Original

Bone Regeneration by Demineralized Dentin Matrix in Skull Defects of Rats

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Abstract: Alveolar bone defects may result in functional and morphological abnormalities. In such cases, bone can be regenerated by grafting fresh autogenous bone into the defect. Subsequently, we focused on the dentin of third molars, which are usually discarded after extraction, or teeth removed for orthodontic therapy. As in bone, type I collagen is the main component of the organic matrix in dentin. Also, it has been proven that the organic matrix of dentin contains osteoinductive bone morphogenetic protein (BMP). It would be safe to perform grafting with the dentin of extracted teeth used as an autogenous bone substitute. Therefore, the objective of the present study was to reuse extracted teeth as a bone substitute material. Demineralized dentin matrix (DDM), an organic material derived from the dentine of bovine teeth, was grafted in rat skull defects to determine its bone regeneration ability and the possibility of its use as a bone substitute. In the present study, new bone formed from the defect margin in the control group, but in the graft group, new bone formation occurred from individual DDM granules within the defect, and not just from the margin. At Week 8, defect repair was limited in the control group. In contrast, most of the defect was covered by osseous tissue in the graft group. In conclusion, DDM may be a useful bone substitute that serves as a scaffold for bone regeneration by inducing a high level of new bone formation soon after surgery.

Key words: Bone regeneration, Demineralized dentin matrix, BMPs, Bone substitute, Micro CT

Introduction

Alveolar bone defects may result in functional and morphological abnormalities. In such cases, bone can be regenerated by grafting fresh autogenous bone into the bone defect. Fresh autogenous bone grafting is widely performed because it has no risk of infection or rejection, and favorable results have been obtained ¹⁾. However, harvesting autogenous bone has disadvantages such as surgical invasiveness, wound infection, chronic pain, nerve paralysis and scarring ²⁾. Also, the volume of bone that can be harvested is limited. Therefore, methods that use a bone substitute material instead of autogenous bone are being investigated.

At present, bone substitute materials consisting of natural bone tissue, such as allogeneic and xenogenic bone, are used for bone grafting. Hydroxyapatite, an artificial biomaterial, is also widely used because of its high tissue affinity and osteoconductive properties ³⁾. However, hydroxyapatite is nonresorbable and

remains in the body for a long period of time ⁴⁾. Hydroxyapatite acts as a scaffold for bone formation, but does not induce new bone tissue. Therefore, bone formation may be delayed ⁵⁾.

Subsequently, we focused on the dentin of third molars that are usually discarded after extraction or teeth removed for orthodontic therapy. As in bone, type I collagen is the main component—accounting for about 20% by weight—of the organic matrix in dentin. Also, it has been proven that the organic matrix in dentin contains osteoinductive bone morphogenetic protein (BMP) ⁶⁾. We have demonstrated that demineralized dentin matrix (DDM) acts as a scaffold for repair of articular cartilage defects ⁷⁾. Moreover, recent studies examine a multi-component technique consisting of a novel side population of multi-purpose stem cells cultured on demineralized bone and dentin matrix, for tissue engineering applications ⁸⁻¹³⁾. The dentin of extracted teeth used as an autogenous bone substitute would be safe for grafting. Therefore, the objective of the present study was to reuse extracted teeth as a bone substitute material.

DDM, an organic material derived from the dentine of bovine teeth, was grafted in rat skull defects to determine its bone regeneration ability and the possibility of its use as a bone

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Figure 1A. DDM powder following lyophilization

Figure 1B. SEM image of DDM powder following lyophilization. ($\times 100$)

substitute.

Materials and Methods

Animals

One hundred and twenty 5-week-old male Wistar rats (CLEA Japan, Inc., Tokyo, Japan) weighing about 100 g were divided into two groups: control group ($n = 60$) and graft group ($n = 60$). Each group was subdivided into the following five groups based on the time after surgery: Weeks 1, 2, 4, 6 and 8 ($n = 12$ each). The rats were maintained under the same conditions, and they had free access to food and water. The present study was conducted according to the Animal Experiment guidelines of Aichi Gakuin University School of Dentistry.

DDM preparation

The DDM used in the study was prepared from bovine lower anterior teeth by slightly modifying the method of Urist et al.¹⁴⁾. Bovine lower anterior teeth were extracted, and after removing the surrounding soft tissue and dental pulp, they were frozen at -80°C for 24 h. After complete demineralization (0.6 N HCl, 1 week), the teeth were washed using distilled water and defatted for 24 h (chloroform/methanol). The teeth were then lyophilized, and DDM was pulverized using FREEZE/MILL (SPEC Industries Inc., MA, USA). In order to make the particle size even, DDM with particle sizes of 250 - 500 μm was collected by sieving (Fig. 1)⁷⁾.

