Effects of Mineral Trioxide Aggregate on Cell Survival, Gene Expression Associated with Mineralized Tissues, and Biomineralization of Cementoblasts

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Abstract

The purpose of this study was to investigate the effects of mineral trioxide aggregate (MTA) on survival, mineralization, and expression of mineralization-related genes of cementoblasts. Immortalized cementoblasts (OCCM) were maintained with Dulbecco modified Eagle medium containing 10% fetal bovine serum. Methyl-thiazol-diphenyl-tetrazolium experiments were performed at 24 and 72 hours to evaluate bioactive components released by MTA (0.002-20 mg/mL) on the cell survival of OCCM. Von Kossa staining was used to evaluate biomineralization of OCCM cells. Images of cementoblasts were taken on day 3 by using inverted microscopy. Gene transcripts for bone sialoprotein (BSP), OCN, collagen type I (COL I), and osteopontin (OPN) were evaluated on days 3 and 5 by using semiquantitative reverse transcriptase polymerase chain reaction. The 20 mg/mL concentration of MTA was toxic for OCCM cells, whereas other concentrations of MTA tested exhibited similar cell numbers when compared with control group, and the 0.02 mg/mL concentration of MTA increased OCCM cell survival at 72 hours. Although an apparent decrease in mineralization was observed in the highest 3 concentrations of MTA used, 0.02 and 0.002 mg/mL concentrations of MTA induced greater biomineralization of OCCM cells than seen in the control. Moreover, increased BSP and COL I mRNA expression was observed at 0.02 and 0.002 mg/mL concentrations of MTA. MTA did not have a negative effect on the viability and morphology of cementoblasts and induced biomineralization of cementoblasts at the concentrations of 0.02 and 0.002 mg/mL. Based on these results MTA can be considered as a favorable material regarding cell-material interaction. *(J Endod 2009;35:513–519)*

Key Words

Cell viability, cementoblasts, mineral trioxide aggregate, mineralization, mRNA expression

Mineral trioxide aggregate (MTA) is a biomaterial that has been investigated for endodontic applications since the early 1990s. MTA was first described in the dental scientific literature in 1993 (1) and was given approval for endodontic use by the U.S. Food and Drug Administration in 1998 (2). Until 2002, only 1 MTA consisting of gray-colored powder was available (3). Because of esthetic concerns, white MTA (WMTA) was introduced as ProRoot MTA in that year, and the compound tetracalcium aluminoferrite was reportedly removed in the white formulation (4, 5). It was reported that WMTA was less soluble, exhibited greater hardness, and was more radiopaque (6).

The characteristics of MTA as far as sealing ability, biocompatibility, ability to set up in the presence of blood, and long-term success when used as a perforation repair material have been investigated in several studies (7–10). According to these study results, MTA materials appear not only to demonstrate acceptable biocompatible behavior, but they also exhibit acceptable in vivo biologic performance when used for root-end fillings, perforation repairs, pulp capping and pulpotomy, and apexification treatment. MTA also creates a biocompatible environment in periodontal tissues and can stimulate cementogenesis when used in the perforation area (6).

Oviir et al (11) examined the effects of MTA in vitro on the proliferation of oral keratinocytes and cementoblasts. They compared WMTA with gray MTA (GMTA), and their data revealed that cementoblast proliferation significantly increased when grown on the surface of WMTA, compared with cementoblasts grown on GMTA. They observed a high degree of biocompatibility with cementoblasts, and they suggested that GMTA and WMTA might significantly contribute to the regeneration of tissue after root perforation repair. In the present study, we used WMTA, because it is preferred in clinics as a result of its esthetic properties.

