

# The Effect of Mineral Trioxide Aggregate on the Mineralization Ability of Rat Dental Pulp Cells: An *In Vitro* Study

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## Abstract

The aim of this study was to investigate the effect of mineral trioxide aggregate (MTA) on cell viability and mineralization ability of rat dental pulp cells. The pulp capping materials, such as MTA, Dycal (Dentsply Caulk, Milford, DE), and Superbond C&B (SB; Sun Medical, Shiga, Japan) were placed on transwell inserts and cultured with rat dental pulp cells. MTA and SB exhibited no cytotoxicity, whereas almost all cells had died after 72 hours of culture with Dycal. MTA significantly stimulated mineralization by 60% compared with the control. MTA and Dycal significantly upregulated by two-fold the level of bone morphogenetic protein (BMP)-2 messenger RNA expression compared with the control. Furthermore, MTA increased BMP-2 protein production by about 40%, whereas Dycal significantly reduced it. Although MTA and Dycal increased the concentration of extracellular calcium by about 0.4 mmol/L, SB had no effect. These results suggest that BMP-2 may play an important role in mineralization stimulated by MTA. (*J Endod* 2008;34:1057–1060)

## Key Words

Bone morphogenetic protein, cytotoxicity, dental pulp cells, extracellular calcium, mineral trioxide aggregate, mineralization

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The repair process after direct pulp capping requires dentin bridge formation on exposed pulp surfaces (1). Calcium hydroxide has been conventionally used but poses problems by causing persistent inflammation of the dental pulp and forming porous dentin bridges (2). On the other hand, the application of adhesive resin cements such as Superbond C&B (Sun Medical, Shiga, Japan) has been attempted (3). However, incomplete hard-tissue formation has been observed, and no long-term clinical results of treatment are available.

Mineral trioxide aggregate (MTA) is a relatively new material that has a wide range of applications, including pulp capping (4, 5), root perforation repair (6–8), and root-end filling (9–12). Direct pulp-capping experiments have reported that MTA induced the formation of dentin bridges with little or no inflammation (4, 5). In addition, MTA has been extensively evaluated for cytotoxicity not only *in vivo* but also *in vitro* and has been reported to be less cytotoxic than other root-end-filling materials (13–15) and other endodontic materials (16). Recently, Tani-Ishii et al. (17) reported that MTA upregulated the expression of type I collagen and osteocalcin in osteoblasts after 24 hours. Although a number of studies have been performed using MTA both *in vitro* and *in vivo*, the precise mechanism of dentin bridge formation after exposure to MTA has not yet been fully elucidated.

Bone morphogenetic proteins (BMPs) have been shown to perform important functions in bone and cartilage formation (18). BMP-2 is expressed along with its receptor in human dental pulp (19). It is also known that recombinant human BMP-2 induces dentin bridge formation on canine amputated pulp (20). We previously reported that BMP-2 accelerated the differentiation of human pulp cells into odontoblasts but did not affect cell proliferation (21). Thus, we hypothesized that BMP-2 is involved in MTA-induced mineralization.

In this study, we aimed to investigate the effect of MTA on cytotoxicity and mineralization in rat dental pulp cells. Furthermore, we examined the effect of MTA on BMP-2 messenger RNA (mRNA) expression and its protein production.

## Materials and Methods

### Cells and Cell-culture Condition

All animal protocols were approved by the Institutional Animal Care and Use Committee of the Health Sciences University of Hokkaido, and experiments were performed in accordance with the university's guidelines for animal experimentation. Dental pulp cells were isolated from the incisors of Wistar rats (6 females, 5 weeks old) as previously described (22). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (Sigma), 10,000 U/mL penicillin (GIBCO; Invitrogen, Grand Island, NY), and 10 mg/mL streptomycin (GIBCO) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. For mineralization experiments, cells were cultured in 50 μg/mL ascorbic acid (AA) and 10 mmol/L β-glycerophosphate (β-GP)-containing media.

