Osteoinductive and Osteopromotive Variability among Different Demineralized Bone Allografts

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ABSTRACT

Objective: The purpose of this investigation was to compare the osteoinductive and osteopromotive potential of two widely used demineralized freeze-dried bone allografts (DFDBA) (Osteotech® DFDBA and LifeNet® DFDBA).

Material and Methods: Twenty-seven male Wistar rats (mean body weight 200 g) were treated with either DFDBA from Osteotech and LifeNet or control for femoral and intramuscular defects and assigned to histological analysis at 2, 4, and 8 weeks postimplantation. Hematoxylin and eosin (H&E), Safranin-O, tartrate-resistant acid phosphatase (TRAP), and osteopontin (OPN) staining were performed. Quantitative analysis of mineralized new bone to total volume (BV/TV) was assessed by micro-computed tomography.

Results: Both allografts demonstrated osteoinductive potential at 2 weeks as assessed by intramuscular bone formation. LifeNet DFDBA displayed continual new bone formation at 4 and 8 weeks, whereas Osteotech particles were fully resorbed by 4 weeks postimplantation. Femur defects demonstrated significantly greater BV/TV at 4 and 8 weeks with higher expression of OPN staining around LifeNet DFDBA particles. TRAP-positive cells were visible in and around both allograft materials.

Conclusion: The results from the present study indicate that variability among allografts exists. In the present, LifeNet DFDBA supported more new bone formation. Further larger animal models or clinical trials are required to validate these findings.

KEY WORDS: bone formation, demineralized freeze-dried bone allograft (DFDBA), LifeNet, osteoinduction, Osteotech

INTRODUCTION

There is a great clinical need for bone grafting materials in implant dentistry. Although autogenous bone remains the grafting material of choice due to its excellent osteoconductive and osteoinductive properties,[1] drawbacks such as longer surgical times, lack of availability, and donor site morbidity necessitates alternatives.[2] In contrast, many bone grafting substitutes such as alloplasts and xenografts are commonly used for maxillary sinus elevation procedures to augment bone around implants and to treat intrabony defects.[3–10] Although these grafting materials are osteoconductive by supporting a three-dimensional scaffold that allows for cell growth, they lack necessary osteoinductive growth factors to recruit bone-forming progenitor cells.[11] One bone grafting biomaterial currently approved by the FDA for use in North America with osteoinductive potential is the demineralized freeze-dried bone allograft (DFDBA).[6–10] DFDBA has been

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used for over two decades[12–19] to fill voids because it possesses osteoinductive growth factors such as bone morphogenetic proteins, which are capable of recruiting mesenchymal progenitor cells and supporting their differentiation to bone forming osteoblasts.[11]

Although the demineralization processing results in access to a multitude of osteoinductive growth factors (as determined by comparison studies to freeze-dried bone allograft),[7] reports suggest that some commercial formations of DFDBA are less osteoinductivity than others.[6,20,21] Schwartz and colleagues tested commercial lots of DFDBA from six different bone banks and found that variability existed when DFDBA was placed in nude mouse muscle. These authors concluded that the variability in osteoinductivity between commercial DFDBA batches may be ascribed to donor age, method of preparation, and/or sterilization.[6,21]

The aim of the present study was to analyze and compare bone formation in association with two widely used commercial DFDBA products (Osteotech® DFDBA and LifeNet® DFDBA). Graft materials were implanted in both intramuscular and femoral defects in rats to determine their osteoinductive potential and ability to promote new bone formation, respectively.

MATERIALS AND METHODS

Animals and Surgical Procedures

Twenty-seven male Wistar rats (mean body weight 200 g) were used with all handling and surgical procedures being in accordance with the policies of the Ethics Committee for Animal Research, Wuhan University, China. Animals had food and water ad libitum with constant temperature at 20 to 25°C.

All operations were conducted under strictly sterile conditions. For surgery, the rats were generally anesthetized with intraperitoneal injection of chloral hydrate (10%, 4 mL/kg body weight). After skin preparation and disinfection, a 10-mm linear skin incision was made in the distal femoral epiphysis of hind limbs bilaterally, and the femoral condyle was exposed to the operative field using a blunt dissection technique. A 3-mm diameter anteroposterior hole was drilled perpendicular to the femoral axis just above the growth plate using a trephine bur at a speed of 1,000 rpm irrigated under saline solution as previously described.[22] The drilled holes were flushed with saline solution and gentamicin infusion successively in order to remove the bone fragments and prevent infection. Then, for the experimental groups, an equal amount of each DFDBA product (10 mg per hole) was implanted into the bone defects. For the blank control, the drilled hole had no treatment. Following closure of the femoral defect, bilateral muscle pouches were made in the gastrocnemius muscle of each animal. Subsequently, an equal mass of DFDBA particles (20 mg per pouch) was implanted intramuscularly, and incisions were sutured in two layers. Postoperatively, penicillin (400,000 IU/mL, 0.1 mL/kg) was injected for 3 days.

