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Characterization of an injectable chitosan-demineralized bone matrix hybrid for healing critical-size long-bone defects in a rabbit model

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Abstract. – BACKGROUND: The effect of injectable demineralized bone matrix (DBM) on bone repair is not known. Here, we tested the hypothesis that injectable DBM can heal a critical-size diaphyseal radius defect in a rabbit model.

MATERIALS AND METHODS: The bone defect was filled with DBM powder, injectable DBM or powdered, freeze-dried powdered allografts. Radiological determination, gross evaluation, histology, and micro-computer tomography was carried out 4, 8, and 12 weeks after the surgery, respectively.

RESULTS: The injectable DBM group yielded better when compared with the freeze-dried powder group ($p < 0.05$). Moreover, biomechanical functionality was restored comparable to normal levels in the injectable DBM group.

CONCLUSIONS: The injectable DBM was as effective in structurally and functionally repairing bone defects as the DBM powder and more effective than the freeze-dried bone powder. Thus, our study supports the use of injectable DBM for bone healing.

Key Words:
Demineralized bone matrix, DBM, Chitosan, Freeze-dried bone, Bone defect

Introduction

Due to the lack of suitable material for bone graft, the main purpose of bone defect treatment is to minimize the scope of the defect. Bone defects constitute a tough challenge as far as scope of remission through orthopedic surgery is concerned, merely because they are not the most feasible bone graft materials. Even though autografts are able to induce and conduct bone without the associated risk of disease transmission, their potential are limited because their use may cause complications, such as hematoma formation, secondary infection, and pain at the donor site[1-3].

Therefore, orthopedic surgeons often choose to use allografts. Allogeneic demineralized bone matrix (DBM) is the most common bone graft material[4]. DBM causes the release of bone morphogenetic protein (BMP) by mesenchymal cells and the secretion of endogenous BMP, which subsequently induces osteogenesis[5,6]. Clinically, rapid promotion of bone growth accompanied by improved healing outcomes has been obtained with DBM, which has been used for spinal fusion and to treat bone tumors[7-11]. In recent years, DBM has also been used for major acetabular reconstruction of revision hip arthroplasty, which confirmed that it could accelerate bone ingrowths and remodeling[12].

The morphology of bone defect is often irregular; hence, the use of particles or rod-like bone graft materials to fill the bone graft will typically result in the shedding of particles or residual dead space, in turn causing failure of the bone defect treatment. On the contrary, injectable materials can be used in the repair of bone defects because of their suitable shape, limited tissue damage, minimally invasive surgery requirements, and reduced complications[13].

DBM is mostly composed of particles or rod-like shapes. However, in conjunction with an adhesive material that allows it to be injectable, DBM can be used to completely fill the defects that have complex geometric shapes. DBM and autologous bone marrow injections have been previously used in the clinic[14,15]. *In situ*-forming gels generated from sodium carboxymethylcellulose (CMC) and poly(ethyleneimine) (PEI) can serve as *in vivo* carriers of DBM[16]. In addition, a composite that utilizes the osteo-inductivity of DBM and the attractive characteristics of polylactide may generally be useful as a tissue-engineered bone substitute[17].

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Chitosan solution is colloid, with adhesion properties, good biocompatibility and safety. It has been widely used in clinical medicine such as blood clotting, wound healing, sustained release of drug and artificial blood vessel. Combination of DBM and medical chitosan to create an injectable DBM material has not been previously reported. Hence, the objective of the current study was to optimize the technique and test the *in vivo* clinical relevance of a DBM and medical chitosan injection for the treatment of critical-size long-bone defects using a rabbit model system.

Materials and Methods

Reagents and Animals

Chitosan was obtained from Qisheng Biological Preparation Co., LTD (Shanghai, China). All animal studies were approved by the Institutional Review Board and Ethics Committee of Chinese PLA General Hospital. Sixty male New Zealand white rabbits (NZWR), six to nine months old and weighing 2.00 ± 0.50 kg was obtained from Animal Center of Academy of Military Medical Sciences, Beijing. The rabbits were kept in separate cages in pathogen free conditions, fed with standard diet and allowed to move freely during the study. Fifty-four rabbits were randomly chosen from the 60 NZWRs and divided into three groups – (1) the DBM-powder, (2) the injectable DBM, and (3) the freeze-dried powder groups (n = 18). Empty control group, without any implant, served as a blank control (n = 6). Fourteen NZWRs were randomly chosen and sacrificed. Cortical bones were taken from both sides of each radius and ulna for preparation of DBM. Cancellous bone was obtained from each condyle of the femur for preparation of freeze-dried bone powder.

Preparation of the DBM Powder, Injectable DBM and Dried Bone Powder

Cortical bones from radius and ulna were crushed into granules of 200-400 μm in diameter, followed with 2 h of dehydration, 1 h of degreasing and 0.5 h of drying. After three times of wash with deionized water; 24 h of decalcification was performed with 0.5 mol/L of hydrochloric acid (HCl). The decalcification was performed at 25°C with oscillation every 30 min. Then the particles were repeatedly rinsed with deionized water until the pH was 7.0. After 2 h of dehydration and 1 h of degreasing, the particles were dried overnight in ventilation hood. The DBM powder was sterilized with 25 KGy of 60 Co radiation and stored at 4°C.

DBM powder was obtained as described above. Injectable DBM was prepared by fully mixture of DBM powder and 20 mg/ml of chitosan gel at a volume ratio of 5:2 (0.7 ml injectable DBM contained 0.5 ml DBM powder and 0.2 ml of chitosan gel). Prepared injectable DBM was stored at –40°C. The ratio was optimized based on previous reported studies on a thermogelling chitosan carrier[18].

Cancellous bone obtained from condyle of the femur was also crushed into granules of 200-400 μm in diameter, followed with 2 h of dehydration, 1 h of degreasing and 0.5 h of drying. The particles were repeatedly rinsed with deionized water until the pH was about 7.0. After 2 h of dehydration and 1 h of degreasing, the particles were dried overnight in ventilation hood. The dried bone powder was sterilized with 25KGy of 60Co radiation and stored at 4°C.