Cementogenesis is a critical event for regeneration of periodontal tissues. Cementoblasts play important roles in repair and/or regeneration of cementum. The histologic response to furcation perforation repair with MTA in dogs showed cementum repair over the material (12). When used as a root-end filling material in monkeys, the results showed new bone formation, and a complete layer of cementum had grown directly against the MTA (13). To understand the interaction of MTA and root cells, an immortalized murine cementoblast cell line isolated from tooth root surface has been used in a few studies (11, 14) regarding in vitro biocompatibility of MTA and cementoblasts. Reports have strongly suggested that the favorable biologic performance exhibited by MTA materials is due to hydroxyapatite formation when these materials are exposed to physiologic solutions. Although the overall results in human studies involving MTA materials are very positive (15–19), further investigation into the biologic and molecular interactions with MTA on the cells within periodontium is needed. It is believed that the investigation of the effects of MTA on cementoblasts on whether it contributes to

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cementogenesis would be northworthy. This knowledge will help elucidate the properties of MTA, especially when in contact with endodontic repair materials. Our study was undertaken to examine the MTA effects on cell survival and on selected genes related to biomineralization to help clarify the mechanisms behind the beneficial biologic effects of MTA.

Materials and Methods

Cell Culture

An immortalized cementoblast (OCCM-30) cell line was used for this study, and methods for isolating these cells have been published previously (20–22). Cells were provided by Dr Martha J. Somerman from University of Washington and were maintained in Dulbecco modified Eagle medium (DMEM; Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal bovine serum (FBS; Biological Industries) containing 100 U/mL penicillin (Biological Industries) and 100 μg/mL streptomycin (Biological Industries) in a humidified atmosphere of 5% CO_2 at 37°C. Cells used in these experiments were between passages 19 and 22.

Preparation of MTA

Various methods were used in the literature to test biocompatibility of MTA materials in different cell lines including osteoblasts, human gingival fibroblasts, periodontal ligament cells, keratinocytes, and cementoblasts (23–31). According to ISO standards, the ratio between the surface of the samples and the volume of medium was 0.5 cm^2/mL. This ratio of medium to sample is called test medium. In the present study, ISO standard (10993-5) was not used. Although it was reported that MTA was not soluble in water (8), bioactive components of MTA might release into liquids and then might affect the cells' behavior in the vicinity of the material. Thus, to evaluate the effects of factors released by WMTA on the cementoblats, a new approach for preparation of WMTA (ProRoot WMTA; Dentsply Endodontics, Tulsa, OK) was introduced in this study. WMTA powder was added to DMEM containing 5% FBS. At a concentration of 20 mg/mL (20 mg MTA:1 mL DMEM), the solution was vortexed until completely suspended, allowed to settle for 10 minutes, and incubated for 24 hours in the incubator (5% CO_2, 37°C) to extract the bioactive content of the WMTA. Supernatant from this preparation was used for treatment, filtering the solution before use with cell cultures. Cells were treated every other day with freshly prepared WMTA solutions.

MTT Experiments

The 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma Chemicals, St Louis, MO) assay was used as previously described (32) to determine proliferation. This assay is based on the reduction of the soluble yellow MTT tetrazolium salt to a blue insoluble formazan product by mitochondrial succinic dehydrogenase. Briefly, for each sample 96-well plates were seeded with a 200 μL suspension of cementoblasts at a concentration of 20,000 cells/well. For viability experiments, cells were allowed to adhere for 24 hours in DMEM with 10% FBS, after which media were changed to MTA (Dentsply Endodontics)-conditioned (20, 10, 2, 1, 0.2, 0.1, 0.02, and 0.002 mg/mL) DMEM with 5% FBS. Media of each concentration of MTA (n = 6) and controls (n = 6) were prepared. The plates were incubated at 37°C for 24 hrs, and 200 μL medium, containing MTT at a concentration of 0.55 mg/mL, was added to each well, after which the plate was incubated for another 4 hours. Each well then was washed with 200 μL of phosphate-buffered saline, and 200 μL of dimethyl sulfoxide was added to each well. To extract and solubilize the formazan, the test plate was agitated by microplate shaker for 30 minutes. Optical density (OD) 590 nm was measured by automatic microplate reader (BioTek Instruments, Winooski, VT).

The worksheets were incorporated into the software Excel (Microsoft Corporation, Redmond, WA) version XP and then recalculated by using the following formula:

$$\text{Cell viability percentage} = \frac{(a - b)}{(c - b)} \times 100$$

where a is the OD 590 nm from test well, b is mean OD 90 nm from blank wells, and c is mean OD 590 nm from control wells (ie, added culture medium).