All animals were subject to treatment with Osteotech DFDBA (Eatontown, NJ, USA) in femoral defect/muscular pouch (n = 6 replicates per three time points), LifeNet DFDBA (Virginia Beach, VA, USA) ($n = 6$ replicates per three time points) and drilled control ($n = 6$ replicates per three time points), respectively. After 2, 4, and 8 weeks postimplantation, rats were sacrificed, and samples were removed and prepared for histological analysis.

Micro-Computed Tomography (μCT) Analysis

The samples were fixed in 4% formaldehyde for 24 hours at room temperature. A μCT imaging system (μCT50, Scanco Medical, Bassersdorf, Switzerland) was used to evaluate new bone formation within the defect region. All femoral samples were placed with the long axis of the drilled channel perpendicular to the axis of the X-ray beam. Scanning was performed at 70 kV and 114 μA with a thickness of 0.048 mm per slice in medium-resolution mode, 1024 reconstruction matrix, and 200 ms integration time. A consistent volume of interest (VOI), which is located in the central 2-mm-diameter region of the 3-mm-diameter defect, was defined to evaluate the level of bone regeneration within the defect. To eliminate the influence of different materials contained in the defect, a low threshold was set at 184. After three-dimensional reconstruction, the bone volume fractions (BV/TV) in defect regions were used to evaluate new bone formation, using a protocol provided by the manufacturer of the micro-CT scanner. All digitalized data and three-dimensional images were generated by the built-in software of the μCT.

Histological Analysis

After μCT imaging, the samples were decalcified in 10% ethylene diamine tetraacetic acid (EDTA), which was replaced twice weekly for 3 weeks at room temperature. Then the femoral samples were dehydrated in a series of

graded concentrations of ethanol from 70% to 100%, whereas the muscular samples started at 30% and were then embedded in paraffin. To get a distinct view of the defect, the orientation and alignment of femurs were carefully considered during paraffin embedding. A series of slices starting at a distance of 1 mm proximal from the end of the growth plate with a length of 2 mm were chosen for evaluation. For analysis of the bone regeneration process within the defect, the central region of the 2.5-mm-diameter defect was defined by analyzing a circular contour as area of measurement per slice, thus to obtain a consistent VOI and to avoid including the native bone margins. Serial sections of 5 μm were cut and mounted on polylysine-coated microscope slides and stained with hematoxylin and eosin (H&E), Safranin-O (Sigma #S2255; Sigma-Aldrich, St. Louis, MO, USA) and tartrate-resistant acid phosphatase (TRAP) (Sigma #387A; Sigma-Aldrich) in accordance with the manufacturer's protocol.

Immunohistochemical Analysis

For immunohistochemical analysis of osteopontin (OPN), the sections of femur defects and muscle pouches were deparaffinized, rehydrated, washed with phosphate buffered saline (PBS), and then incubated with 0.3% hydrogen peroxide for 20 minutes followed by incubation with bovine serum albumin. Then the sections were incubated with primary antibody for OPN (1:100; Boster SA2005, Boster Co., Wuhan, China) for 2 hours at 37°C. Following three washes with PBS, the slices were incubated with a secondary antibody (Zhongshan Biotechnology Co., Ltd, Wuhan, China) for 20 minutes. After washing, the sections were incubated with horseradish peroxidase-conjugated avidin-biotin complex (Zhongshan Biotechnology Co., Ltd) for another 20 minutes. 3, 3-diaminobenzidine tetrahydrochloride (Zhongshan Biotechnology Co., Ltd) was used as the visualization reagent, and the procedure was observed by light microscopy for intensity control. Lastly, the sections were counterstained with hematoxylin.

Statistical Analysis

All data analysis was performed using SPSS software (SPSS, Inc., Chicago, IL, USA), and statistically significant values were adopted as $p < .05$. Because of the sample size chosen, a nonparametric test (Kolmogorov–Smirnov test) was chosen to confirm the asymptotic normality of our data. Mean and standard deviation (SD) were calculated, and we made statistical inference by one-way analysis of variance and Student–Newman–Keuls Test.

