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

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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[1]. 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[1-3]. This concept of using biologic agents to control pulpal response across the existing layer of dentin or the transdentinal approach is a relatively new approach to a long recognized clinical problem[4,5]. 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-

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proaches 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℃ 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

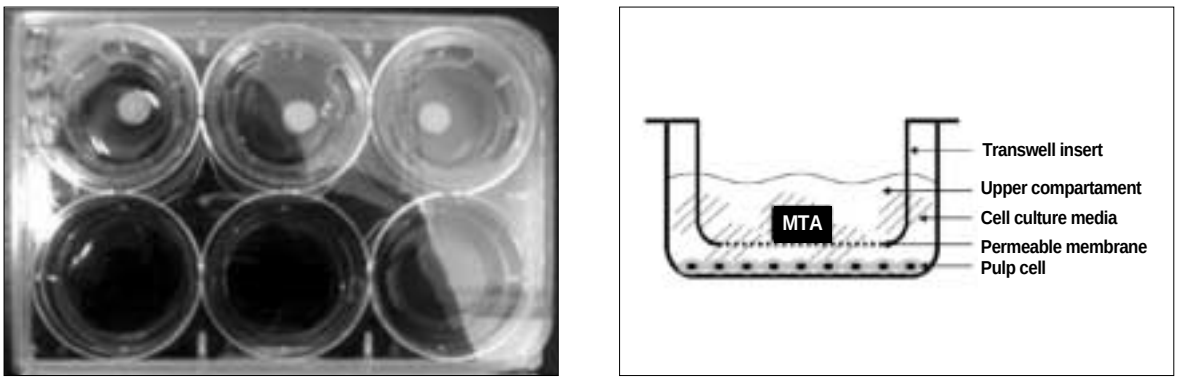


Fig. 1. Photograph and schematic drawing of transwell insert used in the six-well plates to prevent the direct physical contact between the pulp cells and the MTA.

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℃ in a humidified atmosphere of 5% CO_2, 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 3×10^4 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% $AgNO_3$ 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 1×10^5cells 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 $MgSO_4$, 0.1% Triton X-100, 0.1% $NaNO_3$), frozen(-20℃) 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℃ 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℃, 1 cycle of 2 min at 94℃, and 30 cycles of 30 sec at 94℃, 30 sec at 60℃, 30 sec at 72℃, with a final extension of 10 min at 72℃. For caspase 7 and

Table 1. Primers used for RT-PCR

Gene	Primers	Sequence(5'-3')	Size(bp)
Alkaline phosphatease	Forward	GCACCTGCCTTACTAACTCC	626
	Reverse	CATGATCACGRCAATGTCC	
Osteonectin	Forward	ACTGAGAGCCCTCACACTGG	254
	Reverse	CAGCCAACTCGTCACAGTCC	
Osteocalcin	Forward	TGTGTGACCCAGGACTACC	617
	Reverse	CACCACTCATTGTTAGAAAGC	
Collagen type I	Forward	GATTGACCCCAACCAAGG	409
	Reverse	AGTGACGCTGTAGGTGAAGC	
DSPP	Forward	GCAGTGATGAATCTAATGGC	488
	Reverse	CTGATTTGCTGCTGCTGTCTGAC	
GAPDH	Forward	CCAACCTCATCCATGACAACTTTG	464
	Reverse	GTCATACCAGGAAATGAGCTTGACA	

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($P<0.05$).

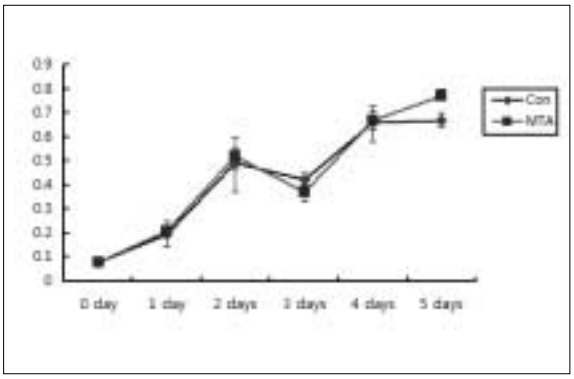


Fig. 2. Survival and proliferation of dental pulp cells on MTA.

