Demineralized deciduous tooth as a source of bone graft material: its biological and physicochemical characteristics

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Objective. To examine structural and physicochemical characteristics of demineralized deciduous tooth powder (DDTP) in relation to demineralization time and to present potential of using DDTP as a bone graft material.

Study design. For structural and physicochemical analysis, scanning electron microscopy, inductively coupled plasma spectrometry, energy-dispersive X-ray analysis, X-ray diffraction analysis, differential scanning calorimetry, and Brunauer–Emmett–Teller surface area analysis were performed. In in vivo experiments, DDTP was grafted in 20 Sprague-Dawley rats’ calvarial defects, and radiographic and histologic examination and histomorphometric analysis were performed.

Results. In vitro studies confirmed physicochemical demands for collagen-based bone graft material, such as lowered calcium content, lowered crystallinity of hydroxyapatite, and exposed organic structures to demineralization. In vivo experiment indicated new bone formation in DDTP-grafted sites and gradual resorption of the grafted particles. Defect closure rate was significantly higher in the 8-week DDTP-grafted group compared with control (P < .05).

Conclusions. Deciduous teeth had structural and physicochemical characteristics suitable for grafting with appropriate demineralization. Bone healing was observed to have successfully occurred in DDTP-grafted sites. (Oral Surg Oral Med Oral Pathol Oral Radiol 2015;120:307-314)

Bone graft is commonly applied in dentistry to induce bone formation, resulting in increased bone thickness and quality that can support oral functions. In the field of pediatric dentistry, bone regeneration could be aimed at filling defects caused by trauma, tumor excision, or congenital defects such as cleft alveolus.

Different bone graft materials have been introduced over time. Among them, autogenous bone is considered to be the most ideal material because it promotes osteogenesis, osteoinduction, and osteoconduction. However, the drawback of using autogenous bone are secondary defects caused at the donor site, limited availability, increased operation time, and inevitable resorption of the graft. To overcome these limits, allogeneic bone, xenogeneic bone, and synthetic bone materials have been developed, but concerns about infection and the high cost associated with allogeneic or xenogeneic bone and low functional activities in synthetic bone limit their usage, especially in adolescents.

Recently, bone graft materials using permanent teeth have come to light, and clinical use and positive safety profile of this material have been confirmed by various studies. Tooth components have been found to be very similar to those of alveolar bone, allowing for novel bone graft materials utilizing the inorganic and organic components of extracted teeth. Studies have indicated that bone graft substitution involving dentin of animals and humans can be used to restore hard tissue defects in the oral and maxillofacial areas.

Although sufficient basis has been developed for using extracted teeth as bone graft materials, permanent teeth can only be used on an individual basis for patients who can provide an extracted tooth, and the number of intact teeth available for extraction is limited. If deciduous teeth collected at the time of natural exfoliation could be used for bone graft, the method would be applicable in many more cases with less cost and sacrifice. Chemical and mechanical properties of permanent and deciduous teeth are different, yet not much study has been performed to establish the possibility of using deciduous teeth as bone graft materials.

Statement of Clinical Relevance

In this study, we provide a basis for assessing the potential use of deciduous teeth as an alternative bone graft material by examining the structural and physicochemical characteristics of demineralized deciduous tooth powder (DDTP) and presenting histologic evidence of bone formation.
In this study, we present the structural and physicochemical characteristics of demineralized deciduous tooth powder (DDTP) in relation to demineralization time, and provide histologic evidence for the potential of using DDTP as a bone graft material.

MATERIALS AND METHODS
This study was approved by the Institutional Review Board of Ewha Womans University Mokdong Hospital (ECT 14-04 B-21).

Sample preparation
Exfoliated deciduous teeth were collected and washed with 4% hydrogen peroxide and 70% ethanol for 10 minutes each and stored at −20°C. All steps, including demineralization, washing, and sterilization, were processed in a vacuum-ultrasonic device (Vacuasonic System, CosmoBioMedicare Co., Seoul, Korea) following manufacturer’s instructions. Teeth were crushed into powders of 800-1000 μm and defatted with ether solution. Contaminants and remaining soft tissues were removed by 4% hydrogen peroxide and 70% ethanol. Demineralization using 0.6N hydrochloride was done for 0, 10, 15, 20, 25, 30, 60, and 90 minutes for in vitro analysis and 15 minutes for in vivo experiment. The sample was then washed with phosphate buffered saline (PBS), sterilized with peracetic acid–ethanol solution, and consecutively washed again with PBS and distilled water. The prepared sample was kept at 4°C before use.