Morphology

Cementoblasts normally appear as cuboidal cells. Possible changes in the shape of cementoblasts (roundedness of cell membrane) and detachment of the cells from the culture plate might be considered morphologic markers of cytotoxicity. Therefore, images of cementoblasts treated with different concentrations of MTA were examined by using phase contrast microscopy (Nikon TS100F; Nikon, Tokyo, Japan) on day 3 after treatment (32).

Mineralization Assay

MTT experiments indicated that the concentration of 20 mg/mL MTA was toxic, so that concentration was excluded from mineralization experiments. Cells were plated at 5×10^4 cells/cm^2 in 24-well plates in DMEM containing 10% FBS. After 24 hours, cells were switched to DMEM containing 5% FBS + mineralization media (MM) (ascorbic acid [50 μg/mL] and β-glycerolphosphate [10 mmol/L]) plus different concentrations of MTA. Mineralization of extracellular matrix was determined on day 8 by von Kossa staining (33). The mineralization experiments were repeated 2 times (triplicate for each experiment) (34).

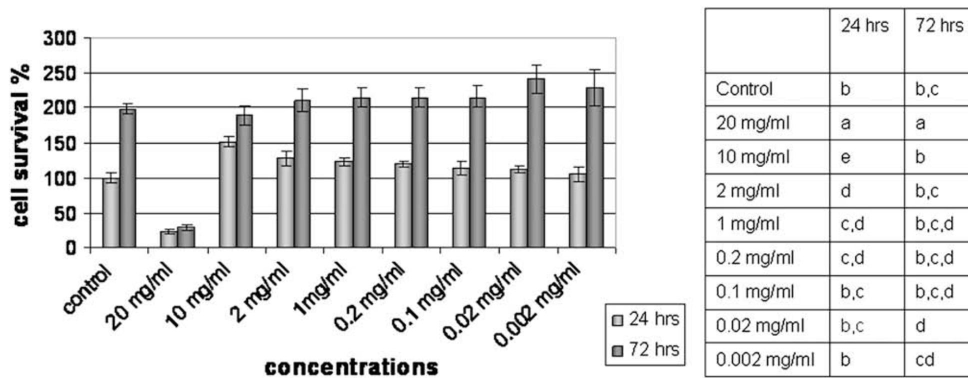
RNA Isolation

Because there was a down-regulation on the biomineralization of OCCM cells at 10 mg/mL concentration of MTA, this group was excluded from RNA isolation.

To determine gene expression, OCCM cells were plated in 60-mm cell culture dishes at 5×10^4 cells/cm^2 and treated with DMEM containing 5% FBS plus different concentrations of MTA after 24 hours. Total RNA was isolated by using monophasic solution of phenol and guanidine isothiocyanate (Invitrogen, Carlsbad, CA) on days 3 and 5. RNA concentration was quantified at 260 nm by spectrophotometer, and RNA samples were stored at –70°C.

cDNA Synthesis for Reverse Transcriptase Polymerase Chain Reaction

cDNA synthesis was performed by using SuperScript First-Strand Synthesis System (Invitrogen) for reverse transcriptase polymerase chain reaction (RT-PCR). cDNA synthesis was carried out in 20 μL containing SuperScript 1× RT Buffer, 5 mmol/L $MgCl_2$, 0.5 mmol/L deoxynucleotide triphosphate (dNTP), 2.5 μmol/L oligo$(dT)_{20}$, 2 μL from 0.1 mol/L DTT, 1 μL RNaseOUT (ribonuclease inhibitor, 40 units/μL) (Invitrogen), 1 μL SuperScript III RT enzyme (200 U/μL), and 1 μg total RNA. RNA samples, dNTPs, and the primer were first combined in a separate tube in 10-μL volume and incubated at 65°C for 5 minutes and held on ice until their addition to the reaction mixture containing the other reaction components. When combined, primers were first annealed at 50°C for 50 minutes and then were incubated at 25°C for 10



