Liquid Nitrogen–Treated Autogenous Dentin as Bone Substitute: An Experimental Study in a Rabbit Model

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Purpose: Different forms of dentin, including untreated, undemineralized, demineralized, boiled, or mixed with other materials, have been evaluated for efficacy as bone substitutes. However, the effects of application of liquid nitrogen–treated dentin for bone grafting remain unknown. The objective of this study was to chronologically evaluate bone healing following grafting with liquid nitrogen–treated dentin in a rabbit model. Materials and Methods: Autogenous dentin treated with liquid nitrogen at −196°C for 20 minutes was used. In 16 New Zealand White rabbits, a bone defect (5 mm in diameter) was created in each femur and randomly grafted with either autogenous dentin (experimental group) or autogenous bone grafts (positive control). In another four rabbits (negative control), a similar defect in each femur was left empty. The rabbits were sacrificed at 2, 4, 8, and 12 weeks. Explants of grafted sites were harvested for histologic and histomorphometric analysis. Results: At 2 and 4 weeks in both the experimental and positive control groups, accelerated formation of new bone was observed, which was undergoing remodeling at 8 and 12 weeks. The mean new bone score was higher in the experimental than in the negative control groups, but this was not statistically significant. Conclusion: The present results demonstrated that liquid nitrogen–treated autogenous dentin has both osteoconductive and osteoinductive properties and therefore has potential as a bone substitute. Oral Craniofac Tissue Eng 2012;2:215–220

Key words: autogenous bone graft, autogenous dentin graft, bone regeneration, bone substitute, liquid nitrogen

In recent years, there has been great interest in the development of bone substitutes that are capable of bone repair. In addition, with advancements in the field of implant dentistry, there is an increased need for research on bone substitutes. The ideal bone substitute should be osteoconductive, osteoinductive, bioresorbable, and biocompatible. Bone substitutes eliminate the morbidity associated with an autogenous donor site, reduce surgical complexity, decrease treatment costs, and improve satisfaction for patients. Clinically, autogenous bone is considered the gold standard for bone grafting because of its osteogenic potential, in addition to its osteoinductivity and osteoconductivity.1 Autogenous dentin also has osteoinductive and osteoconductive properties.2 The dentin’s extracellular matrix provides a scaffold for bone deposition with its most important component—hydroxyapatite—which is highly porous and appropriate for hard tissue regeneration.2 Bone and dentin are organic-inorganic hybrid composites of protein and mineral with superior strength, hardness, and fracture toughness. Dentin is also a calcified tissue, somewhat similar to bone. Dentin may be considered superior to other bone substitutes because, in addition to its...
hydroxyapatite mineral component, it contains more than 20% organic matrix, similar to that of bone. The noncollagen macromolecules represent a very important constituent of dentin’s organic matrix. These macromolecules comprise an extracellular mass of mineralized tissues, which can be classified into seven categories: phosphoproteins, proteoglycans, gamma-carboxy-glutamate (Gla) proteins, acid glycoproteins, growth factors, lipids, and serum proteins. The aim of the present study was to evaluate and compare the efficacy of liquid nitrogen–treated autogenous dentin, autogenous bone, and healing without grafting in enhancing bone regeneration.

**MATERIALS AND METHODS**

**Experimental Animals**
All experimental procedures were carried out in accordance with the ethical principles set forth by the Faculty of Medicine and with the approval of the Ethics Committee for Animal Experimentation (Animal Care and Use Committee, no. PM/12/08/2008/BKA[R]). Twenty New Zealand White male rabbits (average age, 6 months; average weight, 3 to 3.5 kg) were used. The animals were housed together under standard laboratory conditions and permitted free access to food and water throughout the experimental period.

**Preparation of Autogenous Dentin**
Each animal was premedicated according to its weight with an intramuscular injection of Kombitrim (1 mL/10 kg, sulfamethoxazole and trimethoprim; Kela Laboratoria) and meloxicam. General anesthesia was induced by an intramuscular injection of 30 mg/kg of ketamine (100 mg/mL, Troy Laboratories); xylazine (3 mg/kg of 20 mg/mL, Troy Laboratories) was added as an analgesic, sedative, and relaxant. Under sterile conditions, the maxillary and mandibular right central incisors were extracted (Figs 1a and 1b); all soft and hard deposits, anatomical crowns, and cementum were removed (Fig 1c). The remaining root dentin was placed inside a cryotube and frozen in liquid nitrogen (MVE Vapour Shippers, M02-SC4/3V). The freezing process involved 20 minutes in liquid nitrogen and 15 minutes at room temperature (Figs 1d and 1e). The
Dentin was then immersed in a sterile container filled with 0.2 mL of gentamycin and 5 mL of 70% ethyl alcohol for 30 to 60 minutes (Fig 1f). Prior to grafting, the dentin was ground into small particles (2 to 4 mm) with a mortar and pestle. The weight of the particles was approximately 70 mg, as measured with a digital scale.