RESULTS

DFDBA Implantation in Intramuscular Defects

Healing in all animals occurred normally without any further complications or infections from surgical techniques. H&E staining of sections from intramuscular defects revealed that the blank muscle pouches healed naturally (Figure 1, A–C), and both Osteotech DFDBA and LifeNet DFDBA were osteoinductive by demonstrating signs of ectopic bone formation 2 weeks postimplantation (Figure 1, D and G). Interestingly, Osteotech DFDBA began resorption shortly afterwards

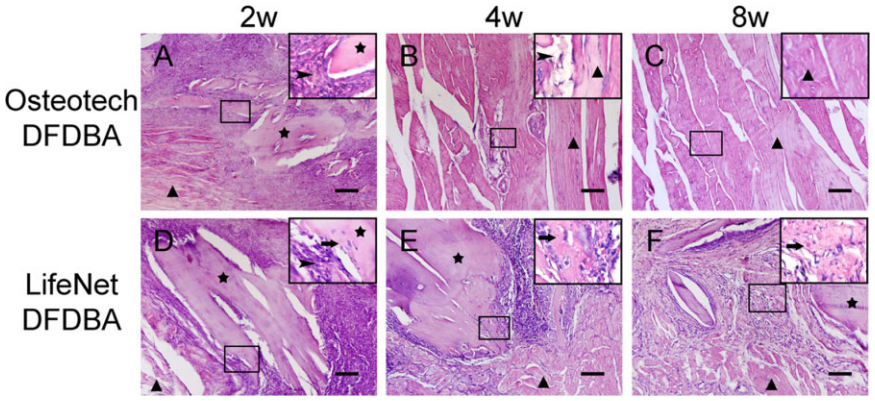


Figure 1 Representative sections of H&E staining revealed that both Osteotech® and LifeNet® demineralized freeze-dried bone allograft (DFDBA) were able to produce ectopic bone formation in rat intramuscular defects at 2 weeks (D and G). Osteotech DFDBA was vacated by inflammatory cells, and by 4 weeks the graft was no longer present (E). Comparatively, abundant osteoid matrix was deposited surrounding the particle of LifeNet DFDBA at 4 and 8 weeks postimplantation (H and I). Asterisk: remnant graft; arrow: newly formed osteoid; arrow head: inflammatory cells; triangle: muscle cells. Scale bar: 100 μm.

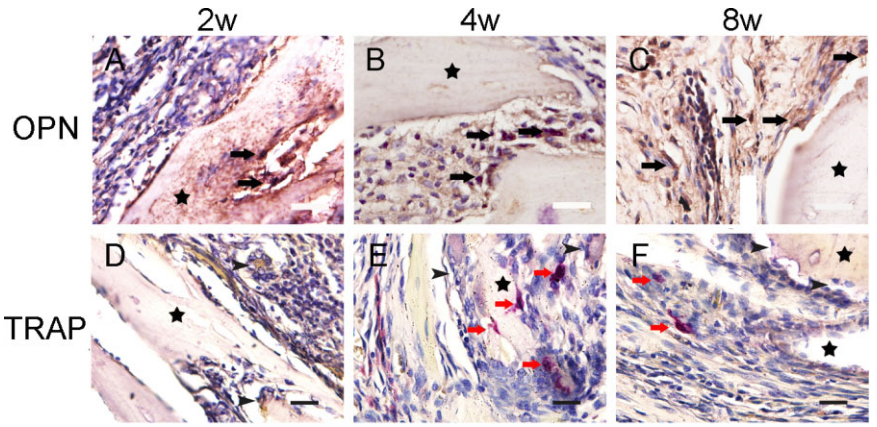


Figure 2 Osteopontin (OPN) immunohistochemical staining and TRAP staining for LifeNet® demineralized freeze-dried bone allograft (DFDBA) implanted in rat intramuscular defects at 2, 4, and 8 weeks. For the middle and advanced time points, LifeNet DFDBA demonstrated large amounts of OPN staining around implanted DFDBA particles that was circumstantial evidence for the formation of osteoid calcification. Osteoclasts and their progenitor cells that were stained in red appeared around the new mineralized bone matrix at 4 and 8 weeks. Asterisk: remnant graft; black arrow: OPN positive cells; black arrow head: osteoblasts and osteocytes; red arrow: TRAP positive cells. Scale bar 25 μm.

and was completely resorbed along with all newly formed bone in all defects by 4 weeks (Figure 1E). Interestingly, abundant new bone formation continued to be deposited surrounding LifeNet DFDBA particles both 4 and 8 weeks postimplantation (Figure 1, H and I), demonstrating a greater osteoinductive ability for these grafting particles. Representative sections of immunohistochemical staining also illustrated a positively strong correlation with OPN expression around LifeNet DFDBA particles (Figure 2, B and C). Beyond that, scattered osteoclast-like cells were observed at 4 and 8 weeks around the osteoid induced by LifeNet DFDBA suggesting the onset of bone remodeling in rat intramuscular defects (Figure 2, E and F).

