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Combined effects of porous hydroxyapatite and demineralized bone matrix on bone induction: \textit{in vitro} and \textit{in vivo} study using a nude rat model

Jae Hyup Lee$^{1,2,4}$, Kyung-Mee Lee$^1$, Hae-Ri Baek$^{1,2}$, Soo-Jeong Jang$^{1,2}$, Ji-Ho Lee$^1$ and Hyun-Seung Ryu$^3$

$^1$ Department of Orthopedic Surgery, Seoul National University School of Medicine, SMG-SNU Boramae Medical Center, Seoul 156-707, Korea
$^2$ Institute of Medical and Biological Engineering, Seoul National University Medical Research Center, Seoul 110-799, Korea
$^3$ Research and Development Center, CGBio Inc., Seong-Nam 462-120, Korea
E-mail: spinelee@snu.ac.kr

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Abstract
Hydroxyapatite (HA) is an osteoconductive material used as a bone graft extender and demineralized bone matrix (DBM) has been used as a source of osteoinductive factors. A combination of DBM and HA is expected to create a composite with both osteoconductive and osteoinductive properties. This study examined the effect of a combination of DBM and HA on osteogenesis both \textit{in vitro} and \textit{in vivo} using an athymic nude rat abdominal muscle pouch model, and evaluated the possibility of HA as a carrier of DBM. Alkaline phosphatase (ALP) staining, ALP assay and measurements of the mRNA expression of ALP and Runx2 by RT-PCR were performed by transplanting human mesenchymal stem cells onto a plate. Five athymic nude rats each were assigned to one of two experimental groups (DBM/HA putty and only HA, i.e. 15 pouches per group). The muscle pouches were filled with DBM/HA putty or only HA. Radiographs were obtained at weeks 4 and 8, postoperatively. The animals were sacrificed at week 8 postoperatively and high resolution microCT was used to confirm the newly formed mineralized tissue. Each pouch was fixed, embedded, sectioned and processed for hematoxylin and eosin staining. The ALP value of the DBM/HA putty was higher than those of HA and control ($p < 0.05$, each). The expression of ALP mRNA appeared higher on the DBM/HA putty than on HA and control. MicroCT and histology examinations of the DBM/HA putty demonstrated the presence of newly generated mineralized tissues but there was no mineralized tissue in the HA cases. In conclusion, the DBM/HA putty indicated osteoblastic differentiation \textit{in vitro} and showed ectopic mineralized tissue formation in the rat abdominal pouch model. These findings indicate that the DBM/HA putty can retain its osteoinductivity and HA can be used as a carrier of DBM.

Introduction

Autogenous iliac crest bone grafts are the gold standard of fusion surgery. Despite its successful use for fusion, the harvesting of an iliac crest graft is associated with morbidity, particularly persistent pain at the graft harvest site in up to one-third of patients [1, 2]. In addition to increasing the operation time and blood loss, the harvesting of an iliac crest bone graft is associated with postoperative complications, such as infection, hematoma, vascular injury and graft site fracture. Moreover,
the amount of autograft is insufficient. Allograft bone products are currently the most common extenders for autogenous bone grafting indications. Allograft bone is available as either mineralized bone tissue or demineralized bone matrix (DBM). DBM is biocompatible and has been used as a source of osteoinductive factors in clinical applications, including long bone defects [3, 4], craniofacial defects [5], tumor surgery [6], joint surgery [7, 8], and enhancement of spinal fusion surgery [9, 10]. However, the fusion rate of DBM is not always uniformly favorable, especially when compared to an autograft [1, 11, 12]. Several pre-clinical and clinical studies demonstrated the efficacy of DBM, and different DBM formulations with different carriers are available [13].

The important property of the ideal carrier of DBM is osteoconductivity. Hydroxyapatite (HA) is biocompatible and osteoconductive, and used as a bone graft extender [14]. Therefore, a combination of DBM and HA might exhibit a combination of both osteoinductivity from the DBM component and osteoconductivity from the HA. The bone formation of the DBM/HA putty on a muscle pouch has not been reported.

