destroys, but also decreases antigenic stimulation and may enhance the release of bone morphogenic proteins (BMPs) (RILEY et al., 1996). BMPs stimulate the local undifferentiated mesenchymal cells to transform into osteoblasts (osteoinduction), and the collagenous framework of the DBM particles allows for migration of the newly regenerated tissue into the site (osteoconduction). Extensive research continues to identify the different BMPs that might be osteoinductive, and these are being readied for clinical application (BOSTROM et al., 1995; COOK et al., 1994; KIRKER-HEAD, 1995; REDDI, 1995). Beyond their role in osteoinduction, certain BMPs and DBM have shown promise in aiding repair of osteochondral defects (LOREDO et al., 1995; TANAKA et al., 1995). The advantages of DBM over other substitutes include inherent osteoinductive capacity (unlike tricalcium phosphate and hydroxyapatite) and its availability in large amounts. A more recent study showed the best bone healing by calf fetal growth plate powder in a rabbit model (BIGHAM-SADEGH et al., 2013).

The aim of this study was to compare the effects of xenogeneic bovine fetal DBM, commercial DBM, cortical autograft and xenogeneic bovine fetal growth plate powder on the healing of an experimental bone defect in a rabbit model.

Materials and methods

Animals. Ten male, 2-year old New Zealand Albino rabbits, weighing 2.0 ± 0.5 kg were included in this study. 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".

Preparation of the calf fetal demineralized bone matrix. DBM was prepared from the midshafts of the long bones of a 4-month-old Holstein calf fetus collected from the local slaughterhouse. The bones were collected aseptically, and the soft tissues were removed before storage at -70 ºC. The bones were later cut into 1 cm pieces with a Stryker saw under 0.9 % normal saline solution lavage. The bone pieces were stored at -70 ºC until further use. The pieces were then thawed in 200-proof ethanol and air dried. All the bones were milled (Universal Mill A-20; Tekmer Co, Cincinnati, OH) and placed through a sieve to collect 2- to 4-mm pieces. The pieces were then decalcified in 0.6 mol/L HCL at 4 ºC for 8 days under constant agitation. Demineralization was evaluated by radiography and calcium analysis (VAIL et al., 1994). Loss of density on radiography was used to subjectively evaluate demineralization. In addition, random samples of DBM were dried at 95 ºC, weighed, and then ashed at 600 ºC for 24 hours. These samples were then dissolved in 0.6 mol/L nitric acid and analyzed by atomic absorption spectrophotometry to determine the percentage of calcium per gram dry weight (% Ca:DW) (FORELL et al., 1993; REDDI and HUGGINS, 1972). Demineralization was considered adequate when samples were no longer visible radiographically and when calcium content was less than 1 % (URIST and STRAKES, 1970). After demineralization, all bone pieces were rinsed in sterile water and placed in phosphate buffered solution overnight. The bone pieces were then rinsed and the pH was adjusted to 7.3. They were placed in ethanol, the ethanol was allowed to evaporate overnight, and the pieces were packaged aseptically and stored at 4 ºC.

Preparation of bovine fetal growth plate powder. All growth plates of the long bones of the 4-month-old Holstein calf fetus were collected, washed three times in 95 % ethanol for 15 min, rinsed in ether for 15 min, and finally air dried overnight. The cleaned and dried growth plates were then milled (Universal Mill A-20; Tekmer Co, Cincinnati, OH) to obtain 400-700 μm granules, and then were air dried and stored in sterile plastic containers at 4 ºC until being used for implantation. This entire process was performed under sterile conditions (except for the milling) and a sample was cultured to demonstrate that the specimens contained no bacterial or fungal contamination.

Cortical autograft granules. During the tibial drilling to create a defect, the protruding granules from beside the drill were collected for further use as autograft cortical granules.

Surgical technique. All the animals were anesthetized by intramuscular administration of 40 mg/kg ketamine hydrochloride and 5 mg/kg xylazine (Alfasan International,

Woerden, the Netherlands). The operative procedure was undertaken under general anesthesia. In all rabbits, the left hind limb, crural region, was prepared for aseptic surgery. The tibia was exposed via a medial approach and five circular bone defects of 4 mm in diameter were made. The drilling was performed with an electrical orthopedic drill under continuous irrigation with 0.9 % normal saline solution. The defects were filled with autograft (as the positive control group), commercial DBM (as experimental group I) (Osteotech Inc., Eatontown, NJ, USA), calf fetal DBM (as experimental group II), calf fetal growth plate powder (as experimental group III), and finally one defect was left empty as a negative control group. The implanted site was changed between materials in each rabbit in a Latin square design.

