Gene Expression of Exposure to Mineral Trioxide Aggregate (MTA) on Dental Pulp Cells

Yu-Seok Choi, Nan-Young Lee, Sang-Ho Lee

Department of Pediatric Dentistry, College of Dentistry, Chosun University

Abstract

Dental pulp cells are assumed to possess the capacity to elaborate both bone and dentin matrix under the pathological conditions following tooth injury. The purpose of this study is to examine the effects of mineral trioxide aggregate (MTA) on various gene expression regarding dentinogenesis and cell viability assay in cultured primary human dental pulp cells. The author also examined the effects of this material on cellular alkaline phosphatase activity as a potential indicator of dentinogenesis. For gene expression on MTA, reverse transcriptase polymerase chain reaction was performed using primer sets of glyceraldehyde-3-phosphate dehydrogenase, type I collagen, alkaline phosphatase (ALP), osteonectin, and dentin sialoprotein after 2 and 4 days. Cell viability assay showed that the proportion of MTA-treated pulp cells which had been exposed for 5 days to MTA was higher than that of the control cells. Among the genes investigated in this study, ALP and osteonectin (SPARC) were increased in MTA treated group than in control. These findings suggest that this dental pulp culture system may be useful in the future as a model for studying the mechanisms underlying dentin regeneration after the treatment with MTA. Exposure to MTA material would not induce cytotoxic response in the dental pulp cells. In addition, MTA could influence the behavior of human pulp cells by increasing the ALP activity and SPARC synthesis.

Key words: Mineral trioxide aggregate (MTA), Dentinogenesis, Alkaline phosphatase activity, Osteonectin (SPARC)

I. INTRODUCTION

After the pulp exposure, pulpal tissue responds by laying down a matrix of tertiary dentin which may be reactionary or reparative, beneath the site of injury. Survived odontoblasts secrete reactionary dentin in response to environmental stimuli. The ability to induce dentinogenesis in a controlled and timely manner around the remaining walls of intact dentin would be therapeutically useful. This concept of using biologic agents to control pulpal response across the existing layer of dentin or the transdental approach is a relatively new approach to a long recognized clinical problem. Stimulation of a specific cellular response in the dentin pulp complex at the site of injury would allow a biologically directed approach to tissue repair rather than a simple mechanical approach. New strategies based on these ap-
approaches will have to address the problems of delivery and control of the bioactive molecules\(^{2,3,5}\).

Calcium hydroxide has been the "gold standard" for inducing pulpal repair\(^6\). Its effectiveness at promoting dentinal bridge formation over small pulpal exposure sites is believed to be related to a combination of antimicrobial activity (attributed to high pH) and its ability to stimulate tertiary dentin formation (attributed to the release of calcium ions). Recently, mineral trioxide aggregate (MTA) has been proposed as an alternative to calcium hydroxide for pulp regeneration. In vitro and in vivo studies suggest that MTA may be more effective at inducing dental hard tissue formation than calcium hydroxide, possibly via a physicochemical reaction in which released calcium ions which react with tissue phosphates to form hydroxyapatite\(^7,8\).

Mineral trioxide aggregate (MTA) is being widely used for fillings the end of root, pulp capping, repairs of root perforation, and other endodontic procedures. Because of its high pH value during its pre-hardening stage, MTA caused lysis of adjacent cell and denaturation of medium protein. After the hardening, MTA shows favorable biocompatibility, with no effect on cell morphology and limited impact on cell growth. MTA has a similar effect to calcium hydroxide when implanted in rat subcutaneous connective tissue\(^9\). When MTA was used as a root-end filling material, fibrous connective tissue and thin layers of hard tissue were formed in direct contact to it\(^10,11\). The formation of cementum and periodontal ligament fibers was also observed on its surface\(^12,13\). MTA stimulates a biologic response in osteoblasts\(^14,15\) and also provides a biocompatible surface for cell adhesion\(^14,16\).

MTA plays the same role with calcium hydroxide on pulp capping\(^3,17\) and apexification\(^13\). MTA has also been successfully used for repairing external root resorption\(^18\) and root perforations\(^6,19\).

Although, MTA has clinically shown to induce pulp tissue regeneration, the mechanisms that MTA influences on cell function are not known. The purpose of present study is to assess the effects of MTA materials on the survival of osteogenic or dentinogenic gene expression in human dental pulp cells. We also examined the effects of these materials on the cellular alkaline phosphatase activity as a potential indicator of dentinogenesis.