Operative Procedure

The rabbits were anesthetized with an intramuscular injection of 50 mg/kg ketamine hydrochloride and 5 mg/kg xylazine. An antibiotic formulation containing 400,000 units/kg penicillin was administered preoperatively and on the first postoperative day. The right forelimb of each rabbit was prepared aseptically for operation. A 3-cm incision was made over the skin of the forelimb, and the radius was exposed by dissecting the surrounding muscles (Supplementary Figure 1A). A 15 mm segmental defect was then created with a low-speed drill by grinding under irrigation with 0.9% sterile saline solution in the midportion of each radius to create a critical-size bone defect (Supplementary Figure 1B and 1C). The periosteum was removed, and 5 mm of the periosteum was stripped from each side of the remaining proximal and distal fragments.

The defect was irrigated with a sterile physiological saline solution. DBM powder (Supplementary Figure 1D), injectable DBM (Supplementary Figure 1E), and freeze-dried powder (Supplementary Figure 1F) was loaded into the syringe and was press-fitted into the defect. The muscles, fascia and skin were separately closed over the defect with absorbable sutures. Fixation of the osteotomized radius was unnecessary because of the fibro-osseous union of the ulna and radius proximal and distal to the surgical site as well as press-fitting of the implant.

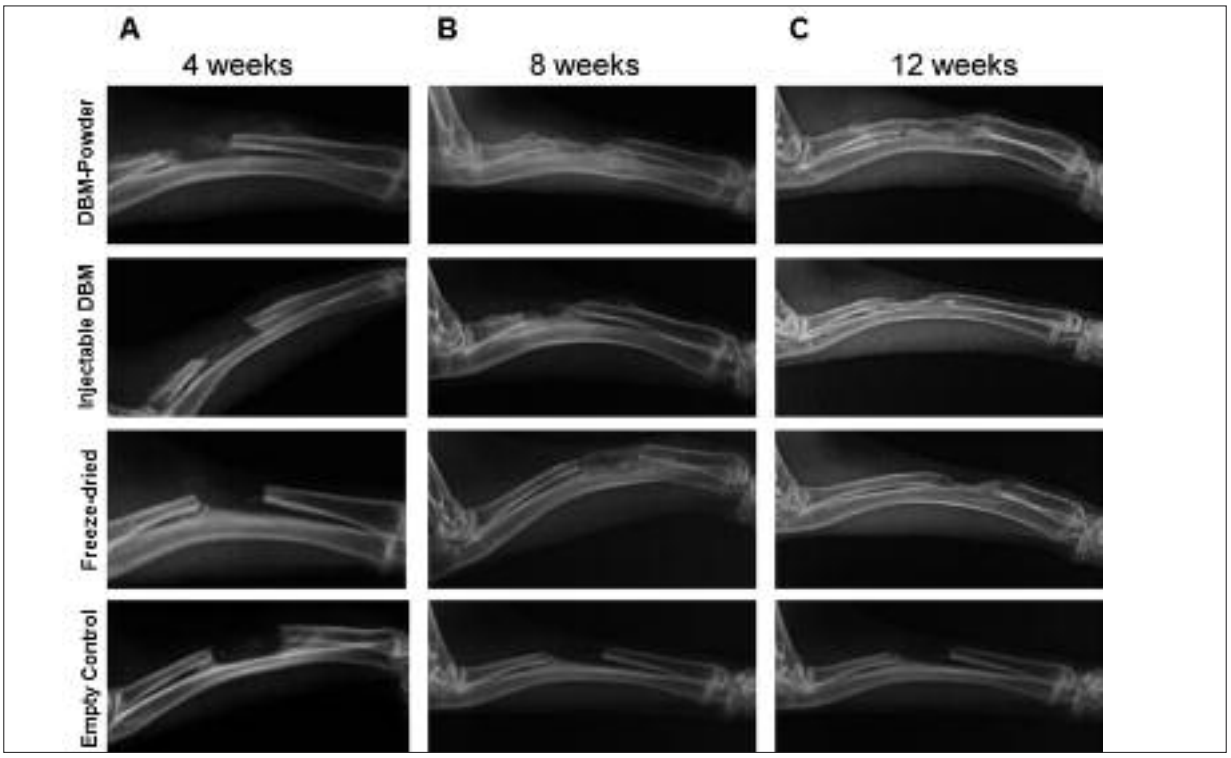

Figure 1. Increased bone formation and bone union were observed in the DBM-powder and injectable DBM groups. Radiographs of treated forelimb at 4 weeks ***(A)***, 8 weeks ***(B)***, and 12 weeks ***(C)*** in DBM-powder group (top panels), injectable DBM group (second from top panels), freeze-dried powder group (second from bottom panels), and empty control group (bottom panels), respectively.

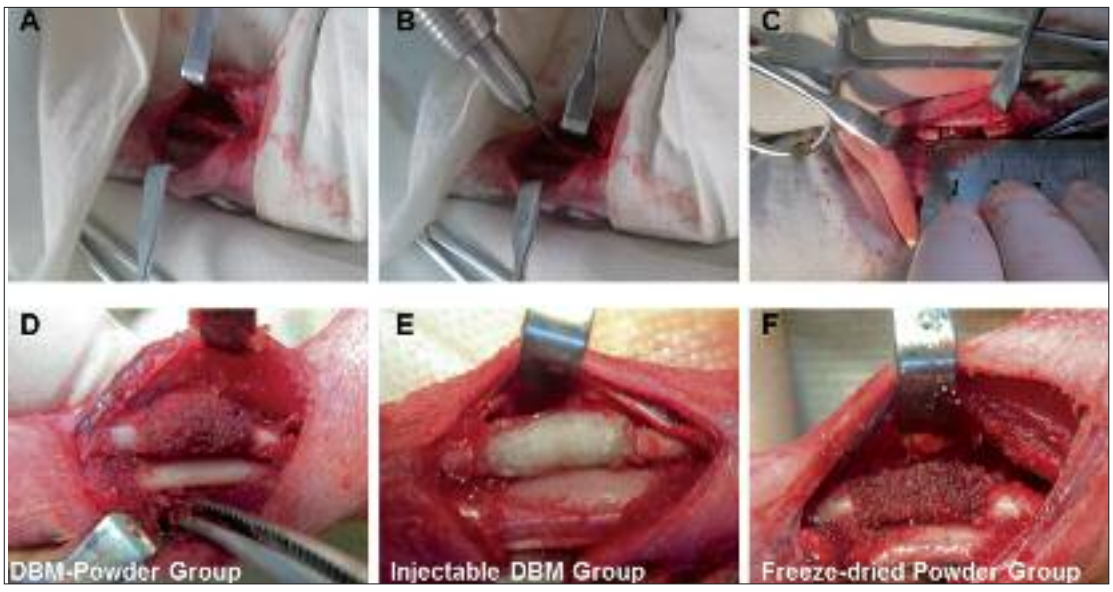

Figure 1. ***(Supplementary)*** Chronological images of the operative procedure. ***A,*** A 3 cm incision was made over the skin of the forelimb, and the radius was exposed by dissecting the surrounding muscles. ***B,*** A 15-mm segmental defect was then created with a low-speed drill by grinding under irrigation with a 0.9% sterile saline solution in the mid-portion of each radius to create a critical-size bone defect. ***C,*** A 15 mm segmental defect was then created with a low-speed drill by grinding under irrigation with a 0.9% sterile saline solution in the mid-portion of each radius to create a critical-size bone defect. ***D, E,*** and ***F,*** The defects were irrigated with a sterile physiological saline solution and the DBM-powder, injectable DBM, and freeze-dried powder, respectively. The DBM and the freeze dried bone particles mixed with blood.