Post operative evaluations

Radiologic evaluation. Lateral view radiographs were taken on the 1st day and then at the 2nd, 4th and 6th weeks post-operatively, using a step-wedge to calibrate radiodensity. The radio-opacity of the implanted area was scored using a range from 0 (minimally opaque) to 4 (most opaque) by an investigator blinded to treatment mode.

Histopathology evaluation. Six weeks after surgery the rabbits were euthanized for histopathology evaluation. The histopathology evaluation was carried out on all harvested specimens. The left hind limb was harvested and dissected free of soft tissues. Sagittal sections containing the defect were cut with a slow speed saw. Each slice was then fixed in 10 % neutral buffered formalin. The formalin-fixed bone samples were decalcified in 15 % buffered formic acid solution and processed for routine histological examination. Two sections, 5 µm in thickness, were cut from the centers of each specimen and then stained with Hematoxylin and Eosin. The sections were blindly evaluated and scored by two pathologists, according to Heiple's scoring system (Table 1) (HEIPLE et al., 1987).

Statistical analysis. The radiologic and histopathology data were compared by Kruskal-Wallis, non- parametric ANOVA, when P-values were found to be less than 0.05, then pair wise group comparisons were performed by the Mann-Whitney U test (SPSS version 17 for windows, SPSS Inc, Chicago, USA).

Results

There were no intraoperative and postoperative deaths during the study. None of the rabbits sustained a fracture of the tibia.

Radiographic findings

14th postoperative day. On the 14th postoperative day statistically significant differences ($P<0.05$) were observed between the positive control ($P = 0.002$), experimental groups I ($P = 0.01$), II ($P = 0.04$) and III ($P = 0.004$) and the negative control group, where the negative control group was significantly inferior to the other groups. In addition,

experimental group I was significantly inferior to the positive control (P = 0.02) and experimental group II (P = 0.03) (Fig. 1A, Table 2).

Table 1. Lane and Sandhu histopathology scoring system modified by Heiple et al. (1987)

Union	
No evidence of union	0
Fibrous union	1
Osteochondral union	2
Bone union	3
Complete organization of shaft	4
Cancellous bone	
No osseous cellular activity	0
Early apposition of new bone	1
Active apposition of new bone	2
Reorganizing cancellous bone	3
Completely reorganization cancellous bone	4
Cortical bone	
Non	0
Early appearance	1
Formation under way	2
Mostly reorganized	3
Completely formed	4
Marrow	
None is resected area	0
Beginning to appear	1
Present in more than half of the defect	2
Complete colonization by red marrow	3
Mature fatty marrow	4
Total points possible per category	
Distal union	4
Cancellous bone	4
Cortex	4
Marrow	4
Maximum score	16

Table 2. Radiographic findings for bone healing at various post-operative intervals

Med (min-max)						P[a]
Postoperative days	Negative control group (n = 10)	Positive control group (n = 10)	Experimental group I (n = 10)	Experimental group II (n = 10)	Experimental group III (n = 10)	
14	1 (0-3)[b]	4 (1-4)	2 (0-3)[c]	2 (0-4)	2 (0-4)	**0.002**
28	1 (0-3)[d]	4 (1-4)	1 (0-3)[e]	1 (0-4)	2 (1-4)	**0.004**
42	2 (0-3)[f]	4 (3-4)	3 (1-3)	2 (2-4)	2 (1-4)	**0.001**

Significant P-values are presented in bold face. [a] Kruskal-Wallis non-parametric ANOVA. [b] There were significant differences between the positive control (P = 0.002), experimental groups I (P = 0.01), II (P = 0.04) and III (P = 0.004) and the negative control group, and the negative control group was significantly inferior to the other groups. [c] There were significant differences between the positive control (P = 0.02), and experimental group II (P = 0.03) and in comparison with them it was the inferior group. [d] There were significant differences between the positive control (P = 0.005), experimental groups I (P = 0.04), II (P = 0.003) and III (P = 0.003) and the negative control group, and the negative control group was significantly inferior to the other groups. [e] There were significant differences between the positive control (P = 0.01), experimental groups II (P = 0.04) and III (P = 0.02), where in comparison with them it was the inferior group. [f] There were significant differences between the positive control (P = 0.009), experimental groups I (P = 0.003), II (P = 0.03) and III (P = 0.003) and the negative control group, where the control group was significantly inferior to other groups.