Structural and physicochemical analysis
Scanning electron microscopy. A scanning electron microscope (S-4800, Hitachi, Ibaraki, Japan) was used to examine the surface structure of DDTP samples demineralized for 0, 10, 30, 60, and 90 minutes.

Inductively coupled plasma spectrometry. For quantitative analysis of calcium in DDTP, inductively coupled plasma spectrometry (ICP; Optima8300, PerkinElmer, MA, USA) was performed for samples demineralized for 0, 10, 30, 60, and 90 minutes.

Energy-dispersive X-ray analysis. Energy-dispersive X-ray analysis (EDS; X’PertPro, Panalytica, Almelo, Netherlands) was performed to examine the relative ratio of chemical elements such as calcium and phosphate (inorganic components) and carbon, nitrate, and oxygen (organic components) from the surface of DDTP samples demineralized for 0, 10, 30, 60, and 90 minutes.

X-ray diffraction analysis. To examine crystallinity change in inorganic components of DDTP according to demineralization time, individual samples were inserted into an analytical glass holder and diffraction patterns were obtained using an X-ray diffractometer (Ultima IV, Rigaku, Tokyo, Japan). After X-ray diffraction analysis (XRD) was performed with 0-, 10-, 30-, 60-, and 90-minute samples, additional XRD was performed with 15-, 20-, and 25-minute samples for more detailed observation between 10 and 30 minutes.

Differential scanning calorimetry. Differential scanning calorimetry (DSC; DSC131 evo, Setaram, Caluire, France) was performed at a constant rate of 2°C/min to examine the phase transition pattern. Phase transition pattern is related to structural stability, which again estimates crystallinity of the material. The analysis was performed with 15-, 20-, and 25-minute samples.

Brunauer–Emmett–Teller surface area analysis. Brunauer–Emmett–Teller (BET) analysis (Quadrasorb, Quantachrome Instruments Co., FL, USA) was performed to obtain surface area and examine pore size distribution in deciduous teeth that had undergone demineralization for 15, 20, and 25 minutes.

In vivo experiment
The experiment was performed in 20 Sprague-Dawley rats that weighed 300 grams (g). Animal selection and management, surgical protocols, and preparation procedures were approved by the Institutional Animal Care and Use Committee of Ewha Medical Center.

Anesthesia. Before surgery, 2% xylazine hydrochloride (Rumpun, Bayer Vetchem-Korea Ltd., Seoul, Korea) was mixed with tiletamine/zolazepam (Zoletil, Virbac S.A., Carros, France) at a ratio of 3:2 and administered by intraperitoneal injection at a dose of 0.01 mL/kg.

Cranial bone defect formation and DDTP graft. A full thickness skin flap was elevated from the midline of the calvarium. Two bicortical cranial bony defects of 5 mm diameter were created on both sides. Care was given to avoid trauma to the dura mater. The experimental side was grafted with DDTP and covered with a thin demineralized dentin sheet protecting the particle in situ, and the control side was left with no graft (Figure 1). Rats were allocated into 2 groups and allowed to heal for 2 (n = 10) or 8 (n = 10) weeks.

Necropsy and sample harvest. Rats were euthanized in a carbon dioxide chamber at 2 weeks or 8 weeks postoperatively. Bone specimens were harvested and fixed in 10% neutral formalin.

Histologic examination. The specimens were decalcified, embedded in paraffin, and sagitally sectioned passing the center of the defect site. Hematoxylin-eosin staining was performed, and histologic examination was done under the optical microscope (BX51, Olympus, Tokyo, Japan).

Histomorphometric analysis. For histomorphometric analysis, the images were captured with a charge-coupled device (CCD) camera (Eclipse 50i, Nikon, Tokyo, Japan) attached to the microscope and analyzed using Image-Pro Plus (Media Cybernetics, Silver Spring, Maryland, USA).
As an indication of bone regeneration capacity, defect closure rate was defined and calculated as follows (Figure 2):

\[
\text{Defect closure rate} = \frac{(\text{Original defect} - \text{Residual defect})}{\text{Original defect}}
\]

**Statistical analysis.** All in vitro analyses except for XRD were done 3 times. Statistical analysis was performed using SPSS Statistics Software Version 20 (IBM Corp., Armonk, NY, USA). Independent sample t test was used and was considered significant at the \( P < .05 \) level.

