

Xenogenic demineralized bone matrix and fresh autogenous cortical bone effects on experimental bone healing: radiological, histopathological and biomechanical evaluation

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Abstract

Background Bone grafting is used to enhance healing in osteotomies, arthrodesis, and multifragmentary fractures and to replace bony loss resulting from neoplasia or cysts. They are source of osteoprogenitor cells and induce bone formation and provide mechanical support for vascular and bone ingrowth. Autografts are used commonly but quantity of harvested bone is limited. The aim of this study is to evaluate autograft and new xenogenic bovine demineralized bone matrix (DBM) effects on bone healing process. **Materials and methods** Twenty male White New Zealand rabbits were used in this study. In group I ($n = 10$) the defect was filled by xenogenic DBM and in autograft group the defect was filled by fresh autogenous cortical graft and fixed by cerclage wire. Radiological, histopathological and biomechanical evaluations were performed blindly and results scored and analyzed statistically.

Results Statistical tests did not reveal any significant differences between two groups on the 14th postoperative day radiographically ($P > 0.05$). There was a significant difference for union on 28th and 42nd postoperative days and for remodeling at on the 56th postoperative day radiologically ($P < 0.05$). Statistical tests did not support

any significant differences between two groups for radiological bone formation ($P > 0.05$). Histopathological and biomechanical evaluation revealed no significant differences between two groups.

Conclusions The results of this study indicate that satisfactory healing occurred in rabbit radius defect filled with xenogenic bovine DBM. Complications were not identified and healing was faster, same as in cortical autogenous grafting.

Keywords Xenogenic DBM · Autogenous cortical bone · Bone healing · Rabbit

Introduction

Bone grafting is used to enhance healing in delayed unions, nonunions, osteotomies, arthrodesis, multifragmentary fractures and to replace bony loss resulting from neoplasia or cysts [1]. Autogenous bone graft is commonly used and is the standard to which allografts and graft substitutes are compared [2–7]. They may provide a source of osteoprogenitor cells (osteogenesis), induce formation of osteoprogenitor cells from surrounding tissues (osteinduction), and provide mechanical support for vascular and bone ingrowth (osteoconduction) [8]. Though autogenous bone grafts have been clinically effective, the additional surgical time required to harvest an autogenous graft, the morbidity associated with its collection, and the limited availability of autogenous bone in some patients, have encouraged the search of suitable bone graft substitutes [5, 9–11]. Therefore, the use of various bone graft substitutes including autografts, allografts, xenografts, polymers, ceramics and some metals have been employed to promote bone reunion [12, 13].

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Allogenic, demineralized bone matrix (DBM) has been used for several decades in human surgery for the treatment of nonunions, osteomyelitis and large defects resulting from benign tumor removal [14]. The process of demineralization with hydrochloric acid destroys, but also decreases antigenic stimulation and may enhance the release of bone morphogenic protein (BMP) [15]. BMPs stimulate local undifferentiated mesenchymal cells to transform into osteoblasts (osteoinduction), and the collagenous framework of the DBM particles allows for migration of 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 [16–19]. Beyond their role in osteoinduction, certain BMPs and DBM have shown promise in aiding repair of osteochondral defects [20, 21]. Advantages of DBM over other substitutes include inherent osteoinductive capacity (unlike tricalcium phosphate and hydroxyapatite) and availability in large amounts. The aim of study reported here was to compare the effects of xenogenic bovine DBM and fresh cortical autogenous bone on the healing of bone defects in rabbits.

Materials and methods

Animals

Twenty male New Zealand Albino rabbits 12 months old and weighing 3.0 ± 0.5 kg were used in this study. The research protocol for this experiment was approved by the Shiraz University research committee.

Preparation of bovine demineralized bone matrix

Demineralized bone matrix, prepared from the midshafts of the long bones of a 2-year-old Holstein cow, were collected from the local slaughterhouse. All bones were collected aseptically, and the soft tissues were removed before storage at -70°C . The bones were later cleared of fascia and cut into 1-cm pieces with a Stryker saw under saline (0.9% NaCl) solution lavage. Bone pieces were stored at -70°C until further use. The pieces were then thawed in 200-proof ethanol and air-dried. All bones were milled (Universal Mill A-20; Tekmer Co, Cincinnati, OH, USA) 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°C for 8 days under constant agitation.