Surgical methods and procedures

The rats were made to inhale diethyl ether, and under general anesthesia, the head was shaved and the surgical field was sterilized. An incision was made on the head to expose the parietal bone covered by the periosteum. Using a bur and micromotor, a 4.3-mm round bone defect was prepared under continuous irrigation with sterile physiological saline. In the DDM group, 10 mg of DDM was placed in the defect, and in the control group, no material was placed in the defect. The periosteum was replaced, and the scalp was sutured using nylon and closed with surgical

adhesive.

For labeling the calcification rate, 0.1 ml/100 g of calcein was administered intraperitoneally 5 and 1 days before dissection. Calcein was dissolved in 2% sodium hydrogen carbonate solution to a concentration of 8 mg/kg (b.w.)/ml. The rats were sacrificed 1, 2, 4, 6 or 8 weeks after surgery and the skull was dissected.

Bone volume (BV) and Bone surface (BS) measurement

Each rat skull was analyzed using a micro CT machine (SMX-225CT, SHIMADZU CORPORATION, Kyoto, Japan). Using image analysis software (TRI/3D-BON, Ratoc System Engineering Co., Ltd., Tokyo, Japan), stereoscopic images were constructed and morphological analysis was conducted. Based on CT values, soft tissue, DDM and hard tissue were differentiated, and only hard tissue was subjected to morphological analysis to measure bone volume (BV) and bone surface (BS)¹⁵⁾.

Histological observation

Specimens for histological observation were prepared as follows. Tissue samples were demineralized using 10% EDTA and embedded in paraffin according to the conventional method. Then, 5- μm -thick serial sections were prepared, stained using hematoxylin-eosin, and observed under a light microscope (ECLIPSE E600, NIKON CORPORATION, Tokyo, Japan).

Calcification rate measurement

In order to measure calcification rate, GMA-embedded polished samples were prepared. Then the samples were observed under an epifluorescence microscope (ECLIPSE E600, Nikon), and calcein double lines labeled in the newly formed bone within the defect area were photographed under $\times 4$ magnification. Using the calcein-labeled samples, calcification rate was measured according to the conventional method^{17, 18)}.

Statistical analysis

Numerical data were expressed as mean $\pm$ standard deviation, and Student t-test was used for statistical analysis.

Figure 2. Stereoscopic micro CT images of bone defects.

Figure 3A. Fluorescent microscopy image at Week 4 for the control group (4×10). In the skull margin of the bone defect (d), slight calcein labeling is seen (e).

Figure 3B. Fluorescent microscopy image at Week 4 for the DDM graft group (4×10). From the skull margin of the bone defect (d) to the center, active calcein labeling is seen (e).

Results

Stereoscopic image analysis by micro CT

Figure 2 shows the chronological changes in bone defects as ascertained by micro CT stereoscopic images. In the control group at Week 1 and 2, slight formation of new bone-like tissue was seen from the margin of the bone defect. When compared to Weeks 1 and 2, the amount of new bone-like tissue in the margin was greater after Week 4. However even at Week 8, defect repair was limited.

On the other hand, in the graft group, new bone-like tissue was actively formed at Weeks 1 and 2, and new bone-like tissue was seen not only at the margin of the defect but also from different locations inside the defect simultaneously. After Week 4, the formation of new bone-like tissue was even more significant at the margin and center, and at Week 8, most of the defect was covered by osseous tissue.

Measurement of calcification rate

Figure 3 shows the fluorescent microscopic images at Week 4. In the control group, only a small amount of labeling was seen at

the defect margin at Week 4, but in the graft group, active labeling was seen from the defect margin to the center at Week 4.

Figure 4 shows the chronological changes in calcification rate at the defect. The chronological changes in calcification rate for the two groups were mostly comparable. In both groups, calcification rate significantly increased from Weeks 1 to 2 (*: $p < 0.05$) and from Weeks 2 to 4 (**: $p < 0.01$). However, from Weeks 4 to 6, the calcification rate significantly decreased (**: $p < 0.01$). No significant changes were seen from Weeks 6 to 8. No significant difference was seen in calcification rate between the two groups at any time period.

BS measurement

Figure 5 shows the chronological changes in BS at the bone defect. In the control group, BS markedly increased from Weeks 2 to 4. No significant changes were seen in BS from Weeks 1 to 2 or after Week 4, but a significant increase was observed from Weeks 2 to 4 (**: $p < 0.01$).

