Clinical Report

Histological Comparison of Autogenous and Allogenic Demineralized Dentin Matrix Loaded with Recombinant Human Bone Morphogenetic Protein-2 for Alveolar Bone Repair: A Preliminary Report

In-Woong Um1, Sang-Ho Jun2, Pil-Young Yun3 and Young-Kyun Kim4,5

1) R&D Institute, Korea Tooth Bank, Seoul, Korea
2) Department of Dentistry, Korea University Anam Hospital, Seoul, Korea
3) Department of Oral and Maxillofacial Surgery, Section of Dentistry, Seoul National University Bundang Hospital, Seongnam, Korea
4) Department of Dentistry and Dental Research Institute, School of Dentistry, Seoul National University, Seoul, Korea

(Accepted for publication, July 5, 2017)

Abstract: The goal of investigation is to examine the histological response to recombinant human bone morphogenetic protein-2 (rhBMP-2)-loaded demineralized dentin matrices (DDMs) of different origins. The evaluation site is chosen between the cover screw and gingiva, as the poor blood supply allows it to simulate a heterotopic condition. We hypothesize that the antigenicity and immunogenicity of the carrier allogenic DDMs are low enough to maintain both the biocompatibility of the scaffold and the activity of the loaded rhBMP-2. Three patients undergoing simultaneous implant placement and receiving a different type of graft were included: allogenic DDM loaded with rhBMP-2 (DDM/rhBMP-2), autogenous DDM/rhBMP-2 and autogenous DDM. Histological specimens were retrieved during the secondary surgery after 3–6 months. In histological examination, particles were encapsulated by dense fibrous tissue without any inflammatory cells. The capsule contained an increased number of cell layers in the DDM/rhBMP-2 (allogenic and autogenous) compared to autogenous DDM. Resorption activity was higher in autogenous DDM/rhBMP-2 than in allogenic DDM/rhBMP-2. Within the limitations of this report, results demonstrated the successful use of DDM as a potential rhBMP-2 carrier. Further studies will be required to confirm the safety and effectiveness of autogenous and allogenic DDM/rhBMP-2.

Keywords: Demineralized dentin matrix (DDM), Allogenic DDM, Autogenous DDM, Recombinant human bone morphogenetic protein-2 (rhBMP-2)

Introduction

In this case report, we compare the histological responses to autogenous and allogenic demineralized dentin matrices (DDMs) loaded with recombinant human bone morphogenetic protein-2 (rhBMP-2) when grafted between the implant screw and gingiva. The assessment is based on tissues procured during secondary surgery. We hypothesize that antigenicity and immunogenicity of autogenous DDM will not reduce either its biocompatibility or the activity of the loaded rhBMP-2.

The protein, rhBMP-2, delivered through a carrier, has demonstrated clinical relevance in the induction of bone formation for maxillofacial/oral applications. Collagen is the most thoroughly documented carrier for exogenous rhBMP-2. In 2007, FDA approved the use of rhBMP-2 soaked into absorbable collagen sponges (ACSs) in the oral and maxillofacial region for the purposes of sinus floor augmentation and extraction socket preservation.

Nevertheless, collagen has a rather limited capacity for controlled release, with most of the absorbed agent released during the first day post-implantation1. Release properties can be improved to a limited extent by cross-linking2. Apart from its limited capacity to control rhBMP-2, the main limiting factor for using collagen scaffolds is their poor mechanical properties. Indeed, collagen scaffolds show no rigidity and shrink due to the cohesive forces that develop as extracellular matrix (ECM) is deposited. Furthermore, collagen is not osteoconductive, and its poor structural integrity reduces its effectiveness in promoting the onlay augmentation of alveolar bone.

In contrast, dentin matrix displays excellent structural integrity and mechanical properties. It contains hydroxyapatite, type I collagen, and other proteins, including growth factors. After removal of the inorganic portion (demineralization), the remaining matrix could be defined as acid-insoluble collagen to which various other proteins are bound3. Histologically, dentin matrix consists of tubes 1–3 μm in diameter (dental tubules), which provide nanopore spaces that increase the surface contact area and can accommodate the rhBMP-2 solution, thereby increasing the capacity of dentin to function as an effective scaffold/carrier for rhBMP-24,5.

With respect to using DDM as an rhBMP-2 carrier, Ike et al. reported in 1998 that exogenous rhBMP-2 adsorbed into pulverized root partially demineralized dentin matrix (PDM) proved to be as osteoinductive as autogenous bone graft6. Murata in 2005 also showed that human DDM particles are osteoinductive, insoluble collagenous matrices, and DDMs might be effective as an rhBMP-2 carrier for bone engineering7. The observations reported in these two studies were later confirmed by in vivo and in vitro studies of Kim et al. and Um et al.8-12.