Demineralization was evaluated with radiography and calcium analysis [22]. Density loss of xenogenic demineralized bone matrix was evaluated radiographically. Also, random samples of DBM were dried at 95°C , weighed,

and then ashed at 600°C for 24 h. These samples were then dissolved in 0.6 mol/l nitric acid and analyzed by atomic absorption spectrophotometry to determine percent calcium per gram dry weight (% Ca:DW) [23, 24]. Demineralization was considered adequate when samples were no longer visible radiographically and when calcium content was less than 1% [25]. After demineralization, all bone pieces were rinsed in sterile water and placed in phosphate buffer 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°C .

Preparation of fresh cortical autogenous bone graft

Fresh autogenous cortical bone was harvested at the time of surgery during the creation of radius bone defect. Then all soft tissues were removed from the harvested bone and used as a fresh autogenous cortical bone graft.

Surgical technique

Animals were anaesthetized with ketamine (40 mg/kg, IM) and xylazine (5 mg/kg, IM). The left forelimb was shaved and prepared aseptically with povidone iodine and the limb draped with sterile drapes. An incision was made directly over the radius; which was exposed by dissection of surrounding muscles. Then an osteoperiosteal segmental defect was created on the middle portion of each radius at least twice as long as the diameter of the diaphysis for creation of nonunion model [26]. The created defects were filled in ten rabbits (group I) with DBM (20 mg/defect) and in other ten rabbits (group II) with same harvested segment of cortical bone and fixed by cerclage wire for prevention of segment dislocation in the grafted area.

Postoperative evaluation

Radiological evaluation

Radiographs of each forelimb were taken postoperatively on 1st day and at the 2nd, 4th, 6th and 8th weeks to evaluate bone formation, union and remodeling of the defect. Results were scored using a modified Lane and Sandhu scoring system [27] (Table 1).

Histopathological evaluation

Eight weeks after operation the rabbits were euthanized pharmacologically for histopathological and biomechanical evaluation. Histopathological evaluation was carried out on

Table 1 Modified Lane and Sandhu radiological scoring system

Bone formation	
No evidence of bone formation	0
Bone formation occupying 25% of defect	1
Bone formation occupying 50% of defect	2
Bone formation occupying 75% of defect	3
Bone formation occupying 100% of defect	4
Union (proximal and distal evaluated separately)	
Nonunion	0
Possible union	1
Radiographic union	2
Total point possible per category	
Bone formation	4
Proximal union	2
Distal union	2
Remodeling	2
Maximum Score	10

five rabbits of each group randomly. Left forelimb were harvested and dissected free of soft tissues. Sagittal sections that contained the defect site were cut with a slow-speed saw. Each slice was then fixed in 10% formalin. The formalin-fixed bone samples were decalcified in 15% buffered formic acid solution and processed for routine histological examination. Two 5-micron thick sections were cut from the centers of each specimen and were stained with hematoxylin and eosin. The sections were individually evaluated and scored by pathologist blinded to the treatment. Scoring system was according to lane and Sandhu modified scoring system by Hieple et al 1987 (Table 2) [28].

Biomechanical evaluation

Mechanical bending test was performed on radial-healed defect of the left forelimb of five rabbits of each group by biomechanical testing machine (Shimatzo, Japan). During the test, the bone ends were placed between two jaws in the testing machine and the load exerted at the grafting area until the failure. The forces, which were needed to break the bones were recorded. Data derived from mechanical testing were expressed as the mean ± SEM (standard error mean) for each group.

Statistical analysis

The radiological and histopathological 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 Mann–Whitney *U* test. The biomechanical data was compared by a Student’s *t*-test (SPSS 15.00).

Table 2 Lane and Sandhu histopathological scoring system modified by Heiple et al. [28]

Union (proximal and distal evaluated separately)	
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
Complete reorganization of cancellous bone	4
Cortical bone	
Non	0
Early appearance	1
Formation under way	2
Mostly reorganized	2
Completely formed	10
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	
Proximal union	4
Distal union	4
Cancellous bone	4
Cortex	4
Marrow	4
Maximum score	20

Results

There was no intraoperative and postoperative death during the study. None of the rabbits sustained a fracture of the radius.