At intervals of 2, 4, 8, and 12 weeks after surgery, X-ray images were obtained from each rabbit of the four groups. In addition, at 4, 8, and 12 weeks after surgery, 6 rabbits from each test group were sacrificed for gross observation, histological analysis, fluorescent labeling, and micro-computer tomography (CT) evaluation.

Radiological Examination

To evaluate bone formation and union as well as remodeling of the defect, standardized anterior–posterior radiographs were taken postoperatively on the first day and 2, 4, 8, and 12 weeks after surgery. An ultra-high definition film, 50 kV and 100 mA, with a constant X-ray-to object-to-film distance of 90 cm were used. The results were scored using the Lane and Sandhu scoring system[19].

Micro-computer Tomography

At 4, 8, and 12 weeks after surgery, 6 rabbits from each test group were sacrificed. Prior to histology analysis, 4 rabbits were randomly chosen for micro-CT using a micro-CT scanner (Explore Locus SP, GE Healthcare Technologies, Denver, MA, USA). The specimens, which contained 15 mm segmental defect and 5 mm of the proximal and distal cortical bone adjacent to the defect, were fixed in 70% (v/v) alcohol. Each bone block was examined with the micro-CT system. Tissue specimens from the implantation zone of the surrounding normal bone were also contained in the micro-CT system for *in vitro* detection. Along the long axis of the specimen, a 45 μm scan was acquired in a consecutive micro-CT imager that included the defects of the implant site and a 5 mm peripheral area as a region of interest. New bone reconstruction was visualized and analyzed by the Microview software.

Gross Observation

Rabbits from the DBM, injectable DBM and freeze-dried groups were sacrificed for gross observation after micro-CT. Healing signs of the radius were observed. The examination and blind scoring of the specimens included the presence of bridging bone, which indicated a complete union (+++), the presence of cartilage, soft tissue, or cracks within the defect, which would indicate a possible unstable union (+ or ++), or complete instability at the defect site, indicating no union (0).

Histopathological Examination

The right forelimb of each animal was harvested and dissected so that it was free of soft tissue. Sagittal sections containing the defect area were cut with a slow-speed saw. The specimens were fixed for 48 to 72 hours with a 10% neutral formalin solution. Each specimen was divided into two parts. One was demineralized for hematoxylin and eosin (H&E) staining, and the other was not demineralized to permit modified Ponceau trichrome staining.

Specimens were harvested, decalcified with 10% EDTA, and embedded in paraffin followed with H&E staining. Sagittal sections (5 μm-thick, 4 sections, with 2 sections from the central region and another 2 sections from the interface between the graft and the defect) were H&E stained. The micrographic images from a light microscope were evaluated using the Lane Sandhu histological scoring system[19]. Non-demineralized specimens were used for modified Ponceau trichrome staining. The samples were embedded for tissue dehydration according to the standard procedure. Non-decalcified sections were subsequently sliced at a thickness of 4 μm and incubated overnight at 42°C before being stained to observe the newly formed bone tissue.

Fluorescent Double-Labeling Observation

Rabbits of the three test groups were subcutaneous injected withfluorescent calcein labeling solution 5 days before sacrifice. Three days later (2 days prior to sacrifice), subcutaneous injection of fluorescent calcein labeling solution were performed again. Fixed specimens were dehydrated with ethanol of a series of concentrations and embedded with paraffin. Sections of 4μm thickness were dried overnight at 42°C followed with xylene resin mounting. Tissue sections were observed under a fluorescence microscope. The osteogenic rates were determined by the fluorescence intensity[20]. The mineral apposition rate (MAR) was defined by the average distance between the two calcein tag lines divided by the number of days (expressed as μm/day).

Biomechanical Assessment

Mechanical testing on the specimens from each treatment group and normal radial bone (for reference values) were performed using a Zwick/Roell 2005 with a crosshead speed of 0.01 mm/s. A load-distance curve was recorded to obtain the mechanical properties; load bearing was calculated with maximum recorded load of the linear portion of the load-distance curve.

Statistical Analysis

Data analysis was performed with SPSS for Windows 17.0 (SPSS Inc., Chicago, IL, USA). The mean values and standard deviations were calculated. The radiological, micro-CT, histopathological and fluorescent double-labeling data were examined using a multifactorial analysis of variance. Differences between the independent variables were evaluated using post hoc tests (Tukey's studentized range or HSD test). The gross evaluation data were compared using a Kruskal-Wallis non-parametric ANOVA. Pairwise group comparisons were performed using the Mann-Whitney U test. A p-value < 0.05 was considered significant. All statistical tests were two-tailed.

Results

Radiological Observations

According to the score criteria of Lane-Sandhu, there were no significant difference between each other of the three treatment groups ($p > 0.05$), but significant differences were observed between the three groups and the empty control group 2 weeks after surgery ($p < 0.05$). Bone formation was observed in the DBM, injectable DBM, and freeze-dried groups, even though the implants were partially absorbed in the latter group. No callus or new bone formation was observed in the blank group (Table I).

Increased bone formation and bone union were observed in groups treated with DBM powder or injectable DBM compared with the other two groups 4 (Figure 1A), 8 (Figure 1B), and 12 weeks (Figure 1C) after surgery. On week 8 after surgery, the animals of the groups treated with DBM powder and injectable DBM presented 75-100% of bone formation, whereas those treated with freeze-dried bone powder displayed 50-75% of bone formation ($p < 0.05$) (Table I and Figure 1).