## RESULTS

**Structural and physicochemical analysis**

**Scanning electron micrograph.** The decalcification process exposed the dentinal tubules with increased size in relation to decalcification time (Figure 3). The sample that was decalcified for 10 minutes revealed a regular arrangement of dentinal tubules and a compact surface between the tubules that contained a mixture of calcium phosphate and dense collagen matrix. As demineralization reached 30 minutes, the surface developed a craterlike porous surface as a result of excess loss of mineralized components. Demineralization time exceeding 30 minutes resulted in total collapse of collagen and loss of structure in samples so that the dentinal tubules could not be found.

**Inductively coupled plasma spectrometry.** With increased decalcification time, the amount of calcium tended to decrease. Significant decrease occurred between 10 and 30 minutes, especially (Table I).

**Energy dispersive X-ray analysis.** As demineralization took place, the relative amount of inorganic elements (calcium and phosphorus) tended to decrease whereas that of organic elements (carbon, oxygen, and nitrogen) tended to increase. From the surface of the teeth, significant change in organic and inorganic components occurred at 10 minutes of demineralization (Table II).

**X-ray diffraction analysis.** A peak of the primary concern was hydroxyapatite (HA). The XRD peaks of HA in samples with different decalcification time were compared for crystallinity. Little change occurred between 0 and 10 minutes; peaks were narrow and displayed a very sharp pattern suggesting high-crystalline structure. Between 10 and 30 minutes, the height and sharpness of peaks started to decrease and widths increased as the peaks started to broaden out, suggesting a decrease in crystallinity. After 30 minutes of demineralization, the samples had changed to a totally amorphous structure (Figure 4).

**Differential scanning calorimetry.** Demineralization shifted thermogravimetry (Tg) toward lower temperature, which implies that demineralization causes loss of structural stability. Tg was 82°C before demineralization. After 15 minutes of demineralization, Tg of deciduous teeth dropped to 55°C, and further demineralization did not lower Tg significantly (Table III).

**BET surface area analysis.** Loss of calcium and degradation of the crystal structure of hydroxyapatite resulted in exposure of organic structures of dentin, which was expressed as increased surface area and porosity. Significant increase of surface area (increase of porosity) of DDTP was found between 15 and 20 minutes (Table IV).

**In vivo experiment**

**Histologic findings.** In 2 weeks, both groups had a slight amount of new bone formation at the peripheral area of the defect. At 2 weeks postoperative, the
The experimental group had favorable tissue response but no remarkable new bone formation. In the 8-week control group, a small amount of new bone formation had occurred from the periphery of the defect, but no bony tissue was observed at the central region of the defect, which was occupied with highly organized connective tissues. In the 8-week experimental group, a large amount of new bone formation had occurred from the defect periphery to the center, closing a large portion of the defect. New bone had also formed around the DDTP particles, forming a direct union with the grafted particles, which were going through gradual resorption (Figure 5).

**DISCUSSION**

Tooth is a composite of organic and inorganic components, containing minerals of the calcium phosphate range, collagen, and other noncollagenous proteins. The main inorganic component is HA, which is known to have an osteoconductive property that makes it a biocompatible bone graft material. The organic component mainly consists of type I collagen, which plays an important role in calcification, and diverse bone growth factors including bone morphogenetic proteins, LIM mineralization protein 1, and insulin-like...
growth factors are known to be present in teeth.\textsuperscript{1,7,11-15} This gives teeth an osteoinductive property.

