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ORIGINAL ARTICLE

Comparison of Bone Regeneration Following Autogenous Bone Grafting and Dentin Transplantation

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SYNOPSIS

This study compared bone regeneration ability following non-demineralized dentin granule transplantation and autogenous bone grafting. A critical-sized bone defect was created at the center of the calvaria in rats, and autologous bone and dentin were transplanted. All specimens were evaluated microradiographically and histologically. Computed tomography (CT) analysis revealed no difference in the amount of hard tissue between the dentin-transplanted group and the autologous bone-grafted group 8 weeks after surgery. Tissue analysis revealed that the defect was almost entirely replaced with new bone in the autogastric bone-grafted group 8 weeks after surgery. About half of the dentin granules remained in the dentin-transplanted group, and the rest were replaced with autologous bone. These results suggest that dentin was slowly replaced by new bone while securing the space at the transplantation site, even after a long period of transplantation.

***Key words**: bone tissue engineering, autologous bone graft, dentin graft, CT analysis, rat calvarial defect*

INTRODUCTION

An artificial bone (*e.g.*, ceramics for biological use, β-tricalcium phosphate [TCP], and hydroxyapatite [HA]) lack osteoproliferation abilities and only show osteoconduction[1]. Autogenous bone grafting remains the gold standard for treatment of bone loss due to trauma, tumors, surgery, and periodontal diseases[2]. Nevertheless, it is widely accepted that the grafted bone often induces significant bone loss at a later stage in the recovery process[3]. There are some drawbacks with this technique, such as the limited availability of bone mass and marked absorption of the grafted bone[4,5].

Recent studies have reported that autogenous tooth bone graft material can provide results equivalent or supe-

rior to those achieved with other bone substitutes[6,7]. Teeth are known to be organic–inorganic hybrids composed of calcium phosphates with collagen and other organic compounds. Dentin, which is a component of teeth, comprises 65% inorganic matter and about 35% organic matter and moisture. Alveolar bone is composed of 65% inorganic matter and about 35% organic matter. Although dentin has a different morphology than bone, it is reported that the components are very similar[8]. The main constituents of inorganic substances are four types of calcium phosphate: HA, TCP, amorphous calcium phosphate (ACP), and octacalcium phosphate (OCP). These substances have osteoconductive potential. Furthermore, about 90% of the organic matter consists of collagen fibers (mainly type I collagen), and these fibers play an important role in osteoinduction in calcification. The remaining organic constituents have also been found to consist of bone growth factors, including bone morphogenetic proteins[9-11].

In this study, we focused on dentin, which is absorbed at a slower rate than autologous bone with the same composition and growth factors as autogenous bone, to cope with the problem of autogenous bone resorption after autogenous bone grafting. In this experiment, autologous bone or dentin was transplanted in the rat skull defect, and bone regeneration ability was evaluated and compared using histological examination and micro-computed tomography (CT).

MATERIALS AND METHODS

1. Preparation of graft dentin

The dentin granules were prepared by collecting teeth from a control group of rats euthanized by intraperitoneal overdose of pentobarbital. Mandibular middle incisor teeth were removed from the rats, and enamel and cementum were removed using a turbine. Soft tissue, such as tooth pulp, was removed with isopropanol, washed, and sterilized. Dry dentin was roughly crushed with a bone mill and formed into fine particles using a mortar and pestle. The dentin particles were sieved with a sieve machine to 75 to 500 μm, which is said to be the optimum range of sizes of the granule of the graft material [12].

2. Electron microscope photography

A field emission-scanning electron microscope (FE-SEM; S-4100, Hitachi High Technologies Corporation, Tokyo, Japan) was used to analyze particle size, pore distribution, and outer surface conditions. Before observation, samples were coated with platinum palladium using E-1030 (Hitachi High Technologies Corporation).