In this preliminary report, we compare the histological responses to autologous and allogenic DDM loaded with rhBMP-2 (DDM/rhBMP-2) grafted during implant placement surgeries, using the response to autogenous DDM (non-loaded with rhBMP-2) as reference. Because
observations reported in these two studies were later confirmed by in vivo and in vitro studies of Kim et al. and Um et al. 9-12.

In this preliminary report, we compare the histological responses to allogenic and autogenous DDM loaded with rhBMP-2 (DDM/rhBMP-2) grafted during implant placement surgeries, using the response to autogenous DDM (non-loaded with rhBMP-2) as reference. Because performing trephine biopsies are impossible in cases where the graft and the implant are placed in a single surgery, we use the tissue located between the gingiva and the cover screw samples for histological assessment. Our working hypotheses, which are based on previous studies, are that (a) allogenic DDM might be an effective carrier for rhBMP-2 and (b) allogenic dentin collagen has low antigenicity; thus, it should elicit only a low level or no immune response, which would otherwise impair the healing process.

Materials and Methods
Fabrication of allogenic and autogenous DDM
Allogenic and autogenous DDMs were both derived according to the patented (Korea Patent Number: 10-1062381) method described by Kim et al. 4. Briefly, DDM powder was procured by pulverizing extracted root dentin to particles 0.3–0.8 mm in diameter, after removing the attached soft tissue and pulp. The pulverized DDM powder was washed using 70 % ethyl alcohol, demineralized by repeated 30-minute incubation periods in 0.6 N HCl to reduce the mineral content to less than 10–30 wt. % leaving type-I collagen as the main constituent, and then dehydrated, defatted and freeze-dried.

Fixation of rhBMP-2
The protein, rhBMP-2 was loaded to the DDM powder by the dip-dry method 12. Briefly, 0.03 g of DDM powder was loaded with a 0.2 mg/ml rhBMP-2 (Cowell, Busan, Korea) solution, in individual 15-ml conical tubes. The mixtures were left to freeze at –70ºC, slotted into a lyophilization glass bottle and then dried in a lyophilizer (ILShin Lab, Seoul,Korea).

Patient selection
This study was approved by the Institutional Review Board of Seoul National University Bundang Hospital (B-1512-328-107). The criterion for choosing patients to undergo placement of DDM/rhBMP-2 was the anticipation of poor blood supply from the host bone and soft tissues. The criteria for recruiting patients for use of allogenic DDM was lack of a sufficient amount of autogenous DDM and the presence of wall defects requiring repair. We excluded patients with poor plaque control and untreated chronic periodontitis, smokers, alcoholics, and patients with systemic diseases.

Three healthy patients were recruited for this study, after signing written consent forms. They underwent simultaneous DDM graft and implant placement without using membrane on the lower left canine with allogenic DDM/rhBMP-2 (male, 56 years old), the lower central incisors with autogenous DDM/rhBMP-2 (female, 68 years old), and the lower left second molar with autogenous DDM (female, 52 years old). After respective healing periods of six, two and a half, and three and a half months, specimens were retrieved from the space between the implant cover screw and gingiva at the time of the secondary

Figure 1. Allogenic DDM/rhBMP-2 application on lower left canine (Case 1)
A: Extraction. Flap reflection, cleaning, and removing all granulation after extraction of lower left canine.
B: Immediately after extraction. Placement of implant (diameter: 3.8 mm, length: 13 mm) ensuring a secure initial stability.
C: Immediately after implant placement. Allogenic DDM/rhBMP-2 is packed into the lingual wall gap and along the labial wall. No membrane is used.
D: After 6 months. Well-organized tissue is found over the cover screw after reflection of flap during the secondary prosthetic surgery, 6 months after implant and bone graft placement.
E: After 6 months. The tissue between the reflected gingival flap and the cover screw is typically removed during the secondary surgery in order to uncover the cover screw of the implant, and was chosen as biopsy material for this study.
F: After 6 months. The 3 × 3 mm sized specimen that is finally procured for histological evaluation.
Case 1: Simultaneous implant and allogenic DDM/rhBMP-2 placement on lower left canine

A 56-year old male patient was recruited who had experienced intermittent pain, swelling, and mobility of his lower left canine for more than three years. After reviewing his medical history and performing a preliminary clinical examination, a digital panoramic radiography and cone beam computerized tomography (CBCT) scan were taken for diagnosis and treatment planning. The chosen surgery and processed for histological evaluation.
There were clear border lines separating the graft from the remaining cortex had been patched up by the allogenic DDM/rhBMP-2 powder. After the primary surgery showed that the missing part of the labial implant manufacturer’s instructions. A CBCT image taken immediately after the surgery reveals a completely repaired labial bone. The demarcated border between the remaining labial cortex and the repaired bone has almost disappeared. The volume and shape of the alveolus have remained as seen in Figure 1D.

