Effects of a calcium phosphate cement on mineralized nodule formation compared with endodontic cements

Hidehiro OGATA¹, Makoto HAYASHI¹, Hiromasa TSUDA², Naoto SUZUKI², Masao MAENO³, Akiyoshi SUGAWARA⁴ and Bunmai OGISO¹

¹Department of Endodontics, Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan
²Department of Biochemistry, Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan
³Department of Oral Health Sciences, Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan
⁴Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan
Corresponding author, Makoto HAYASHI; E-mail: hayashi-m@dent.nihon-u.ac.jp

The aim of this study was to investigate mineralizing ability of a premixed calcium phosphate cement (premixed-CPC) compared to mineral trioxide aggregate (MTA) and zinc oxide eugenol cement (SuperEBA) in ROS17/2.8 cells. The measurements of cell proliferation, alkaline phosphatase (ALPase) activity and mineralized nodule formation in the presence or absence (control) of the test materials were performed using a cell culture insert method with the test materials placed on a porous membrane of culture plate insert. Mineralized nodules were detected by staining with alizarin red, and the calcium content of the mineralized nodules was determined quantitatively using a calcium assay kit. Premixed-CPC and MTA indicated significantly higher cell proliferation, ALPase activity, mineralized nodule formation, and calcium content in nodules than those of SuperEBA (p<0.05). The present results suggest that premixed-CPC has the same mineralizing ability as MTA.

Keywords: Calcium phosphate cement, Endodontic cement, Biocompatibility, Mineralization

INTRODUCTION

The majority of endodontic failures occur as a result of leakage of irritants into the periapical tissues¹⁻³. Ideal orthograde or retrograde filling materials should seal the pathways of communication between the root canal system and its surrounding periodontal tissues⁴. Critical pathways of communication between the root canal orthograde or retrograde filling materials should seal the time of MTA is relatively long, according to manufacturers’ instructions, and the setting handling property when this cement was prepared 3 to 4 h after mixing⁶. Furthermore, endodontic materials should be easily manipulated, have adequate working time and quick setting time, and that the presence of moisture does not affect their sealing ability.

Some examples of the endodontic materials are gutta-percha, zinc oxide eugenol-based cements, composite resin, glass ionomer cements, water settable cements, gold foil, polycarboxylate cements, polyvinyl cements, and amalgam¹⁻³. Unfortunately, most of them have shown different levels of weakness in biocompatibility, leakage, solubility, handling properties, moisture incompatibility, and cost. Mineral trioxide aggregate (MTA) materials developed in 1993 by Torabinejad et al.⁵ have overcome most of these weaknesses and have been in widespread use clinically. However, MTA has disadvantages: it does not have good handling property when this cement was prepared according to manufacturers’ instructions, and the setting time of MTA is relatively long, i.e. 3 to 4 h after mixing⁶. Calcium phosphate cements (CPC) have become useful materials in a variety of dental and medical applications because of the combination of setting properties and biocompatibility. The application of calcium phosphate materials as a bone substitute or bone graft may be traced back to Albee’s report⁷ which described that a triple calcium phosphate compound placed in a bony defect promoted osteogenesis or new bone formation. Brown and Chow⁸ described that certain combinations of finely powdered calcium phosphates, when mixed with water, harden like a cement with chemical composition and crystal structures similar to that of teeth and bone. Their article is the first report about CPC consisting of equimolar amounts of tetracalcium phosphate (TTCP, Ca₄(PO₄)₂O) and dicalcium phosphate anhydrous (DCPA, CaHPO₄). In vitro studies and animal models have indicated that CPC was useful as a filler/sealer in root canal treatment⁹⁻¹¹. Sugawara et al.¹² reported that the glycero-CPC paste showed better biocompatibility than a number of presently used root canal filling or sealing materials. Takagi et al.¹³ also reported the feasibility of formulating premixed-CPCs that are stable in a package, resist washout, and will harden only after being delivered to the defect site. Glyceral was used as the liquid because the CPC hardening reaction does not occur in a water-free glyceral environment. Hydroxypropyl methylcellulose (HMC) and Na₂HPO₄ (disodium hydrogen phosphate) was added to improve the paste cohesiveness and accelerate cement hardening, respectively. This premixed-CPC exhibited good handling properties, set in an aqueous environment, and formed hydroxyapatite as the final product¹⁴. The results of a preliminary in vivo study using a rabbit model showed that the material had good biocompatibility¹⁵. The above results suggest that the premixed-CPC has many desirable properties as a root-end filling or
root repair material, and it may overcome the disadvantages of MTA. Because there have been no studies comparing cultured osteoblastic cell activities on premixed-CPC and other endodontic materials. This study was conducted to analyze cell proliferation, alkaline phosphatase (ALPase) activity and mineralized nodule formation of a premixed-CPC as a new root-end-filling or root repair material with MTA as the gold standard control.