Table 3. Histopathology findings (sum of histopathology criteria) for bone healing in various groups

Med (min-max)						P[a]
	Negative control group (n = 10)	Positive control group (n = 10)	Experimental group I (n = 10)	Experimental group II (n = 10)	Experimental group III (n = 10)	
Sum of histopathology criteria	7 (5-7)[b]	9 (7-15)	9 (8-12)	9 (7-13)	8 (6-13)	**0.01**

Significant P-values are presented in bold face. [a] Kruskal-Wallis non-parametric ANOVA. [b] There were significant differences between the positive control (P = 0.02), experimental groups I (P = 0.04), II (P = 0.03) and III (P = 0.03) and the negative control group, and the negative control group was significantly inferior to the other groups.

28th postoperative day. Significant differences (P<0.05) were observed on the 28th postoperative day, whereby the negative control group was significantly inferior to the positive control (P = 0.005), experimental groups I (P = 0.04), II (P = 0.003) and III (P = 0.003). Experimental group I was significantly inferior to the positive control (P = 0.01), and experimental groups II (P = 0.04) and III (P = 0.02) (Fig. 1B, Table 2).

42nd postoperative day. On the 42nd postoperative day statistically significant differences ($P<0.05$) were observed whereby the negative control group was significantly inferior to the positive control ($P = 0.009$), and experimental groups I ($P = 0.003$), II ($P = 0.03$) and III ($P = 0.003$) (Fig. 1C, Table 2).

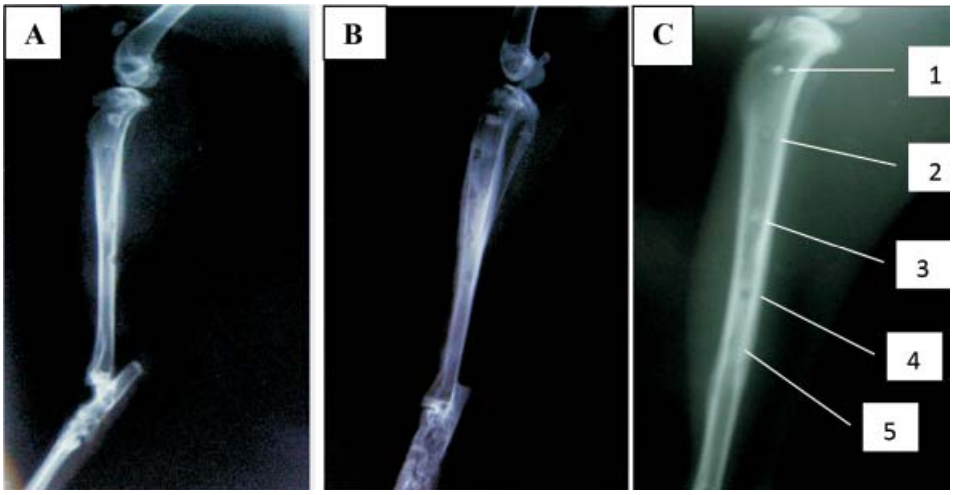

Fig. 1. Lateral and cranio-caudal view radiology evaluation on the 14th (A), 28th (B) and 42nd (C) postoperative days. Note to fig. C. 1) positive control group 2) experimental group I, 3) experimental group II, 4) experimental group III, 5) negative control group

Histopathology findings. Histopathologically there was a statistically significant difference between the histopathological sections of the lesions of the negative control group in terms of bone healing criteria and the positive control ($P = 0.02$), and experimental groups I ($P = 0.04$), II ($P = 0.03$) and III ($P = 0.03$), where the negative control group was significantly inferior to the other groups (Table 3). None of the grafted materials elicited any significant inflammatory reaction. As shown in Fig. 2 (A, B, C, D and E), by the end of 6 weeks post-surgery, histological examination demonstrated the presence of regenerated bone with the typical structure of the trabecular bone in the defect site of all groups, except the negative control group. The defected area of the negative control group was filled with prominent fibrous tissue (Fig. 2A).