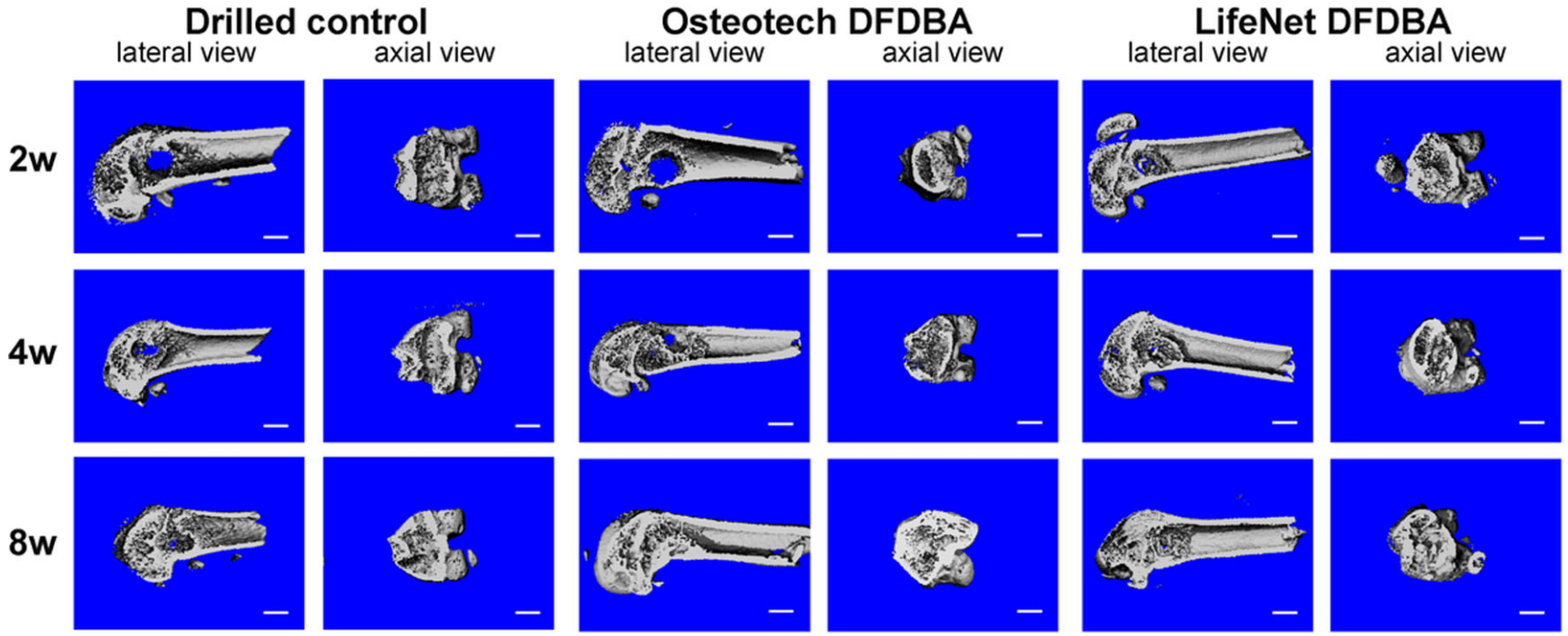
μCT Analysis of Femoral Defects

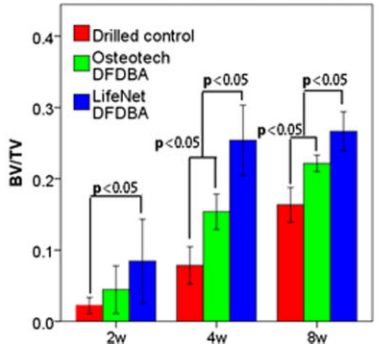
In the two experimental groups, the femoral defects were filled with minor compact cortical bone formation after 2 weeks, and the cortical bone gradually increased over time and fully formed after 8 weeks. Though the drilled control defect decreased in size throughout the experiment length, complete healing was not reached. Representative images of bone formation performed by three-dimensional reconstruction for each group are shown in Figure 3. Some minor mineralization was visible in the femoral defect after 2 weeks postimplantation for LifeNet DFDBA particles when compared with Osteotech DFDBA and control; however, results were not significant (Figure 3). At both 4 and 8 weeks postimplantation, significantly more mineralized new bone formation was quantified in defects filled with LifeNet DFDBA by analysis of μCT data ($p < .05$) (Figure 3).