	24 hrs	72 hrs
Control	b	b,c
20 mg/ml	a	a
10 mg/ml	e	b
2 mg/ml	d	b,c
1 mg/ml	c,d	b,c,d
0.2 mg/ml	c,d	b,c,d
0.1 mg/ml	b,c	b,c,d
0.02 mg/ml	b,c	d
0.002 mg/ml	b	cd

Figure 1. Effects of different concentrations of MTA on cementoblast viability at 24 and 72 hours. Different letters in each column represent significantly different groups ($P < .05$).

minutes followed by another 50°C for 50 minutes of incubation before heating at 85°C for 5 minutes. cDNA samples were chilled on ice and spun down before the addition of 1 μL RNase H and incubation for 20 minutes at 37°C. cDNA synthesis reactions were stored at −20°C until use.

RT-PCR

Semiquantiative RT-PCR was performed by using specific primers of collagen type I (COL I-F: 5′-GCAACATTGGATTCCCTGGACC-3′; COL I-R: 5′-GTTCACCCTTTT CTCCCTTGCC-3′), bone sialoprotein (BSP-F: 5′-GA GACGGCGATAGTTCC-3′; BSP-R: 5′-AGTGCCGCTAACTCAA-3′), osteocalcin (OCN-F: 5′-TGAACAGACT CCGGCG-3′; OCN-R: 5′-GATACCGTAGATGCGT TTG-3′), and osteopontin (OPN-F: 5′-TTTACAGCCTGCACCC-3′; OPN-R: 5′-CTAGCAGTGACGGTCT-3′), which might play important roles in cementogenesis, and for normalization glyceraldehyde-3-phosphate dehydrogenase (GAPDH-F: 5′-ACCACAGTCCATGCCATCAC-3′; GAPDH-R: 5′-TCCACCACCCTGTTGCTGTA-3′) primers. After the initial denaturation at 94°C for 3 minutes, RT-PCR was performed with 30 cycles of 45 seconds of denaturation at 94°C, 50 seconds of annealing at 54.5°C, and 90 seconds of extension at 72°C followed by 1 step of final extension at 72°C for 10 minutes. Amplification reactions were performed in a final volume of 25 μL containing 1 μL cDNA, 25 mmol/L of each dNTP,, 50 pmol of each forward and reverse primer, 1.5 mmol/L $MgCl_2$ (Bioron GmbH, Ludwigshafen, Germany), 1× reaction buffer [(160 mmol/L $(NH_4)_2SO_4$, 670 mmol/L TrisHCl (pH 8.8), 0.1% Tween-20)] (Bioron GmbH), and 1 U of *Taq* DNA polymerase (Bioron GmbH). The RT-PCR products (18 μL) were resolved by electrophoresis in 2% agarose gels stained with ethidium bromide. Gel photographs were taken by using a gel documentation system. Results were reproduced in 2 separate experiments. Densitometric analysis was performed for mRNA expressions of COL I, BSP, OCN, and OPN by using Scion Image analysis, normalized with GAPDH.

Statistical Analysis

For MTT assays, one-way analysis of variance and Tukey honestly significant difference multiple comparison tests were used to compare different concentrations of MTA and control.

Results

Cell Viability Experiments

Statistically significant decrease ($P < .05$) was observed in 20-mg/mL concentration group (Fig. 1) when compared with other groups at 24 and 72 hours, and it was thought that 20 mg/mL concentration of MTA was toxic for OCCM cells. The concentrations of MTA including 10, 2, 1, and 0.2 mg/mL increased cell survival of cementoblasts significantly when compared with the control group at 24 hours. At 72 hours, 0.02 mg/mL and 0.002 mg/mL concentrations of MTA remarkably increased OCCM cell survival when compared with the other concentrations of MTA.

Morphology of the OCCM-30 Cells

It was noted that only the highest concentration of MTA (20 mg/mL) was cytotoxic (Fig. 2), whereas the other groups were observed as healthy and similar with the control group. Cells were rounded in shape and detached from the culture plate surface in 20-mg/mL concentration group.

