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Original

Effect of a novel fluorapatite-forming calcium phosphate cement with calcium silicate on osteoblasts in comparison with mineral trioxide aggregate

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Abstract: We compared the effects of treatment with fluorapatite-forming calcium phosphate cement (FA-forming CPC) containing tricalcium silicate (TCS) and those of mineral trioxide aggregate (MTA), the gold standard endodontic cement, on cultured osteoblast-like cells (ROS 17/2.8 cells; ROS cells). The FA-forming CPC powder consisted of 61.29% $CaHPO_4$, 32.26% $CaCO_3$, and 6.45% NaF. One part TCS was combined with nine parts FA-forming CPC powder to make FA-forming CPC with TCS. A 1.5-M phosphate solution was mixed as a cement liquid with a powder/liquid ratio of 2.22. Cell culture was carried out using cell culture inserts, whereby each test material was put on a porous membrane insert in the cell culture plate. Proliferation, morphologic changes, and alkaline phosphatase activity in ROS cells were measured in the presence of FA-forming CPC with TCS and MTA and compared. The logarithmic growth phase and cellular morphologic changes in ROS cells were identical in all experimental groups. Additionally, no significant difference in alkaline phosphatase activity was noted in ROS cells exposed to FA-forming CPC with TCS and those exposed to MTA. In conclusion, FA-forming CPC with TCS has characteristics identical to those of MTA under the present experimental conditions and may thus be useful for endodontic applications.
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Keywords: calcium phosphate cement; fluorapatite; calcium silicate; cell proliferation; alkaline phosphatase activity; osteoblast.

Introduction

Most endodontic failures result from leakage of irritants from pathologically involved root canals into periradicular tissues (1). The ideal filling materials should seal off communication pathways between the root canal system and surrounding tissues (2). Mineral trioxide aggregate (MTA) is a promising biomaterial that is widely used in various endodontic treatments due to its excellent biocompatibility, superior sealing, and ability to set in the presence of blood (3). However, a disadvantage of MTA is its difficult handling properties, particularly its relatively long setting time (4). Thus, materials more suitable for endodontic treatment are needed.

Calcium phosphate cement (CPC) containing an equimolar mixture of tetracalcium phosphate (TTCP),

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i.e., $Ca_4(PO_4)_2O$, and dicalcium phosphate anhydrous (DCPA), $CaHPO_4$, has self-hardening characteristics and forms hydroxyapatite (HA) as an end-product (5). CPC is of interest as a bone graft biomaterial in dental and medical applications, due to its setting properties, biocompatibility, and osteoconductivity (6,7). It is important to note that CPC of different compositions can form various end-products in addition to HA, such as dicalcium phosphate dihydrate, octacalcium phosphate, and fluorapatite (FA) (8). Because CPC end-products may control resorption rates, CPC that results in resorption rates suitable for diverse clinical applications can be developed. Takagi et al. developed self-hardening CPCs based on DCPA and CaO or $CaCO_3$ and reported that the addition of sodium fluoride (NaF) to these CPCs also resulted in formation of FA and CaF_2 (9).

For endodontic treatments such as root-end filling, perforation repair, pulp-capping, and pulpotomy, it is desirable to use CPC that is biocompatible and osteoconductive, yet non-bioresorbable, in soft and hard tissue. Fully or partially fluoridated HA materials were reported to significantly lower solubility in acids (10) and to promote bone formation in rat tibia and dog mandible (11,12). Moreover, the addition of tricalcium silicate (TCS) improved sealing ability and raised the pH of CPC to a level comparable to that produced by MTA (13). Hence, FA-forming CPC with TCS is likely to have considerably lower resorbability than HA-forming CPC, as well as good sealing ability and sufficient alkalinity.

We previously reported that setting time was shorter for FA-forming CPC with TCS than for MTA. In addition to formation of FA, it had adequate diametral tensile strength and pH and was biocompatible with rat subcutaneous connective tissue (14). The effect of FA-forming CPC with TCS on the proliferation and alkaline phosphatase (ALP) activity of osteoblasts has not been determined. Therefore, proliferation, morphologic changes, and ALP activity induced by the novel endodontic cement FA-forming CPC with TCS and by MTA were evaluated and compared in the present study.