Histological and Immunohistochemical Observation of the Femoral Defect

In accordance with the micro-CT data, representative zones of the femoral defect are shown in Figures 4–6 to demonstrate histological evidence of new bone formation. At 2 weeks postoperation, fibrous ossification participated in the ongoing bone formation with more osteoblasts visible in the entire area filled with LifeNet DFDBA (Figure 4, F and J). Conversely, bone regeneration was confined in the periphery of the drilled blank defect (Figure 4A). OPN immuno-histochemical staining also demonstrated a positive correlation with newly formed bone surrounding LifeNet DFDBA particles (Figures 4K, 5K, and 6K). Positive OPN staining was detected in osteogenic active areas in both experimental groups by 2 weeks (Figure 4, G and K) demonstrating early onset of osteogenesis. Noticeably in both groups, few TRAP-positive multinucleated giant cells could be detected at 2 weeks; however, at 4 and 8 weeks, these cells became more prominent in the environment (as indicated by arrows in H and L of Figures 5 and 6).

At 4 weeks postimplantation, new bone was formed continuously, and osteoclasts appeared in all three groups (Figure 5). More newly formed bone around the grafting particles was present around LifeNet DFDBA when compared with Osteotech DFDBA filled defects and the drilled blank. In all three groups,





	2 weeks			4 weeks			8 weeks		
	Mean	SD	CI(95%)	Mean	SD	CI(95%)	Mean	SD	CI(95%)
Drilled blank	0.022	0.006	0.016-0.028	0.078	0.013	0.065-0.092	0.163	0.012	0.151-0.176
Osteotech® DFDBA	0.045	0.017	0.027-0.062	0.154	0.012	0.141-0.167	0.222	0.006	0.215-0.228
LifeNet® DFDBA	0.084	0.029	0.054-0.115	0.254	0.025	0.228-0.280	0.266	0.014	0.252-0.281

Figure 3 Three-dimensional reconstruction of longitudinal section and cross-section images by micro-computed tomography (μCT) at 2, 4, and 8 weeks postoperation of a rat cavitary defect in the femur. At 2 weeks, little to no new bone formation was present in defects treated with Osteotech®, LifeNet® demineralized freeze-dried bone allograft (DFDBA), and the drilled blank. At 4 and 8 weeks postimplantation, significantly more new bone formation was present for defects treated with LifeNet DFDBA (scale bar 3 mm). Quantitative analysis of mineralized new bone formation from μCT revealed significantly more newly formed mineralized tissues in LifeNet DFDBA at 4 and 8 weeks postimplantation. Data were shown as mean ± SD. $^{*}p < .05$.