### The Preparation of Test Materials

The test materials used in this study were MTA (ProRoot MTA; Dentsply Tulsa Dental, Johnson City, TN), adhesive resin cement (Superbond C&B), and calcium hydroxide-containing cement (Dycal; Dentsply Caulk, Milford, DE). These materials were

prepared according to the manufacturers' instructions. Each sample (diameter, 6 mm; thickness, 3 mm) was allowed to set for 24 hours at 37°C in 100% humidity. Rat dental pulp cells ( $1 \times 10^5$  cells/well) were seeded in 24-well plates containing 2 mL of culture medium and allowed to attach overnight. The test materials were placed on the bottoms of 6.5-mm diameter transwell inserts (Falcon, Franklin Lakes, NJ), which fit into the wells of a 24-well cell culture plate. The transwells contained permeable membranes (0.4  $\mu$ m pore size) and were used to prevent direct physical interaction between cells and the specimens while allowing for soluble compounds from the specimens to reach the cells. The transwells, each with a specimen that has been set for 24 hours, were placed over the 24-well plates containing cells and then were treated for 12 days. Culture media were changed every 3 days. Cells cultured with empty transwells served as the negative control.

**The Cell Viability Assay**

The cells were cultured in DMEM for 5, 10, 24, and 72 hours with each material as described previously. Cell viability was determined by a modified 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (WST-8 assay; Dojindo, Kumamoto, Japan), and data are presented as a percentage of viability values seen under control culture conditions. The assay is based on the cleavage of tetrazolium salt WST-8 to formazan by cellular mitochondrial dehydrogenase (23). The absorbance of the dye was measured at 450 nm by using a Model 680 microplate reader (Bio-Rad, Hercules, CA).

**Quantitative Alizarin Red Staining**

The cells were cultured as described previously in 50  $\mu$ g/mL AA and 10 mmol/L  $\beta$ -GP-containing media for 12 days. Cells were stained with 40 mmol/L Alizarin Red S (Sigma), pH 4.2, for 10 minutes with gentle agitation as described previously (24). Alizarin Red S staining is specific for calcium deposition (25). The amount of Alizarin Red S was determined according to an Alizarin Red S standard curve.

**Real-time Polymerase Chain Reaction**

The cells were cultured in DMEM containing 50  $\mu$ g/mL AA and 10 mmol/L  $\beta$ -GP for 0, 5, and 24 hours with each material as described earlier. The total RNA was extracted by using RNeasy (Qiagen, Chatsworth, CA), and 1  $\mu$ g RNA was reverse transcribed with standard reagents (Invitrogen). Real-time polymerase chain reaction (PCR) was performed on a volume of 15  $\mu$ L containing 1.5  $\mu$ L (50 ng) complementary DNA and 13.5  $\mu$ L master mix containing 7.5  $\mu$ L mix (SYBR Green PCR Master Mix, Invitrogen), 0.75  $\mu$ L each primer (10 pmol/L), and 4.5  $\mu$ L diethyl pyrocarbonate-treated water using an ABI PRISM 7500 Sequence Detection System Thermal Cycler (Applied Biosystems, Foster City, CA). The rat specific primer sequences were as follows: BMP-2 (215 bp), forward 5'-CGGAAGCGTCTTAAGTCCAG-3', reverse 5'-CATGCCCTTAGGGATTTTGA-3'; the control primers specific for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 450 bp) were forward 5'-TCCACCACCCTGTGCTGTA-3', reverse 5'-ACCACAGTCCATGCCATCAC-3'. The program was set at 50°C for 2 minutes and 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 34 seconds. The reaction product was quantified by Sequence Detection Software V1.3.1 (Applied Biosystems) with GAPDH as the reference gene. Data are expressed as relative quantity, and differences are shown as the expression ratio of the normalized target gene according to the software results.