To evaluate the possibility of HA as a carrier of DBM, this study examined the osteoinductive properties of the DBM/HA putty and only HA in vitro using human mesenchymal stem cells as well as in vivo using an established athymic nude rat model.

Material and methods

In vitro study

The DBM putty with porous HA granules (DBM/HA putty) was provided by CG-Bio (CG Bio Inc., Korea). DBM powder was made from a human femur. Institutional review board approval was obtained for this study. After removing the soft tissues surrounding the bone at iced purified water, the epiphysis on both sides was removed using a band saw to divide the diaphysis. The internal blood and fat etc were removed using a saline solution (Choongwae, Korea). Subsequently, the bone was cut to a size of 1.5 cm and made to the cut graft form. The residual cells were removed by a chemical treatment with a 3% hydrogen peroxide (Junsei, Japan) and ethanol (Daejung, Korea) solution. The cortical bone treated both mechanically and chemically was made into powder form using a bone mill (IKA, Germany). The resulting bone powder underwent a demineralization process through 0.6 N of an HCl (J.T Baker, USA) solution, after which its pH was adjusted to 7.4 using a buffered solution of phosphoric acid (Sigma, USA). The powder was dried to a water level ⩽ 6% using a bone mill. The resulting bone powder particles and porous HA were prepared using the polymeric sponge method [14]. HA powder as a starting material was synthesized according to the ASTM F 1185 standards. These had a three-dimensional interconnected pore structure, of which the interconnected pore size was 300 μm and the porosity was 85%. These were divided into small granules using a zirconia ceramic knife (Kyocera Co., Japan). In this study, porous HA granules with a particle size of 0.6–1.0 mm (HA, figure 1) were used for combination with the DBM powder or as a control. The putty was made by mixing to scale the DBM powder particles and porous HA granules in sterile distilled water. The putty was then placed into a syringe by each volume.

Human bone marrow mesenchymal stem cells were purchased from Lonza company and cultured in DMEM (low glucose) containing 10% fetal bovine serum (FBS, Gibco BRL, USA) and 1% antibiotic-antimycotics (Gibco BRL, USA) in humidified air containing 5% CO₂ at 37°C. The BMMSCs from the fourth passage were used in the following experiments.

Osteogenic differentiation. For osteogenic differentiation, 3 × 10⁴ cells/well in DMEM + 10% FBS were transferred to each well of a 24-well plate and cultivated overnight to allow all the cells to attach. The study was divided into four subgroups according to the presence of DBM/HA and culture media. The first group was cultured in the osteogenic media in the presence of the DBM/HA putty. The second group was cultured on the osteogenic media in the presence of HA. The third group was cultured on conventional media (negative control (NC)). The fourth group was cultured on the osteogenic media (positive control (PC)).

After the adherence period, the cell culture inserts (8 μm pore size) with 140 mg CG Bio-DBM and 14 mg HA were placed carefully into each well and then medium was changed in a DMEM supplemented with dexamethasone (10⁻⁸ M), β-glycerophosphate (10 mM) and ascorbic acid-2-phosphate (100 μM) (Sigma, St Louis, MO, USA). The cells were cultured for 7 and 21 days and the medium was changed twice a week.

ALP staining. For ALP staining, the cultured cells were washed twice with a phosphate-buffered saline solution (PBS, Welgene, USA), fixed with 4% paraformaldehyde for 5 min, and stained with naphthol AS-MX phosphate alkaline solution buffer (Sigma, Munchen, Germany) and fast blue RR salt (Sigma-Aldrich, Brondby, Denmark) for 20 min at room temperature.
temperature. The reaction was quenched by removing the alkaline solution and washing with distilled water.

ALP staining was performed on the four different groups of osteogenic media with the DBM/HA putty, osteogenic media with HA, conventional media (NC), and osteogenic media (PC) for 7 days.