Mineralization of the OCCM-30

Because 20 mg/mL concentration of MTA was toxic (Fig. 3), this concentration was not used in the mineralization experiments. Brown staining is indicator of mineralized-like nodules (33). Apparent induction in brown staining was observed in 0.02 and 0.002 mg/mL MTA-treated OCCM cells when compared with untreated control group. Lack of brown staining means no mineralization was observed in 10, 2, and 1 mg/mL concentrations of MTA.

Gene Expression Results

BSP. On day 3, there were no differences among the groups (Fig. 4). On day 5, there were apparent increases in BSP expression in 0.02 and 0.002 mg/mL concentrations of MTA in comparison with control group (5% FBS group), parallel with mineralization results.

OPN. There was no difference on day 3. Decreased OPN transcripts were noted in the groups treated with MTA except 0.2 and 0.002 mg/mL concentrations of MTA groups when compared with control group on day 5.

COL I. Increased COL I mRNA expression was noted in 0.1, 0.02, and 0.002 mg/mL MTA concentrations on day 3. On day 5, COL I transcript was higher only in 0.02 and 0.002 concentrations of MTA when compared with control group.

OCN. No differences were observed among the groups on day 3; however, slight decreases were noted in the MTA-treated groups in comparison with untreated control group on day 5.

Discussion

In the present study, bioactive components released from WMTA into DMEM containing 5% FBS were used to evaluate the effects of these components on the cementoblasts. MTA is reported to be not soluble in water (8); however, the results of this study demonstrated that released

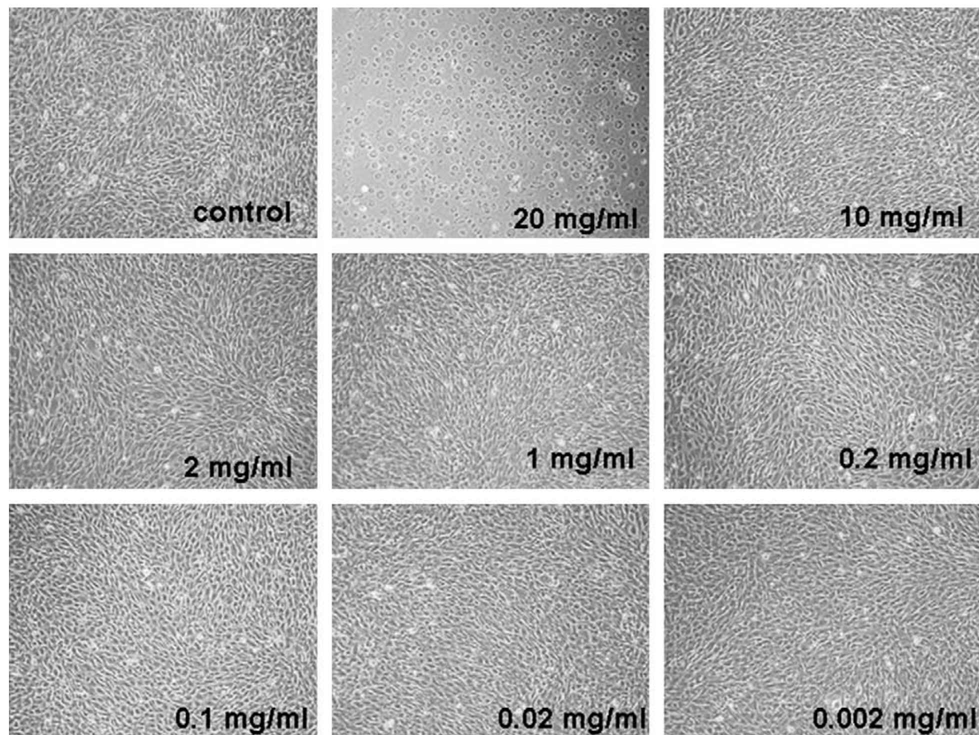


Figure 2. Microscopic appearance of cells treated with/without MTA on day 3. Note rounded and detached cells from plate surface at 20 mg/mL concentration of MTA. Other groups look similar to control group.

components might affect cell survival, mineralization, and the genes associated with mineralized tissues in cementoblasts.