Materials and Methods

Test materials

All chemicals were of reagent grade and were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). FA-forming CPC powder without TCS consisted of 61.29% (percentage mass) DCPA ($CaHPO_4$), 32.26% calcium carbonate ($CaCO_3$), and 6.45% sodium fluoride (NaF), as described previously (15). TCS was prepared by combining 3 M CaO with 1 M fumed silica (SiO_2) in water and stirring for 8 h. The slurry was filtered, dried, and then heated at 1,450°C. The resulting powder was ground to a median size of 20 μm. To prepare FA-forming CPC powder with TCS, one part TCS was added to nine parts FA-forming CPC powder. A 1.5-M sodium phosphate solution (pH 5.6) was used as the cement liquid. A powder/liquid (P/L) ratio of 2.22 was chosen so as to produce pastes with workable consistencies, as previously described (15). MTA (ProRoot MTA, Dentsply Tulsa, Johnson City, TN, USA) was prepared according to the manufacturer's instructions and used as the gold standard endodontic cement.

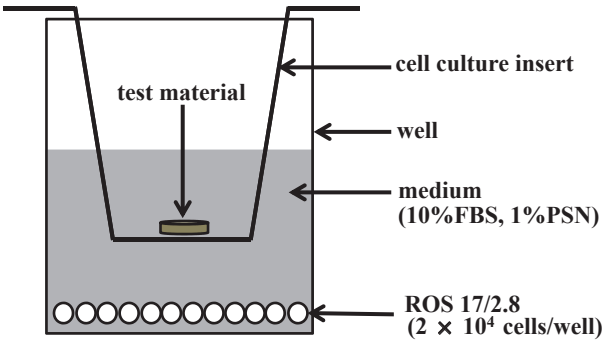

Fig. 1 Schematic representation of a culture plate with a cell culture insert.

Each sample of test material (diameter, 3 mm; thickness, 0.5 mm) was allowed to set for 24 h at 37°C and 100% humidity and put in 700 μL of α-minimal essential medium (α-MEM; Gibco BRL, Rockville, MD, USA) for 3 days, as previously described (16).

Cell culture

The rat clonal osteoblast line ROS 17/2.8 (hereafter, ROS cells) was used in this study. The cells were maintained in growth medium consisting of α-MEM supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA) and 1% (vol/vol) penicillin–streptomycin–neomycin solution (PSN; Sigma Chemical, St. Louis, MO, USA) under standard culture conditions (37°C, 100% humidity, 95% air, 5% CO_2), as previously described (17).

Cell proliferation and morphology

Cell proliferation in the presence of the test materials was measured using 24-well cell culture plates. Each well contained a culture plate insert with a porous membrane (pore size, 3 μm; BD Falcon, Franklin Lakes, NJ, USA), as previously described (17). Briefly, ROS cells were plated at a density of 2.0×10^4 cells per well in 500 μL α-MEM containing 10% (vol/vol) FBS. After incubation for 24 h, a culture plate insert with one pellet of the test

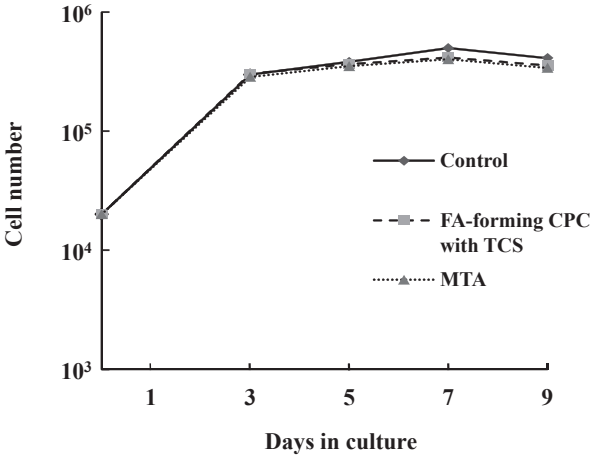

Fig. 2 Effects of FA-forming CPC with TCS, MTA, and control on proliferation of ROS 17/2.8 cells. Proliferation and viability of ROS cells at 3, 5, 7, and 9 days of culture were determined using a Cell Counting Kit-8.

material was put into each well (Fig. 1). Cells cultured without the test material served as the control. Cells were enumerated using a Cell Counting Kit-8 (Dojindo Molecular Technologies Inc., Kumamoto, Japan) after 3, 5, 7, and 9 days, at which time points the medium was replaced with fresh medium containing 10% (vol/vol) cell-counting reagent after observation of changes in cell morphology by light microscopy (DIAPHOT, Nikon, Tokyo, Japan). After further incubation for 1 h, a microtiter plate reader (Titertek Multiskan Plus, Flow Laboratories, McLean, VA, USA) was used to measure intensity of the reaction products at 450 nm. The number of cells was calculated from the absorbance value on the basis of a standard curve.