Radiographic findings

There was 25% bone formation in some rabbits in group I and group II on 14th postoperative day. Although there was union in some rabbits of group I, there was no evidence of union in group II. Remodeling was not found in either group. Statistical tests did not support any significant difference (Table 3, *P* > 0.05) (Fig. 1).

There was 50–75% bone formation in some rabbits of group I and 0–25% bone formation in some rabbits of group II on 28th postoperative day. Although there was some union

Table 3 Radiological findings at 2nd week

	Median (min–max)		<i>P</i> ^a
	Group I (<i>n</i> = 10)	Group II (<i>n</i> = 10)	
Bone formation	0 (0–0)	0 (0–1)	0.11
Proximal union	0 (0–0)	0 (0–1)	0.36
Distal union	0 (0–0)	0 (0–0)	1.000
Remodeling	0 (0–0)	0 (0–0)	1.000

^a Kruskal–Wallis non-parametric ANOVA

Fig. 1 Radiographs of forelimb on 14th postoperative day. (a) Xenogenic DBM. b autograft)



in most rabbits of group II, remodeling was not seen in all rabbits of either groups. There was a statistically significant difference only for union at the 28th postoperative day in the radiological signs of bone healing ($P < 0.05$). When pairwise group comparisons were performed by Mann–Whitney *U* test, group II was found to be superior to group I (Table 4, $P = 0.008$ and $P = 0.03$) (Fig. 2).

There was 75–100% bone formation in all rabbits in group I and 50–75% bone formation in all rabbits of group II on 42nd postoperative day. Although there was some union in all rabbits of both groups and some remodeling in group I. There was a statistically significant difference only for union at the 42nd postoperative day in the radiological

signs of bone healing ($P < 0.05$). When pairwise group comparisons were performed by Mann–Whitney *U* test, group II was found to be superior to group I (Table 5, $P = 0.01$) (Fig. 3).

There was 100% bone formation and union in group I and 75–100% bone formation and some union in group II on 56th postoperative day. There were 25–50% points remodeling in the two groups. Group II was statistically superior to group I only in terms of radiological callus remodeling ($P < 0.05$). When pairwise group comparisons were performed with Mann–Whitney *U* test, the group II was superior to group I (Table 6, $P < 0.03$) (Fig. 4).

Histopathological findings

Histopathologically there was no statistically significant difference between the groups in terms of cancellous and cortical bone, union and marrow formation. None of the grafted materials elicited a significant inflammatory reaction. In the group II the chondroblastic differentiation zone was observed (Table 7, $P > 0.05$) (Fig. 5).

Biomechanical findings

There was no statistically significant difference between two groups in terms of biomechanical bending test (Table 8, $P > 0.05$).

Table 4 Radiological findings at 4th week

	Median (min–max)		<i>P</i> ^a
	Group I (<i>n</i> = 10)	Group II (<i>n</i> = 10)	
Bone formation	1 (1–1)	1 (1–1)	0.006
Proximal union	0 (0–0)	1 (1–1) ^b	0.004
Distal union	0 (0–0)	1 (0–1) ^c	0.006
Remodeling	0 (0–0)	0 (0–0)	1.000

Significant *P*-values are presented in bold face

^a Kruskal–Wallis non-parametric ANOVA

^b $P = 0.008$ (compared with group I by Mann–Whitney *U* test)

^c $P = 0.03$ (compared with group I by Mann–Whitney *U* test)

Fig. 2 Radiographs of forelimb on 28th postoperative day. (a Xenogenic DBM, b autograft)



Table 5 Radiological findings at 6th week

	Median (min–max)		<i>P</i> ^a
	Group I (<i>n</i> = 10)	Group II (<i>n</i> = 10)	
Bone formation	2 (1–3)	2 (1–2)	0.11
Proximal union	1 (0–1)	2 (1–2) ^b	0.008
Distal union	1 (0–1)	2 (1–2)	0.01
Remodeling	1 (0–1)	1 (0–1)	0.17

Significant *P* values are presented in bold face

^a Kruskal–Wallis non-parametric ANOVA

^b *P* = 0.01 (compared with group I by Mann–Whitney *U* test)

Fig. 3 Radiographs of forelimb on 42nd postoperative day. (a Xenogenic DBM, b autograft)



Discussion

In this study a radius defect model was created to compare healing of bovine DBM implant as a new xenograft and fresh autogenous cortical bone graft in the rabbit model. This model has been reported previously

suitable because there was no need for internal or external fixation that can influence the healing process [29]. The osteoperiosteal segmental defect was created in middle portion of radius at least twice as long as the diameter of diaphysis to produce nonunion model and prevent spontaneous healing [26].