3. Surgical procedure

Male Sprague–Dawley rats (age, 8 weeks; weight, 300–350 g) were used for transplantation. The rats were anesthetized with a 30-mg/kg intraperitoneal injection of pentobarbital sodium (Somnopentyl; Kyoritsu Seiyaku, Tokyo, Japan); in addition, 0.2 mL of local anesthetic (1% xylocaine / epinephrine 1:100,000; Astra-Zeneca, Tokyo, Japan) was injected into the surgical sites before the start of surgery. The surgical areas were shaved and disinfected with povidone iodine (Isodine Surgical Scrub; Meiji, Tokyo, Japan). Skin incision and subperiosteal dissection were then performed. After the flap was raised, a critical-sized bone defect (diameter, 8 mm; depth, 1.0 mm) was created at the center of the skull using a bone trephine bur (external diameter, 9 mm) under running water. The rats were divided into 3 groups for the experiments: in one group, the defect was filled with autologous bone graft; in another, the defect was filled with dentin granules; and in the control group, the defect was not filled with any material. Finally, the flap was repositioned and sutured. The skin was sutured with 3-0 silk (Natsume Seisakusho Co., Tokyo, Japan). The

animals were euthanized at 4, 6, and 8 weeks after surgery (n = 3 at each time interval per group). All procedures in this study were approved by the Animal Experiment Committee of Osaka Dental University and conformed to the guidelines described in the Guiding Principles for the Use of Laboratory Animals (approval nos. 14-01001・15-07008).

4. Micro-CT imaging of animal specimens

The harvested cranial bone defects were imaged using high-resolution micro-CT (SMX-100CT SHIMADZU, Kyoto, Japan). Each specimen was fixed vertically by the sample holder and placed in the micro-CT specimen chamber. The scanner voltage was set at 34 kV and current at 30 mA with no aluminum filter in place. Serial coronally oriented tomograms were reconstructed from the raw images. The number of slices was set at 25 slices. High-resolution three- dimensional (3D) images were constructed using Ratoc System Engineering. The bone regenerated at the 8-mm defect was scored according to the criteria[3].

5. Bone volume and total volume analysis

New bone was measured using the TRI/3D-BON 3D analysis routine using the obtained CT values. In the 3D analysis, the total volume (TV, cm^3) and bone volume (BV, cm^3) were measured directly. The volumetric density was calculated as BV/TV (%).

6. X-ray diffraction analysis

Autologous bone and dentin were characterized using the powder X-ray diffraction system (XRD; XRD-6100, Shimadzu, Kyoto, Japan). XRD patterns were obtained under the following conditions: 40.0 kV, 30.0 mA, scan rate of 2 degrees/min with a step size of 0.05 degrees, 3–80 degrees. The crystal phase was characterized using the database from the International Centre for Diffraction Data.

7. Histological analysis

At the end of the observation period (4, 6, and 8 weeks after surgery), the animals were lethally exsanguinated and euthanized under intravenous anesthesia with sodium pentobarbital. For all cases, after observing the presence or absence of macroscopic abnormality in each organ and tissue, the surrounding vegetable surrounding organs and tissues (including the skull) were removed and fixedly stored in 10% neutral buffered formalin solution. After immobilization, acid demineralization was performed on the buried vegetable surrounding organs and tissues (including the skull) to prepare a paraffin block, and the skull-buried area was cut into coronal sections. Three sections 3 μm in thickness were produced at intervals of 800 μm in order from the cross-section after confirming the part where the end of the implanted plant was confirmed. Each section was subjected to hematoxylin and eosin staining, and subsequently examined. Tartrate-resistant acidic phosphatase (TRAP) staining was also performed in the dentin-transplanted group.

RESULT

1. Electron microscope images

Autologous bone (Fig. 1(A)) and dentin (Fig. 1(B)) were observed under an electron microscope. In autogenous bone, the material was finely divided into particles. The particles overlapped and formed a porous structure. Unlike autogenous bone, elongated particles were observed in dentin. Fibrous, overlapping particles and more pores were observed.

2. XRD analysis

XRD analysis of materials before and after surgery was performed. In the autologous bone-grafted group, the crystalline structures observed before

transplantation were similar to those observed after transplantation (Fig. 2(A)). In the dentin-transplanted group, the peak observed before transplantation displayed a sharp pattern, but the peak of the graft after transplantation was similar to that of autogenous bone (Fig. 2(B)).