Because of its hydrophilic characteristic, moisture in the surrounding tissue acts as an activator of a chemical reaction in MTA (1). Arens et al (35) recommended covering the MTA with a wet cotton pellet and a layer of intermediate restorative material (IRM) for 1–3 days to encourage setting. Sluyk et al (36) used saline-moistened Gelfoam (Pfizer Inc, New York, NY), and they indicated that this simulated the moist clinical environment present in the periodontal and bone tissues adjacent to the perforation site. They also recommended a matrix

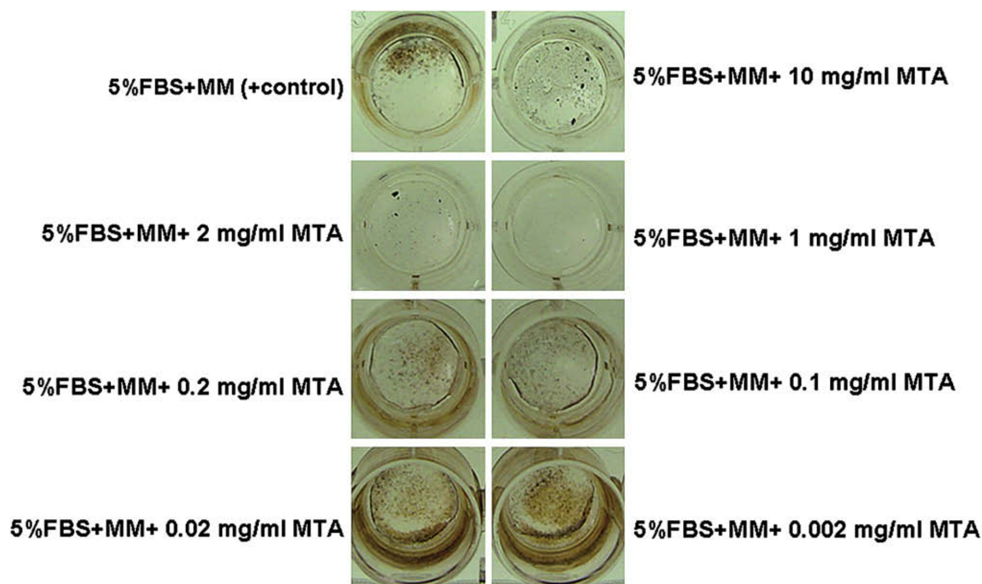


Figure 3. Von Kossa staining was used to determine mineral nodule formation on day 8. For these studies, cells were cultured in media containing the following: (*a*) 5% FBS + MM (ascorbic acid [AA, 50 μg/mL] and β-glycerolphosphate [BGP, 10 mmol/L]); (*b*) 5% FBS + MTA (10 mg/mL) + MM; (*c*) 5% FBS + MTA (2 mg/mL) + MM; (*d*) 5% FBS + MTA (1 mg/mL) + MM; (*e*) 5% FBS + MTA (0.2 mg/mL) + MM; (*f*) 5% FBS + MTA (0.1 mg/mL) + MM; (*g*) 5% FBS + MTA (0.02 mg/mL) + MM; (*h*) 5% FBS + MTA (0.002 mg/mL) + MM. Note increased mineral nodule formation at 0.02 and 0.002 mg/mL concentrations of MTA-treated cementoblasts.

