Effects of Mineral Trioxide Aggregate Mixed with Hydration Accelerators on Osteoblastic Differentiation

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Abstract

Introduction: Despite good physical and biological properties, mineral trioxide aggregate (MTA) has a long setting time. A hydration accelerator could decrease the setting time of MTA. This study assessed the biocompatibility of MTA mixed with hydration accelerators (calcium chloride and low-dose citric acid) and investigated the effect of these materials on osteoblast differentiation. **Methods:** Cell viability was evaluated by the EZ-Cytox assay kit (Daeil Lab Service, Seoul, Korea). The gene expressions of osteocalcin and bone sialoprotein were detected by reverse-transcription polymerase chain reaction and real-time polymerase chain reaction. The mineralization behavior was evaluated with alizarin red staining. **Results:** There was no statistically significant difference in cell viability between experimental groups. The messenger RNA level of osteogenic genes significantly increased in MTA mixed with hydration accelerators compared with the control and MTA mixed with water. MTA mixed with the hydration accelerators resulted in similar mineralization compared with MTA mixed with water. **Conclusions:** Hydration accelerators increase the osteogenic effect and show a similar effect on the mineralization of MTA, which may have clinical applications. *(J Endod 2014;40:2019–2023)*

Key Words

Calcium chloride, citric acid, hydration accelerator, mineral trioxide aggregate

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The ideal root-end filling material should be biocompatible with normal tissues, have high sealing ability, allow periapical tissue regeneration, effectively inhibit pathogenic microorganisms, possess sufficient radiopacity to distinguish the material from surrounding tissue, and have excellent workability and handling properties (1–4).

Materials suggested as root-end filling materials include amalgam, composite resin, glass ionomer cements, reinforced zinc oxide–eugenol cements, superethoxybenzoic acid, and mineral trioxide aggregate (MTA) (5–8). Among these materials, MTA has superior biocompatibility, antibacterial capability, osteoconduction ability, ability to stimulate cytokine release from cells that actively promote hard tissue formation, and sealing ability, with clinical applications being documented (1, 9–17). Despite these good physical and biological properties, MTA has a long setting time (18–21).

MTA is a powder mixture consisting of hydrophilic particles. In the setting process, calcium and hydroxide ions are released from the particle surface of the calcium silicates. Once the critical concentration of ions is reached, the nuclei of the crystallized hydration products appear. Finally, hydration of MTA results in a colloidal gel that solidifies to a hard structure (22). So, adding hydration accelerators such as calcium chloride ($CaCl_2$) (23–25), low-dose (0.1%) citric acid (26), Na_2HPO_4 (7, 27), and calcium lactate gluconate (22, 28, 29) can decrease the setting time.

MTA mixed with hydration accelerators displays a decreased setting time and good biocompatibility with various cells (22–29). However, the effects of MTA mixed with hydration accelerators on osteogenic differentiation and mineralization behavior have not been investigated. The purpose of this study was to investigate the effect of MTA mixed with hydration accelerators ($CaCl_2$ and low-dose citric acid) on the differentiation and mineralization of osteoblasts.

Materials and Methods

Cell Culture

MC3T3-E1 cells derived from mouse osteoblasts were cultured in alpha-minimum essential medium (α-MEM; GIBCO, Grand Island, NY) with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Invitrogen). The cells were cultured until subconfluence at 37°C in a humidified atmosphere containing 5% CO_2. To induce osteogenic differentiation, the cells were incubated in an osteoinduction medium containing α-MEM supplemented with 2% FBS, antibiotics, 50 μg/mL ascorbic acid, and 10 mmol/L beta-glycerophosphate.

Preparation of Material Extracts

MTA was mixed in accordance to the manufacturer's instructions under aseptic conditions with or without hydration accelerators, and discs were prepared using a sterile cylindric polyethylene tube 8 mm in diameter and 3 mm in height. Three different groups were formed according to the hydration accelerators used. In group 1, the samples were prepared by mixing white ProRoot MTA (Dentsply Tulsa Dental, Tulsa, OK) with distilled water. In groups 2 and 3, MTA was mixed with 10 wt% $CaCl_2$ and 0.1 wt% citric acid, respectively, in the same weight ratio (Table 1). To obtain the complete setting, discs were kept for 6 hours at 37°C and 95% relative humidity. After setting,

TABLE 1. Composition of the Tested Materials

Group	Powder	Liquid
1	MTA (1 g)	Distilled water (0.3 mL)
2	MTA (1 g) with 10 wt% $CaCl_2$ (0.1 g)	Distilled water (0.33 mL)
3	MTA (1 g)	0.1 wt% CA (0.3 mL)

CA, citric acid; $CaCl_2$, calcium chloride; MTA, mineral trioxide aggregate.

discs were demolded and exposed to ultraviolet light for 1 hour on each surface to ensure sterility and transferred into conical centrifuge tubes. Discs were incubated in 10 mL α-MEM containing 2% or 10% FBS and antibiotics at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours. The supernatant was collected, and all material extracts were sterilized using 0.20-μm filters (Minisart; Sartorius Stedim Biotech, Goettingen, Germany).

Cell Viability Assay

MC3T3-E1 cells were seeded in 96-well culture plates at a density of 1×10^4 cells per well and preincubated in α-MEM containing 10% FBS and antibiotics for 24 hours to allow attachment of the cells before adding the material extracts. The cells were then exposed to the material extracts for 48 hours. The cell viability was examined using an EZ-Cytox enhanced cell viability assay kit (Daeil Lab Service, Seoul, Korea) according to the manufacturer's recommendations. Briefly, 10 μL Ez-Cytox (tetrazolium salts) was added to the medium, and the cells were incubated at 37°C for 3 hours. The absorbance was measured at 420 nm with a background subtraction of 650 nm using a VERSAmax multiplate reader spectrophotometer (Molecular Devices, Sunnyvale, CA).