3. Histological evaluation

1) 4-week postoperative group

No inflammatory cells were found in the autologous bone-grafted, dentin-transplanted, and control groups. In the autologous bone-grafted group, more than half of the autologous bones transplanted were substituted with new bone and many connective tissues were observed (Fig. 3(A)). In the dentin-transplanted group, many osteoblasts were found around the transplanted dentin and replaced with new bone. Although osteoclasts began to absorb dentinal granules, little replacement with new bone was observed and dentin granules remained (Fig. 3(B), Fig. 4). In the control group, connective tissue was found in the defective part (Fig. 3(C)).

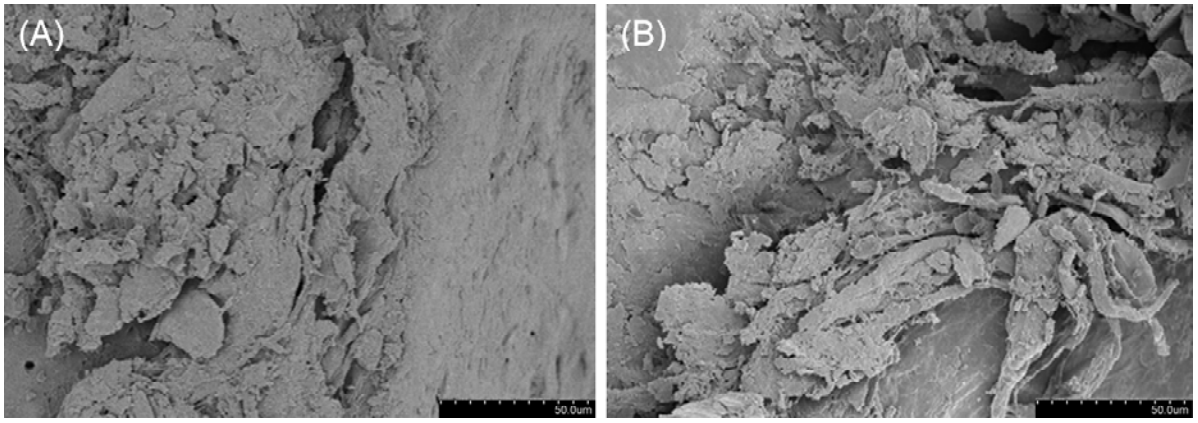

Fig 1 Scanning electron micrographs of the autologous bone-grafted group (A) and dentin granule-transplanted group (B). Original magnification: ×1000.

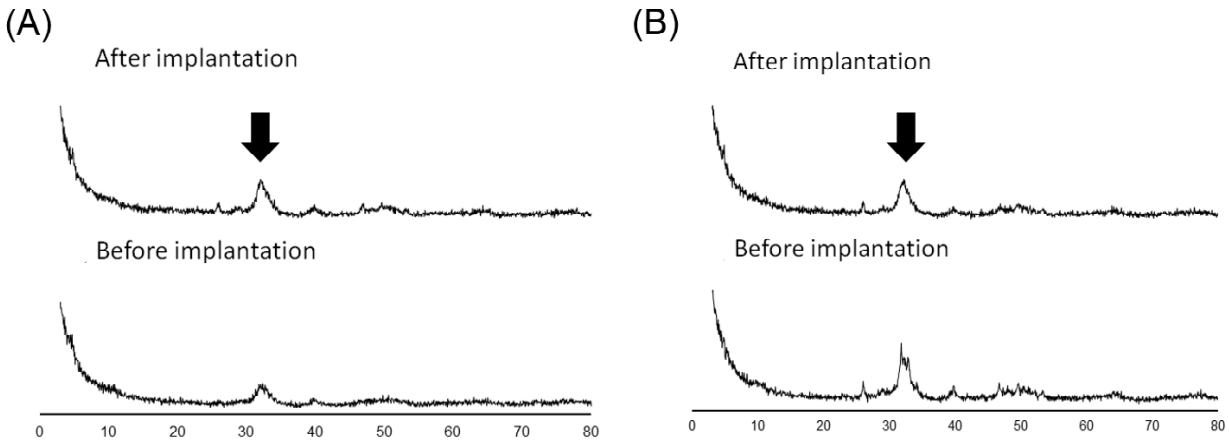

Fig 2 X-ray diffraction pattern of samples before (A) and after (B) the implantation.
(Left： Autologous bone-grafted group, Right： Dentin-transplanted group)
Arrow: specific peak of apatite at 32.9°

