Demineralized Dentin Matrix as a Carrier of Recombinant Human Bone Morphogenetic Proteins: in Vivo Study

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Abstract: This study aimed to evaluate the efficacy of rabbit demineralized dentin matrix (DDM) as a recombinant human bone morphogenetic protein-2 (rhBMP-2) carrier using the subcutaneous tissues of mice and rabbit calvarial critical-sized defects. DDM of rabbit, combined with rhBMP-2 (DDM/rhBMP-2) was transplanted into the subcutaneous tissues of 6 mice and 6 rabbit calvarial critical-sized defects (DDM = 0.03 g, control; DDM/rhBMP-2 = 0.03 g of DDM, 0.2 mg/ml, 5.0 μg of rhBMP-2, experimental). Both DDM and DDM/rhBMP-2 was transplanted into the left and right subcutaneous tissues of mice symmetrically. For rabbits, 4 round critical-sized defects (8 mm diameter) were formed on the exposed skull. DDM was transplanted into the 2 defects on the left sides (n = 12) and DDM/rhBMP-2 into the right sides (n = 12). Two animals among 6 mice and 6 rabbits were sacrificed respectively at the 1, 2, and 4 experimental weeks for the histological and histomorphometrical evaluations with hematoxylin and eosin staining. Tissues from rabbits were imaged via micro-computed tomography (μCT). DDM/rhBMP-2 in mice induced new bone formation at 2 weeks and maturation with bone marrow at 4 weeks. DDM/rhBMP-2 in rabbit calvarium induced new bone formation remarkably at 4 weeks 21.77 ± 47.99% compared to the DDM. These observations suggest that DDM could be considered a potential carrier of rhBMP-2. The rhBMP-2 loaded on DDM enhanced bone formation.

Key words: Bone formation, Demineralized dentin matrix (DDM), Recombinant human bone morphogenetic protein-2 (rhBMP-2), rhBMP-2 carrier

Introduction

Human demineralized dentin matrix (DDM) is fabricated from the tooth that is traditionally discarded after extraction. Human DDM is one of the most acid-insoluble collagenous scaffolds, containing non-collagenous proteins (NCPs) such as bone morphogenetic protein (BMP), in addition to a mineral phase, and is an ideal bone substitute. Of clinical importance, DDM-based scaffolds are reprocessed, acellular, and nanoporous type I collagen.

Dentin has been characterized as the biologic composite of collagen matrix filled with nanometer-sized calcium-deficient, carbonate-rich apatite crystalites. The composition of the extracellular matrix of dentin consists of about 70 wt.% (40–45 V.%) mineral component (carbonated hydroxyapatite (HA)), 20 wt.% (30 V.%) organic component (mainly type I collagen), and the remaining 10 wt.% (20–25 V.%) water.

Fibrillar type I collagen accounts for about 90% of the dentin organic matrix, while the remaining 10% consists of non-collagenous proteins such as phosphoproteins and several growth factors such as BMPs and transforming growth factor-beta (TGF-β), and forms a fibrous three-dimensional network structure which build up the dentin matrix. Compared to bone, the collagen matrix in dentin is more interwoven with numerous crossing of fibrils. Dentinal collagen fibrils network serves a variety of structural roles including shaping and organizing extracellular matrices; providing a scaffold for mineral formation and deposition; cells adhesion and differentiation; and preserving the structural, mechanical, and functional integrity of the dentin. Fibril-forming collagen is characterized by a hierarchical assembly of substructures that consist of three-polypeptide chains, which assemble into fibrils with diameters in the range of 10–500 nm, and the fibrils further assemble into fibers.

Dentin has numerous micro pores and dentinal tubules, and the diameter of tubes varies between 2 and 4 μm. The number of dentin tubules is 18,000–21,000 tubules/mm\(^2\). They are more numerous in the inner third layer than the outer third layer of the dentin with the volume fraction (porosity) of average 3.47 ± 1.46% that is lesser than that of the natural human bone (6.2%)\(^7\)–\(^10\).