Table 6 Radiological findings at 8th week

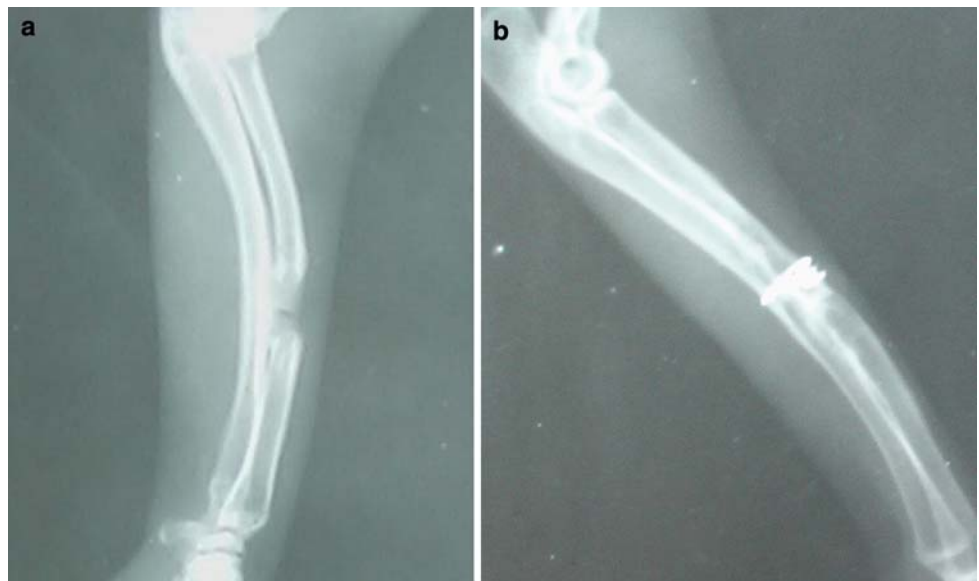
	Median (min–max)		<i>P</i> ^a
	Group I (<i>n</i> = 10)	Group II (<i>n</i> = 10)	
Bone formation	2 (0–3)	2 (1–3)	0.13
Proximal union	1 (0–2)	2 (1–2)	0.9
Distal union	1 (0–2)	1 (1–2)	0.1
Remodeling	1 (1–1)	1 (0–2) ^b	0.007

Significant *P*-values are presented in bold face

^a Kruskal–Wallis non-parametric ANOVA

^b *P* = 0.03 (compared with group I by Mann–Whitney *U* test)

Fig. 4 Radiographs of forelimb on 56th postoperative day. (a) Xenogenic DBM, (b) autograft)

**Table 7** Histopathological findings at 8th week

	Median (min–max)		<i>P</i> ^a
	Group I (<i>n</i> = 5)	Group II (<i>n</i> = 5)	
Union	2 (1–2)	1 (1–2)	0.2
Cortical bone	1 (0–3)	1 (0–2)	0.9
Cancellous bone	1 (1–3)	1 (0–3)	0.6
Bone marrow	1 (0–2)	1 (0–2)	1.000

^a Kruskal–Wallis non-parametric ANOVA

Fig. 5 Histopathological evaluation of a Xenogenic DBM implantation. Note the chondroblastic differentiation in grafted area (white arrow) (H&E × 100) and b cortical bone autograft