Bone union was shown in groups treated with DBM powder, injectable DBM and freeze-dried powder 4, 8, and 12 weeks after the operation, which was not evident in the blank group. In addition, bone union in DBM-powder and injectable DBM groups 4, 8, and 12 weeks after surgery was more prominent than that in the freeze-dried group. The bone formation and bone union in the injectable DBM group were equal to that in the DBM-powder group at 4, 8, and 12 weeks. The bone formation and bone union was increased in the DBM and injectable DBM groups compared with the freeze-dried group at 4, 8, and 12 weeks (Table I and Figure 1). At 4, 8, and 12 weeks after surgery, there was no significant difference between the DBM-powder group and injectable DBM group, but significant differences were shown between the DBM-powder group and freeze-dried powder group and between the injectable DBM group and freeze-dried group, and pairwise comparisons indicated significant differences among the three treatment groups ($p < 0.05$), according to the Lane-Sandhu scoring criteria (Table I and Figure 1).

Quantitative Evaluation of micro-CT

Quantitative evaluation of bone formation was performed using post-mortem micro-CT, and the results were similar to those from the radiological examination (Table II and Figure 2). Bone formation and scaffold degradation were quantified separately. There was no significant difference in new bone volume between the DBM powder group and the injectable DBM group (p

Table I. Radiographical scores at various post-operative intervals and Sandhu X-ray score comparisons (mean ± SD, n = 6).

Post-operative weeks	DBM-powder	Injectable DBM	Freeze-dried powder	Empty control
2	1.67 ± 0.52	1.50 ± 0.55	1.33 ± 0.52	0.33 ± 0.52*
4	5.00 ± 1.27	5.00 ± 0.89	3.67 ± 0.52	1.33 ± 0.52#
8	9.00 ± 0.89	8.67 ± 0.82	6.17 ± 1.17	2.50 ± 0.55#
12	11.50 ± 0.55	11.17 ± 0.75	8.67 ± 0.82	3.68 ± 0.82#

*There were no significant differences among the DBM, injectable DBM and freeze-dried groups ($p > 0.05$), but significant differences were observed between the DBM, injectable DBM and Freeze-dried groups with the empty group ($p < 0.05$). #There was no significant difference between the DBM group and the injectable DBM group, but significant differences were observed between the DBM group and the freeze-dried group and between the injectable DBM group and the freeze-dried group; in addition, significant differences were observed in pair wise comparisons among the DBM, injectable DBM and freeze-dried groups ($p < 0.05$).

Table II. Comparison of the new bone volume (mm^3) (mean ± SD, n = 4) and percent bone union achieved at different study points.

Postoperative weeks	DBM-powder group*/(percent bone volume/total volume§)	Injectable DBM group	Freeze-dried group#
4	48.63 ± 9.45/40.3%	39.93 ± 8.93/33.1%	20.44 ± 4.67/16.9%
8	92.28 ± 21.32/76.5%	77.38 ± 16.62/64.2%	50.26 ± 10.68/41.7%
12	108.71 ± 24.85/90.2%	103.80 ± 17.35/86.1%	66.94 ± 22.36/55.5%

*There was no significant difference between the DBM group and the injectable DBM group ($p > 0.05$). #There were significant differences observed between the DBM group and the freeze-dried group and between the injectable DBM group and the freeze-dried group ($p < 0.05$). §Total volume, 120.58 mm^3, was calculated as $II \times r^2 \times h = 3.14 \times (1.6)^2 \times 15$; where r = 1.6 mm and h = 15 mm.