## II. MATERIALS AND METHODS

1. MTA specimen

A commercial brand of white ProRoot MTA (Dentsply Maillefer, Ballaigues, Switzerland) was used for this study. The cement was prepared according to the manufacturer’s specifications, and their manipulation was carried out in a laminar flow hood under aseptic conditions. The pellets of cement were allowed to set for 24 hours at 37°C in a humidified CO\(_2\) incubator for complete hardening. Subsequently, the pellets were sterilized using EO gas. The diameter of each pellet was approximately 1 cm. In one set of the specimens, the fresh pellet was immediately placed over a transwell insert measuring 24 mm in diameter (Corning, NY, USA) that fitted one well of a six-well cell culture plate, as depicted (Fig. 1). The transwell insert contained a permeable membrane (0.4 um-pore size) and was

![Fig. 1. Photograph and schematic drawing of transwell insert used in the six-well plates to prevent the direct physical contact between the pulp cells and the MTA.](image-url)
used to prevent direct physical interaction between the cells and the specimens while allowing for soluble compounds from the specimens to reach the cells (Fig. 1). Untreated controls were cells which were cultured in a six-well plate, with transwell inserts, but without the MTA specimen.

2. Cultures of dental pulp cells

All teeth used in this study were immature third molars extracted during normal treatment of 16-year-old patients. The teeth were all normal, freshly extracted, and used with the patient's informed consents. Immediately after the extraction, the teeth were swabbed with 70% (v/v) alcohol and then washed with sterile phosphate-buffered saline (PBS, 0.01 M, pH 7.4). The teeth were then transferred into a laminar flow tissue culture hood in order to perform the rest of the procedures under sterile conditions. The apical part of the teeth was removed with sterile scalpels and the dental pulps were gently removed with forceps. Dental pulps were minced with scalpels and rinsed with PBS. The explants were cultured in 100 mm diameter culture dishes (Becton Dickinson Labware, Lincoln Park, NJ) in modified Eagle's medium (MEM) (GIBCO) containing 10% heat inactivated fetal bovine serum (FBS), 100 ug/ml of streptomycin, and 100 U/ml of penicillin. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂, 95% air. The culture medium was changed every other day. When the dishes get confluent, cell were collected by trypsinization (0.2% trypsin and 0.02% EDTA) and subcultured.

3. Cell Viability Assay (MTT assay)

Cells were cultured in 24-well plates at a density of 3 x 10⁴ cells/well. Twenty-four hours later, cells were treated with MTA for 12 hrs or 24 hrs. Cell viability was determined by using the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cell proliferation assay (measured at 570 nm).

4. Von Kossa staining

After dental pulp cells were fixed with 70% ethanol for 1 hr, the culture dishes were washed with distilled water and then they were treated with 1% AgNO₃ for 20 min. After they were washed with distilled water, they were treated with 2.5% sodium thiosulfate for 5 min. The samples were then examined without counterstaining or after Mayer’s hematoxylin counterstaining.

5. Alkaline phosphatase activity

Cells were seeded in 6-well plates at a density of 1 x 10⁵ cells per well and cultured in complete medium containing MTA or transwell only. After 2 or 4 days in culture, the cell layers were rinsed with PBS, scraped into 1 ml buffer (10 mM TRIS, HCl, 5 mM MgSO₄, 0.1% Triton X-100, 0.1% NaNO₃), frozen (-20°C) and thawed three times, sonicated for 5 min to disrupt cell membranes, and centrifuged (4,000 g) at 4°C for 15 min. ALP activity was determined by the hydrolysis of p-nitrophenyl phosphate in 2-amino-2-methyl-1-propanol buffer (pH 10.4) at 37°C for 30 min. Absorbance at 405 nm was measured with a spectrophotometer (Bio-Rad 3550 microplate Reader). ALP activity was corrected for the total protein content (determined by the Bradford method with bovine serum albumin as a standard) and was expressed as nano moles of para nitrophenol per milligram of total protein.

6. Isolation of total RNA and RT-PCR analysis

The total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The integrity of RNA was verified following electrophoresis on denaturing agarose gel.

Semi-quantitative RT-PCR analysis was used to confirm the expression of several genes. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and RT-PCR was performed using SuperScript One-Step RT-PCR Systems (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Specific primers used for RT-PCR are shown in Table 1. RT-PCR was performed by using the following conditions: One cycle of 30 min at 50°C, 1 cycle of 2 min at 94°C, and 30 cycles of 30 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C, with a final extension of 10 min at 72°C. For caspase 7 and
GAPDH, PCR reactions were performed for 25 cycles. The PCR products were then run on 1.5% agarose gels and visualized by ethidium bromide staining.