**ALP activity assay.** After osteogenic differentiation for 7 and 21 days, the protein contents and ALP activities were measured. The differentiated cells were washed twice with PBS and scraped into 0.2% triton X-100 in PBS. After sonication, an aliquot of each cell suspension was subjected to protein quantification using a Bradford assay (Biorad). The same sonicated cell suspension was centrifuged at 13,000 rpm for 10 min at 4 °C, and an aliquot of the supernatant was assayed for the ALP activity using p-nitrophenylphosphate as a substrate. The activity was defined as the amount of p-nitrophenol released after incubation for 30 min at room temperature and normalized to the total protein concentration. The ALP activity was measured on each individual sample of four and repeated four times.

**RT-PCR.** The total RNA was extracted from the hMSCs and differentiated toward osteogenic differentiation after 7 and 21 days using an easy-blue reagent (Intron Inc., Korea) according to the manufacturer’s instructions. Two micrograms of the total RNA were reverse-transcribed using Super Script III Reverse Transcriptase (Invitrogen, Carsbad, USA) in a 20 μl volume. PCR was performed using the following primers: ALP (5′ TGG AGC TTC AGA AGC TCA ACA CCA, 3′ ATC TCG TTG TCT GAG TAC CAG TCC); GAPDH (5′ CCA GAA CAT CAT CCC TGC CTC TAC, 3′ GGT CTC TCT CTT CTT). The PCR conditions were as follows: initial denaturation at 94 °C for 3 min, 34 cycles of amplification by denaturation for 30 s at 94 °C annealing at the temperature specified for each pair of primers for 30 s, and extension for 60 s at 72 °C. The PCR products were resolved on 1.5% agarose gel. The PCR bands were analyzed by densitometry measurements using Kodak imaging software (Kodak, Japan). To compare the relative quantity of the RT-PCR reactions, the PCR products were normalized by the transcription level of GAPDH. We collected the whole RNA of the four samples and performed RT-PCR once.

**In vivo ectopic bone formation**

**Surgical model.** Five male athymic nude rats (Crl: NIH Foxn1, 180–200 g, 7 weeks) were used. The animals were housed at 24 °C with a 12 h light/12 h dark cycle and had free access to water and a commercial diet ad libitum. Surgery was performed under general anesthesia (intraperitoneal injection with zoletil and rompun). This study was approved by the Standing Ethical Committee at the Laboratory for Animal Research at the Clinical Research Institute of our hospital, and the investigation was conducted in conformity with the ‘Guiding Principles for Research Involving Animals and Human Beings’ as adopted by The American Physiological Society. The abdomen was shaved, disinfected and the skin was incised. Each rat had six implant sites (figure 2). Implantation of the DBM/HA putty was performed in a muscle pouch of the nude rats abdominal wall for 8 weeks (n = 15). Porous HA granules only (HA) were used as the negative controls (n = 15).

**Radiological study.** The plane anteroposterior radiographs of the abdomen were taken 4 weeks after surgery to examine the newly formed bone formation around the DBM powder or HA granules. Additional plain radiographs were obtained from all surviving animals at 8 weeks after surgery. After the animals were sacrificed 8 weeks after surgery, a micro-CT scan (Skyscan 1172, Kontich, Belgium) of harvested specimens were obtained. The scanned images were reconstructed in the coronal and sagittal planes.

**Histological study.** On the eighth week, the test subjects were sacrificed and the abdominal tissues were extracted. The implants were then dipped in a 5% nitric acid solution for 4 days for decalcification and placed in a paraffin block. The block was cut into the size 4 μm and dyed with hematoxylin and eosin (H & E) after which the formation of new bone around the HA granule was observed by optical microscopy.

The other specimens were fixed for 5 days in 10% neutral buffered formalin. The specimens were then cut in the sagittal plane to include the HA granule and surrounding calcific tissue. The undecalcified tissues were embedded in methylmethacrylate, sectioned using a diamond cutter (EXAKT300p, EXAKT Co. German) and stained with hematoxylin and eosin. The sections were studied under an optical microscope.
Figure 3. Effect of the DBM/HA putty on the alkaline phosphatase (ALP) activity in MSCs. ALP staining (A), (a) DBM/HA putty, (b) HA, (c) negative control (NC), (d) positive control (PC)) and quantification of ALP activity (B) in cultured MSCs for 7 days. ALP staining and activity were increased by the DBM putty compared to the control at 7 days. The results are expressed as the means ± STD of each individual sample of four tests performed four times (n = 4).