**Enzyme-linked Immunosorbent Assay for BMP-2**

The cells were cultured in DMEM containing 50  $\mu$ g/mL AA and 10 mmol/L  $\beta$ -GP for 24 hours with each material as described earlier. The quantities of BMP-2 in the cell-free culture media were determined by a

Quantikine BMP-2 Immunoassay (R&D Systems Inc, Minneapolis, MN) according to the manufacturer's protocol. The peroxidase-substrate color reaction was assessed by using a Model 680 microplate reader set to a wavelength of 540 nm. The optical density of each assay aliquot was used to calculate the concentration of BMP-2 in picograms per well.

**The Determination of the Calcium Concentration in Culture Media**

The cells were cultured in DMEM containing 50  $\mu$ g/mL AA and 10 mmol/L  $\beta$ -GP for 0, 2, 5, 10, and 24 hours with each material as stated earlier. The calcium concentration in the culture media was determined colorimetrically by the *o*-cresolphthalein complexone method (Calcium C-test; Wako, Osaka, Japan). The absorbance of the dye was measured at 570 nm by using a Model 680 microplate reader. The concentration of calcium was determined according to a calcium standard curve.

**Statistical Analysis**

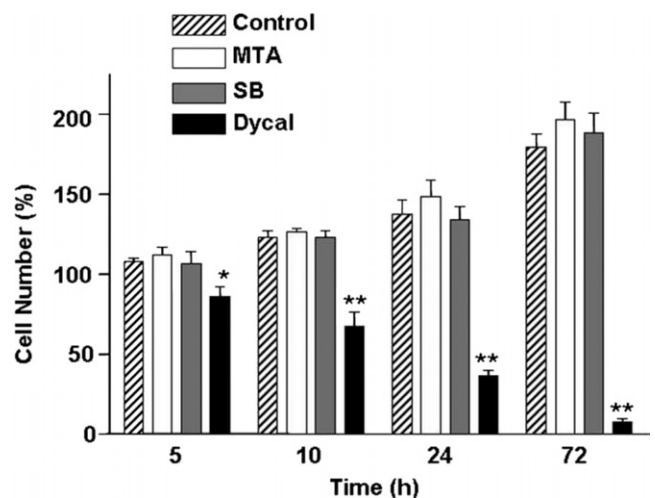
Statistical analysis was performed with data obtained from three independent experiments. The data are expressed as mean  $\pm$  standard deviation and were analyzed using a one-way analysis of variance followed by the Tukey test. Statistical significance was established at  $p < .05$ .

**Results**

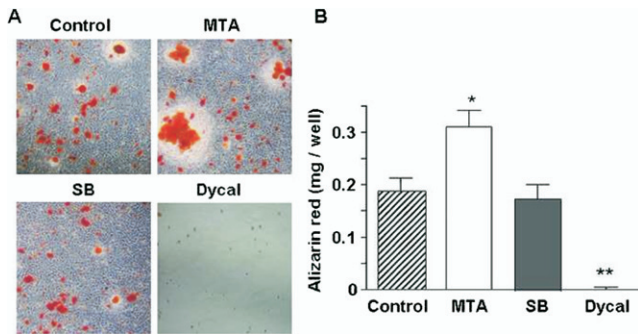
The cell viability of rat dental pulp cells was assessed after exposure to MTA, SB, and Dycal for 5, 10, 24, and 72 hours (Fig. 1). MTA and SB exhibited no cytotoxicity for up to 72 hours after culture. However, after 5 hours of culture in the presence of Dycal, cells numbers were significantly decreased, and almost all cells had died within 72 hours.

Next, the effect of each capping material on mineralization was examined by Alizarin Red S staining. MTA clearly increased the area of calcified nodules, whereas no mineralization was observed in Dycal-treated cells (Fig. 2A). The quantification of Alizarin Red S staining of calcified nodules showed a similar increase with control and SB-treated groups, whereas staining associated with MTA exceeded that amount by about 60% (Fig. 2B).