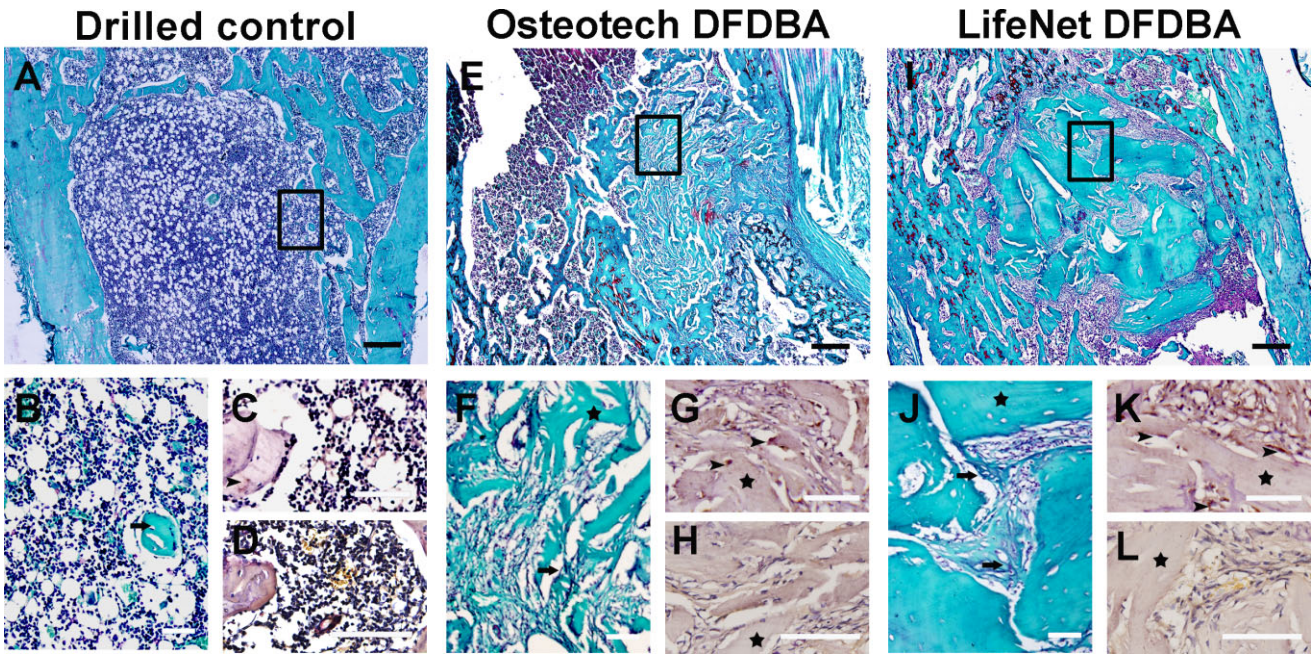


Figure 4 Representative sections of Safranin-O staining (A, B, E, F, I, and G), osteopontin (OPN) immunohistochemical staining (C, G, and K), and TRAP staining (D, H, and L) demonstrating the healing of femoral defects at 2 weeks postoperation. Fibrous ossification participated in the ongoing bone formation. More osteoblasts were visible in the LifeNet® demineralized freeze-dried bone allograft (DFDBA) filled defect. Asterisk: remnant graft; black arrow: osteoblasts and osteocytes; black arrow head: OPN positive cells. Black scale bar: 500 μm; white scale bar: 100 μm.

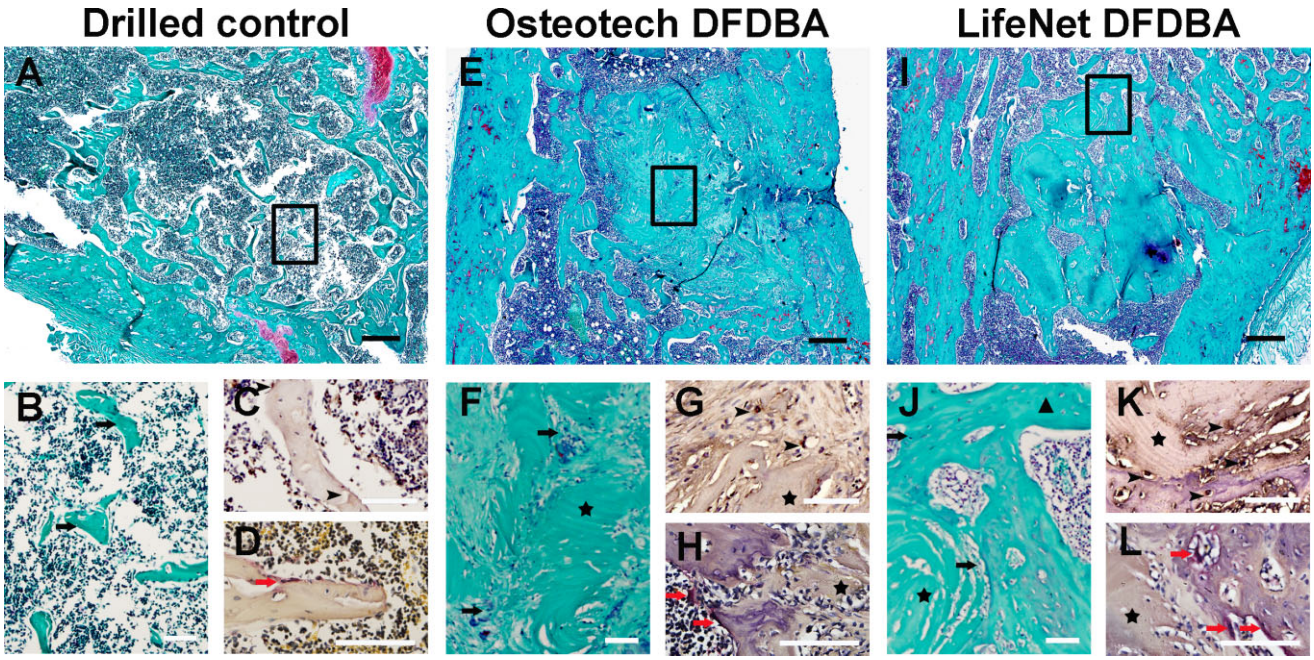


Figure 5 Representative sections of Safranin-O staining (A, B, E, F, I, and G), osteopontin (OPN) immunohistochemical staining (C, G, and K), and TRAP staining (D, H, and L) demonstrating the healing femoral defects at 4 weeks postoperation. New bone formation was continuously formed in all groups. Activated bone remodeling was present in both groups as demonstrated by the presence of osteoclasts in both treatment groups. Asterisk: remnant graft; black arrow: osteoblasts and osteocytes; black arrow head: OPN positive cells; triangle: mature mineralized new bone; red arrow: TRAP positive cells. Black scale bar: 500 μm; white scale bar: 100 μm.