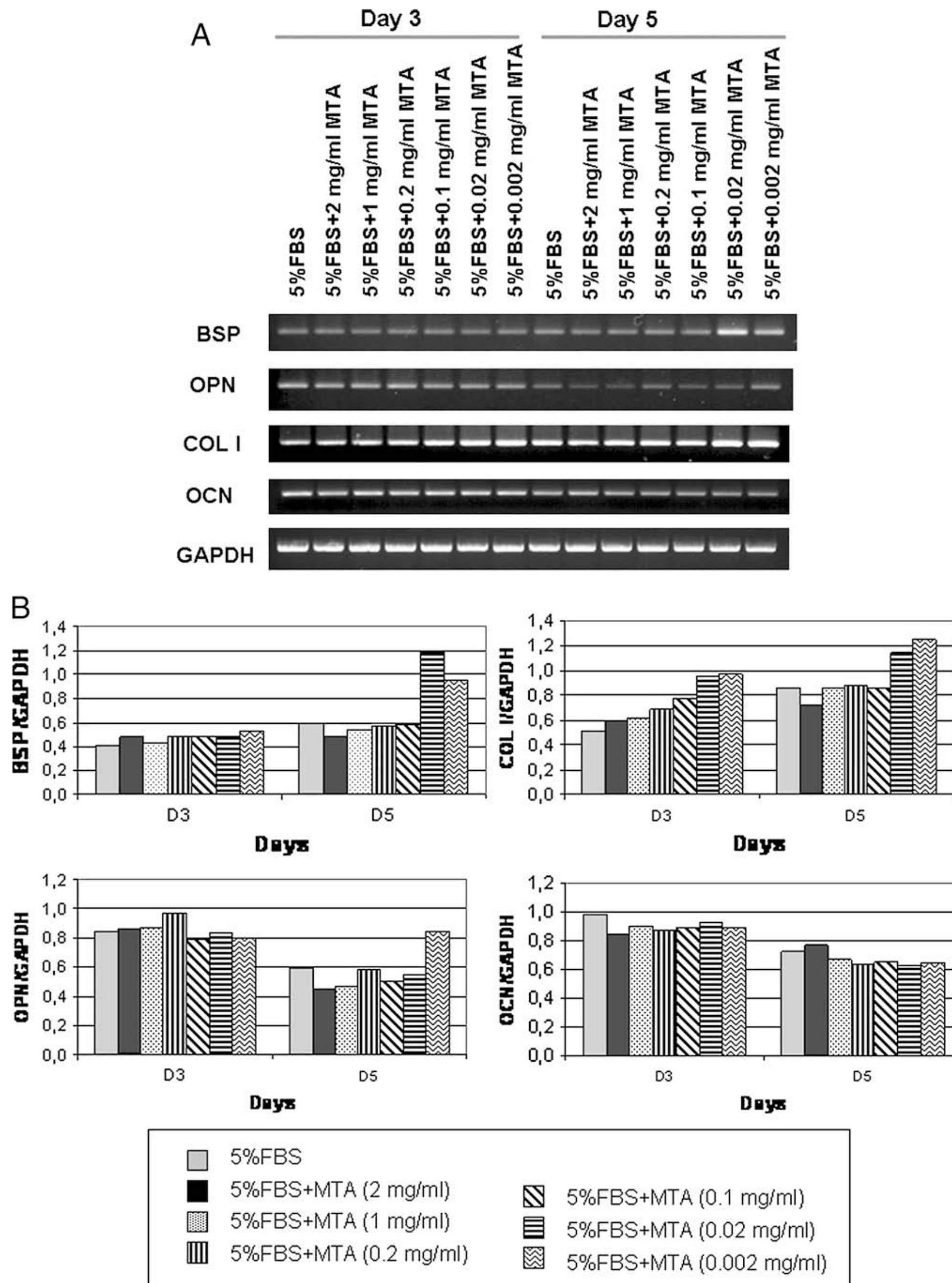


Figure 4. (*a*) mRNA expression with RT-PCR for mineralized tissue associated proteins. GAPDH was used as housekeeping gene. Increased BSP and COL I mRNA expressions were observed at 0.02 and 0.002 mg/mL concentrations of MTA-treated cementoblasts. (*b*) Normalization was expressed as gene/GAPDH.

moistened with anesthetic solution before placement of the MTA into the perforation defect to provide the wet environment needed by the MTA. In this study, if MTA disks were prepared according to the ISO standards (10993-5) for experiments, MTA would be set up in dry conditions and would contact with air (dry environment). However, in clinical conditions, MTA needs wet environment for initiation of chemical reaction (1). Because we thought that this method would better mimic the clinical conditions, different concentrations of MTA in DMEM containing 5% FBS were used. We speculate that this technique is more realistic than the other methodologies because the released bioactive components of WMTA might be different in DMEM before or after setting. Furthermore, the difference in the released bioactive components might interact with surrounding tissues differently.