Since 1967, it has been known that rabbit DDM induces bone formation in the intramuscular pockets and that animal DDM can induce ectopic bone formation in subcutaneous and intramuscular pockets in rodents\(^11\)–\(^14\). Moreover, another report indicated that DDM induced bone and cartilage formation independently, while demineralized bone matrix (DBM) induced cartilage, bone, and marrow formation at 4 weeks in the back skin of nude mice\(^15\). Bessho et al. successfully isolated BMP from human dentin matrix\(^16\). Although the human dentin-derived BMP was different from the human bone-derived BMP, the 2 types had similar functions in the body.

Recombinant human bone morphogenetic protein-2 (rhBMP-2), a potent exogenous osteoinductive cytokine, has long been considered a promising avenue for bone regeneration and is commercially available for the treatment of oral-maxillofacial defects. However, the ideal carrier system has not yet been identified. Like other growth
A carrier for rhBMP-2 should meet several requirements: 1) Enhancing the activity of the protein by maintaining a certain rhBMP-2 concentration in the defective area for a sufficient time to allow for new bone formation\(^\text{21-25}\). 2) Good affinity between BMP and its carrier to maintain sustained and prolonged BMP release. 3) Ease of sterilization and biodegradability with no immunogenicity\(^\text{20}\). 4) Supporting bone growth by having an appropriate porous structure to allow for cell infiltration and in growth. 5) Retaining the biological activity of soluble BMP\(^\text{21-25,26}\).

Despite varying levels of success, the major components of human bone collagen and HA are preferred BMP carriers, such as the DBM\(^\text{27-29}\). DBM is obtained from native allogenic bone sources. The fact that collagen is present in DBM in addition to the natural apatite structure may make DBM a more effective carrier for rhBMP-2 than tricalcium phosphate (TCP) or HA\(^\text{30}\). However, DBM as a carrier has not gained popularity because of the risk of both immunogenicity and disease transmission\(^\text{30}\).

However, a report in 1998 suggested that human root dentin prepared from extracted teeth could be recycled for use as a carrier of rhBMP-2 because it induces new bone formation in the periodontium\(^\text{30}\). A later report in 2005 showed that the osteoinductive matrices of human DMM particles could be effective carriers of rhBMP-2 for bone engineering\(^\text{30}\). Since then, DMM powder has shown great potential as an effective carrier for rhBMP-2 based on in vitro and in vivo studies in 2014 and 2015\(^\text{22,23}\). Recently, human DMM/rhBMP-2, applied to the rabbit calvarial defect, has shown superior results for bone formation compared to Bio-Oss/rhBMP-2 and DDM alone\(^\text{30}\).

Based on these previous studies, we hypothesized that the exogenous rhBMP-2 has a synergistic effect with endogenous growth factors of DDM. The aim of this study is to evaluate DDM as a potential carrier for rhBMP-2 using mice and a rabbit calvarial critical-sized defect model.

**Materials and Methods**

The ethics committee for in vivo study of the Dankook University Hospital approved this research (IRB DKU-17-038). Teeth extracted from healthy rabbits were collected from the animal laboratory at Dankook University Hospital.

**Preparation of Demineralized Dentin Matrix (DDM)**

Extracted rabbit teeth were soaked in 70% ethyl alcohol and cleaned by removing soft tissues of the periodontium, pulp, and caries. After dividing cleaned teeth into the crown and root, the root portion was collected and prepared for DDM. Crushed particles (300 to 800 µm) were soaked in distilled water and hydrogen dioxide solution, and the remaining foreign substances were removed by ultrasonic cleaner. The cleaned particles were dehydrated with ethyl alcohol and subjected to defatting using ethyl ether solution. The particles were then demineralized for 30 minutes in 0.6 N HCl. The demineralized particles were lyophilized, and sterilized with ethylene oxide gas.