Using deciduous teeth for graft material has many benefits over permanent teeth. With permanent teeth, using one’s own teeth for bone graft material is only possible for patients who have any intact teeth that can be extracted, such as third molars, teeth from orthodontic extraction cases, or periodontally involved teeth. On the other hand, deciduous teeth are naturally gained in all individuals during the mixed dentition age: No surgical trauma or stress is caused in the process, it is economical, and the number of deciduous teeth that can be gained from an individual is abundant. Patients who can potentially benefit from this procedure include those with cleft lip and palate who require treatment of alveolar clefts. In treating these patients, ideal timing for secondary alveolar bone grafting is at the mixed dentition age, before eruption of permanent canines.\textsuperscript{16} The timing coincides with the point at which most deciduous teeth are undergoing exfoliation. If the patient’s own deciduous teeth be collected during this time and used as the bone graft material for alveolar

### Table II. EDS results of each samples of different demineralization time. Significant increase of organic component and also decrease of inorganic component at surface of tooth occurs at 10 min of demineralization

<table>
<thead>
<tr>
<th></th>
<th>0 min</th>
<th>10 min</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
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<tbody>
<tr>
<td>C</td>
<td>22.34 ± 2.32</td>
<td>48.76 ± 3.02</td>
<td>49.74 ± 2.85</td>
<td>50.92 ± 0.95</td>
<td>46.8 ± 1.35</td>
</tr>
<tr>
<td>N</td>
<td>4.88 ± 1.34</td>
<td>23.79 ± 3.23</td>
<td>26.1 ± 2.78</td>
<td>26.02 ± 4.02</td>
<td>26.42 ± 3.34</td>
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<tr>
<td>O</td>
<td>47.36 ± 0.78</td>
<td>26.78 ± 2.36</td>
<td>24.12 ± 1.39</td>
<td>23.06 ± 3.21</td>
<td>26.71 ± 2.97</td>
</tr>
<tr>
<td>P</td>
<td>9.08 ± 0.56</td>
<td>0.2 ± 0.07</td>
<td>0.06 ± 0.03</td>
<td>0.02 ± 0.01</td>
<td>0.07 ± 0.04</td>
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<tr>
<td>Ca</td>
<td>16.35 ± 1.58</td>
<td>0.47 ± 0.12</td>
<td>0.02 ± 0.02</td>
<td>0.03 ± 0.02</td>
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Data are expressed in percent weight.

EDS, energy-dispersive X-ray analysis; C, carbon; N, nitrogen; O, oxygen; P, phosphorus; Ca, calcium.

Fig. 4. X-ray diffraction analysis graphs according to demineralization time of demineralized deciduous tooth powder. Narrow peaks with high-intensity, sharp patterns suggest high crystalline structure. Decrease in crystallinity occurs between 10 and 30 min, and the structure becomes amorphous in 30 min. A, 0 min; B, 10 min; C, 15 min; D, 30 min.
Table III. DSC results of deciduous teeth and permanent teeth according to demineralization time. Significant change of phase transition pattern occurs at 15 min, and it is assumed that significant loss of crystallinity and structural stability of hydroxyapatite started after 15 min. Change of $T_g$ is not significant for longer demineralization times.

<table>
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<tr>
<th>Decalcification time (min)</th>
<th>$T_g$ (°C)</th>
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<tr>
<td>0</td>
<td>82 ± 2.6</td>
</tr>
<tr>
<td>15</td>
<td>55 ± 1.8</td>
</tr>
<tr>
<td>20</td>
<td>55 ± 1.2</td>
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<tr>
<td>25</td>
<td>52 ± 2.1</td>
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*Table IV. Surface area of deciduous teeth according to demineralization time. Significant increase of surface area occurs at 20 min of demineralization.*

<table>
<thead>
<tr>
<th>Decalcification time (min)</th>
<th>Surface area (g/m²)</th>
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<tbody>
<tr>
<td>15</td>
<td>3.671 ± 0.082</td>
</tr>
<tr>
<td>20</td>
<td>6.795 ± 0.132</td>
</tr>
<tr>
<td>25</td>
<td>5.622 ± 0.115</td>
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On the other hand, if resorption of the grafted material occurs too fast, it may lower bone fusion or healing rates. Porosity is another factor that can influence new bone formation. It is known that porous HA is more resorbable and osteoconductive than dense HA. A porous surface provides increased surface for cell adhesions and allows bone ingrowth into the material. However, minimum stiffness should be maintained because osteogenic differentiation and activity has been reported to decrease on substrates of lower stiffness.