**Surgery**

After the autogenous dentin was prepared and while the animals were still under general anesthesia, the approach to the shaft of the femur was done based on a procedure of Piermattei and Gordon. Under constant irrigation with saline (sodium chloride 0.9%, Surgical Sucker-OP Flex), two round bone defects, each 5 mm in diameter, were created using a low-speed trephine bur in the lower third of the shaft of the femur, just above the knee, bilaterally (Figs 2a and 2b). The harvested bone was kept and used as autogenous grafting material (Fig 2c). The first 16 rabbits were randomly divided into four groups according to experimental time (one in each group to be sacrificed at 2, 4, 8, and 12 weeks). One defect in each of these rabbits was randomly grafted with 70 mg autogenous dentin (experimental) (Figs 2d and 2e), and the other defect was grafted with 70 mg autogenous bone (positive control) (Fig 2f). In the last four rabbits, both of the defects (one in each femur) were left empty, with no graft material, and served as negative control. These rabbits were sacrificed at 2, 4, 8, and 12 weeks. The specimen samples were harvested from the grafted site with 2 mm of normal bone and immediately immersed in 4% buffered paraformaldehyde for at least 72 hours before decalcification with ethylenediaminetetraacetic acid. The processed tissues were embedded in paraffin; 4-µm-thick sections were obtained and stained with hematoxylin and eosin.

**Histomorphometric Methods**

Computer-assisted histomorphometry was performed to determine the volume of newly formed bone in each defect. The values for mean percentage of new bone were subjected to statistical analysis; a nonparametric two-tailed sample test (Wilcoxon rank test) was used to compare the experimental and positive control groups, and the Mann-Whitney test was used to compare the experimental and negative control groups. The threshold for significance was set at $P < .05$.  

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**Fig 2** Surgical procedures for grafting with liquid nitrogen–treated autogenous dentin (experimental) and autogenous bone (positive control).

**Fig 2a** The 5-mm-diameter cylindric defect was first outlined with a trephine bur.

**Fig 2b** The defect has been created.

**Fig 2c** Harvested bone specimens, kept to be used in the positive control sites.

**Fig 2d** Autogenous dentin particles after treatment with liquid nitrogen.

**Fig 2e** Defect grafted with the dentin particles.

**Fig 2f** Defect grafted with autogenous bone.
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RESU LTS

Clinical Findings
Postoperative healing was uneventful. The surgical defects treated with dentin (experimental) and autogenous bone (positive control) were partially filled with newly formed bone. In empty defect (negative control) sites, healing was seen only at the periphery. At 12 weeks, it was difficult to distinguish between the defect site and the host bone in both experimental and positive control sites, whereas in negative control sites, the healing was incomplete, with areas left unfilled by bone.

Microscopic Findings
At 2 weeks, newly formed woven bone was observed in both the experimental (Fig 3a) and positive control groups (Fig 3b). The newly formed bone trabeculae were fused to the dentin particles. Numerous osteoblasts were seen at the periphery of the newly formed bone in the experimental and positive control sites. This newly formed bone appeared denser and more compact in the autogenous dentin–treated defects than in the negative control defects (Fig 3c).

Histomorphometric Findings
At 2, 4, 8, and 12 weeks, there was no significant difference in the mean percentage of new bone between the experimental and positive control groups (P > .05) (Table 1). There was also no significant difference in mean new bone percentage between the experimental and negative control groups (P > .05) (Table 2). However, there was a clinically significantly greater amount of newly formed bone in the experimental group than in the negative control group.

DISCUSSION
Many investigations of bone grafting with autogenous demineralized dentin matrix have achieved excellent results, most likely because dentin contains bone...
morphogenetic proteins.6–10 A few studies that utilized pieces of teeth or pure dentin were unsuccessful, which may be a result of the methods used. These observations suggest that the method of preparation of bone grafting material is crucial to ensure effective bone healing. For this reason, a novel technique of treating dentin in liquid nitrogen prior to grafting was attempted in this experiment. The authors found that undemineralized dentin appeared to enable its own replacement/resorption by new bone formation, indirectly suggesting that dentin enhances bone regeneration at sites of bone healing.

The osteoconductive property of processed dentin has been evaluated previously.3 In that study, the dentin fragments were boiled in distilled water. Implantation of the processed dentin into rats’ femurs stimulated formation of new bone. Another study used undemineralized dentin; the particulate dentin was heated in a furnace at 950°C for 30 minutes, mixed with plaster of Paris, and compared with Bio-Oss (de-mineralized bovine bone matrix). They concluded that the combination of particulate dentin and plaster of Paris was a viable bone substitute.11 In the present study, the rabbits’ root dentin was successfully retrieved and preserved for in vivo use as a bone substitute. The autogenous dentin was treated with liquid nitrogen in a one-cycle protocol.12,13 This process did not compromise the osteoinductive and osteoconductive properties of dentin, as evidenced by the substantial amount of new bone that had formed at the experimental defect sites.