The results of the present study revealed that MTA did not decrease cell survival except for 20 mg/mL concentration. Moreover, the lowest 2 concentrations of MTA used induced biomineralization of OCCM cells. In the literature, results of cytotoxicity tests demonstrated that MTA was significantly less toxic than the other root-end filling materials currently used. The findings of this study were confirmed by another study with human gingival fibroblasts and L929 cell lines (37). It was also reported that when human osteosarcoma cell lines U2OS and other human osteosarcoma cell lines (Saos-2) were incubated with GMTA, it displayed biocompatibility (38–43). GMTA has also been reported to be biocompatible with mouse cementoblasts (14).

Tani-Ishii et al (44) investigated the effects of MTA on the expression of bone extracellular matrix proteins including COL I, OCN, and

BSP during hard tissue formation in osteoblastic MC3T3-E1 cells. In their study, MTA caused an up-regulation of COL I and OCN mRNA expression after 24 hours. Their results showed that in the presence of MTA, cells were grown faster and produced more mineralized matrix gene expressions in osteoblasts. In our study, MTA also apparently induced COL I and BSP of cementoblasts at the concentrations of 0.02 and 0.002 mg/mL, parallel to mineralization results.

Yasuda et al (45) examined the effects of MTA on the mineralization ability of rat dental pulp cells, and they demonstrated that MTA significantly stimulated mineralization by 60% compared with the control, regulating bone morphogenetic protein-2 mRNA expression. Moreover, Thomson et al (14) investigated the effects of MTA on cementoblast growth and OCN production, and they demonstrated that cementoblasts maintain expression of OCN in the presence of MTA. Although our methodology was different to test the effects of MTA on the cementoblasts, our results regarding increased mineralization were consistent with their results.

Exact regeneration in the injured area of periodontium including cementum, periodontal ligament, and bone is essential in the repair of perforation areas or root ends. Regeneration of periodontal attachment is possible with new cementum, new periodontal ligament, and new bone. In our study, we tested the effects of MTA on cementogenesis with OCCM. The results indicated that MTA not only maintained expression of mineralized tissue associated markers, but it also regulated the mRNA expression and mineralization of cementoblasts. We believe that this study provides valuable information regarding the effects of MTA on cementoblasts.

Several root-end filling materials including amalgam, IRM, and Super-EBA, currently in use, failed to obtain regeneration of periodontal attachment (12). Pitt Ford et al (12) used amalgam, IRM, and Super-EBA, and they demonstrated inflammatory reaction without new cementum in the histologic section of the root ends of monkey teeth. The critical point was the lack of cementogenesis. In our study, we did not test the previously tested other materials but determined the cytotoxic level of MTA first and then evaluated biomineralization of cementoblasts. On the basis of the results of this study, MTA supports cementoblast genotype and phenotype except 20 mg/mL MTA concentrations.

COL I, BSP, OCN, and OPN are important proteins in mineralized connective tissues. The expression of BSP is highly specific for mineralized tissues including bone and cementum (33). Highest BSP expression is found in the newly synthesized and regenerated bone matrix (33, 46). In the present study, we observed increased BSP expressions and mineralization at the 0.02 mg/mL and 0.002 mg/mL concentrations. On the basis of the results of this study, MTA induces mineralization and mineralized tissue associated proteins mRNA expression, which play a critical role in cemental repair and regeneration, namely cementogenesis. These results emphasize that MTA can be called a cementoconductive and/or cementoinductive material by regulating cementoblast cell behavior. It can be stated that MTA has favorable properties regarding biologic response of the cells within the periodontium. Further studies are required to clarify the bioactive components releasing from the MTA material.

On the basis of the results of this experimental study, the following conclusions were drawn:

(1) MTA did not have a negative effect on the cell survival and morphology of cementoblasts.
(2) MTA was not toxic for cementoblasts except 20 mg/mL concentration in DMEM.
(3) MTA induced biomineralization of the cementoblasts at the concentrations of 0.02 and 0.002 mg/mL.
(4) MTA up-regulated BSP and COL I mRNA expression of cementoblasts at the concentrations of 0.02 and 0.002 mg/mL.

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