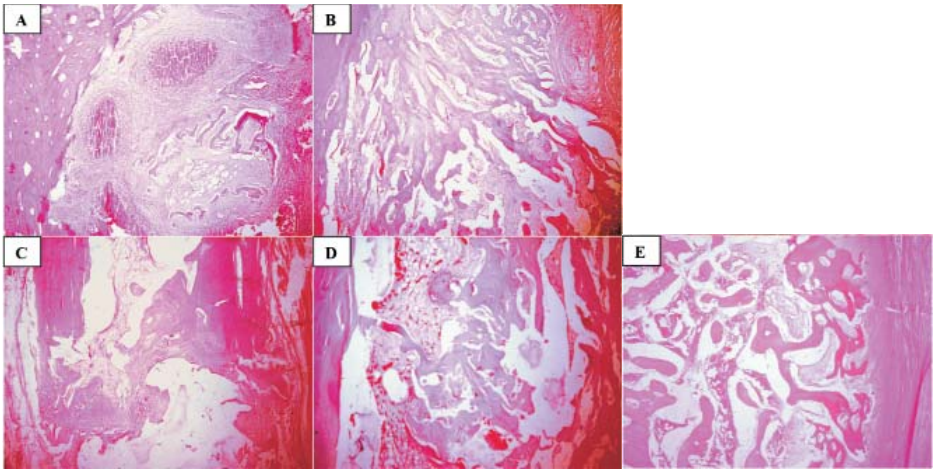

Fig. 2. After 42 days more fibrous tissue was seen in the defected area in the negative control group (A). In other groups histopathology findings showed the normal structure of the trabecular bone in the defect area, with various phases of remodeling. B) the positive control group C) experimental group I, D) experimental group II, E) experimental group III (H&E, 10x)

Discussion

In this study, the positive control, experimental groups I, II and III groups demonstrated superior osteogenic potential in healing of the tibial bone defects in the rabbit model by radiology and histopathology. However, the negative control group was inferior to the positive control, experimental groups I, II and III at this stage. Autogenous bone still remains the "golden standard" of bone graft material in all facets of orthopedic surgery and is commonly used as a standard to which allografts and graft substitutes are compared (ALEXANDER, 1985; ALEXANDER, 1987; BRINKER et al., 1997; FITCH et al., 1997; FOX, 1984; McLAUGHLIN and ROUSH, 1998). Usage of autografts diminishes the risk of infectious disease transmission, and the osteoconductive, osteoinductive, and osteogenic properties of the graft are optimal. Moreover, there is no immune response after implantation of an autograft, enhancing its ability to incorporate into its new site (LOHMANN et al., 2001; POKORNY et al., 2003). In the present study, the positive control group (autograft) was found to be the best implant on radiology evaluation. In addition, on histopathology evaluation it showed an intensive, properly thickened trabecular bone and it did not elicit any inflammatory reaction.

The bone inductive activity of the demineralized bone matrix (DBM) has been well established (CHALMERS et al., 1975; DAHNERS and JACOBS, 1985; EINHORN et al., 1984; GEPSTEIN et al., 1987; HULTH et al., 1988; LINDHOLM et al., 1988; TULI and SINGH, 1978;

URIST, 1965; URIST et al., 1967). It has been shown that addition of autologous bone marrow and/or autograft to DBM provides an immediate source of osteogenic precursor cells at the implant site that has been claimed to be responsible for providing an additional biochemical contribution to osteogenesis (URIST, 1965; URIST et al., 1967; BURWELL, 1985). DBM also appears to support new bone formation through osteoconductive mechanisms (MARTIN et al., 1999). The primary osteoinductive components of DBM are a series of low-molecular-weight glycoproteins that include bone morphogenetic proteins (BMPs). Decalcification of the cortical bone exposes these osteoinductive growth factors buried within the mineralized matrix, thereby enhancing the bone formation process (URIST et al., 1979). These proteins promote the chondroblastic differentiation of the mesenchymal cells, followed by new bone synthesis by endochondral osteogenesis (URIST et al., 1983; URIST et al., 1979). In this study, it was found that the results of the experimental groups I (commercial-DBM), II (calf fetal DBM) and III (calf fetal growth plate) were not statistically significantly different at 6 weeks post-injury in comparison with the positive control group. It seems the grafted xenogeneic commercial-DBM, calf fetal DBM and calf fetal growth plates, have osteoinductive activity, possibly by releasing some BMPs, similar to that of the autogenous cortical bone grafts. However, previous studies have shown that a cortical autograft has more osteoconductive properties and less osteoinductive activity than DBM materials (BAUER and MUSCHLER, 2000; KHAN et al., 2005). DBM also appears to support new bone formation through osteoconductive mechanisms (MARTIN et al., 1999). No significant differences were found in histopathology evaluation between the animals in the different biomaterial groups (with the exception of the negative control group) and none of the graft materials elicited a significant inflammatory reaction. It has been reported that the demineralization process destroys the antigenic materials in bone, making DBM less immunogenic than a mineralized allograft, (GUIZZARDI et al., 1992) and a cortical autogenous bone graft does not induce an immunological reaction by the host (BAUER and MUSCHLER, 2000). Therefore, the authors did not observe any inflammatory reaction in the experimental groups I (commercial-DBM) and II (calf fetal DBM).