**Characterization of Demineralized Dentin Matrix (DDM) Microparticles**

The range of particle size of DDM is from 300 to 800 µm in diameter with a median size of 500 µm. Microscopic observations demonstrated that the basic dentin microtexturing was preserved after demineralization that starts at the surface and progresses to the interior of dentin particle\(^\text{30}\) (Fig. 1). Dentin demineralization with 0.6 N HCl results in the elimination of the major part of the mineral phase and immunogenic components, while retaining a very low fraction of minerals (5–10 wt.%), the majority of type I collagen, and NCPs, providing an osteoconductive and osteoinductive scaffold containing several growth factors\(^\text{30}\) (Fig. 1). Structurally, dentinal tubules are enlarged and dense collagen matrix is loosened (Fig. 2). The amount of extracted proteins in DDM was 0.29 wt.% (2.89 mg/g)\(^\text{30}\).

X-ray diffraction (XRD) analysis found that low crystalline structures, domain sizes, and high Ca/P ion dissolution of the DDM were similar to those of autogenous bone with calcium phosphate. These included HA (Ca/P = 1.75), TCP (Ca/P = 1.46), amorphous calcium phosphate (ACP, Ca/P = 1.32), and octacalcium phosphate (OCP, Ca/P = 1.24) with the plate-like crystals\(^\text{37,38}\).

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**Figure 1. Cross-sectional image of DDM microparticle**

Surface demineralized area was shown in shallow hematoxylin and eosin staining from the surface (arrow head). Enlarged dentinal tubules and loosened collagen matrix at surface may provide spaces for cellular infiltration and protein exchanges. The interior of the particle remained mineralized in deep hematoxylin and eosin staining (asterisk). (Hematoxylin and eosin staining. Scale bar = 100 µm) (arrow head = surface of demineralized dentin, asterisk = core of the dentin).

**Figure 2. Scanning electron microscope (SEM) images of the dentinal surface of DDM particle**

A: Normal dentin before demineralization. Micropores are dentinal tubules (asterisk, diameter: 1.0–3.0 µm, approximately 20,000 tubes/mm2), and mineralized intertubular dentin (arrow head) among dentinal tubules. B: Dentin after demineralization with 0.6N HCl. Enlarged dentinal tubules (asterisk) and loosened collagen (arrow head) with enhanced surface micro-roughness or micro-texture to help easy release of endogenous proteins, which may promote growth and differentiation of osteoblasts. (Scale bar = 5.0 µm) (asterisk = dentinal tubule, arrow head = intertubular dentin).
Combination of Demineralized Dentin Matrix with rhBMP-2 (DDM/rhBMP-2)

The rhBMP-2 (0.2 mg/ml, approximately 50 μg, Cowell BMP, Busan, Korea) was fixed to 0.3 g of DDM by placing both into 15-ml conical tubes. The mixtures were frozen in a deep freezer at -70 ºC and then fixed in a lyophilizer (ILShin Lab, Seoul, Korea).

Implantation of DDM and DDM/rhBMP-2 in subcutaneous tissues of mice

In this study, 6 mice aged 4 weeks, weighing 15–20 g were used. These animals were bred with a free supply of cubed diet and water under average ambient temperature conditions of 22 ºC and a 12-hour shade cycle. We provided general anesthesia through intra-peritoneal administration of pentobarbital sodium (Nembutal, 43 mg/kg, Dainabot Co., Tokyo, Japan) that had been diluted in sterile water for injection, and then disinfected and isolated the surgical site. Two bilateral incisions were made in the dorsum, and a subcutaneous pocket was formed on both sides. In the control group (n = 6 mice), grafts of DDM (0.03 g, control) were applied on the left side while grafts of DDM/rhBMP-2 (0.03 g of DDM, 0.2 mg/ml, 5.0 μg of rhBMP-2) were applied on the right side in the experimental group (n = 6 mice). Two mice were sacrificed at the 1, 2, and 4 experimental weeks for histological evaluation.

The specimens were procured en bloc and fixed in 10% neutral buffered formalin, decalcified with 10% formic acid, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE) and Masson's trichrome (MT). Animal studies were conducted to test DDM as rhBMP-2 carriers. In order to follow the ethical considerations in animal experiments, a reduction was implemented according to the 3R’s.
After μCT analysis, the 12 specimens from each group were histologically and histomorphometrically analyzed (Bruker microCT). μCT images of calvarium were reconstructed and analyzed using three-dimensional (3D) structure analysis software (NRecon, Bruker microCT, CTAn, dataviewer, BrukermicroCT). μCT images of calvarium were reconstructed and morphometrically analyzed.