Statistical analysis

Statistical analysis was performed using the SPSS statistical package. The data were analyzed by one-way analysis of variance (ANOVA) using a Kruskal–Wallis test followed by Fisher’s exact test. P values < 0.05 were considered significant.

Results

In vitro results

Measurement of ALP activity. The hMSC were exposed to the DBM/HA putty and cultured for 7 days in a culture medium containing ascorbic acid, beta-glycerophosphate and dexamethason. ALP staining and an ALP assay were carried out to verify the osteogenic differentiation capacities of the two groups. Seven days after differentiation, ALP staining of the DBM/HA putty group and HA group showed a greater increase in staining intensity than the positive control group. The ALP assay for the DBM/HA putty group and HA group showed a 12.1-fold higher ALP activity than the positive control group. Moreover, the DBM/HA putty group showed a 1.5-fold higher ALP activity than the HA group (p < 0.05, each) (figure 3).

ALP gene expression. After differentiating the MSCs for 7 days in the bone differentiation culture, the ALP gene was analyzed by PCR. PCR revealed 1.2 and 1.8 times more ALP generation in the DBM/HA putty group than in the positive control and HA groups, respectively, but not significant (figure 4).

In vivo results

Plane radiographic findings. From the plane radiographs taken at the fourth and eighth weeks, mineralized calcified tissue formation was observed even at the fourth week in the DBM/HA group, and calcified tissue was observed at the 8th week (figure 5). However, no radio-opaque lesion was observed in the HA only group. This shows that HA does not induce ectopic bone formation. On the other hand, ectopic mineralized calcified tissue was formed when DBM/HA was used.

Micro-CT results. Eight weeks after implantation, no obvious bone or cartilage formation was observed in the HA group as expected (figure 6(A)). In contrast, in the central area, the DBM/HA putty group displayed mineralized calcified tissue formation (figure 6(B)) in all 15 samples (100.0%). The rate of mineralized calcified tissue formation in the DBM/HA putty group was significantly higher than that in the HA group (P < 0.00001).

Histological results. While new tissue formed around the HA in the HA only group according to the decalcified H & E histology, no mineralized tissue was observed (figure 7(A)). However, the newly formed mineralized ectopic calcific tissues were observed in multiples around the HA from the group transplanted with DBM (figure 7(B)), and such ectopic calcific tissue was observed in all slides. In addition, even in the undecalcified slides, newly formed tissue was observed around HA, which is distinct from the soft tissue for the group transplanted with HA only (figure 8(A)). For the group transplanted with DBM, there was variety of sizes of newly formed ectopic mineralized calcific tissues around HA. Therefore, osteoinductivity was observed (figure 8(B)).
Discussion

The efficacy of DBMs differs according to the age and gender of the donor, particle size, or preparation method [13, 15–17]. The osteoinductivity of DBM is due to growth factors within the matrix. However, the quantity of growth factors including BMP-2, 4 and 7 are varied. This is why some authors have suggested that DBM is insufficient for inducing new bone formation [18]. Porous HA ceramics as bone graft substitute has been used extensively in the field of orthopedic surgery, dental surgery and plastic surgery because it is biocompatible and has good osteoconductivity.