In this study using the vacuum-ultrasonic device, demineralization time of the DDTP was set at 15 minutes for in vivo study. Results of scanning electron microscopy, ICP, and EDS indicated that the relevant demineralization time for collagen-based bone graft material of deciduous tooth is between 10 and 30 minutes. Also, DSC, XRD, and BET provided further information that 15-20 minutes of demineralization prepared DDTP with appropriate mineral content, crystallinity of hydroxyapatite, and organic structure for partially mineralized organic graft material. Considering studies of other authors, it is interesting that deciduous tooth shows similar physico-chemical characteristics with permanent tooth except for faster demineralization and larger surface area (data are not shown). Further study should be done to clarify differences between permanent and deciduous tooth as bone graft substitutes.

In the in vivo experiment, two 5-mm calvarial defects were created bilaterally in the same rat to allot one to the experimental group and the other to the control group. This was to minimize any bias arising from different general conditions or healing capacities of individual rats. In a systemic review by Vajgel et al., it was suggested that calvarial defects with a diameter of 5 mm could be considered as a critical size defect. Our histologic examination on the DDTP-grafted areas revealed that by 8 weeks, successful bone healing and bone remodeling occurred within the defect and most DDTP particles underwent resorption, being replaced by new bone. A direct union was formed between the new bone and the grafted material, confirming that the bony remodeling was being achieved by osteoconduction.
Whether bone formation that occurred in our experiment involves osteoinduction has not been investigated. However, there are reported evidences that organic materials included in teeth promote osteoinduction. Induction of cartilage and bone by dentin demineralized in citric acid was reported in 1986, and several animal studies have reported that demineralized dentin induces differentiation of undifferentiated mesenchymal cells into osteoblasts and stimulates bone formation. In an experiment study by Inoue et al.,

\[\text{dentin was implanted into subcutaneous connective tissue, periodontal ligaments, femoral muscles, and rectus abdominis muscles of rats and it was reported that dentin induced chondrogenesis, which is the first step in endochondral bone formation. Ike and Urist implanted human demineralized dentin particles into femoral muscles of nude mice and observed bone induction. It has been suggested that the bone morphogenetic proteins within the demineralized dentin induce differentiation of undifferentiated mesenchymal cells into osteoblasts and other growth factors promote and maintain the osteogenic cascade.}\]

Limitations of our study include interrupted time interval between in vitro samples and a small number of samples in the in vivo study. Property changes examined at shorter intervals of demineralization time would give a better understanding and suggestion on the best demineralization time for DDTP. Also, a more in-depth analysis of the organic components of DDTP should be conducted. Excellent bone healing effect would be

\[\text{Fig. 5. Postoperative 8-week examination of control and experimental group (A); the defect of the experimental group (box) is completely closed with the newly formed bony bridge underlying the graft material (×40) and neither samples of the control group show complete bony closure (arrow). B, The remaining defect of the control group is filled with loose connective tissue (arrow). C, Most of the demineralized deciduous tooth powder (DDTP) is resorbed and replaced with new bone and the remnants of the DDTP are in direct union with the new bone (arrowhead). There is no abnormal immunologic response around the DDTP (×200). D, DDTP particle; NB, new bone.}\]

\[\text{Table V. Defect closure rate. Defect of the graft site is significantly closed with new bone at 8 weeks postoperative}\]

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>DDTP grafted</th>
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<tbody>
<tr>
<td>2 week</td>
<td>0.22 ± 0.09</td>
<td>0.38 ± 0.12</td>
</tr>
<tr>
<td>8 week</td>
<td>0.32 ± 0.11</td>
<td>0.78 ± 0.22*</td>
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\[\text{DDTP, demineralized deciduous tooth powder.}\]

*Statistically significant difference compared with sham (P < .05).

†Statistically significant difference compared with 2 week (P < .05).
expected if organic substances within the teeth are released slowly through an appropriate demineralization process and release of noncollagenous proteins within the teeth are encouraged to play their role.

CONCLUSIONS

Deciduous teeth have structural and physicochemical characteristics suitable for grafting with appropriate demineralization, with benefits over permanent teeth such as better availability and higher surface area for cell adhesions. Demineralization enhances the osteoinduction capacity of tooth material by exposing organic substances within the teeth to the surface, increasing porosity and surface area, and decreasing crystallinity. From rat calvarial defect models, bone healing as a result of osteoconductance was observed to have successfully occurred using DDTP.

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REFERENCES


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