Expression of Mineralization Markers during Pulp Response to Biodentine and Mineral Trioxide Aggregate

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Abstract

Introduction: The purpose of this study was to compare the cell viability of dental pulp cells treated with Biodentine (Septodont, Saint-Maur, France) and mineral trioxide aggregate (MTA) and the *in vitro* and *in vivo* expression of mineralization markers induced by the 2 materials. **Methods:** Human dental pulp cells isolated from 6 permanent teeth were stimulated with Biodentine and MTA extracts. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, and quantitative reverse-transcriptase polymerase chain reaction was used to determine the expression of mineralization markers. Specimens of teeth from dogs treated with Biodentine and MTA after pulpotomy were used to determine the presence of osteopontin and alkaline phosphatase by immunohistochemistry and runt-related transcription factor 2 by immunofluorescence. **Results:** No significant differences in cell viability were found between MTA and Biodentine extracts and controls after 24 and 48 hours ($P > .05$). After 48 hours, osteopontin (*SPP1*), alkaline phosphatase (*ALP*), and runt-related transcription factor 2 (*RUNX2*) expression was higher in MTA and Biodentine than in controls ($P < .05$). Osteopontin staining was more intense and spread over a greater number of areas in Biodentine than in MTA samples ($P < .0001$). Alkaline phosphatase staining of a mineralized tissue bridge was significantly different between materials ($P < .0001$), but no difference in alkaline phosphatase staining of pulp tissue was found between MTA and Biodentine ($P = .2$). Also, no significant difference in the number of cells labeled for runt-related transcription factor 2 by immunofluorescence was observed between materials ($P > .05$). **Conclusions:** Biodentine stimulated similar markers as MTA, but staining was more intense and spread over a larger area of the pulp tissue. *(J Endod 2016;42:596–603)*

Key Words

Biodentine, dental pulp, mineralization, mineral trioxide aggregate, pulpotomy

Mineral trioxide aggregate (MTA) (ProRoot MTA; Dentsply Tulsa Dental, Johnson City, TN) has been extensively used in endodontics to protect pulp tissue after pulpotomy for carious and traumatic exposures. Biodentine (Septodont, Saint-Maur, France) is a novel root-end filling material, which is a powder consisting mainly of tricalcium silicate (Ca_3SiO_5), zirconium oxide, and calcium carbonate and the mixing liquid is composed of water, calcium chloride, and a hydrosoluble polymer (1). Similar to MTA, Biodentine has been shown to release calcium hydroxide (2), induce reparative dentin synthesis (3), and have antibacterial activity (4). Biodentine offers an additional advantage over MTA in that it can be used as a temporary enamel substitute and permanent dentin substitute (5). Because this new material comes in contact with both pulp and periapical tissues, biocompatibility is crucial (6).

Biodentine has shown tissue compatibility and exhibited tissue bridge formation similar to that of MTA (7). Human clinical cases using Biodentine for conservative and radical endodontic treatments showed satisfactory results regarding the induction of mineralization (8–10). However, the differentiation of pulp cells into odontoblastlike cells (11) and the mineralization (3, 12) induced by this material are not fully known.

Osteopontin (gene name *SPP1*), alkaline phosphatase (gene name *ALPL*), bone sialoprotein (gene name *IBSP*), dentin sialophosphoprotein (gene name *DSPP*), and dentin matrix protein 1 (gene name *DMP1*) have been used as markers of differentiation in osteoblasts and odontoblasts to investigate the mechanisms of mineralization (13–16). Alkaline phosphatase is found in the early stages of osteoblast and odontoblast differentiation and provides inorganic phosphate to form hydroxyapatite (ie, the first step of mineralization) (17). Osteopontin is an extracellular matrix acidic glycoprotein that has been implicated in many physiological and pathological events, including the maintenance or reconfiguration of tissue integrity during inflammatory processes (18). The runt-related transcription factor 2 (gene name *RUNX2*) has been linked to bone and dental mineralization and plays a primary role in the differentiation of osteoblasts and homeostasis of mineralized tissues (19, 20).

However, further *in vivo* studies with Biodentine are needed to elucidate the mechanism of action for biostimulation because differences have been reported between Biodentine and MTA. Thus, this study aimed to compare the cell viability of dental pulp cells treated with Biodentine and MTA and the expression of mineralization markers *in vitro* and *in vivo* induced by both materials.

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Materials and Methods

Explant Cell Culture

Teeth extracted for orthodontic reasons were obtained after informed patient consent (n = 6) and under institutional review board approval (process # 25315013.0.0000.5419). Human dental pulp cells were isolated from collected teeth and grown in medium consisting of Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and amphotericin. Cells from the third through fifth passages were used for experimentation.

Preparation of Materials

This study is in accordance with ISO 10993-5:2009 (21). The extraction method was used to put the materials (Table 1) in contact with the cells tested. Two cylindric matrices 10 mm in diameter and 5 mm in height were prepared from metallic matrix bands. The materials were mixed according to the manufacturer's instructions on a sterile glass slab in a laminar flow cabinet and introduced into the sterile matrices using sterile instruments for a volume of 47.5 mm^3.