In this study, the DBM/HA putty showed higher ALP activation for osteoblastic differentiation of human mesenchymal stem cells than HA, which has high osteoconductivity. This suggests that transplanting the DBM/HA putty is advantageous in bone formation. In addition, the level of ALP gene expression was also higher for the DBM/HA putty than HA, which confirms that the DBM/HA putty is advantageous for osteoblastic differentiation. In the ectopic bone formation test using an abdominal muscle pouch model for athymic nude rats, ectopic bone formation was not observed in the group transplanted with HA only. On the other hand, ectopic calcified tissue was generated and DBM osteoinductivity was observed in all 15 cases of the group transplanted with the DBM/HA putty. Therefore, when HA was used with DBM, it did not affect the efficacy of DBM for osteoinduction and the composite was more advantageous for bone formation than the HA group. This confirms that HA can be used effectively as a DBM carrier. Ozturk et al [19] examined the effect of a combination of DBM and a HA/TCP mixture on the healing of rat radius segmental defects, and reported that the DBM group showed the best healing response. The combined use of the DBM and HA/TCP mixture did not improve bone healing, and produced similar results to the control group. Moreover, they reported that the osteoinductive properties of

Figure 5. Plane radiographic findings. The arrows indicate the newly formed mineralized calcified tissues on the DBM inserted sites. (A) 4 weeks after surgery. (B) 8 weeks after surgery.

Figure 6. Ecotropic bone formation model using athymic nude rats. (A) No obvious bone or cartilage formation was observed in any of the HA samples. (B) DBM/HA displayed definitive mineralized calcified tissue formation in all 15 samples. The arrows indicate the newly formed mineralized calcified tissues on the DBM inserted sites.
Figure 7. Decalcified histologic findings. Hematoxylin and eosin staining. (A) HA only group. The arrows indicate HA (left, \( \times 40 \), right, \( \times 200 \)). (B) DBM/HA group. The arrows indicate HA and the arrow heads indicate the newly formed mineralized calcific tissues. (left, \( \times 40 \), right, \( \times 200 \)).

Figure 8. Undecalcified histologic findings. Hematoxylin and eosin staining. (A) HA only group. The arrows indicate HA. The bi-directional arrows indicates newly formed tissues around HA (left, \( \times 25 \), middle, \( \times 40 \), right, \( \times 100 \)). (B) DBM/HA group. The arrows indicate HA and the arrow heads indicate the newly formed mineralized calcific tissues. (left, \( \times 25 \), middle, \( \times 40 \), right, \( \times 100 \)).

DBM were inhibited by the HA/TCP mixture. However, the present data are different from that reported by Ozturk in that the carrier of DBM used in this study was HA alone, and the osteoinductivity of the DBM/HA group was better than the HA group in the ectopic bone formation model. The environment of the segmental bone defect model and that of ectopic bone formation in the muscle pouch model are totally different for bone formation. The reason for the difference is unclear and the results should not be extrapolated from one model to another model.
The reason why the osteoconductiveivity of DBM was higher than that of the bone graft extender, which has osteoconductiveivity only, is that the residual calcium in DBM acts as a nucleus for calcium phosphate deposition making the calcification of the bone easier [20], and a matrix, such as proteoglycan or collagen, allows easier cell attachment or its saturation through osteoconductiveivity [21]. In addition, growth factors, such as BMP, etc, stimulate bone formation by inducing mesenchymal stem cells [20]. Therefore, DBM with a carrier that has high osteoconductiveivity can be advantageous of its relatively higher osteoconductiveivity than DBMs mixed with glycerol, collagen and gelatin. However, DBM could not generate completely remodeled bony tissue as in the rat ectopic bone formation model of rhBMP-2 [22] which suggests that DBM has far lower osteoconductiveivity than rhBMP-2. Therefore DBM can more easily be detected with osteoconductiveivity in the body than that of HA which has osteoconductiveivity only, but in cases as cortical bone, nonunion site, and posterolateral fusion model of vertebrae which have difficulties in osteogenesis by DBM only, it was determined that there are limits in expecting successful bony union that can be observed in rhBMP-2. So it is deemed that reservation and caution are required when using the procedure clinically.

Conclusion

The DBM/HA putty showed clearer osteoblastic differentiation in an in vitro study using the human mesenchymal stem cells than HA only. In addition, the mineralized calcific tissue was formed even in the ectopic bone formation model using athymic nude rats, confirming the presence of osteoconductiveivity. Moreover, the osteoconductiveivity of the DBM/HA putty suggests that HA can be used as a carrier of DBM.

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References