In the graft group, BS increased with time. No significant

Figure 4A. Comparison of changes in calcification rates in each week.

Figure 4B. Comparison of chronological changes in calcification rates of control and graft groups.

Figure 5A. Comparison of changes in BS in each week.

Figure 5B. Comparison of chronological changes in BS of control and graft groups.

A

B

Weeks after operation	1	2	4	6	8
Control Group	0.03±0.009	0.22±0.07	0.94±0.12	1.46±0.26	2.00±0.38
Graft Group	0.79±0.1	1.01±0.17	3.12±0.28	4.16±0.21	4.91±0.20

*** = p < 0.001

Figure 6A. Comparison of changes in BV in each week.

Figure 6B. Comparison of chronological changes in BV of control and graft groups.

A

B

Figure 7A. Histological image at Week 1 for the control group (HE, 4×10). In the skull margin of the bone defect (d), small amount of bone formation (c) is observed.

Figure 7B. Histological image at Week 8 for the control group (HE, 4×10). Bone formation (c) is limited to the defect margin (d).

A

B

Figure 8A. Histological image at Week 2 for the DDM graft group (HE, 4×10). DDM (a) formed a dense structure with narrow tubes, and formation of bone tissue (c) is seen on the surface of DDM (a).

Figure 8B. Histological image at Week 8 for the DDM graft group (HE, 4×10). Bone tissue (c) is formed at the defect margin (d) and around DDM (a) granules.

Figure 9A. Histological image at Week 1 for the DDM graft group (HE, 10×10). DDM (a) formed a dense structure with narrow tubes, and formation of osteoid tissue (b) is seen on the surface of DDM (a).

Figure 9B. Histological image at Week 2 for the DDM graft group (HE, 20×10). Formation of bone tissue (c) is seen on the surface of DDM (a).

Figure 9C. Histological image at Week 4 for the DDM graft group (HE, 20×10). Formation of bone tissue (c) bridging DDM (a) is seen.

Figure 9D. Histological image at Week 6 for the DDM graft group (HE, 10×10). Formation of bone tissue (c) surrounding DDM (a) is seen.

changes were seen in BS from Weeks 1 to 4, but a significant increase was seen after Week 4 (**: $p < 0.01$). A significant intergroup difference was seen at Weeks 1 and 2 (**: $p < 0.001$) and Week 8 (**: $p < 0.05$), but not at Weeks 4 and 6. Particularly at Weeks 1 and 2, the BS of the graft group was significantly greater than that of the control group.

BV measurement

Figure 6 shows the chronological changes in defect BV. In the control group, BV slightly increased with time. No significant change was seen in BV from Weeks 1 to 2 or after Week 4, but a significant increase was seen from Weeks 2 to 4 (*: $p < 0.05$). However, significant changes were seen from Weeks 4 to 8 (**: $p < 0.01$).

In the graft group, defect BV increased with time. No significant change was seen from Weeks 1 to 2, but significant changes were seen after Week 2 (**: $p < 0.01$). Comparing the two groups, the BV for the graft group was significantly greater at all time points (***: $p < 0.001$). When compared to the control group, the BV for the graft group was high soon after surgery,

and there was about 2.5-fold difference at Week 8.

Histological observation

Figures 7 and 8 show HE-stained images of both groups. In the control group, a small amount of bone formation was seen at the defect margin from Week 2, but the entire defect was filled with fibrous tissue, and even at Week 8, bone formation was limited to the defect margin (Fig. 7).

In the graft group, the grafted DDM formed a dense structure, and many dentinal tubules were seen. At Week 2, much fibrous tissue was seen among DDM granules, and bone tissue formation was seen not only at the defect margin, but also on the surface of the DDM granules. Also at Week 8, fibrous tissue was mostly absent, and bone tissue had formed at the defect margin and around DDM granules (Fig. 8).

Figure 9 shows enlarged HE images of the graft group. At Week 1, a small amount of osteoid tissue was seen on the surface of DDM (Fig. 9A). At Week 2, bone formation was partially seen on the surface of DDM (Fig. 9B). At Week 4, active bone formation bridging the DDM granules was seen, and the new bone was

trabecular in appearance (Fig. 9C). At Weeks 6 and 8, active bone formation was observed, and DDM granules were connected by osseous tissue (Fig. 9D).