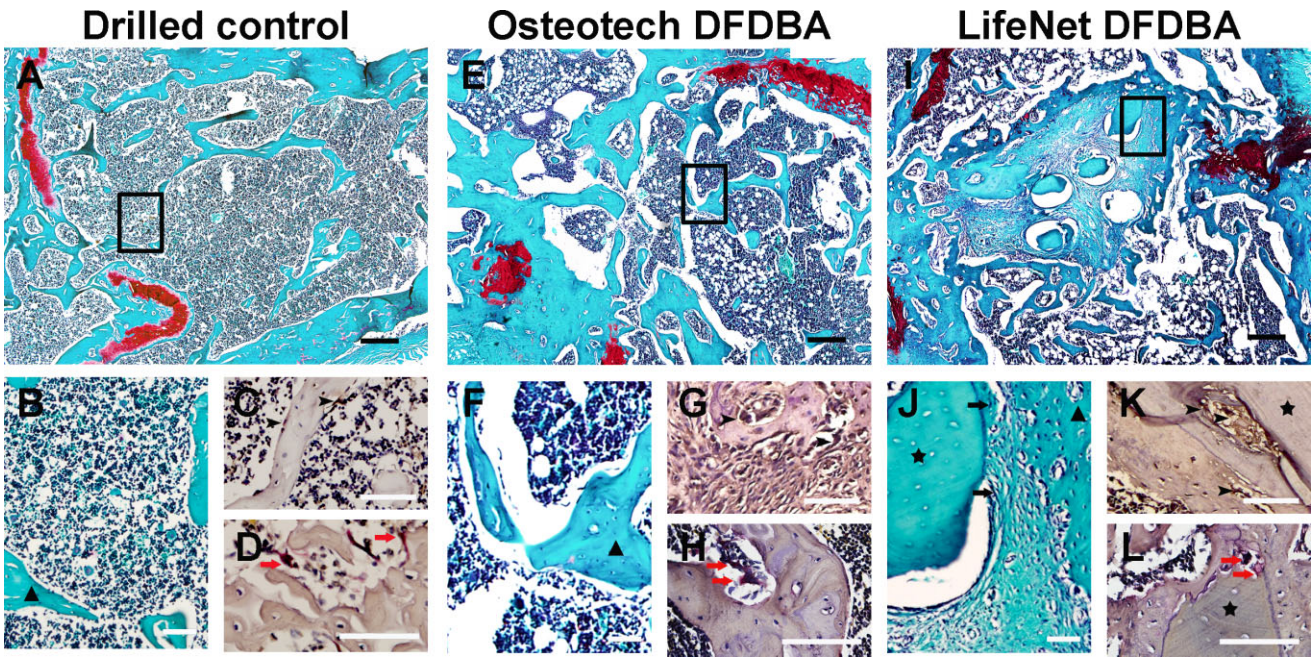


Figure 6 Representative sections of Saffranin-O staining (A, B, E, F, I, and G), osteopontin (OPN) immunohistochemical staining (C, G, and K), and TRAP staining (D, H, and L) demonstrating the healing of femoral defects at 8 weeks postoperation. Asterisk: remnant graft; black arrow: osteoblasts and osteocytes; black arrow head: OPN positive cells; triangle: mature mineralized new bone; red arrow: TRAP positive cells. Black scale bar: 500 μm; white scale bar: 100 μm.

TRAP-positive multinucleated giant cells were observed in close association with the newly formed trabeculae at 4 and 8 weeks, indicating an activated bone remodeling process with both active osteoblasts and osteoclasts present.

Large amount of mineralized new bone existed in both experimental groups at 8 weeks postoperation. In accordance with the μCT data, Safranin-O staining demonstrated more new bone formation surrounding LifeNet DFDBA particles at 4 and 8 weeks post implantation in a femur defect as indicated by the black arrow and triangle in Figures 5F and 6F. Osteotech DFDBA were partially resorbed 8 weeks postimplantation as indicated in Figure 6, A and B.

DISCUSSION

For the past three decades, DFDBA has been used alone or in combination with other regenerative modalities in implant dentistry and periodontal therapy.[23] Becker and colleagues were one of the first to compare the bone inducing ability of DFDBA in comparison with other bone grafting alternatives.[12–15] Although not as potent for bone regeneration as autogenous bone, it is now widely accepted that DFDBA supports regeneration of periodontal and osseous tissues due to its excellent combination of osteoconduction and osteoinduction.[11,24,25] The presence of growth factors contained within DFDBA[24,26,27] allows for mesenchymal cell migration and supports their future differentiation to bone forming osteoblasts.