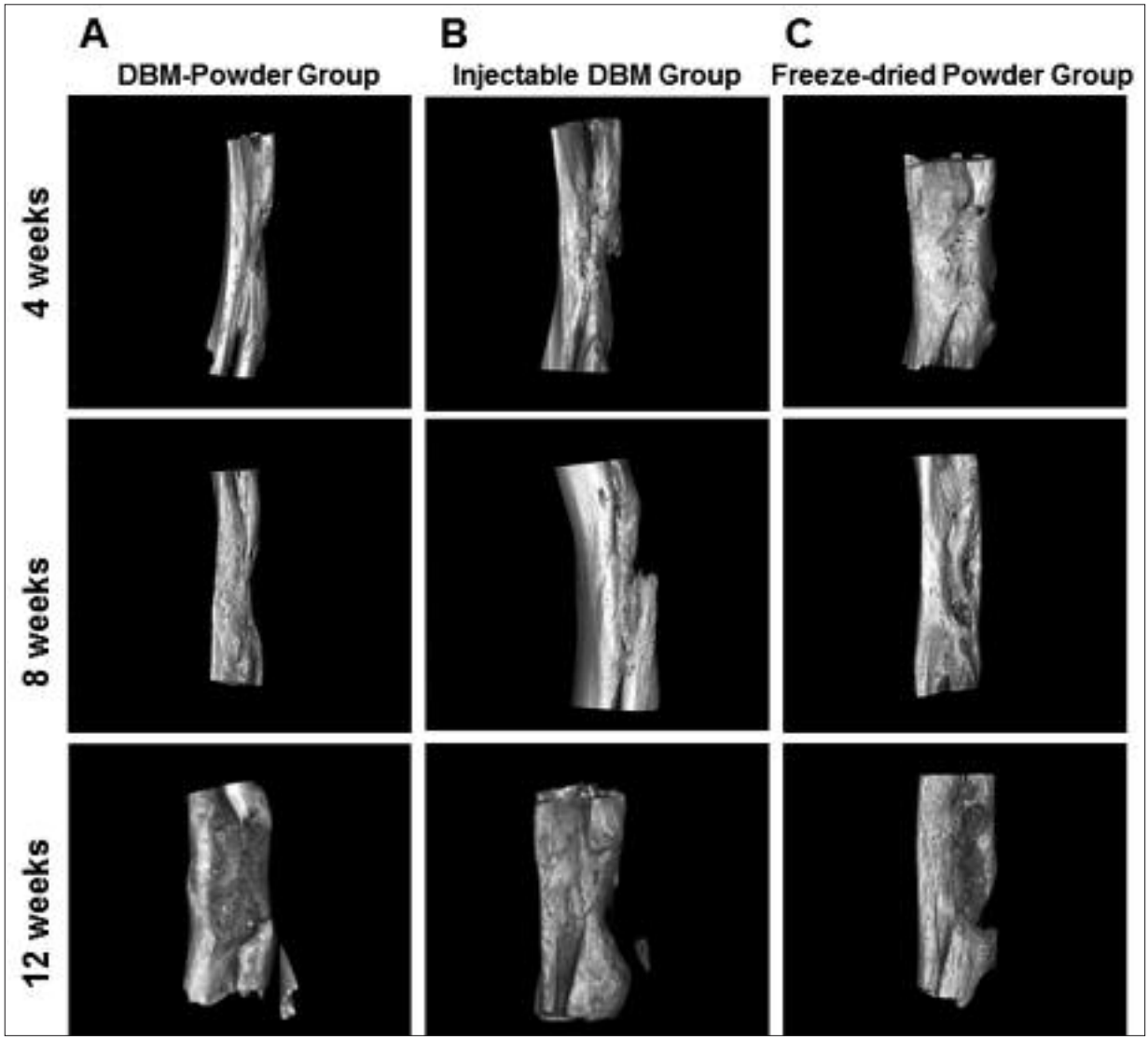

Figure 2. Quantitative micro-computer tomography confirmed increased bone formation and bone union in the injectable DBM and DBM-powder groups in comparison to the freeze-dried powder group. Images were obtained post-mortem of treated forelimb at 4 weeks ***(A)***, 8 weeks ***(B)***, and 12 weeks ***(C)*** in DBM-powder group (top panels), injectable DBM group (middle panels), and freeze-dried powder group (bottom panels), respectively. No significant differences were observed in new bone volume between the DBM-powder group and the injectable DBM group. However, significant differences were observed between the aforementioned and the freeze-dried powder group.

> 0.05). New bone volume of the DBM-powder was significantly larger compared with that of the freeze-dried group 4, 8, and 12 weeks after the operation ($p < 0.05$). Similarly, new bone volume of the injectable DBM group was significantly larger compared with that of the freeze-dried group 4, 8, and 12 weeks after the operation ($p < 0.05$).

Gross Findings

Forelimbs of the animals displayed different degrees of swelling. Approximately 1 or 2 weeks after surgery, there were no signs of infection at the incision region. Four weeks after implantation, fibrous tissue adhesion was observed in all of the implants and surrounding tissues. The DBM-powder and injectable DBM groups showed a small amount of callus and new bone formation.

No absorption of the implant materials was seen in the DBM-powder and injectable DBM groups, as indicated by dense, fibrous tissue encapsulating the adhesion area. The near and far ends were connected to the host bone fiber, and there was a small amount of callus and new bone formation. The implant materials in the freeze-dried group were visibly absorbed, as indicated by a dense, fibrous tissue parcel and surrounding adhesions that were easier to separate than those of the DBM-powder and injectable DBM groups.

Eight weeks after surgery, cartilage, soft tissue and a greater amount of callus and new bone formation were present within the defect in the DBM, injectable DBM, and freeze-dried groups. At the same time point, the DBM and injectable DBM groups showed bridging bone, which indicated a complete union and the suitable shaping of bone. The bone defect of the freeze-dried group was filled with new bone, and bone remodeling was visible. There was a small amount of new bone formation in the empty group.

Differences between each of the three test groups with each other were not significant at the same time point after surgery ($p > 0.05$). However, significant differences in the grading were shown at different time points within the same group ($p < 0.05$) (Table III).

Histopathological Findings

The defect areas (same specification of the defect site) of the animals in all the groups showed various amounts of new bone formation (Figure 3). However, the defects of the animals in the empty group contained the smallest amounts of new bone, and the defects were often filled with a mixture of fibrous connective tissue and cartilage (data not shown).

In the DBM powder group, four weeks after the operation, trabecular bone growth was strong and disordered and was formed by many types of bone tissue with many chondrocytes (Figure 3A, *upper panel*). At the same time, the injectable DBM group had trabecular derangement, vigorous growth, and proliferation of granulation tissue. Additionally, trabecular bone was formed, and cartilage cells were generated (Figure 3B, *upper panel*). However, the freeze-dried group bone contained fibrous connective tissue, trabecular bone and bone tissue (Figure 3C, *upper panel*). The histological scores in the DBM or injectable DBM groups were significantly higher than those in the freeze-dried group. Furthermore, the scores of the rabbits in the DBM group were significantly higher than those of the injectable DBM group (Table IV).

Eight weeks after surgery, the samples in the DBM and injectable DBM groups demonstrated mature lamellar bone formation. The osseous cells were arranged regularly, and the trabecular bone was thick. A uniform distribution of woven bone alterations developed into the mature lamellar bone (Figure 3A and 3B, *middle panels*). In

Table III. Gross scores of healing at different time points (n = 6).

Post-operative weeks	DBM-powder§				Injectable DBM§				Freeze-dried powder§			
	–	+	++	+++	–	+	++	+++	–	+	++	+++
4	0	2	4	0	0	3	3	0	0	4	2	0*
8	0	0	3	3	0	0	4	2	0	0	3	3*
12	0	0	0	6	0	0	1	5	0	0	1	5*

*There were no significant differences in pair wise comparisons among the DBM, injectable DBM, and freeze-dried groups ($p > 0.05$); §There were significant differences at different time points within the same group ($p < 0.05$).