Results

Histological findings in Subcutaneous Tissues of Mice

The DDM implants were surrounded by a dense fibrous capsule and no inflammatory cells were observed (Fig. 3A). Collag enolytic resorption, either enzymatic or osteoclastic, with cellular infiltration was observed after 2 weeks (Fig. 3B). Higher magnification of Fig. 3B showed representative resorption of DDM particle that were filled with giant cells (Fig. 3C). Increased deposits of osteoids were observed on the surface of DDM particles with a marrow like structure between the particles observed at 4 weeks (Fig. 3D).

On the surface of the DDM/rhBMP-2 implant, multiple collagenolytic resorption was observed with active cellular infiltration at 1 week (Fig. 3E). At 2 weeks, newly formed osteoids were deposited on the surface of the implant with highly proliferated and phenotypically transformed lining cells (Fig. 3F). As shown via higher magnification at 2 weeks, the characteristics of new osteoids on the surface of DDM/rhBMP-2 implants showed typical cartilaginous bone formation with several chondrocytes and new vascular invasion into the collagenolytic resorbed space (Fig. 3G). At 4 weeks, newly formed bones were bridged and amalgamated between the implant with Howship's lacuna, and embedded osteocytes were observed. The loose areolar tissues between the particles had changed into bone marrow-like structures with new osteoids deposited along the surface in the DDM/rhBMP-2 group (Fig. 3H).

Implantation of DDM and DDM/rhBMP-2 in rabbit calvarial defects

Six male rabbits (2.50–3.00 kg body weight) were maintained in cages at an ambient room temperature of 21 °C with ad libitum access to water and a standard laboratory pellet diet. Rabbits were anesthetized via intramuscular injection (5 mg/kg body weight) of a 4:1 solution of ketamine hydrochloride (Ketalar, Yuhan Co., Seoul, Korea) and xylazine (Rompun, Bayer Korea, Seoul, Korea). The surgical site was shaved and scrubbed with iodine. For the calvarial defect model, an incision was made in the sagittal plane across the cranium, and a full thickness flap was reflected to expose the calvarial bone. Four round defects with 8 mm diameters were created symmetrically from the central synostosis in each rabbit calvarium using a trephine drill (3i Implant Innovations Inc., Palm Beach Gardens, FL, USA). Each 2 round defect received the following treatments: 1) DDM control (0.03 g of DDM, 0.2 mg/ml, 5.0 μg of rhBMP-2) on the right 2 defects (n = 12). 2) DDM/rhBMP-2 (0.03 g of DDM, 0.2 mg/ml, 5.0 μg of rhBMP-2) on the left 2 defects (n = 12). 2) DDM/rhBMP-2 (0.03 g of DDM, 0.2 mg/ml, 5.0 μg of rhBMP-2) on the right 2 defects (n = 12). All surgical sites underwent primary closure using 4-0 Monosyn (glyconate absorbable monofilament, B. Braun, Aesculap, PA, USA) on the right 2 defects (n = 12). Two rabbits were sacrificed at 1, 2, and 4 weeks after implantation for radiological and histological evaluation. As a result, it was possible to compare the statistical significance between DDM group (n = 4) and DDM/rhBMP-2 (n = 4) at each time point.

Micro computed tomography (μCT) analysis

Calvarial en bloc specimens were fixed in 10% buffered formalin solution for imaging and scanned using a SkyScan 1172 CT system (Bruker microCT, Kontich, Belgium) in a high-resolution scanning mode (pixel matrix, 683×2,000×1,048; pixel size, 10.89 μm) under standard conditions. Using three-dimensional (3D) structure analysis software (NRecon, Bruker microCT, CTAn, dataviewer, BrukermicroCT), μCT images of calvarium were reconstructed and morphometrically analyzed