To elucidate the mechanism of mineralization stimulated by MTA, we analyzed its effect on BMP-2 mRNA expression by real-time PCR



**Figure 1.** The effect of direct pulp-capping materials on the cell viability of dental pulp cells. Rat dental pulp cells were cultured with each direct pulp-capping material for up to 72 hours. Cell viability was assessed by modified MTT assay at indicated time points. The bar represents the mean  $\pm$  standard deviation ( $n = 3$ , \* $p < .05$ , \*\* $p < .01$  compared with the control).



**Figure 2.** The effect of direct pulp-capping materials on mineralization of dental pulp cells. Rat dental pulp cells were cultured in DMEM supplemented with AA and  $\beta$ -GP with each direct pulp-capping material for 12 days. (A) Representative photographs of calcified nodules stained with Alizarin Red S (original magnification  $\times 200$ ). (B) The quantification of Alizarin Red S staining. The bar represents the mean  $\pm$  standard deviation ( $n = 3$ , \* $p < .05$ , \*\* $p < .01$  compared with the control).

(Fig. 3A). After 5 and 24 hours of culture with MTA or Dycal, BMP-2 mRNA expression was significantly enhanced by about two-fold compared with the control. However, the level of BMP-2 mRNA in SB-treated cells was similar to that in the control. In addition, the level of BMP-2 protein in the culture medium was determined by enzyme-linked immunosorbent assay (Fig. 3B). BMP-2 protein production in MTA-treated cells was significantly higher, by 40%, compared with that in the control or SB-treated cells. Conversely, BMP-2 protein production in Dycal-treated cells was significantly lower than that in the control. The calcium concentration in culture media with MTA or Dycal treatment increased to about 2.2 mmol/L after 2 hours and thereafter remained

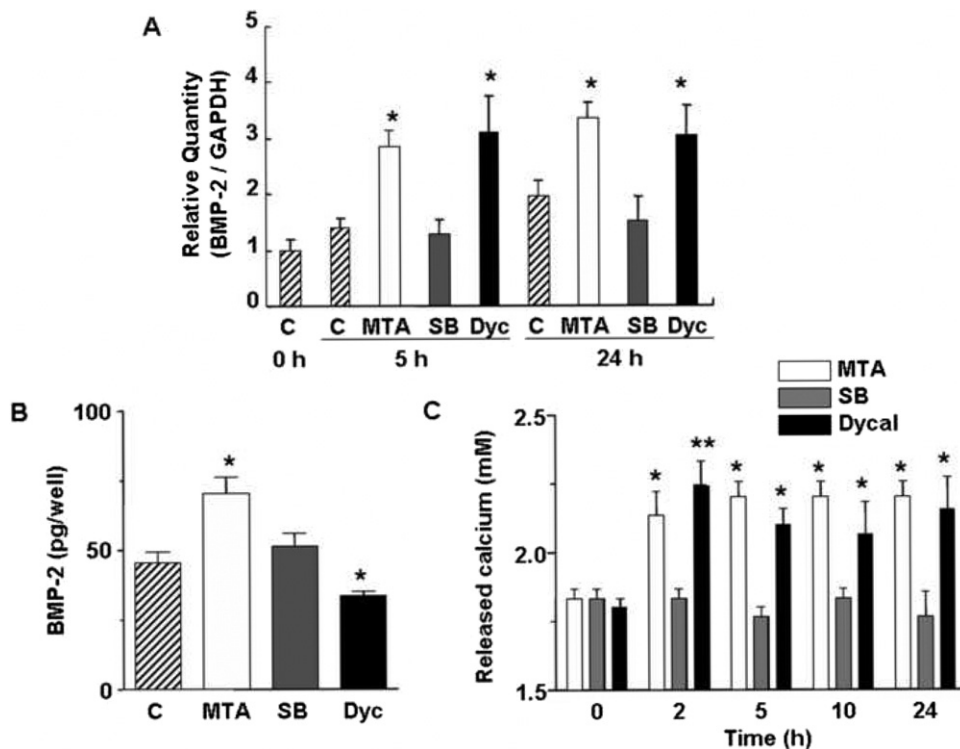
almost unchanged until 24 hours, whereas that of SB treatment remained constant at about 1.8 mmol/L (Fig. 3C).