The focus of this study was to compare DFDBA from two well-established and widely used companies. Both Osteotech and LifeNet have been studied extensively, but due to the best of our knowledge, no study has directly compared their osteoinductive or osteopromotive potential. The findings from the present study demonstrate that both products are osteoinductive; however, the results demonstrate a greater potential for LifeNet DFDBA with more new bone formation taking place in intramuscular and femur defects.

The osteoinduction model was chosen to determine up to which time point DFDBA particles could support new bone formation in intramuscular regions. Interestingly at 4 weeks, the Osteotech DFDBA was completely absent from the intramuscular region indicating complete resorption of their particles. At the 4 and 8 weeks, OPN, which is one of the most abundant noncollagenous proteins in bone and also invariably found in ectopic calcifications of soft tissues,[28,29] was positive in the LifeNet DFDBA filled muscle pouches (Figure 2, B and C). This phenomenon suggested that the osteoinduction of LifeNet DFDBA activated the biomineralization and

abundant secretion of OPN (Figure 2). TRAP staining revealed that at 4 and 8 weeks osteoclast-like cells and progenitor cells in the LifeNet DFDBA filled pouches (Figure 2, E and F) were absent to participate in the bone remodeling process. However, no Osteotech DFDBA was available to demonstrate osteoclast-like resorption of these particles (date not shown). Based on the results from the present study, a time point of up to 4 weeks might be best suitable to test osteoinduction in a small rodent model with further time points supporting greater osteoinductive potential.

Following intramuscular osteoinduction experiments, healing of femoral defects filled with DFDBA particles was analyzed. In present study, we sought to characterize the effects of different demineralized bone allografts on pure bone defects in a rat femoral model. Though the femoral defect may physically infiltrate with a high number of mesenchymal progenitor cells, this model was chosen because it is commonly employed for testing bone grafting materials[22,30,31] and also represented an area with self-healing capacity to some degree that is similar to the healing of socks after tooth extraction. The μCT and histological results revealed up to 8 weeks; the drilled control achieved incomplete healing in both cortical bone and cancellous bone, which characterized bone regeneration centralized in the periphery of the defect (Figures 3 and 6). The μCT results demonstrated and supported the finding that the better osteoinductive material was also able to increase the speed and quality of new bone formation in complete bone defects (Figure 3). As a kind of bone matrix proteins and a sign of mature osteoblast, OPN was highly expressed in the new generated bone of LifeNet DFDBA filled defect demonstrating a more dynamic healing process. LifeNet DFDBA did show TRAP-positive cells around grafting particles at 4 and 8 weeks with TRAP suggesting that osteoclast-mediated remodeling started shortly after implantation.

The results from the present study demonstrate that large variability exists between DFDBA products, which is possibly caused by their processing and/or sterilization procedures. Although variability exists between different batches of DFDBA within a tissue bank,[25] in this study, a greater variability existed among company stocks of DFDBA. Further research aimed to determine the effects of processing, sterilization, and particle size as possible reasons for the variability among DFDBA products are necessary.

In a position paper describing tissue banking of bone allografts used for periodontal regeneration, the authors raised the issue of variability among DFDBA preparations.[32] Although most bone banks adhere to the guidelines of the American Association of Tissue Banks, these authors concluded that the issue is complicated by the fact that tissue banks do not use identical methods for DFDBA preparations and the methods used by bone banks are proprietary information, making it difficult for the clinician to evaluate which procedures are best for preserving bone inducing ability during DFDBA processing. While other authors suggest particles in the range of 125 to 1000 microns improves surface area and packing density and thus new bone formation,[33] other authors have shown that various methods of sterilization by radiation or ethylene oxide have shown to affect the osteoinductive ability of DFDBA to a greater extent.[34–36]

The search for the ideal graft material is still ongoing. Consequently, the evolution of grafting materials has resulted in the use of various combinations of either bone grafts combined with guided bone regeneration, various collagen and noncollagen membranes, and growth factors/regenerative agents. The results from the present study as well as future studies are necessary to provide the clinician with optimal allograft preparations that will maximize their osteoinductive and bone forming potential.

CONCLUSION

The results from the present study demonstrate that both Osteotech DFDBA and LifeNet DFDBA are osteoinductive by promoting new bone formation in rat intramuscular defects. At 4 weeks, however, LifeNet sustained new bone formation, whereas Osteotech DFDBA particles were completely resorbed. The results from rat femur defects also demonstrated the ability to form new bone in defects treated with LifeNet DFDBA when compared with Osteotech DFDBA and the drilled control at 4 and 8 weeks postimplantation. The combination of these experiments suggests that LifeNet DFDBA is capable of supporting greater new bone formation as a result of its greater osteoinduction; however, further studies including larger animal models or clinical trials are required to validate these findings.

CONFLICT OF INTEREST

The authors report no conflict of interest for this study. This project was supported by Program for New

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