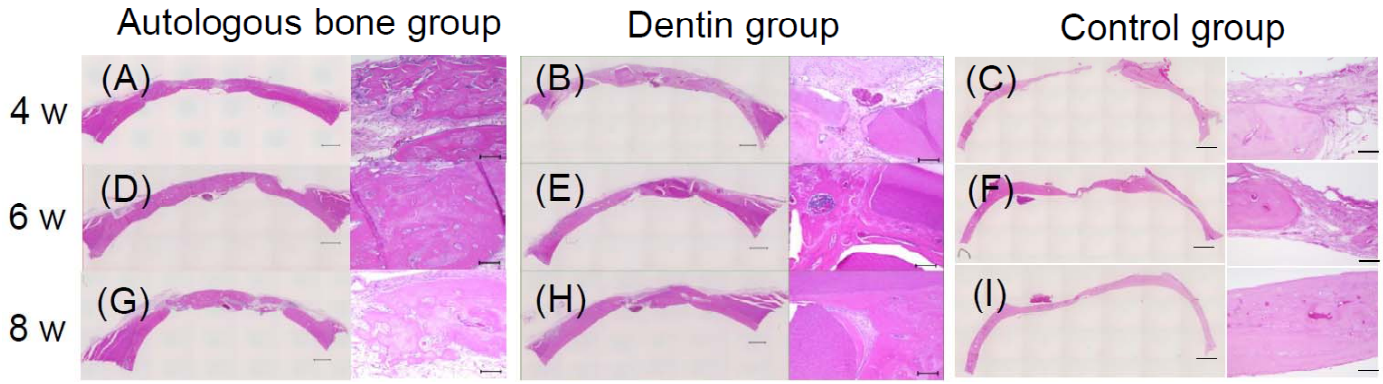

Fig 3 Histological micrographs of Hematoxylin and eosin-stained sections from each group at 4, 6, and 8 weeks after surgery. (A),(D),(G) : Autologous bone-grafted group, (B),(E),(H) : Dentin-transplanted group, (C),(F),(I) : Control group (Left: Original magnification: ×4; Right: Original magnification: ×20)

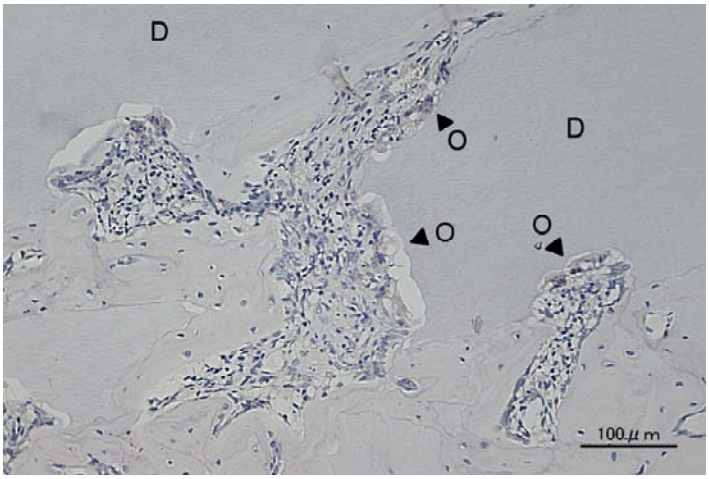

Fig 4 Histological micrographs of tartrate-resistant acidic phosphatase-stained sections from the dentin-transplanted group 4 weeks after surgery. (Original magnification: ×20) D : Dentin, O : Osteoclast.