### Discussion

In this study, MTA exhibited no cytotoxicity, and mineralization was significantly increased compared with the control. To test cell viability, we placed the test material on transwell inserts to prevent its direct contact with dental pulp cells because Camilleri et al. (26) reported that direct contact between cement and cells inhibited cell growth. On the other hand, we could not find any difference in the cytotoxicity between the set and freshly mixed MTA in the preliminary experiments. Therefore, the findings in this study can be applied to the clinical situation.

Our results showed that MTA and SB had no effect on cell viability. These results are in good agreement with previous reports (13–16). In contrast, Dycal exhibited strong cytotoxicity, and almost all cells had died after 72 hours. Further study will be necessary to examine whether Dycal causes indiscriminate cell death or selective cell death. Our finding conflicts with a report that Dycal showed little or no cytotoxicity (17). It is considered that this was because of differences in conditions for preparing the test material and in the test material-to-culture medium ratio. Although Tani-Ishii et al. (17) studied the cytotoxicity using the materials (diameter 2 mm, thickness 1 mm) and 3 mL of the culture media, we used the materials (diameter 6 mm, thickness 3 mm) and 2 mL of the culture media. Furthermore, the setting time of materials is 48 hours in their report, whereas it is 24 hours in our report.

MTA significantly increased BMP-2 mRNA expression and its protein production compared with the control after 5 hours and 24 hours, respectively. Guven et al. (27) recently reported that MTA significantly stimulated BMP-2 protein production in dental pulp fibroblasts. Fur-



**Figure 3.** The expression of BMP-2 in rat dental pulp cells exposed to each direct pulp-capping material. (A) Real-time PCR for BMP-2 in dental pulp cells exposed to each material for indicated time periods. (B) The levels of BMP-2 protein in culture media exposed to each material for 24 hours were determined by enzyme-linked immunosorbent assay. (C) The concentration of calcium in culture media of rat dental pulp cells exposed to each material for indicated time periods was examined by using the calcium C-test. The bar represents the mean  $\pm$  standard deviation ( $n = 3$ , \* $p < .05$ , \*\* $p < .01$  compared with the control). C; control.

thermore, Ham et al. (28) found that MTA-treated teeth were more immunoreactive to BMP-2 than control teeth in a pulp-capping experiment. These results suggest that BMP-2 may be involved in reparative dentin formation after pulp capping using MTA. The expression of BMP-2 is expressed as a relative quantity of the normalized target gene, thus the remaining viable cells treated with Dycal expressed relatively more BMP-2 mRNA than the control cells. However, the amount of BMP-2 protein production was lower than that in the control. This discrepancy appears to have resulted from an obvious decrease in the number of cells at 24 hours because of the toxicity of Dycal.

The effect of MTA on cell differentiation is still being elucidated. Bonson et al. (29) observed that MTA induced alkaline phosphatase activity in periodontal ligament cells on days 5 to 13. Moreover, MTA upregulated mRNA expressions of type I collagen and osteocalcin after 24 hours (17). In this study, MTA induced BMP-2 mRNA expression as early as 5 h. These results raise the possibility that BMP-2 produced by dental pulp cells acted on them in an autocrine fashion, thereby inducing the differentiation of pulp cells.

The addition of MTA or Dycal to the culture medium increased the concentration of calcium by approximately 0.4 mmol/L after 2 hours. Extracellular calcium ions have been reported to induce BMP-2 expression (30). By using real-time PCR, we observed that MTA as well as Dycal induced a similar degree of increase in BMP-2 mRNA expression, suggesting that an increased level of calcium ions might influence the expression of BMP-2.

Taken together, these results indicated that MTA exhibited no cytotoxicity and stimulated mineralization in rat dental pulp cells in vitro. We further found that BMP-2 mRNA expression and its protein production in MTA-treated cells were significantly increased compared with those in the control. Our results suggest that BMP-2 may play an important role in mineralization stimulated by MTA.

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