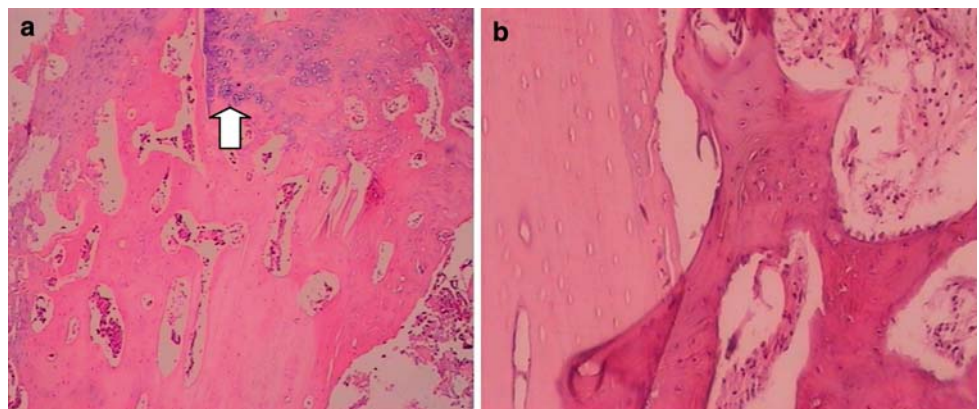


Table 8 Biomechanical three point bending test findings (mean \pm SEM)

Group	I	II	<i>P</i> value
Maximum load (kgf)	9.04 \pm 0.97	6.9 \pm 0.56	0.09

SEM standard error mean

The bone inductive activity of DBM has been well-established [30–38]. The addition of autologous bone marrow and/or autograft to DBM provides an immediate source of osteogenic precursor cells at the implant site that may provide an additional biochemical contribution to osteogenesis [37–39]. DBM also appears to support new bone formation through osteoconductive mechanisms [40]. Autogenous bone graft is commonly used and is the standard, to which allografts and graft substitute are compared [2–7]. The primary osteoinductive component of DBM is a series of low-molecular-weight glycoproteins that includes the BMPs. The decalcification of cortical bone exposes these osteoinductive growth factors buried within the mineralized matrix, thereby enhancing the bone formation process [41]. These proteins promote the chondroblastic differentiation of mesenchymal cells, followed with new bone synthesis by endochondral osteogenesis [41, 42]. In this study, it was found that the results of group I was not statistically significant after the 8 weeks in comparison with group II. It proves that the grafted xenogenic bovine DBM has osteoinductive (by releasing the some BMPs) activity same as autogenous cortical bone graft. However it was found that cortical autograft has more osteoconductive properties and less osteoinductive activity [43, 44]. DBM also appears to support new bone formation through osteoconductive mechanisms [40]. There were not any significant differences in histopathological evaluation between two groups 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 mineralized allograft [45] and the cortical autogenous bone graft does not induce immunological reaction by the host [43]. Therefore, we did not observe any inflammatory reaction in group I and group II. We observed chondroblastic differentiation zone in histopathological evaluation of group I. Urist showed chondroblastic differentiation from mesenchymal cell by bone morphogenetic proteins [41, 42]. It was understood that the chondroblastic differentiation in group I was related to BMPs releasing from grafted bovine DMB.

In biomechanical evaluation, group I was superior to group II, but there is not any statistically significant difference between two groups. It has been reported that cortical autogenous bone graft remains a combination of

necrotic and new bone for a prolonged period and leads to reduction in mechanical strength [46]. Moreover, experimental studies have shown that osteoinductive bone protein growth factors combined with DBM produce biomechanically enhanced fusions as compared to autograft alone [47–50]. A number of well-controlled studies in a well-established and validated animal model of posterolateral spine fusion have demonstrated the suitability of various forms of DBM as a graft extender and, in some cases, as a graft enhancer and a graft substitute [40, 51]. The results of this study indicate that satisfactory healing occurred in rabbit radius defect filled with xenogenic bovine DBM. Complications were not identified and healing was faster, same as in cortical autogenous grafting. The use of xenogenic bovine DBM is an acceptable alternative to cortical autogenous graft and could reduce the morbidity associated with harvesting autogenous graft during surgery. Further studies are needed to evaluate the long-term effects of DBM implantation on bone healing to document the use of this graft substitute in various clinical situations. DBM has a number of additional advantages that make it an attractive bone graft alternative. It is cost-effective and is readily available from tissue banks.

Conflict of interest statement The authors declare that they have no conflict of interest related to the publication of this manuscript.

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