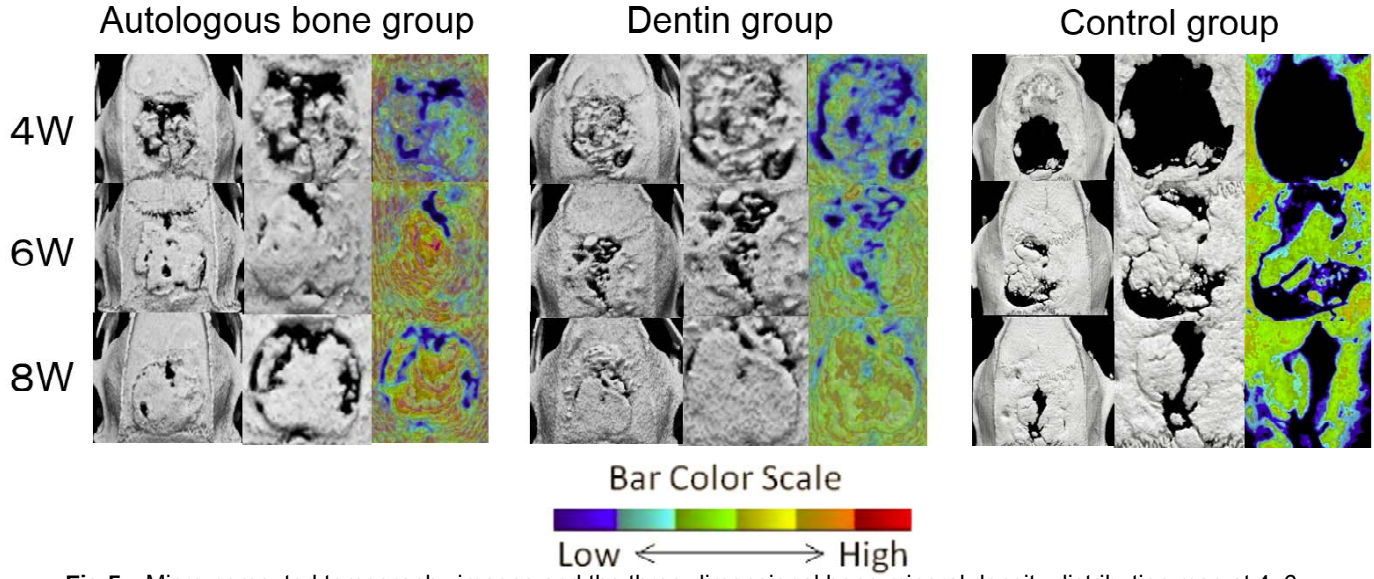

Fig 5 Micro-computed tomography images and the three-dimensional bone mineral density distribution map at 4, 6, and 8 weeks after surgery.
(A) : Autologous bone-grafted group, (B) : Dentin-transplanted group, (C): Control group

2) 6-week postoperative group
In the autologous bone-grafted group, most autologous bone transplanted was absorbed and replaced with new bone (Fig. 3(D)). In the dentin-transplanted group, most of the transplanted dentin granules remained, and replacement with new bone was not performed as much as in the autogenous bone-grafted group. (Fig. 3(E)) In the control group, new bone was formed from around the existing bone, but most of the connective tissue was found (Fig. 3(F)).

3) 8-week postoperative group
In the autologous bone-grafted group, almost all the transplanted autogenous bone was substituted by new bone (Fig. 3(G)). In the dentin-transplanted group, nearly half of the transplanted dentin granules were absorbed and replaced with new bone. It was confirmed that dentin granules were in the process of replacement of newly formed bone and that the formation of new bone continued, compared to the replacement of newly formed bone in autologous bone almost 8 weeks after surgery (Fig. 3(H)). In the control group, bone regeneration occurred from the side of the dura of the cerebrum, and newly formed bones were observed thinly as a whole (Fig. 3(I)).