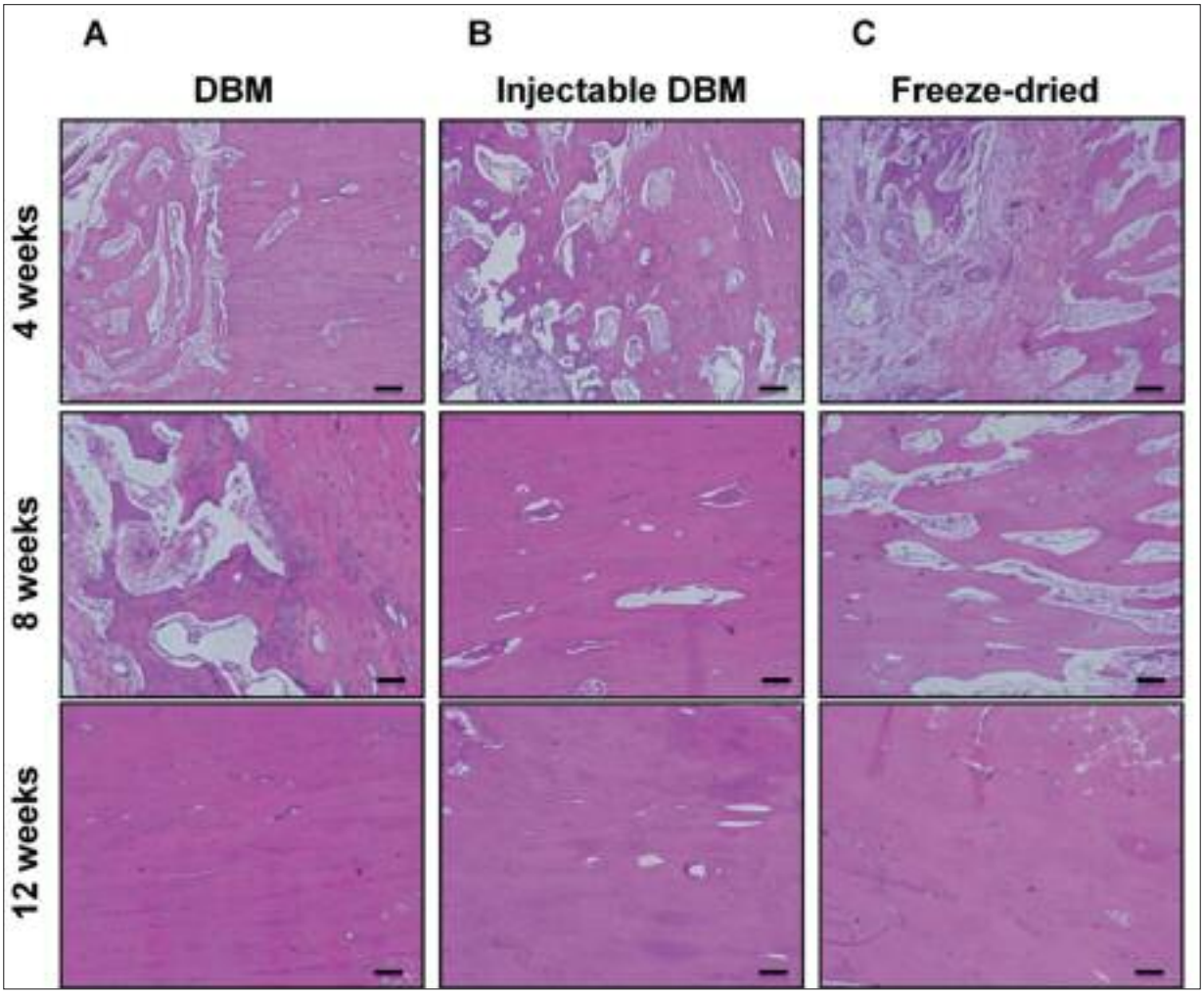

Figure 3. H&E staining of tissue samples showed differential amount of new bone formation in the different experimental groups, with the maximum observed in the injectable DBM group. Images shown are representative of DBM group ***(A)***, injectable DBM group ***(B)***, and freeze-dried group ***(C)*** after 4 weeks (top panels), 8 weeks (middle panels), and 12 weeks (bottom panels), respectively. Images were obtained at 100x magnification. Scale bars are 50 μm.

the freeze-dried group, normal trabecular and woven bone were uniformly formed within the defects (Figure 3C, *middle panel*). The histological scores of the DBM and injectable DBM groups were significantly higher than those of the animals in the freeze-dried group. Furthermore, the scores in the DBM group were not significantly higher than those of the injectable DBM group (Table IV).

Twelve weeks after surgery, the regenerated bone completely spanned the defect and produced a full histological union in the DBM and

Table IV. Histological scores at various post-operative intervals (mean ± SD, n=6).

Postoperative weeks	DBM-powder	Injectable DBM	Freeze-dried powder
4	5.83 ± 0.75	5.50 ± 0.55	4.33 ± 0.52*
8	7.17 ± 0.75	7.00 ± 0.89	5.83 ± 0.75§
12	9.50 ± 1.38	8.67 ± 1.21	7.67 ± 0.82§

*Among the DBM, injectable DBM, and freeze-dried groups, the differences were statistically significant, $p < 0.05$; §The difference between the DBM group and the injectable DBM group was not significant, $p > 0.05$. The difference between the DBM group or the injectable DBM group and the freeze-dried group was not significant, $p < 0.05$.

injectable DBM groups (Figure 3A and 3B, *bottom panels*). In these two groups, most cortical bone was completely regenerated, the normal relationship of the cortical bone and marrow cavity was restored, and the normal structure of the trabecular bone was restored. On the other hand, the freeze-dried group demonstrated extensive mature cord formation and lamellar and incomplete regeneration of the cortical bone (Figure 3C*, bottom panel*). The histological scores were similar to those at 8 weeks (Table IV).

Non-demineralized specimens were used for modified Ponceau trichrome staining. Four weeks after surgery, in the DBM group, the bone graft area was surrounded by a trabecular bone mass, and the edge of the bone matrix formation was a deeper color, indicative of vigorous growth. In the injectable DBM group, a new bone junction was visible with many osteoid and bone cells, small bone trabeculae, and many disordered bone cells. In the freeze-dried group, more trabecular bone interstitial fibrous tissues were present but with thinner trabecular bone; in addition, the trabecular osteoid strip edge was visible in the cells and matrix lining the bone edges, and fewer osteoblasts were present than in the other two groups.

After 8 weeks, the DBM and injectable DBM groups exhibited an increased deposition of fibrous tissue, formation of woven bone, thickening of the trabecular osteoid strip edge, and many osteoblasts. There was more bone cartilage formation in these two groups, whereas in the freeze-dried group, the number of trabecular bone edges increased, and bone cartilage formation was occasionally observed, although it was rare compared with the bone cartilage formation in the DBM or injectable DBM groups.

After 12 weeks, in the DBM (Figure 4A) and injectable DBM groups (Figure 4B), cortical bone was extensively formed, new bone was transformed, and many bone cells were present. In comparison, in the freeze-dried group, many mature lamellar bones and only a small amount of cortical bone formed (Figure 4C).

Fluorescent Double-Labeling

As shown in Figure 5 and Table V, in the DBM group, fluorescent labeling revealed that the new bone formation was substantial only after 4 weeks, and the MAR was higher than that of the freeze-dried group. In the injectable DBM group, the new bone formation was also substantial, and the MAR was higher than that of the freeze-dried group. Between the DBM group and the injectable DBM group, there was no significant difference. In the freeze-dried group, the new bone formation was higher, but the MAR was the lowest of the three groups.