### III. RESULTS

1. **Cell Viability Assay (MTT assay)**

   The results of cell viability are shown in Fig. 2. The incubation time did not influence the cell growth after 5 days of culture. The proportion of MTA treated pulp cells exposed for 5 days to MTA was higher than the control cells. However, there was no difference in the percentages of live cells between the two groups. According to this result, exposure to MTA material would not induce cytotoxic response in the dental pulp cells.

2. **Von Kossa staining**

   Von Kossa staining did not reveal formation of calcium-positive mineralization nodule on both groups (Data was not shown).

3. **Alkaline phosphatase activity**

   Alkaline phosphatase activity of each group was increased gradually, but MTA induced slight levels of alkaline phosphatase activity, and did the same to control pulp cells (Fig. 3). Maximal enzyme activity was detected after 4 days in culture for both cell populations, while this expression was delayed for 2 days with MTA.

4. **RT-PCR analysis**

   MTA had no effect on alkaline phosphatase expression after 2-day incubation. However, the expression of alkaline phosphatase was enhanced after 4-day incubation (Fig. 4). This result corresponds to the alkaline phosphatase activity assay. Like the effect on expression of alkaline phosphatase, MTA also increased DSPP and collagen type I expression in all the tested periods. However, there was no difference in the expression levels between the two groups (P<0.05). Interestingly, the expression of osteonectin of MTA treated pulp cells exposed for 4 days to MTA was statistically higher than that of the control cells (P<0.05).

### Table 1. Primers used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>Size (bp)</th>
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<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>Forward</td>
<td>GCACCTGCCCTTACTAACCTCC</td>
<td>626</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CATGATCAGGRCAATGTCC</td>
<td></td>
</tr>
<tr>
<td>Osteonectin</td>
<td>Forward</td>
<td>ACTGAGAGCCCTCACACTGG</td>
<td>254</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAGCCAACTCGTCACAGTCC</td>
<td></td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>Forward</td>
<td>TGTGTGACCAGGGACTACC</td>
<td>617</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CACCACCTCATTGTTAGAAAGC</td>
<td></td>
</tr>
<tr>
<td>Collagen type I</td>
<td>Forward</td>
<td>GATTTGACCCCAACCAAGG</td>
<td>409</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>AGTGACGCTGTAGGTGAAGC</td>
<td></td>
</tr>
<tr>
<td>DSPP</td>
<td>Forward</td>
<td>GCAGTGATGAATCTAATGTC</td>
<td>488</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTGATTGTGTGCTGTAGTGCTGAC</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>CCAACCTCATCCATGACAACTTTG</td>
<td>464</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTCAATACGGAATGAGCCTGACA</td>
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Fig. 2. Survival and proliferation of dental pulp cells on MTA.

Fig. 3. Effects of MTA on alkaline phosphatase activity.

Fig. 4. A. Transcript alteration by MTA. B. Intensities of bands mRNA shown in A were normalized with respect to those for GAPDH.
IV. DISCUSSION

Since its introduction to clinical dental practice in 1993, mineral trioxide aggregate (MTA) has gained acceptance as the material of choice for several clinical procedures. It has been shown to be an effective pulp capping material\(^6,17\). In addition, MTA has been used to seal and repair root perforations\(^18,19\), and to create an apical barrier in teeth with open apices\(^10,11\). Success as a root-end filling material has also been reported\(^12,13\). MTA seems to be an adequate material for these procedures because of its sealing ability when compared with other materials such as amalgam, IRM or SuperEBA\(^12,20\). Several investigators have demonstrated that MTA is biocompatible with the surrounding tissues\(^13,14\). It has been shown to promote osteoblastic activity\(^15\) and to be less cytotoxic than amalgam, IRM or SuperEBA\(^12,20\). Furthermore, MTA has been found to possess antibacterial activity\(^21\).

The author hypothesized that MTA would induce dental pulp cells to express genes associated with dentin formation. This is based on studies in both human and dogs in which dentin deposition was histologically observed on MTA\(^12,17\). Our results indicated that dental pulp cells are capable of expressing genes which indicate mineralization, especially alkaline phosphatase and osteonectin. Alkaline phosphatase (ALP) is a phosphate-releasing protein, and its activity is considered as an important indicator of bone formation and a phenotypic marker of osteoblasts\(^22\). The observation that alkaline phosphatase transcript levels peak prior to any detectable increase in alkaline phosphatase activity following exposure to MTA was expected; however, the finding that enzyme activity levels increased after a decline in transcript levels was unexpected. Further studies will be needed to be performed for a more careful examination of the correlation of transcript and protein stability with the respect to the regulation of functional levels of phosphatase activity\(^23\).