The exposed implant and the alveolar defect were augmented with allogenic DDM/rhBMP-2 (Fig. 1C). The graft was placed and carefully packed into the destroyed area of the lingual wall, while ensuring that it extended to the surface of the lateral buccal wall, without applying excessive pressure. The muco-periosteal flap was replaced and stabilized with monofilament, non-resorbable sutures without a covering membrane. During the postoperative phase, the patient was protected from infection by administration of prophylactic antibiotics, analgesics and anti-inflammatory medication. Following a healing period of 7-10 days, sutures were removed. Clinical and radiographic examinations were undertaken after 6 months, when the patient underwent secondary surgery; under local anesthesia, a similar incision was made at the lingual side of the alveolar crest. A split-thickness flap was raised (Fig. 1D) and tissues over the cover screw were retrieved with a #12 surgical knife for histological examination (Fig. 1E, F).

All routine prosthetic procedures were performed according to the implant manufacturer’s instructions. A CBCT image taken immediately after the primary surgery showed that the missing part of the labial cortex had been patched up by the allogenic DDM/rhBMP-2 powder. There were clear border lines separating the graft from the remaining labial cortex and the lingual cortex (Fig. 2A). After fourteen months with a provisional prosthesis, the alveolar bone appeared completely repaired in both volume and shape. The border at the lower labial defect had completely disappeared, indicating good incorporation of the graft into the alveolar bone. Even though the new labial cortical bone was still thin, it seemed to be fully supported by cancellous bone and marrow (Fig. 2B).

**Case 2: Simultaneous implant and autogenous DDM/rhBMP-2 on lower central incisors**

Full thickness flaps were lifted to expose the knife-edge alveolar ridge of both lower central incisors. Ridge split procedure was performed to secure the labio-lingual site for implant placement (diameter: 3.8 mm, length: 13 mm; Daemul, Seoul, Korea) (Fig. 3A). The lower central incisors gaps created by ridge split and implant placement were filled with autogenous DDM/rhBMP-2 (Fig. 3B). During the secondary surgery, two and a half months after graft placement, well organized tissues over the cover screw were retrieved for histological examination (Fig. 3C, D).

A CBCT image taken 6 months after surgery showed a completely repaired labial bone. The demarcated border between the remaining labial cortical bone and the repaired bone was still visible (Fig. 4).

**Case 3: Simultaneous implant and autogenous DDM placement on lower second molar**

Two implants were placed on the extraction socket of the lower left second molar (Fig. 5A) together with autogenous DDM powder to repair the defects (Fig. 5B). During the secondary surgery, which was performed three and a half months post-implantation, the flap was reflected and the tissues over the cover screw were procured for histological evaluation (Fig. 5C).

A CBCT image of the second molar taken after the first surgery showed that the graft had patched up the lingual defect (Fig. 5D). Three and a half months post-implantation, i.e., at the time of secondary surgery, the repair of the lingual defect had progressed and the border line between the defect and the graft was no longer visible (Fig. 5E). Even though the repaired lingual cortical bone had not yet matured, it seemed to be well incorporated. After 9 months with a provisional prosthesis, the repaired bone had been remodeled into a mature cortical bone supported by cancellous bone and marrow (Fig. 5F).

**Results**

**Clinical outcomes**

In all three cases (allogenic DDM/rhBMP-2, autogenous DDM/rhBMP-2, and autogenous DDM), a secure primary implant stability was achieved, while no postoperative complications were observed. The healing process was uneventful and no clinical signs of inflammation and/or infections were observed. During the secondary surgery (2.5-6 months after graft) the DDM covering the cover screw had transformed

| Table 1. Histological comparison of allogenic DDM/rhBMP-2, autogenous DDM/rhBMP-2, and autogenous DDM |
|-----------------|-----------------|-----------------|-----------------|
| Age/Sex         | 56/M            | 68/F            | 52/F            |
| Graft site      | lower left canine | lower central incisors | lower left second molar |
| Inflammatory Cells | -              | -              | -              |
| Fibrous Capsule | +              | +              | +              |
| Cell layer      | 3–4            | 3–4            | 1–2            |
| Surface Resorption | +            | ++             | -              |