The material prepared was kept in the laminar flow cabinet under ultraviolet light for 1 hour from the initial setting and then placed separately in polypropylene tubes with 5 mL incomplete DMEM medium and stored refrigerated for 72 hours before the experiment. The remaining material was discarded; the extracts were filtered, and 1:10 and 1:100 serial dilutions were prepared from the initial extracts (1:1). The 1:1, 1:10, and 1:100 solutions were stored refrigerated until use.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium Bromide Assay: Cell Viability

1×10^5 cells/well were plated in a 96-well plate, preincubated for 12 hours in serum-free media, and then treated with different MTA and Biodentine extracts for 24 and 48 hours. A tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (0.5 mg/mL, Sigma M-5655 MTT Colorimetric Assay; Sigma-Aldrich, St Louis, MO) was added to each well and incubated at 37°C for 3 hours in the dark. The absorbance in each well was determined at 570 nm using a microplate reader (μQuanti; Bio-tek Instruments, Inc, Winooski, VT). Cell viability in each group was expressed as a percentage and compared with the control group (culture medium). Data were analyzed using 1-way analysis of variance followed by the Bonferroni post test (α = 0.05).

Quantitative Reverse-transcriptase Polymerase Chain Reaction: Expression of Mineralization-related Genes

SPP1, *IBSP*, *DSPP*, *ALPL*, *DMP1*, and *RUNX2* messenger RNA levels were assayed by quantitative reverse-transcriptase chain reaction using fluorescent markers. After stimulation with MTA and Biodentine extracts, cells were harvested for the extraction of total RNA by the column method using thiocyanate guanidine (RNEasy; Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. Total RNA was estimated using 2 μL of each sample with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE).

Next, reverse transcription was performed for complementary DNA synthesis followed by polymerase chain reaction. Primers and probes for *SPP1* (Hs00959010_m1), *IBSP* (Hs00173720_m1), *DSPP* (Hs00171962_m1), *ALPL* (Hs01029144_m1), *RUNX2* (Hs00231692_m1), and *DMP1* (Hs01009391_g1) were obtained commercially and are proprietary; thus, sequences are not available (TaqMan Gene Expression Assay; Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (Hs 02758991_g1) and beta-actin (Hs 01060665_g1) were used as reference genes. Quantitative reverse-transcriptase polymerase chain reactions were performed in duplicate using a StepOne Plus real-time PCR system (Applied Biosystems).

Amplification was performed under the following conditions: activation of AmpliTaq Gold Enzyme (Applied Biosystems) at 95°C for 2 minutes followed by 40 cycles at 95°C for 1 second for DNA denaturation and 60°C for 20 seconds for primer annealing and polymerization. The results were analyzed based on cycle threshold values. Distilled deionized water was used as a negative control of each primer probe pair. Relative expression was calculated based on the equation $2^{-\Delta\Delta Ct}$.

Relative messenger RNA expression was compared among the groups using 1-way analysis of variance followed by the Tukey test (α = 0.05).

Acquisition of Slides after *In Vivo* Pulpotomy

The slides used in this section were obtained from the specimen database at the Children's Clinic Department, School of Dentistry of Ribeirão Preto, University of São Paulo, Brazil, in accordance with ISO 7405:2008 (22). This study was approved by the Institutional Animal Research Ethics Committee (process #2013.1.643.58.5).

In total, 87 specimens of the upper second and third premolars and the lower second, third, and fourth premolars from four 12-month-old male and female Beagle dogs treated with MTA (n = 35) and Biodentine (n = 52) were evaluated 120 days after pulpotomy.

Immunohistochemistry: *In Vivo* Osteopontin and Alkaline Phosphatase Staining

To determine the expression of mineralization-related markers, immunohistochemistry assays for osteopontin and alkaline phosphatase were conducted in the Biodentine (n = 46) and MTA (n = 29) groups.

The slides were deparaffinized, hydrated in a decreasing ethanol series, and kept in phosphate-buffered saline (PBS). Next, tissue sections were microwaved (7 × 12 seconds at 2-minute intervals) with sodium citrate buffer (pH = 6.0) for antigen retrieval. After temperature stabilization, the slides were washed with PBS (3×) for 5 minutes, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 40 minutes. Slides were further washed with PBS (3×) for 5 minutes, and nonspecific binding sites were blocked with 5%

TABLE 1. Composition and Manufacturer (city and country) of Materials Evaluated

Brand name	Material	Manufacturer
Biodentine	Powder: tricalcium silicate, dicalcium silicate, calcium carbonate, calcium oxide, iron oxide, zirconium oxide Liquid: calcium chloride, hydrosoluble polymer	Septodont (Saint Maurdes Fosses, France)
ProRoot MTA White	Powder: Portland cement (75%), bismuth oxide (20%), calcium sulfate dihydrate (5%), tricalcium silicate, dicalcium silicate, tricalcium aluminate, tetracalcium aluminoferrite Liquid: water	Dentsply Tulsa Dental (Johnson City, TN)