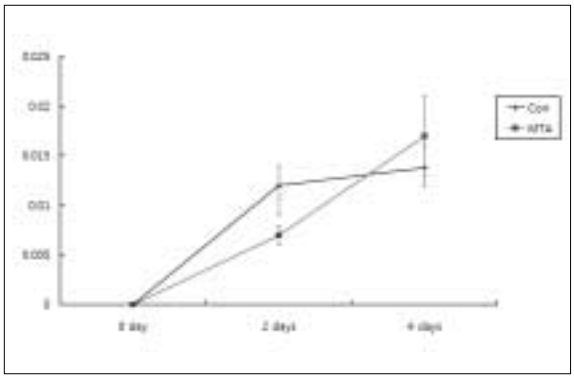
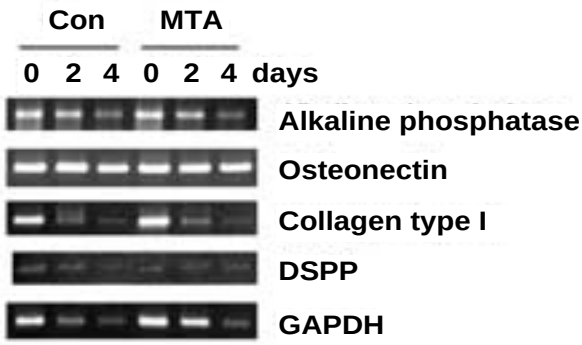
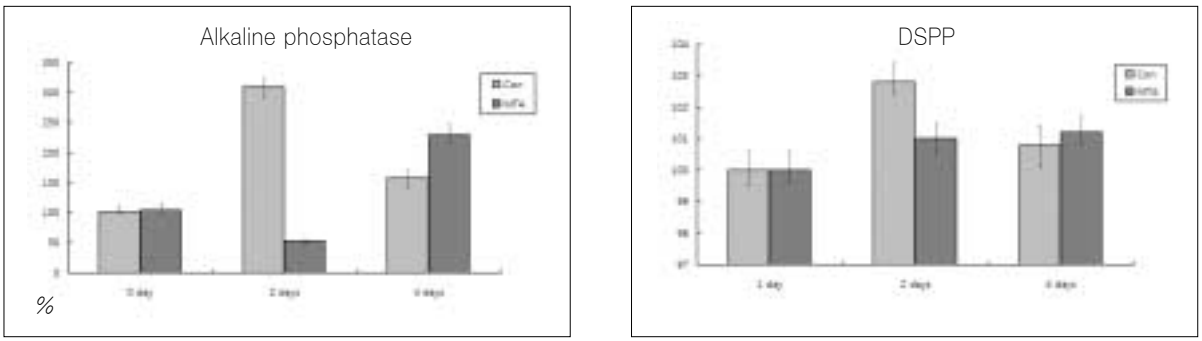
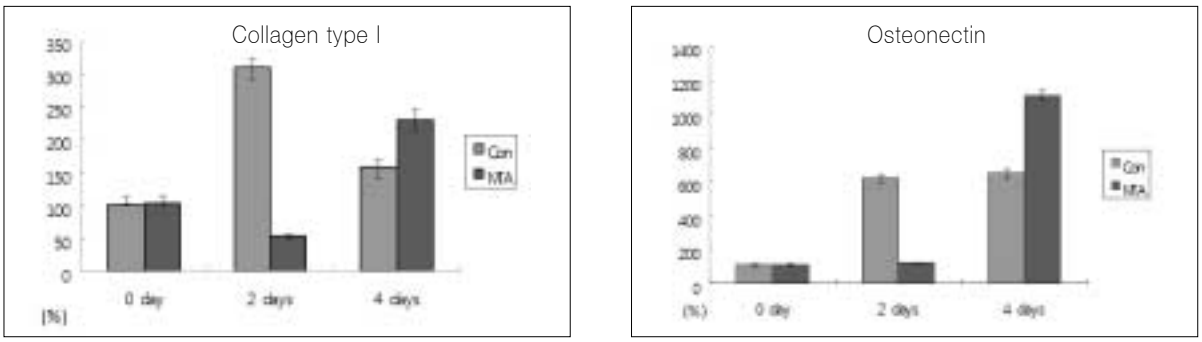


Fig. 3. Effects of MTA on alkaline phosphatase activity.





A



B

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 materia[16,17]. 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[23]. 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[24]. 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