Biomechanical Function

As shown in Table VI, by the end of 12 weeks the compressive pressure values (indicative of biomechanical functionality) of the DBM-powder (340.9 ± 5.3 N) and injectable-DBM (343.7 ± 6.5 N) groups closely resembled the reference

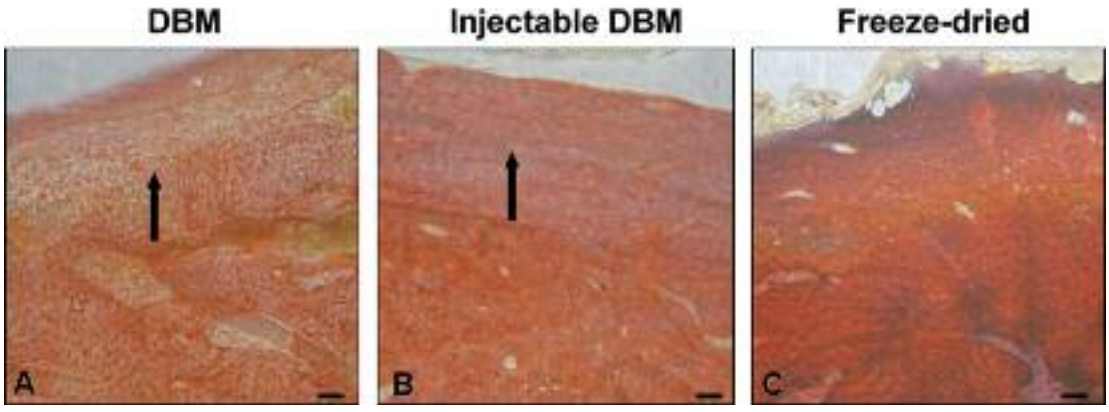

Figure 4. DBM-powder and injectable DBM groups showed prominent cortical bone formation and promiscuous amounts of bone cells in comparison to largely lamellar bone growth observed in the freeze-dried powder group. Modified Ponceau trichrome staining after 12 weeks showed cortical bone generation (*black arrow*) in the DBM-powder and injectable DBM groups (***A*** & ***B***, respectively), in comparison to mature lamellar bone formation in the freeze-dried powder group ***(C)***. Images were obtained at 200x magnification. Scale bars are 50 μm.

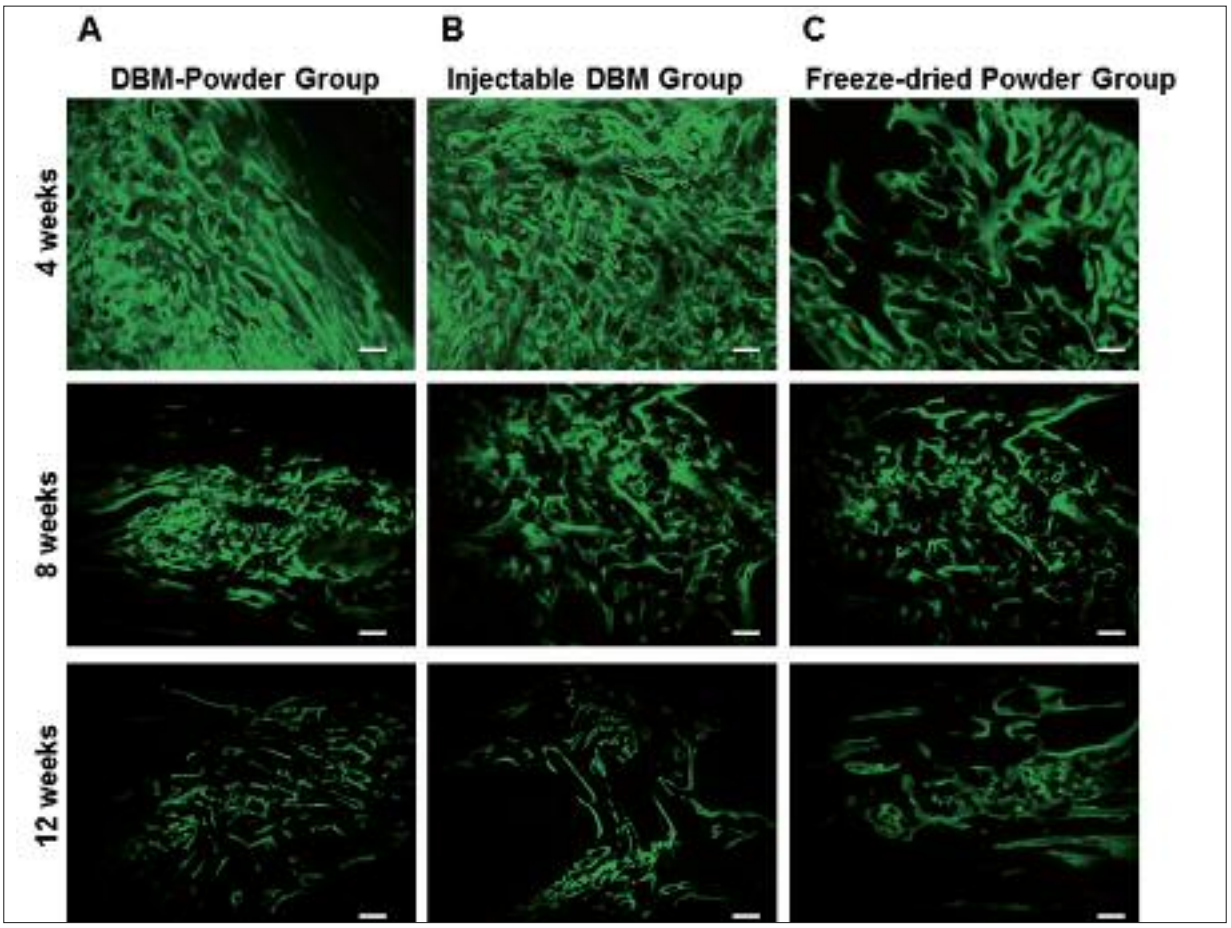

Figure 5. Osteogenic rate was higher in the injectable DBM and DBM-powder group in comparison to the freeze-dried powder group. A fluorescent double-labeling strip was used to determine the osteogenic rates. The mineral apposition rate (MAR) (units μm/d) was defined by the average distance between the two calcein tag lines divided by the number of days. Images shown are representative of DBM group ***(A)***, injectable DBM group ***(B)***, and freeze-dried group ***(C)*** after 4 weeks (*top panels*), 8 weeks (*middle panels*), and 12 weeks (bottom panels), respectively. Images were obtained at 100 × magnification. Images were obtained and analyzed using the OsteoMeasure fluorescent double-labeling analysis system; a magnification of 100x was used to calculate the average distance between the two mark lines. Scale bars are 50 μm.

values obtained from normal radial bone (381.3 ± 4.1). This suggested that injectable-DBM almost recapitulated normal mechanical property or tensile strength.