A defect size of 5 mm in diameter was chosen for this experiment because it was considered a reasonable size for efficient dentin grafting. It has been shown that 5 mm is not a critical-size defect,14 but creation of a defect larger than 5 mm in the rabbit femur increases the risk of fracture.

The present result was not in agreement with the results of Bang,15 who found that the bone induction process is slowed and the yield of new bone is low when the implanted dentin is undemineralized. However, the present study used a new method for treating autogenous dentin (liquid nitrogen), and satisfactory results were obtained.

### Table 1

<table>
<thead>
<tr>
<th>Time since surgery/graft type</th>
<th>Mean % new bone ± SD</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentin</td>
<td>22.65 ± 1.8138</td>
<td>−1.473</td>
<td>.141</td>
</tr>
<tr>
<td>Bone</td>
<td>24.25 ± 2.6901</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentin</td>
<td>31.25 ± 1.6862</td>
<td>−1.604</td>
<td>.109</td>
</tr>
<tr>
<td>Bone</td>
<td>32.92 ± 3.8922</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentin</td>
<td>30.85 ± 4.9359</td>
<td>−0.816</td>
<td>.440</td>
</tr>
<tr>
<td>Bone</td>
<td>32.25 ± 2.3473</td>
<td></td>
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<tr>
<td>12 wk</td>
<td></td>
<td></td>
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<tr>
<td>Dentin</td>
<td>39.47 ± 6.6274</td>
<td>−0.730</td>
<td>.465</td>
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<tr>
<td>Bone</td>
<td>41.15 ± 6.7993</td>
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</table>

n = 4 graft sites/group.  
SD = standard deviation.  
All comparisons not significant (nonparametric Wilcoxon signed rank test).

### Table 2

<table>
<thead>
<tr>
<th>Time since surgery/graft type</th>
<th>Mean % new bone ± SD</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentin</td>
<td>22.65 ± 1.8138</td>
<td>−1.852</td>
<td>.064</td>
</tr>
<tr>
<td>Empty</td>
<td>6.8 ± 0.7071</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentin</td>
<td>31.25 ± 1.6862</td>
<td>−1.852</td>
<td>.064</td>
</tr>
<tr>
<td>Empty</td>
<td>18.5 ± 0.7071</td>
<td></td>
<td></td>
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<tr>
<td>8 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentin</td>
<td>30.85 ± 4.9359</td>
<td>−0.235</td>
<td>.814</td>
</tr>
<tr>
<td>Empty</td>
<td>30.7 ± 2.8284</td>
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<tr>
<td>12 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentin</td>
<td>39.47 ± 6.6274</td>
<td>−1.852</td>
<td>.064</td>
</tr>
<tr>
<td>Empty</td>
<td>27.5 ± 0.2828</td>
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Dentin group: four defects/group; negative control group: two defects/group.  
SD = standard deviation.  
All comparisons not significant (nonparametric Mann-Whitney test).
In the present study, the clinical results demonstrated that the liquid nitrogen–treated autogenous dentin accelerated bone regeneration in bone defects in a manner similar to that of autogenous bone grafts. These observations suggest that dentin prepared in this form could be used as a bone substitute. The autogenous dentin and bone graft particles were partially resorbed and replaced by newly formed bone. This phenomenon indicated that the treated dentin used in the study has the properties of an ideal bone substitute. These include biocompatibility, bioresorbability, and gradual replacement that begins with fusion between the newly formed bone and graft particles. The early presence of a large quantity of osteoblasts along the periphery of newly formed bone at 2 weeks in experimental sites indicated that the liquid nitrogen–treated autogenous dentin matrix induced deposition and migration of osteogenic cells, as well as differentiation of the undifferentiated mesenchymal cells into osteoblast cells. In addition to osteoinduction, dentin matrix has osteoconductive properties characterized by bone growth via apposition from the surrounding bone. This process provides a scaffold for new bone formation.

Based on the histologic and statistical findings, there was no significant difference in the quantity of new bone formation between the experimental and positive control groups in all experimental periods. There was also no statistically significant difference in new bone formation between the experimental and negative control groups at all experimental times because the sample size in the negative control groups was relatively small; however, clinically, there was a significant difference in new bone formation between the experimental and negative control groups at 2, 4, and 12 weeks.

The present results confirmed that liquid nitrogen–treated autogenous dentin has bone regeneration properties comparable to those of autogenous bone. This finding opens up a new avenue to explore the use of autogenous dentin prepared from clinically sound human teeth extracted for orthodontic reasons and/or surgical impaction. Furthermore, the materials used in the treatment of dentin with liquid nitrogen are uncomplicated and inexpensive. The present results indicate that the treated autogenous dentin graft could be used as a bone substitute for enhancing bone regeneration.

ACKNOWLEDGMENTS

This study was supported by a grant from University of Malaya (project no. PS072/2009A). None of the authors have any conflicts of interest.

REFERENCES