Histological and histomorphometric analysis

After μCT analysis, the 12 specimens from each group were demineralized in 10% formic acid for 14 days and then embedded in paraffin. From serial sections of 5 μm thickness through the horizontal plane of the circular calvarial defects, 2 sections that contained whole defects were selected and stained with HE and MT. The digital images were obtained and new bone area was calculated by percentage of bone area to tissue area of 8 mm diameter (NBA%; new bone area%, BA: bone area, TA: tissue area, NBA% = BA/TA × 100) that was determined by using an image analysis program (Kappa Image Base Metreo, Kappa Optronics GmbH, Gleichen, Germany). Statistical analysis of Histomorphometric results in rabbit calvarium was performed using the Graphpad PRISM 5.0 software (San Diego, CA). Data are expressed as mean ± S.D. The significance level of the treatment effects was determined using a paired T-test to compare the means of each group. Values of p<0.05 were considered to be statistically significant.
Discussion

This study was designed to determine whether DDM might serve as a potential carrier for exogenous rhBMP-2. We hypothesized that the exogenous rhBMP-2 showed synergistic effects with endogenous growth factors of DDM based on previous studies.

DDM is prepared by acid extraction of the root dentin. It results in the elimination of the major part of the mineral phase and the immunogenic components of dentin, but retains collagen that provides a structured osteoconductive scaffold, and a soluble protein fraction comprising several growth factors and BMPs. It is also common to retain a very low fraction of mineral bone phase after the demineralization step (approximately 10%). Because 70–75% extrabifibrillar mineral was removed rapidly, while the remaining intrabifibrillar mineral (25%) demineralized at a significantly slower rate as depicted in Fig. 1.

The acid treatment of dentin might have increased the volume fraction (porosity) occupied by the dentinal tubules from an average porosity of 3.47 ± 1.46% to more than 5.88 ± 2.24% that is very similar to that of natural human bone (6.2%)\(^{19,32,41}\). Collagenolytic resorption by enzymes or osteoclasts increased surface area and porosity that accounts for triggering of the induction cascade by releasing of endogenous growth factors in DDM which induce phenotypic transformation of fibroblasts (Fig. 3). Once dentin is resorbed, more widened dentinal tubule and more loosened dense collagen fibers serve as a channel for releasing essential endogenous proteins such as BMPs, which may promote growth and differentiation of osteoblasts (Fig. 3).

In the mice, the new osteoids on the surface of DDM/rhBMP-2 implants at 2 weeks showed cartilaginous bone formation with several chondrocytes and new vascular invasion into the collagenolytic resorbed space that is consistent with the previous studies of Reddi and Murata (Fig. 3).

At early stage of graft (1 and 2 weeks), in addition to the osteoinduction on the surface of DDM particle, the collagenolytic or osteoclastic resorption of the DDM particle was more prevalent in DDM/rhBMP-2. This may be primarily due to the release of exogenous growth factors delivered by dentin matrix that may act both osteoinductive function and osteoclastic dentin resorption. By this resorption and cellular infiltration into the space, the osteoinductive functions of each DDM/rhBMP-2 particle might be enhanced by the releasing of the endogenous growth factors in the DDM matrix at later stage (4 weeks) (Figs. 5 and 6).

In the rabbit calvarial defects, the bone regeneration from the edge of the defect was increased in a time-dependent manner in both groups. As shown in Fig. 5, bone formation in DDM/rhBMP-2 occurred not only from the defect margins (osteocclusion) but also around the center of defect (osteoid formation) at 4 weeks. The DDM particles in the center of defect showed replacement of dentin matrix with newly formed bone that result in the increased amount of bone formation. The resorption of dentin matrix, either enzymatically or phagocytically, seemed to be triggered by exogenous rhBMP-2 and may result in the release of endogenous growth factors in DDM, which, in turn, initiates the coupling with osteoblastic bone formation\(^{42}\). These observations from mice and rabbit indicate that exogenous rhBMP-2 acted synergistically with the endogenous growth factors of DDM (Figs. 5 and 6).

In terms of DDM as a potential rhBMP-2 carrier, Ike & Urist showed that exogenous rhBMP-2 adsorbed onto human DDM was osteoinductive as autogenous bone in the thigh muscles of mice\(^{20}\). Also reported positive results using human DDM as a carrier for...
rhBMP-2 in the subcutaneous tissue of mice. They both indicated that as the large quantity of induced bone developed, the complete resorption and replacement of root dentin matrix (carrier) occurred. We paid attention to the relationships between the exogenous BMP and dentin resorption that results in the replacement with bone because we have not found the increased resorption of DDM alone in our in vivo and clinical studies before\(^{20,31}\).