Discussion

To evaluate the potential effect of injectable DBM on bone healing , a defect model was estab-

Table V. Mineral apposition rate at various post-operative intervals (mean ± SD, n = 6).

Post-operative weeks	DBM-powder group	Injectable DBM group	Freeze-dried powder group
4	3.28 ± 0.23	3.20 ± 0.19	2.27 ± 0.15
8	3.46 ± 0.25	3.48 ± 0.25	2.83 ± 0.21
12	4.12 ± 0.35	4.02 ± 0.42	3.31 ± 0.23

Within each interval, the difference between the DBM powder group and the injectable DBM group was not significant, $p > 0.05$. The differences between the DBM powder group or the injectable DBM group and the freeze-dried group were significant, $p < 0.05$.

Table VI. Comparative mechanical functionality at 12 weeks post-operative (mean ± SD, n = 3).

Normal bone	DBM-powder group	Injectable DBM group	Freeze-dried powder group
381.3 ± 4.1 N	340.9 ± 5.3 N	343.7 ± 6.5 N	73.4 ± 5.2 N

The difference between the DBM powder group and the injectable DBM group was not significant compared to the reference normal bone, $p > 0.05$. The differences between the DBM powder group or the injectable DBM group and the freeze-dried group were significant, $p < 0.05$.

lished in the radial bone of rabbits[21-24]. In rabbit long bone, a defect with a length of 15 mm is an established critical-size defect (CSD)[21-24]. Forelimb comprises ulna and radius; the ulna can support the traumatized forelimb, so the radius bone can have a defect in the middle without the need for internal or external fixation. In comparison to creating the segmental defect with an oscillating saw (where a Hohmann retractor is placed between ulna and radius to protect the ulna)[24], we created the defect with a low-speed drill.

Our hypothesis that an injectable DBM used in a critical-size defect in the radial bone of a rabbit could not negatively affect bone formation was based on the nature of medical chitosan as a viscous colloid that could be added to DBM. Our radiological, micro-CT, histological and fluorescent double-labeling examinations supported our hypothesis in that (1) bone healing was not decreased when medical chitosan was used concurrently with DBM, and (2) in the samples treated with injectable DBM, bone healing was significantly superior to that in the samples treated with freeze-dried powder.

One potential limitation of clinical use of DBM is its particulate nature, making difficulty in handling[25]. Our results suggest that this problem can be adequately circumvented as injectable bone materials can take on a satisfactory shape and meet the needs of different bone defects. Other studies have also used different delivery vehicles to ease the handling of DBM[18,26-28]; however, had limitation in profound off-target effects or handling concerns. Hence, injectable DBM provided a functional alternative with improved handling characteristics. Our data revealed that injectable DBM was effective in repairing bone defects and that the injectable DBM was as effective as the DBM powder in the repair of bone defects and was better than the freeze-dried powder.

DBM powder is widely used in the clinic for bone regeneration because of its effect on osteoinductivity and osteoconductivity[10,29,30] and results in a composite of noncollagenous proteins, growth factors, and collagen[31]. Although the high chitosan content in the DBM composite can inhibit the osteoinduction of the DBM powder[18], our study shows that injectable DBM containing a low ratio of chitosan permits inductive bone formation without significantly compromising the ability of DBM to repair bone defects.

Use of chitosan was based on the fact that chitosan is a biologically renewable, natural cationic polymer that is (1) biocompatible, (2) non-toxic, (3) non-antigenic, and (4) biofunctional. Being a natural polymer, chitosan has a hydrophilic surface that allows it to promote cell adhesion, differentiation, and proliferation, without evoking any significant immune reaction[32,33]. In addition, previous studies had shown that Chitosan hybrids were osteoconductive and could enhance bone formation both *in vitro* and *in vivo*[34]. However, chitosan by itself is unstable and mechanically weak, and will be unable to maintain a predefined shape for transplantation as a result of swelling[35]. Hence, our rationale of using DBM-Chitosan hybrid as an injectable bone material uses the advantages of both components in a way that also circumvents the individual component's individual usage as a bone material.

An ideal bone repair material should facilitate (1) bone conduction-matrix scaffold for the ingrowth of bone conduction; (2) osteoinductivity by conversion of mesenchymal cells into osteoblasts to promote bone formation; (3) provide osteoblasts to promote bone formation[36]. Allogeneic bone material have all the aforementioned properties; however, clinical bone banks require deep frozen, defatted, freeze-drying, irradiation sterilization, which severely compromises the bioactivity and osteoinductivity of frozen allogeneic bone graft materials. Our data also demonstrates that injectable DBM for bone repair is more effective than the powder of the freeze-dried cancellous bone because DBM calcium salt causes bone morphogenetic protein (BMP) and osteogenic factors to combine, thereby providing strong osteoinduction. Subsequent-

ly, these osteogenic-promoting substances in combination with the bone matrix collagen can induce the mesenchymal cells to differentiate into cartilage cells and the osteoblasts to form cartilage and bone tissue. Additionally, DBM has a natural porous structure but is also conducive to BMP release and new bone and other tissue ingrowths, which also potentiate bone growth[37,38].

Our findings demonstrate that allografts and DBM remain favorable alternatives to iliac crest bone grafting. However, such iteration would require future research endeavors to further answer the following: (1) If these allograft are as effective as autografts, which are considered the most ideal graft material? (2) What is the underlying mechanism of new bone formation (intramembranous or endochondrial) by this method? (3) Whether the healed fracture post-DBM allograft regains functional strength and other biomechanical activities? Our current research endeavors are focused on answering the aforementioned.

Conclusions

Injectable DBM was equally as effective in repairing bone defects and restoring biomechanical function as the DBM powder; both were more effective than the freeze-dried bone powder. The injectable DBM formulation improves the handling of the DBM powder and can meet the needs of different bone defects. This result suggests that injectable DBM is an attractive alternative for the reconstruction of major diaphyseal defects in the long bones. Our findings will potentially lead to the iterative cycle of system modeling, hypothesis generation, and systematic experimentation that will help us find useful material for healing bone fracture.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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