Based on the radiology and histopathology findings in the present study, healing of the defects of the animals in the negative control group was not very efficient, and the defect area was filled with fibrous tissues and rarely with cartilage instead of osseous tissue. BARNES et al. (1999) indicated that the chondrocytes derived from the mesenchymal progenitors proliferate and synthesize a cartilaginous matrix until all the fibrous granulation tissue is replaced by cartilage. Where cartilage production is deficient, fibroblasts fill the region with generalized fibrous tissue. Discrete cartilaginous regions progressively grow and merge to produce a central fibrocartilaginous plug between the fractured fragments that splints the fracture.

Histopathology evaluation did not show any significant differences after 6 weeks between the grafted materials groups in the present study; however, statistical differences between the different groups were expected. Probably there were earlier significant differences between the histology features of the lesions in the different biomaterial groups in the previous postoperative intervals, but the authors did not perform histopathology evaluation at earlier postoperative intervals due to ethical committee limitations. Therefore, histopathology studies of such lesions at earlier stages of fracture healing are highly recommended. Such studies could include the inflammatory, proliferative and remodeling phases of fracture healing and investigate the type of inflammatory cell constituents, osteoblast proliferation and maturation, angiogenesis, collagen synthesis, the presence or absence of cartilaginous materials, the quantity and quality of mineralization and many other criteria.

Conclusion

On the basis of the radiology and histopathology findings of the present study, the autograft, commercial-DBM, calf fetal DBM and calf fetal growth plate groups demonstrated superior osteogenic potential in healing of tibial bone defect in a rabbit model. However, the control group was inferior to the autograft, commercial-DBM, calf fetal DBM and calf fetal growth plate powder groups at this stage.

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Declaration of interest

All authors declare that there is no conflict of interests.

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BIGHAM-SADEGH, A., I. KARIMI, S.-E. SHARIATI, A. ORYAN: Učinak kortikalnog autopresatka, komercijalnog pripravka demineralizirane koštane matrice, demineralizirane koštane matrice telećeg ploda i praška od epifizealnog diska telećeg ploda na cijeljenje kostiju u kunića. Vet. arhiv 85, 23-36, 2015.

SAŽETAK

Trauma, razvoj anomalija, infekcije i patološka stanja mogu dovesti do različitih defekata kostiju. Njihovo cijeljenje i obnova izgubljenih tkiva predstavlja veliki izazov za kirurge i istraživače. Ovo istraživanje poduzeto je sa svrhom da se prosude učinci kortikalnog autopresatka, komercijalne demineralizirane koštane matrice, demineralizirane koštane matrice telećeg ploda i praška od epifizealnog diska telećeg ploda na cijeljenje

kostiju na kunićjem modelu. Pet okruglih defekata učinjeno je električnim svrdlom na goljeničnoj kosti u 10 bijelih novozelandskih kunića. Kontrolni defekt ostavljen je prazan dok su ostali bili ispunjeni različitim biomaterijalima. Za procjenu cijeljenja defekta učinjene su radiografske slike stražnjih nogu prvog, drugog, četvrtog i šestog tjedna nakon ranjavanja. Operirane goljenične kosti uzete su 42. dana nakon operacije te pretražene patohistološki. Radiološki i patohistološki nalazi pokazali su da autopresadak, komercijalni pripravak demineralizirane koštane matrice, demineralizirana koštana matrica telećeg ploda i prašak od epifizealnog diska telećeg ploda imaju dobar učinak na cijeljenje defekta goljeničnog zgloba u pokusnih kunića. Cijeljenje defekta u kunića kontrolne skupine bilo je slabije.

Ključne riječi: kortikalni autopresadak, demineralizirana koštana matrica, telećI plod, cijeljenje kostiju, kunić