4. 3D color-mapping (3D map) and micro-CT inspection

Fig. 5 shows the 3D-reconstructed micro-CT image and BMD distribution map. Each experimental group was analyzed at three timepoints of 4, 6, and 8 weeks after surgery, and the BMD of each group is shown in the 3D map. The color scale of BMD is expressed as red and orange for high BMD values, yellow and green for middle BMD values, and blue and purple for low BMD values. In the transplanted part of the autologous bone-grafted and dentin-transplanted groups 4 weeks postoperatively, the bone defect was filled with hard tissue in the new bone. In contrast, low to moderate BMD values (blue) were observed in the control group. The micro-CT image showed that no hard tissue was formed in the defect. Micro-CT images showed high BMD values (red–orange color) in both the autogenous bone-grafted and dentin-transplanted groups 6 weeks postoperatively. This indicated that the graft material was replaced with new bone and hard tissue was present in the grafted part. In the control group, hard tissues were formed from the existing bone margin towards the center of the defect, and the low to middle BMD values (blue–green color) was shown. The proportion of low BMD values (blue) increased in the autologous bone-

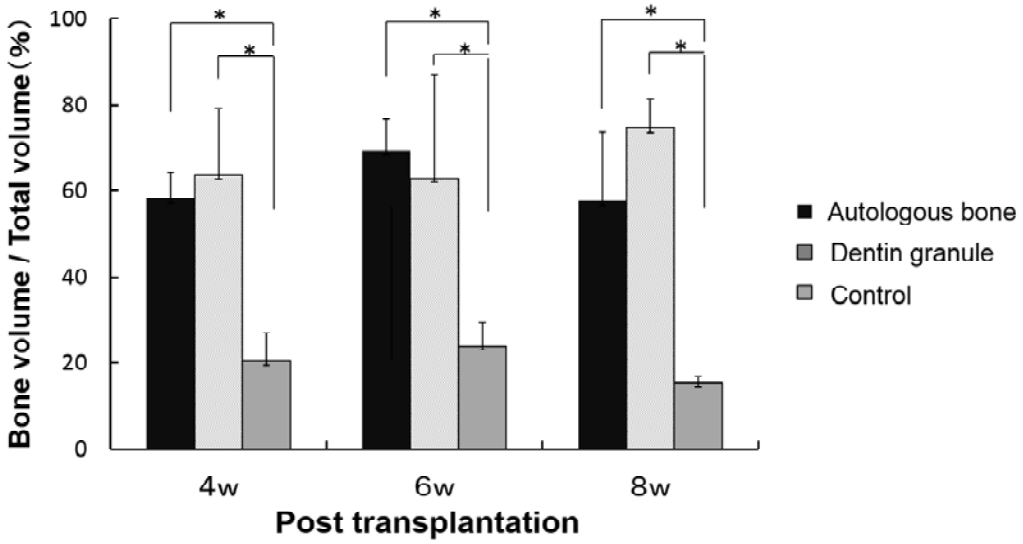

Fig 6 Analysis of bone volume to total volume ratio (BV/TV) (%) of each group to evaluate the quantity of new bone at 4, 6, and 8 weeks after surgery. ($*p < 0.05$)

grafted group 8 weeks postoperatively. This suggested a decrease in hard tissue at the graft site from 6 weeks to 8 weeks. In the dentin-transplanted group, the proportion of middle and high BMD values increased. In the control group, low BMD values were similar at 6 weeks.

5. BV/TV analysis

In the determination of new bone tissue using image analysis software (Ratok Engineering), the average volume ratio of new bone is obtained by dividing the BV by the TV. The regeneration rate of new bone relative to the total amount of bone loss was 58.2% for the autologous bone-grafted group and 63.8% for the dentin-transplanted group at 4 weeks, while it was 20.4% in the control group. At 6 weeks, the regeneration rate was 69.4% in the autologous bone-grafted group, 62.9% in the dentin-transplanted group, and 24.0% in the control group. At 8 weeks, the regeneration rate was 57.7% in the autologous bone-grafted group, 74.7% in the dentin-transplanted group, and 15.4% in the control group. There was a significant difference in the autogenous bone-grafted group and the dentin-transplanted group compared with the control group in each week (Fig. 6).