ALP activity

ALP activity in the presence of the test materials was measured using previously described methods (17). Briefly, ROS cells were seeded onto 24-well microplates at a density of 2×10^4/well for up to 9 days, using cell culture inserts, as described above (Fig. 1). At each time point, 500 μL of enzyme assay solution (8 mM *p*-nitrophenyl phosphate, 12 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.1 M glycine-NaOH buffer; pH 10.5) were added to the cells in each well, and the plate was incubated for several minutes at 37°C. The enzymatic reaction was terminated by the addition of 500 μL of 0.1 M NaOH. The amount of *p*-nitrophenol released by the enzymatic reaction was determined by measuring absorbance at 405 nm using a microtiter plate reader. ALP activity was defined as the amount of *p*-nitrophenol produced per minute.

Data analysis

The results of ALP activity were statistically analyzed using one-way analysis of variance and the Bonferroni test of an average of three replicates. A P value of <0.05 was considered to indicate statistical significance. Data are expressed as means ± SD.

Results

Cell proliferation and cell morphology

Proliferation of ROS cells was determined in the presence of FA-forming CPC with TCS, MTA, or control for up to 9 days of culture (Fig. 2). The logarithmic growth phase was equal in all cultures at 3 and 5 days after seeding. Light micrographs of ROS cells were obtained at each time point. In all experimental groups, ROS cells grew and formed a confluent monolayer up to day 9 of culture. There were no morphologic differences between the test materials and control at any time point (Fig. 3).

ALP activity

ALP activity was determined in ROS cells up to day 9 of culture. The only significant difference observed was between the control and MTA groups at 3 days of culture (Fig. 4).

Discussion

MTA is widely used in endodontic treatment because of its superior sealing ability and biocompatibility (3). However, MTA has inadequate handling properties and long setting time (4). Therefore, we developed a novel endodontic cement, FA-forming CPC with TCS.

To minimize incidences of local and systemic adverse effects, the biocompatibility of endodontic materials should be investigated in *in vitro* and *in vivo* tests before their clinical application (18). Many parameters can be used to characterize the biocompatibility of an endodontic material, including genotoxicity, carcinogenicity, cytotoxicity, histocompatibility, and antimicrobial effects (18). Numerous cell culture studies have evaluated cytotoxic reactions induced by endodontic materials. These studies focused on the evaluation of growth inhibition, synthesis of DNA, RNA, and protein, and alterations in cell morphology (19-23). In this study, responses of osteoblast-like cells to FA-forming CPC with TCS were evaluated and compared with MTA in relation to proliferation, morphologic alterations, and ALPase activity.

Proliferation of ROS cells in the presence of FA-forming CPC with TCS was similar to that in the presence of the control or MTA. A previous study found that MTA was one of the least cytotoxic dental materials (3). The present results indicate that FA-forming CPC