Reverse-transcription Polymerase Chain Reaction and Quantitative Real-time Polymerase Chain Reaction

MC3T3-E1 cells were seeded in 6-well culture plates at a density of 2×10^5 cells per well and preincubated in growth medium for 24 hours. The cells were then exposed to a differentiation medium containing material extracts for 1 and 3 days. Untreated MC3T3-E1 cells were used as a control. The total RNA was isolated from the cultures using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The complementary DNA was synthesized using a Maxime RT PreMix Kit (iNtRON Biotechnology, Seongnam, Korea). Each reaction consisted of an initial denaturation at 95°C for 1 minute followed by denaturation at 95°C for 30 seconds, annealing at a temperature optimized for each primer pair for 30 seconds, and extension at 72°C for 30 seconds. After the required number of cycles (25–30 cycles), the reactions underwent a final extension at 72°C for 5 minutes. The primer sequences were osteocalcin (OCN), (F) 5′-CTCCTGAGTCTGACAAAGCCTT-3′, (R) 5′-GCTGTGACATCCATTACTTGC-3′; bone sialoprotein (BSP), (F) 5′-ACACTTACCGAGCTTATGAGG-3′, (R) 5′-TTGCGCAGTTAGCAATAGCAC-3′; and beta-actin, (F) 5′-TTCTTTGCAGCTCCTTCGTTGCCG-3′, (R) 5′-TGGATGGCTACGTACATGGCTGGG-3′. Each polymerase chain reaction (PCR) product was loaded onto 1.5% agarose gels by electrophoresis and visualized by ethidium bromide staining. Quantitative real-time PCR was conducted using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) in triplicate in the MiniOpticon Real-Time PCR system (Bio-Rad, Hercules, CA). The thermal cycling conditions were as follows: 15 minutes at 95°C followed by 40 cycles of 95°C for 10 seconds, 58°C for 15 seconds, and 72°C for 20 seconds. The melting curve analysis was conducted in 0.2-second intervals from 65°C–95°C. Primer sequences were OCN, (F) 5′-GCAATAAGGTAGTGAACAGACTCC-3′, (R) 5′-GTTTGTAGGCGGTCTTCAAGC-3′; BSP, (F) 5′-AAGCAGCACCGTTGAGTATGG-3′, (R) 5′-CCTTGTAGTAGCTGTATTCATCCTC-3′; and beta-actin, (F) 5′-ACCCACACTGTGCCCATCTAC-3′, (R) 5′-GCCATCTCCTGCTCGAAGTC-3′. All the primers were synthesized by Bioneer (Daejeon, Korea). All quantified values were normalized to an endogenous control beta-actin. The data for gene expression were analyzed by the $\Delta\Delta$Ct method (30).

Mineralization Assay

MC3T3-E1 cells were cultured in 24-well plates at a density of 1×10^5 cells per well with differentiation medium containing material extracts for 7 and 14 days. Mineralization of MC3T3-E1 cells was observed by alizarin red staining. After 7 and 14 days of culture, each cell layer was rinsed with phosphate buffered saline, fixed with formaldehyde (Sigma-Aldrich, St Louis, MO), and stained with 0.5% alizarin red (pH = 4.2) at 37°C. Finally, they were photographed and observed with a microscope (CKX41SF; Olympus Optical Co Ltd, Tokyo, Japan). Staining density was quantified using ImageJ (Version 1.47; National Institutes of Health, Bethesda, MD).

Statistical Analysis

Each experiment, containing triplicate independent samples, was repeated at least twice, and qualitatively identical results were obtained. One-way analysis of variance followed by the Tukey post hoc test was used to determine any statistically significant differences according to the test materials with the use of the SPSS 18.0 software program (SPSS, Chicago, IL). Differences were considered significant at $P < .05$.

Results

Cell Viability

The effect of different material extracts on the cell viability of MC3T3-E1 cells was shown in Figure 1. No statistical difference in cell viability between groups was evident ($P > .05$).

Effect of MTA Mixed with Hydration Accelerators on Osteogenic Gene Expression

To investigate the effect of MTA mixed with hydration accelerators on osteogenic differentiation of MC3T3-E1 cells, the expressions of osteogenic genes were measured. Reverse-transcription PCR showed an increased expression of OCN and BSP in MTA mixed with hydration accelerators compared with the control and MTA mixed with water on day 1. Also, the band density of OCN and BSP was increased in the experimental groups compared with the control on day 3 (Fig. 2).

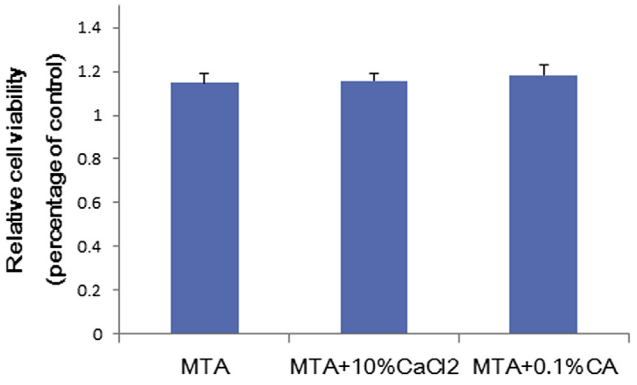

Figure 1. The effect of MTA mixed with hydration accelerators on cell viability. Results are expressed as the relative cell viability (percentage of control, $n = 10$). There was no statistical difference between the groups ($P > .05$).

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