DISCUSSION

1. Transplantation of dentin granules

Experiments involving the transplantation of dental pulp stem cells are common, but comparisons of autologous dentin granule transplantation and autologous bone granule transplantation have not been reported under non-demineralized and same granule size conditions [13]. Therefore, we generated dentin granules and autologous bone granules under the same conditions. The size of the particles was not limited to a uniform particle size, but different sizes varying between 100 μm to 1000 μm were decided to be transplanted.

As reported by Dr. Sakai *et al*., small particles are replaced immediately in bone, and large particles secure space and promote blood vessel invasion [14]. Regarding demineralization of dentin particles, it has been reported that osteoblasts tend to invade owing to swelling of the dentinal tubules due to demineralization, but degeneration of collagen I and BMP protein growth factors occurs due to decalcification [15]. In this study, transplantation was performed without demineralization to make maximum use of growth factors.

2. XRD analysis

XRD analysis of grafts obtained from the dentin-transplanted group 8 weeks after transplantation was performed. The crystalline structure of the mineral components of rat autogenous bones displayed low crystallinity. The peak for dentin displayed a very sharp pattern, indicating that the tooth crown only partially contains enamel [16]. In the dentin-transplanted group, the linearity of XRD changed before and after transplantation, and a peak attributable to dentin was observed in the graft, but it became a straight line close to autogenous bone. This suggested that dentin remained 8 weeks after transplantation, suggesting that new bone is positively formed even after 8 weeks.

3. Histological evaluation and micro-CT evaluation

Based on histological findings and micro-CT evaluations, skeletal-like structures were observed in the dentin-transplanted and autologous bone-grafted groups compared with the control group. In the dentin-transplanted group, osteogenesis was observed at sites where existing osteoblasts, such as bone and dura, easily attach, and newly formed bone was observed at the center of the defect in the early stages. Osteoclasts were also observed adhered to the periphery of dentin. This characteristic suggests that dentin is an

excellent material as new bone is formed around the existing bone and the central part, compared with other scaffold materials from which new bone is formed from the periphery of the existing bone. These results show that the main components of the dentin inorganic substance are four calcium phosphates that have osteoconductive capacity: HA, TCP, ACP, and OCP. Furthermore, about 90% of the organic matter is composed of collagen fibers (mainly type I collagen), and these fibers are thought to play an important role in osteoinduction in calcification [17, 18]. In the autologous bone-grafted group 8 weeks after transplantation, almost all the transplanted bone was replaced with newly formed bone, but dentin granules remained in the dentin-transplanted group. From these results, it was suggested that after transplantation of dentin, the space of the transplanted site was secured for a long time while it was slowly replaced with new bone, suggesting that there is an advantage that is not present in autograft bone grafting. From the above, although the amount of new bone is not different between the dentin-transplanted and the autologous bone-grafted groups 8 weeks after transplantation, the dentin-transplanted group can retain a large amount of bone 8 weeks after transplantation

CONCLUSION

In this study, the wound healing process of the control, autologous bone-grafted, and dentin-transplanted groups at 4, 6, and 8 weeks after surgery was evaluated. Micro-CT findings revealed a significant difference in the bone mass ratio (BV/TV evaluation) between all groups. This is because the hard tissue of the autologous bone tissue decreased 8 weeks after the operation, but the hard tissue content of the dentin tissue was maintained a high level for a long time, even 8 weeks after the transplantation. From the above, it was confirmed that transplantation of dentin exhibits almost the same level of bone regeneration as autogenous bone grafting and is extremely useful as a long-term bone regenerating material.