M: Male, F: Female, -: absence, +: presence, ++: increased presence
Figure 5. Autogenous DDM application on lower left second molar (Case 3)
A: Immediately after implant placement. Two implants are placed on the extraction socket of the lower left second molar.
B: Immediately after graft. The defect is patched up by autogenous DDM powder.
C: After 3.5 months. Three and a half months later, the flap is reflected during the secondary surgery and the tissues over the cover screws are procured for histological evaluation.
D: Immediately after implant placement and graft. A CBCT image taken immediately after the first surgery reveals that the lingual defects have been patched up by the autogenous DDM.
E: After 3.5 months. A CBCT image taken at the time of the second surgery (three and a half months after implantation) reveals a complete repair of the lingual defect. The border line between the defect and the graft has disappeared.
F: After 9 months. A CBCT image taken after nine months with a provisional prosthesis reveals a repaired lingual cortical bone seemingly well incorporated and remodeled into cortical bone, supported by cancellous bone and marrow.

Figure 6. Histological comparison of autogenous DDM, autogenous DDM/rhBMP-2 and allogenic DDM/rhBMP-2 particles using tissues from the area between the implant cover screw and gingiva.
A: Case 1. After 6 months. Allogenic DDM/rhBMP-2 particle surrounded by dense fibrous connective tissues in close contact with the particle, consisting of multiple cell layers of activated cells, which are infiltrating into the DDM. The image is reminiscent of the resorption remodeling procedure. A single isolated space filled with infiltrating cells can be seen (Masson's trichrome stain, scale bar = 200 μm).
B: Case 2. After 2.5 months. The surface of an autogenous DDM/rhBMP-2 particle, which is being infiltrated by activated cells at multiple locations. Two isolated spaces filled with infiltrating cells can be observed. (Hematoxylin-Eosin Stain, scale bar=500 μm)
C: Case 3. After 3.5 months. Autogenous DDM particle surrounded by dense fibrous connective tissues, which are in close contact with the particle, leaving no gaps. The fibrous capsule seems to consist of inactive, flat fibroblast-like cells (Hematoxylin-Eosin Stain, scale bar=500 μm).

into woven bone-like structures, which were easily removed with a surgical knife (Figs. 1D, 3C, 5C). It had the form of a complex consisting of particles and dense fibrous tissue resembling well-organized, woven bone-like structures (Fig. 1F). CBCT images of each patient showed that the volume and shape of the labial cortex had been maintained at the time of the secondary
surgery (Figs. 4, 5E). After respective periods of 14, 6 and 9 months with a provisional prosthesis, mature cortical bone was seemingly observed, fully supported by cancellous bone and marrow (Figs. 2B, 5F).

**Histological results**

Each tissue was evaluated with respect to inflammatory cell presence, fibrous capsule formation, cell layer structure, and surface resorption. The results were summarized in Table 1, while a detailed analysis was provided in the next three paragraphs.

**Allogenic DDM/rhBMP-2**

No inflammatory cells or foreign body reactions were seen around the particle. The particle was surrounded by a dense fibrotic capsule consisting of 3-4 cell layers. Activated cells from the fibrous capsule were infiltrating the periphery of the DDM matrix. Masson's trichrome staining revealed that isolated spaces within the matrix had already been filled with infiltrating cells (Fig. 6A).

**Autogenous DDM/rhBMP-2**

There were no gaps between the DDM particle and the fibrous capsule, and no inflammatory cells were detected. The dense fibrotic capsule had 3-4 cell layers that seemed to be activated and displayed an osteoblast-like phenotype. The DDM particle exhibited multiple irregularly shaped surfaces infiltrated by activated cells. These areas seemed to be the result of resorption. Isolated spaces in the DDM filled with infiltrating cells were also observed. These spaces were reminiscent of DDM undergoing collagenolytic resorption and their presence suggested that remodeling was taking place (Fig. 6B).

**Autogenous DDM (without rhBMP-2)**

The histological features of the sample gathered from the patient that had grafted autogenous DDM in soft tissue were representative of its proven biocompatibility, osteoinductivity and osteoconductivity properties. Neither inflammatory cells (e.g., multinucleated cells) nor surface resorption was observed around the particles. Dense fibrous connective capsules surrounded the particles consisting of one or two cell layers of flat fibroblast-like cells (Fig. 6C).

**Discussion**

The aim of this preliminary report was to compare the histological responses to autogenous and allogenic DDM/rhBMP-2 with the response to autogenous DDM (without loaded rhBMP-2). We hypothesized that DDM is a very effective BMP carrier and that allogenic DDM can also carry BMPs that will be effectively functional upon release, because the antigenicity and immunogenicity of this scaffold are such that they will illicit little or no immune response, and will therefore be unable to impede the healing process.