A major collagenous protein found in dentin, DSP and DPP, plays a regulatory role in the mineralization of dental hard tissue. The production of DSP and DPP is restricted to cells that function in a mineralizing capacity, including osteoblasts, odontoblasts, and cementoblasts\(^24\). The strong expression of DSPP gene expression by dental pulp cells on both MTA and control is suggestive that the process of dentin formation can be proceeded under experimental conditions. These results support MTA as being dentin-conductive by allowing the expression of genes and proteins which are consistent with the dentinogenesis process. The results of this investigation compare positively with the results of other studies which indicate a favorable biologic response by MTA comparing with other endodontic materials\(^9,13,17\).

Changes in expression patterns of several transcripts associated with hard-tissue formation were also detected. Among the genes investigated in this study, ALP and osteonectin are the most strongly associated with mineralization. ALP activity is essential for normal osteoblast function\(^15\), and it is generally considered as an early marker of mineralization. In this study ALP expression by dental pulp cells at days 2 and 4 appeared on MTA and control substrates. These results suggest again that MTA is permissive for dentinogenesis gene expression.

Osteonectin (SPARC) is a calcium binding matricellular glycoprotein expressed by odontoblasts but not by the pulp cells\(^24,25\). Furthermore, an increased level of SPARC was detected during the healing process of pulp tissue\(^26\). ALP is an enzyme usually detected in the mineralized tissue forming cells\(^22,27\). Thus, an increase in SPARC and ALP synthesis might be considered as a marker of pulp cell differentiation.

SPARC is a kind of phosphorylated glycoprotein, which is associated with tissue development, tissue remodeling, and repair\(^28\). It has been recently shown that odontoblasts and unique dental pulp cells expressing SPARC, demonstrated increased expression of SPARC in the initial stage of tertiary dentin formation\(^24-26\). Although the function of SPARC in dentin formation is still unknown, it is suggested that odontoblasts may release SPARC to stimulate proliferation of some fraction of pulp cells to replace the injured tissue due to dental caries or cavity preparation\(^29\). SPARC may also regulate the production of extracellular matrix and matrix metalloproteinases\(^29,30\) which might be involved in the modulation of matrix for dentinogenesis. Moreover, SPARC has been shown to play a role in differentiation of some cell types\(^31\). Thus, upregulation of SPARC and
ALP activity in dental pulp cells by the treatment of MTA, as demonstrated in this study, might also reflect the changes in their function or differentiation of these cells.

In conclusion, this dental pulp culture system may be useful in the future as a model for studying the mechanisms underlying dentin regeneration after the treatment of MTA. Exposure to MTA material would not induce cytotoxic response in the dental pulp cells. MTA could influence the behavior of human pulp cells by increasing the ALP activity and SPARC synthesis.

REFERENCES


국문초록

Mineral Trioxide Aggregate(MTA)에 의한 치수세포의 유전자 발현변화

최유석, 이난영, 이상호
조선대학교 치과대학 소아치과학교실

치아 치수 세포는 치아 손상에 따르는 병리적인 상황에서 골과 상아질 기질을 형성하는 능력을 가진 것으로 생각된다. 본 연구에서는 MTA가 사람 치수세포의 성장에 미치는 영향과 상아질 형성에 관여하는 유전자의 발현을 유도하는지를 알아보고자 하였다. 또한 상아질 형성의 잠재적 지표인 alkaline phosphatase(ALP) activity에 미치는 영향을 평가하였다. 유전자 발현 검사를 위해 glyceraldehyde-3-phosphate dehydrogenase, type I collagen, alkaline phosphatase, osteonectin(SPARC), and dentin sialoprotein primer set을 이용하여 MTA 처리 2일과 4일 후 reverse transcriptase polymerase chain reaction(RT-PCR)을 시행하였다. cell viability assay에서 5일간 MTA에 노출된 치수 세포의 비율이 대조군보다 높았다. 대조군에 비해 MTA를 처리한 군에서 ALP와 SPARC가 증가되었다. 이상의 결과를 종합하여 보면, 이 연구에 사용한 dental pulp culture system은 MTA를 포함한 치과재료의 처리 후 치수세포의 성장과 분화 그리고 상아질 형성 유도 기전을 연구하는 데 유용한 모델로 사용할 수 있다. MTA 처리는 치아 치수세포에 세포독성을 유도하지 않으며, ALP 활성도와 유전자 발현 그리고 osteonectin (SPARC) 유전자 발현을 증가시켜 수복상아질을 형성할 것으로 사료된다.

주요어: Mineral trioxide aggregate(MTA), 상아질 형성, Alkaline phosphatase activity, Osteonectin (SPARC)