Discussion

Experimental bone defect

When investigating bone repair in an experimental bone defect, it is necessary to suppress the natural osseous healing of the bone defect. One of the factors affecting bone repair is defect size¹⁸⁾, and a critical size defect is an experimental defect where natural osseous healing does not occur¹⁹⁾. Freeman and Turnbull prepared 2-mm bone defects in the temporal bone of albino Wistar rats weighing 500 g and reported that the defects were not repaired by new bone even after 12 weeks^{20, 21)}.

In the present study, a 4.3-mm bone defect involving the periosteum was prepared on the skull of each 5-week-old rat, and even at Week 8, the defect was not repaired, and only minimal new bone formation was seen at the defect margin. When compared to past studies, the defects in the present study were larger, and even after 8 weeks, the size of the defects did not decrease. Therefore, the effects of natural repair by new bone formation were minimal in the present study.

DDM

The weight ratio of the organic matrix in dentin is about 20%, and as in bone, type I collagen is the main component. The organic matrix includes osteoinductive bone morphogenetic protein (BMP)²²⁾. It has also been clarified that demineralized dentin originating from various mammals induces undifferentiated mesenchymal cells to differentiate into osteogenic cells and then into cartilage and bone²³⁾. Although Reddi documented osteoinduction with particle sizes of 74 to 420 μm ^{24, 25)}, our previous study demonstrated particle sizes of 250 to 500 μm were highly efficient in osteoinduction⁷⁾. In the present study, to ensure post-grafting stability, the particle size of DDM was set at 250 to 500 μm .

Bone generation by DDM grafting

In the present study, osteoid tissue formation was seen at the bone defect from Week 2 of DDM grafting, and osseous tissue formation was seen at Week 4. At Week 8, the bone defect was filled with osseous tissue. It has been reported that similar *in vivo* bone formation was seen with demineralized bone and DDM^{11, 26-28)}. The results of the present study showed that, like demineralized bone, DDM is capable of forming new bone.

With regards to the site of initiation of bone formation, micro CT findings showed that new bone formed from the defect margin in the control group, but in the graft group, new bone formation occurred simultaneously from different locations within the defect, not just from the margin. The BS in the graft group was greater than that in the control group soon after surgery. Also, histological

findings showed that bone formation advanced adjacent to DDM. No significant difference was seen in the calcification rate between the graft and control groups. Therefore, when grafting DDM granules in a bone defect, bone formation simultaneously occurs from individual DDM granules, and due to the subsequent increase in bone surface (BS) and bone volume (BV), bone repair advanced faster in the graft group when compared to the control group.

According to unpublished data from our laboratory, the bioactivity of BMP stored at room temperature for more than 10 years has been confirmed. Hence, teeth that are removed at a young age can be processed to prepare DDM, and DDM can be stored frozen using liquid nitrogen for long periods and later thawed to be used as a graft material. Because dentin is autogenous tissue, there is no risk of infection or immunogenic problems, and grafting is possible without invasive harvesting of autogenous bone. Furthermore, because third molars and teeth that are removed for orthodontic therapy are reused, grafting is possible without secondary invasion. Therefore, DDM has advantages such as high bioaffinity, easy harvesting, osteoinduction and no risk of infection, and can be considered as a safe grafting material that may be used instead of autogenous bone graft.

In 1991, a protein with BMP-like activity was isolated from human dentin, but the NH_2 -terminal amino acid sequence did not resemble that of the known BMP family²⁹⁾. Moreover, amelogenin-polypeptides with chondrogenic / osteogenic-inducing activity were isolated from normally processed bovine dentine in 1999³⁰⁾, but unrelated to therefore, has been unclear until now. We believe that BMP-like molecules and amelogenin-polypeptides might be synergistic factors involved in chondrogenic / osteogenic capability of dentin. Tissue engineering of new bone relies on the combination and application of osteoconductive, osteoinductive, and osteogenic elements. Natural scaffolds, such as demineralized bone and dentin matrix, contain collagenous networks with growth factors such as bone morphogenetic protein-2. Stem cells from readily available sources, including discarded adipose tissue, have the propensity to differentiate into bone. Recent studies examine a multi-component technique consisting of a novel side population of adipose stem cells cultured on demineralized bone and dentin matrix, for tissue engineering applications⁸⁻¹⁰⁾. In the present study, we investigated the extent to which DDM induces bone repair by implanting DDM derived from bovine teeth grafted in rat skull defects. The new bone formed from the defect margin in the control group, but in the graft group, new bone formation occurred from individual DDM granules within the defect, and not just from the margin. At Week 8, most of the defect was covered by osseous tissue in the graft group. In conclusion, DDM may be a useful bone substitute that serves as a scaffold for bone regeneration by inducing a high level of new bone formation soon after surgery.

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