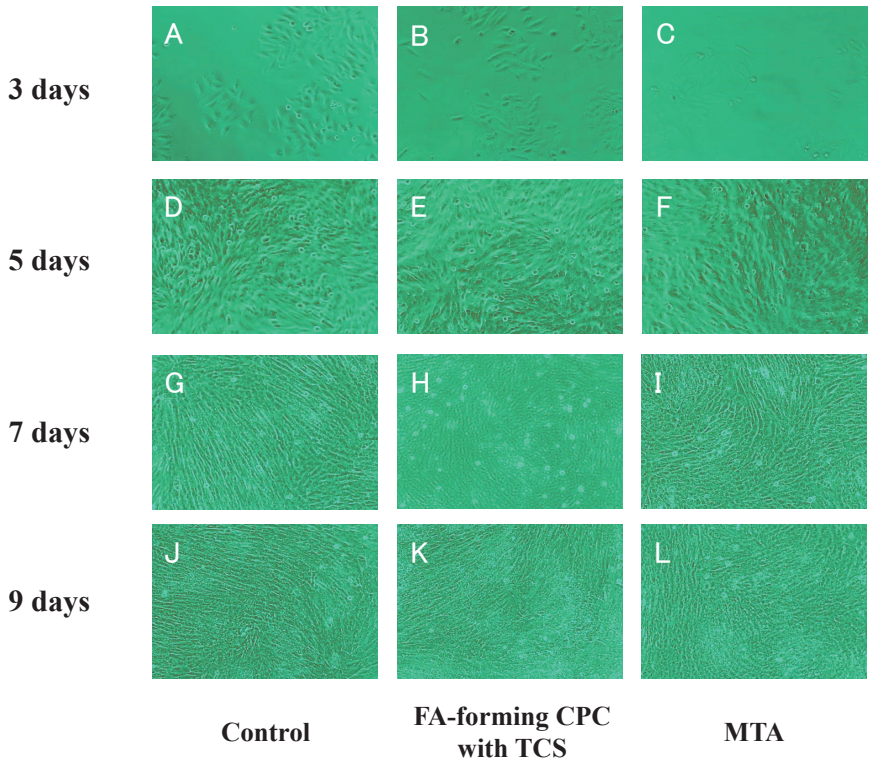

Fig. 3 Morphology of ROS 17/2.8 cells after incubation with FA-forming CPC with TCS, MTA, and control (original magnification, ×100). Control: A, D, G, and J; FA-forming CPC with TCS: B, E, H, and K; MTA: C, F, I, and L.

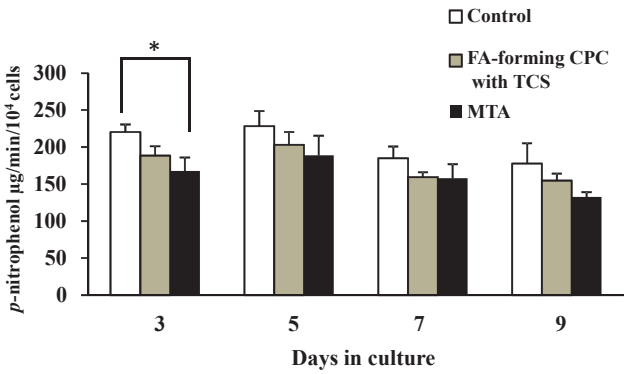

Fig. 4 Effects of FA-forming CPC with TCS, MTA, and control on ALP activity in ROS 17/2.8 cells. ALP activity was defined as the amount of *p*-nitrophenol produced per minute. Value are means ± SD. *Significant difference ($P < 0.05$).

with TCS had excellent biocompatibility, similar to that of MTA. We recently reported that FA-forming CPC with TCS and MTA were highly biocompatible with rat subcutaneous connective tissue (14). The proliferation of ROS cells observed in the present study is consistent with our previous finding. In aqueous conditions, FA-forming CPC with TCS is almost entirely converted to FA within 24 h (14). FA typically has very high crystallinity, as compared with HA, and good biocompatibility with not only osteoblasts but also dental pulp cells, mesenchymal cells, and endothelial cells (24,25). The excellent biocompatibility of FA-forming CPC plus TCS with ROS cells is attributable to its conversion to FA.

Furthermore, FA-forming CPC with TCS, MTA, and the control had equally favorable biocompatibility and no effect on cell morphology. Biocompatibility with cell morphology may also be due to the conversion to biocompatible FA. Haglund et al. (26) reported that MTA caused lysis of adjacent cells and denaturation of medium proteins because, when freshly mixed, MTA releases various cytotoxic chemical by-products. However, when set, MTA is biocompatible with cells. Therefore, all test materials were allowed to set for 24 h in 100% humidity and then put in α-MEM for 3 days before cell culture, for the assessment of cell morphology in set test materials.

ALP hydrolyzes the ester bond of organic phosphate compounds under alkaline conditions and is important in bone calcification (27). In this study, ALP activity was used to assess cell differentiation in test materials. The only significant difference in ALP activity was between the control and MTA groups at 3 days of culture. Lui et al. (28) reported that FA showed good biocompatibility with osteoblast-like MG-63 cells, and reported long-

term growth and differentiation of these cells. Because FA-forming CPC with TCS is converted to biocompatible FA, our findings regarding ALP activity in ROS cell are in agreement with their results (28). The statistically significant difference between the control and MTA groups at 3 days of culture is likely due to the small amount of residual chemical by-products of MTA, which may be present for up to 3 days of culture, even if MTA is adequately immersed in α-MEM before cell culture.

In conclusion, FA-forming CPC with TCS and MTA—the gold standard endodontic cement—had similar effects on proliferation, morphology, and ALP activity in ROS cells. FA-forming CPC with TCS may thus be useful for endodontic applications, although additional studies of its sealing ability and characteristics in animal models of endodontic procedures are necessary before clinical application.

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