1. Yamamura T : Differentiation of pulpal cells and inductive and influences of various matrices with reference to pulpal wound healing. J Dent Res, 64:530-540, 1985.
2. Tziafas D : Basic mechanisms of cytodifferentiation and dentinogenesis during dental pulp repair. Int J Dev Biol, 39:281-290, 1995.
3. Tziafas D, Smith AJ, Lesot H : Designing new treatment strategies in vital pulp therapy. J Dent, 28:77-92, 2000.
4. Smith AJ, Cassidy M, Perry H, et al. : Reactionary dentinogenesis. Int J Dev Biol, 39:273-280, 1995.
5. Smith AJ, Lesot H : Induction and regulation of crown dentinogenesis: embryonic events as a template for dental tissue repair?. Crit Rev Oral Biol Med, 12:425-37 2001.
6. Murray PE, Windsor LJ, Smyth TW, et al. : Analysis of pulpal reactions to restorative procedures, materials, pulp capping, and future therapies. Crit Rev Oral Biol Med, 13:509-520, 2002.
7. Ham KA, Witherspoon DE, Gutmann JL, et al. : Preliminary evaluation of BMP-2 expression and histologic characteristics during apexification with calcium hydroxide and mineral trioxide aggregate. J Endod, 31:275-279, 2005.
8. Sarkar NK, Caicedo R, Ritwik P, et al. : Physicochemical basis of the biologic properties of mineral trioxide aggregate. J Endod, 31:97-100, 2005.
9. Holland R, Souza V, Nery MJ, et al . : Reaction of rat connective tissue to implanted dentin tubes filled with mineral trioxide aggregate or calcium hydroxide. J Endod, 25:161-166, 1999.
10. Regan JD, Gutmann JL, Witherspoon DE : Comparison of Diaket and MTA when used as root-end filling materials to support regeneration of the periradicular tissues. Int Endod J, 35:840-847, 2002.
11. Economides N, Pantelidou O, Kokkas A, et al. : Short-term periradicular tissue response to mineral trioxide aggregate(MTA) as root-end filling material. Int Endod J, 36:44-48, 2003.
12. Torabinejad M, Hong CU, Lee SJ, et al. : Investigation of mineral trioxide aggregate for root end filling in dogs. J Endod, 21:603-608, 1995.
13. Torabinejad M, Pitt Ford TR, McKendry DJ, et al. : Histologic assessment of MTA as root end filling in monkeys. J Endod, 23:225-228, 1997.
14. Koh ET, McDonald F, Pitt Ford TR, et al. : Cellular response to Mineral Trioxide Aggregate. J Endod, 24:543-547, 1998.
15. Koh ET, Torabinejad M, Pitt Ford TR, et al. : Mineral trioxide aggregate stimulates a biological response in human osteoblasts. J Biomed Mater Res, 37:432-439, 1997.
16. Zhu Q, Haglund R, Safavi KE, et al. : Adhesion of human osteoblasts on root-end filling materials. J Endod, 26:404-406, 2000.
17. Aeinehchi M, Eslami B, Ghanbariha M, et al. : Mineral trioxide aggregate(MTA) and calcium hydroxide as pulp-capping agents in human teeth: a preliminary report. Int Endod J, 36:225-231, 2003.
18. White C Jr, Bryant N : Combined therapy of mineral trioxide aggregate and guided tissue regeneration in the treatment of external root resorption and an associated osseous defect. J Periodontol, 73:1517-1521, 2002.
19. Holland R, Filho JA, de Souza V, et al. : Mineral trioxide aggregate repair of lateral root perforations. J Endod, 27:281-284, 2001.
20. Keiser K, Johnson CC, Tipton DA : Cytotoxicity of mineral trioxide aggregated using human periodontal ligament fibroblasts. J Endod, 26:288-291, 2000.
21. Estrela C, Bammann LL, Estrela CR, et al. : Antimicrobial and chemical study of MTA,

Portland cement, calcium hydroxide paste, Sealapex and Dycal. Braz Dent J, 11: 3-9, 2000.
22. Kuru L, Griffiths GS, Petrie A, et al. : Alkaline phosphatase activity is upregulated in regenerating human periodontal cells. J Periodontal Res, 34:123-127, 1999.
23. Bonson S, Jeansonne BG, Lallier TE : Root-end filling materials alter fibroblast differentiation. J Dent Res, 83:408-413, 2004.
24. Reichert T, Storkel S, Becker K, et al. : The role of osteonectin in human tooth development: an immunohistological study. Calcif Tissue Int, 50:468-472, 1992.
25. Begue-Kirn C, Smith AJ, Loriot M, et al. : Comparative analysis of TGF beta s, BMPs, IGF1, msxs, fibronectin, osteonectin, and bone sialoprotein gene expression during normal and *in vitro*-induced odontoblast differentiation. Int J Dev Biol, 38:405-420, 1994.
26. Itota T, Nishitani Y, Sogawa N, et al. : Alteration of odontoblast osteonectin expression following dental cavity preparation. Arch Oral Biol, 46:829-834, 2001.
27. Wlodarski KH, Reddi AH : Alkaline phosphatase as a marker of osteoinductive cells. Calcif Tissue Int, 39:382-385, 1986.
28. Yan Q, Sage EH : SPARC, a multicellular glycoprotein with important biological functions. J Histochem Cytochem, 47:1495-1506, 1999.
29. Tremble PM, Lane TF, Sage EH, et al. : SPARC, a secreted protein associated with morphogenesis and tissue remodeling, induces expression of metalloproteinases in fibroblasts through a novel extracellular matrix-dependent pathway. J Cell Biol, 121:1433-1444, 1993.
30. Francki A, Bradshaw AD, Bassuk JA, et al. : SPARC regulates the expression of collagen type I and transforming growth factor-beta1 in mesangial cells. J Biol Chem, 274:32145-32152, 1999.
31. Bassuk JA, Birkebak T, Rothmier JD, et al. : Disruption of the Sparc locus in mice alters the differentiation of lenticular epithelial cells and leads to cataract formation. Exp Eye Res, 68: 321-331, 1999.

국문초록

Mineral Trioxide Aggregate(MTA)에 의한 치수세포의 유전자 발현변화

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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 assay(세포 생존력 측정)에서 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 사람 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)