Kim et al. reported that human DDM showed larger amounts of rhBMP-2 release than HA and TCP in the subcutaneous tissue of mice, and showed that the expression of osteonectin was found to be significant in the dentinal tubules and in the stromal cells of the DDM/rhBMP-2 implant\(^{20}\). Um et al. performed similar experimental studies on human DDM/rhBMP-2 (0.2 mg/ml concentration) using a rabbit calvarial model and reported that human DDM has greater potential as an rhBMP-2 carrier compared to anorganic bovine bone\(^{29}\). This might be due to the binding capacity and slow time-release kinetics related to the collagenous nature and innate microporous structures of the dentinal tubules in DDM.

This resorption of DDM has been clinically investigated previously in the clinical studies. In the preliminary human clinical report, the DDM/rhBMP-2 (0.2 mg/ml concentration) exhibited multiple irregularly shaped surfaces infiltrated by activated cells, as well as isolated spaces that filled with infiltrating cells. These spaces were consistent with collagenolytic resorption with fibroblast infiltration and were reminiscent of DDM undergoing resorption by multinucleated giant cells\(^{20}\).

In the randomized clinical study, it was shown that residual DDM within the implanted area at 3 to 6 months after graft was 8.95% compared with 17.08% of Bio-Oss\(^{20}\). In a retrospective clinical study of DDM/rhBMP-2 (0.2 mg/ml concentration) for alveolar bone augmentation after implant removal, 10–11 months after surgery, histomorphometric analysis showed 14.98 ± 10.09% newly-formed bone, 6.22 ± 5.5% residual DDM, and 60.86 ± 18.66% soft tissue components in the tissues\(^{20}\).

This might be interpreted that as a large quantity of induced bone developed, the increased resorption and replacement of the dentin matrix (carrier) occurred that was indicated by Ike & Murata\(^{20,31}\). With previous experimental and clinical reports, our observations in mice and rabbits support the hypothesis that the exogenous rhBMP-2 showed synergistic effects with endogenous growth factors of DDM, whereas low concentration of rhBMP-2 might also facilitate osteogenesis in regions distant from the resident bone such as the center of the skull defect.

In limitations of this study, particularly dose- and concentration-dependent biologic assays of DDM should be performed with more number of experimental samples to achieve higher statistical power of experimental results.

Even though rhBMP-2 exerts its effect in a dose-dependent manner, BMPs shows a therapeutic dose barrier, below which carriers do not function better with the addition of rhBMP-2 and a threshold dose above without any additional benefits\(^{20,31}\). Approximately, the general requirement of rhBMP-2 varied from 5 μg for mice (in muscle), 50 μg for rabbit (femur defect), 400 μg for dogs (ribs) and milligram quantities of rhBMP-2 for human (spinal fusion)\(^{29}\). As it was observed that the initial burst release of rhBMP-2 is not inevitably required for osteoinduction, we adopted a single dose according to the report of Alam, et al.\(^{29}\) to reduce the total amount of rhBMP-2 instead of supraphysiologic dosage\(^{20,31}\).

Based on the observations of this study, it is possible that DDM/rhBMP-2 contains endogenous and exogenous growth factors, shows, and promotes enhanced osteogenesis compared to DDM alone. Probably this promising observation might be due to the exogenous rhBMP-2, released from the DDM carrier at an early stage, that enhance collagenolysis to provide spaces for exchanging proteins. Subsequently, many endogenous proteins are released from the DDM to promote cellular proliferation and differentiation. This may account for the prominent bone formation in DDM/rhBMP-2 compared to DDM alone and support DDM as a potential carrier for rhBMP-2. Further studies are necessary to determine the most suitable conditions for demineralization, concentration of rhBMP-2, and particle size for clinical applications in alveolar bone repair.

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Conflicts of Interest

The authors have declared that no conflict of interests exists.

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