REFERENCES

1) Nampo T, Watahiki J, Enomoto A, Taguchi T, Ono M, Nakano H, Yamamoto G, Irie T, Tachikawa T, Maki K. A new method for alveolar bone repair using extracted teeth for the graft material. J Periodontol 2010;81:1264-1272.
2) Mahuna PC, Gandhi HS, Farrokhyar F, Harnett BE, Dore EM, Roberts B. Anatomically safe and minimally invasive transcrestal technique for procurement of autogenous cancellous bone graft from the mid-iliac crest. Can J Surg 2011;54:327.
3) Li P, Honda Y, Arima Y, Yasui K, Inami K, Nishiura A, Hashimoto Y, Matsumoto N. Interferon-γ enhances the efficacy of autogenous bone grafts by inhibiting postoperative bone resorption in rat calvarial defects. J Prosthodont Res 2016;60:167-176.
4) Borstlap WA, Heidbuchel KLWM, Freihofer HPM, Kuijpers-Jagtman AM. Early secondary bone grafting of alveolar cleft defects. A comparison between chin and rib grafts. J Craniomaxillofac Surg 1990;18:201-205.
5) Zins JE, Whitaker LA. Membranous versus endochondral bone: Implications for craniofacial reconstruction. Plast Reconstr Surg 1983;72:778-784.
6) Jun SH, Ahn JS, Lee JI, Ahn KJ, Yun PY, Kim YK. A prospective study on the effectiveness of newly developed autogenous tooth bone graft material for sinus bone graft procedure. J Advanced Prosthodont 2014;6:528-538.
7) Kim YK, Yun PY, Um IW, Lee HJ, Yi YJ, Bae JH, Lee J. Alveolar ridge preservation of an extraction socket using autogenous tooth bone graft material for implant site development: Prospective case series. J Advanced Prosthodont 2014;6:521-527.
8) Uno H, Neda M, Taru H, Kohno N. Carbon and oxygen isotope analyses of bioapatite in dental enamel : Toward ecological restoration of fossil mammals. The Palaeontolog Soc Japan 2013;94: 33-43.
9) Bono N, Tarsini P, Candiani G. Demineralized dentin and enamel matrices as suitable substrates for bone regeneration. J applied biomater functio mater 2017;15:e236-e243.
10) Kamal M, Andersson L, Tolba R,

Al-Asfour A, Bartella AK, Gremse F, Rosenhain S, Hölzle F, Kessler P, Lethaus B. Bone regeneration using composite non-demineralized xenogenic dentin with beta-tricalcium phosphate in experimental alveolar cleft repair in a rabbit model. J Transl Med 2017;15:263.
11) Kim Y-K, Lee J, Um I-W, Kim K-W, Murata M, Akazawa T, Mitsugi M. Tooth-derived bone graft material. J Korean Assoc Oral and Maxillofac Surg 2013;39:103-111.
12) Koga T, Minamizato T, Kawai Y, Miura K-i, Takashi I, Nakatani Y, Sumita Y, Asahina I. Bone regeneration using dentin matrix depends on the degree of demineralization and particle size. PLoS One 2016;11:e0147235.
13) Zeng D, Zhang X, Wang X, Cao L, Zheng A, Du J, Li Y, Huang Q, Jiang X. Fabrication of large-pore mesoporous ca-si-based bioceramics for bone regeneration. Int j nanomed 2017;12: 8277.
14) Sakai K, Hashimoto Y, Baba S, Nishiura A, Matsumoto N. Effects on bone regeneration when collagen model polypeptides are combined with various sizes of alpha-tricalcium phosphate particles. Dent Mater J 2011;30: 913-922.
15) Sato D, Murata M, Sasaki T, Akazawa T, Arisue M. Bone Induction by Human Demineralized Dentin Matrix/ Recombinant Human BMP-2 Complex Implant. J Jap Soc Oral Implantol 2002;15: 403 411.
16) Kim YK, Kim SG, Yun PY, Yeo IS, Jin SC, Oh JS, Kim HJ, Yu SK, Lee SY, Kim JS, Um IW, Jeong MA, Kim GW. Autogenous teeth used for bone grafting: A comparison with traditional grafting materials. Oral Surg, Oral Med, Oral Path, Oral Radiol 2014;117:e39-e45.
17) Gao J, Symons A, Bartold P. Expression of transforming growth factor-beta 1 (TGF-β1) in the developing periodontium of rats. J Dent Res 1998;77:1708-1 716.
18) Schmidt-Schultz TH, Schultz M. Intact growth factors are conserved in the extracellular matrix of ancient human bone and teeth: a storehouse for the study of human evolution in health and disease. Biol Chem 2005;386:767-776.

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