All three DDMs were encapsulated with dense fibrous connective tissue consisting of multiple cell layers. Specifically, autogenous and allogenic DDM/rhBMP-2 had capsules with 3-4 cell layers, while autogenous DDM without rhBMP-2 was surrounded by a capsule with 1-2 cell layers. Moreover, both types of DDM/rhBMP-2 (autogenous and allogenic) displayed surface resorption or infiltration of cells via the periphery of the matrix, indicating an induction of cellular activities by the exogenous rhBMP-2. Finally, isolated cavities filled with infiltrating cells, which were reminiscent of the process of collagenolytic resorption that takes place during natural bone remodeling, were observed in both autogenic and allogenic DDM/rhBMP-2. These might be a result of the exogenous rhBMP-2 targeting cells around the DDM particle.

It has been shown that rhBMP-2 needs to be carried by a biomaterial matrix to attain maximal efficacy. These matrices must possess adequate porosity to allow cell and blood vessel infiltration, appropriate mechanical stability to withstand compression and tension, biocompatibility, biodegradability, amenability to sterilization, adhesiveness to adjacent bone, affinity for BMPs, and the ability to retain the protein for a sufficient period of time so that it will be able to augment the repair process. The main role of the delivery system for rhBMP-2 is to retain the factor at the site for a prolonged period of time.

In 2007, FDA approved the use of rhBMP-2 soaked into ACSs in the oral and maxillofacial region for the purposes of sinus floor augmentation and extraction socket preservation. Successful studies in animals had paved the way for the testing of ACS loaded with rhBMP-2 in humans for maxillary sinus floor augmentation and alveolar ridge augmentation. In humans, Howell et al. and Boyne et al. reported that grafts with 0.43 mg/ml rhBMP-2 absorbed in ACSs were well tolerated locally and systemically with no adverse events in local ridge preservation and augmentation. Since then, the effectiveness of ACSs loaded with other BMPs and growth factors, such as transforming growth factor (TGF) and fibroblast growth factor (FGF), has also been examined.

Nevertheless, collagen has rather limited controlled release capacity, with most of the release occurring in the first day. Apart from its limited capacity to control rhBMP-2, the main limitations of collagen scaffolds derive from their poor mechanical properties.

In addition to the bone forming capacity of DDM, Ike et al. in 1998 and Murata in 2005 also examined the potential of using DDM as a BMP carrier. Ike et al. reported that exogenous rhBMP-2 adsorbed into partly demineralized dentin from a pulverized partly demineralized root (allogenic PDM/rhBMP-2) dose-dependently induced heterotopic bone formation and that PDM provided the minerals and the matrix for adsorption of rhBMP-2, proving that allogenic PDM can be as osteoinductive as autogenous bone. Murata demonstrated that human DDM particles are osteoinductive, insoluble collagenous matrices and DDM might be an effective rhBMP-2 carrier for bone engineering.

Kim et al. compared the release kinetics of rhBMP-2 loaded on three different scaffolds: tricalcium phosphate (TCP), inorganic bovine bone and human DDM. They also measured the osteonectin expression to determine the efficacy of rhBMP-2 carried by each scaffold. They concluded that human DDM powder might be an effective scaffold for rhBMP-2, as it displayed the highest release-to-loaded rhBMP-2 ratio, the lowest release speed and the highest expression to induce osteonectin expression, resulting in augmented mature bone formation. In 2005 and 2006, Um et al. reported that human DDM/rhBMP-2 induced a higher degree of bone formation than TCP when grafted in the dorsal muscle pouch of nude mice and rabbit calvarial defects, respectively. The histological observations of our study were consistent with those of Kim et al. and Um et al.
remodeling was taking place. The immune reaction to allogenic DDM, even if existent (no inflammatory cells were detected), did not seem to reduce rhBMP-2 activity.

In conclusion, the advantage of using the tissue between the implant cover screw and the gingiva as biopsy material is that, due to its anticipated poor blood supply, it simulates the conditions found in heterotopic sites more closely compared to samples taken from an orthotopic site. Thus, it allows for a better clinical evaluation of the reactions that take place in heterotopic sites. As this is only a preliminary report on the response to DDM/rhBMP-2, further studies are required to guarantee the safety and efficacy of DDM as a suitable carrier for rhBMP-2. Despite its limitations, this case report strongly suggests that allogenic and autogenous DDMs may be effective rhBMP-2 carriers.

Acknowledgements
This study was supported by a grant from the Korean Health Technology R&D Project received through the Korea Health Industry Development Institute (KHIDI), and funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI15C3136).

Conflict of Interest
The authors have declared that no conflict of interests exists.

References