MATERIALS AND METHODS

Cell culture
The rat clonal cell line ROS 17/2.8 was used as the osteoblasts in this study. The cells were maintained in a growth medium consisting of α-minimal essential medium (α-MEM; Gibco BRL, Rockville, MD, USA) containing 10 volume fraction % heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA) and 1% volume fraction penicillin-streptomycin-neomycin solution (PSN; Sigma Chemical, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Preparation of test materials
Test materials used in this study (Table 1) were a premixed-CPC (courtesy of Drs. Laurence Chow and Shozo Takagi, Paffenbarger Research Center, ADAF at NIST, MD, USA), MTA (ProRoot®MTA, Dentsply Tulsa, TN, USA) and eugenol-based cements (SuperEBA, Harry J. Boworth Company, IL, USA). The premixed-CPC was prepared by mixing of equimolar amounts of TTCP and DCPA with a glycerol liquid that contained 30 mass fraction % Na₂HPO₄ (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 0.55 mass fraction % HMC (Wako Pure Chemical Industries Ltd.) at a powder-to-liquid mass ratio of 4 as previously described. MTA and eugenol-based cements were prepared according to manufacturers’ instructions.

Each pellet (3 mm diameter and 0.5 mm thickness) was allowed to set for 24 h at 37°C in 100% humidity, and placed in α-MEM (0.7 mL) for 3 days as previously described by Haglund et al., Saidon et al. and Takita et al.

Determination of cell proliferation
The measurement of cell proliferation in the presence of the test materials was performed using 24-well cell culture plates, and each well had a culture plate insert with a porous bottom (3 μm pore size) (BD Falcon, Franklin Lakes, NJ, USA). ROS 17/2.8 were seeded onto the plates at an initial density of approximately 2.0×10⁴ cells per well in 0.5 mL α-MEM containing 10 volume fraction % FBS. The cells were incubated for 24 h to allow adhesion, and then a culture plate insert with one pellet of the test material was placed into each well (Fig. 1). Cells cultured without test material served as the negative control. The number of cells was determined using a Cell Counting kit 8 (Dojindo Molecular Technology, Inc., Kumamoto, Japan).

Table 1  Materials used in this study

<table>
<thead>
<tr>
<th>Material (manufacturer)</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premixed-CPC (courtesy from Paffenbarger Research Center, ADAF at NIST, Maryland, USA)</td>
<td>Paste: Tetracalcium phosphate, dicalcium phosphate anhydrous, glycerol, hydroxypropyl methylcellulose, disodium hydrogen phosphate; Liquid: Distilled water</td>
</tr>
<tr>
<td>MTA (ProRoot®MTA, Dentsply Tulsa, Tennessee, USA)</td>
<td>Powder: Tricalcium silicate, bismuth oxide, dicalcium silicate, tricalcium aluminate, calcium sulfate dehydrate or gypsum; Liquid: Distilled water</td>
</tr>
<tr>
<td>Eugenol-based cements (SuperEBA, Harry J. Boworth Company, Illinois, USA)</td>
<td>Powder: Zic oxide 60%, alumina 34%, natural resin 6%; Liquid: Ortho ethoxy, benzoic acid 62.5%, eugenol 37.5%</td>
</tr>
</tbody>
</table>

Fig. 1 Schematic representation showing the culture plate with cell culture insert. Cell culture insert: samples were placed. Well (24-well microplates): ROS 17/2.8 were incubated for 24 h to allow adhesion, and then a culture plate insert with one pellet of test material was placed into each well.
Technologies Inc., Kumamoto, Japan) at 3, 5, 7 and 9 days. At the specified time periods, the medium was replaced with fresh medium containing 10 volume fraction % Cell Counting reagent, and the incubation continued for 1 h. After incubation, the absorbance of the reaction products was measured at 450 nm with a microtitre plate reader (Titertec Multiskan Plus, Flow Laboratory, McLean, VA, USA). The cell number was calculated from the absorbance value relative to a standard curve.

**Determination of ALPase activity**
The cells were plated on 24-well microplates at a density of 2×10^4 cells/well for up to 12 days. Two hundred microlitres of enzyme assay solution (8 mM p-nitrophenyl phosphate, 12 mM MgCl₂, 0.1 mM ZnCl₂, 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 enzyme reaction was terminated by the addition of 50 mL of 0.1 M NaOH. The amount of p-nitrophenol released by the enzymatic reaction was determined by measuring the absorbance at 405 nm in the microtiter plate reader. One unit of ALPase activity was defined as the amount required for the liberation of 1.0 µmol p-nitrophenol/min. The enzyme activity was recorded as milliunits (mU)/10^4 cells.

**Determination of mineralized nodule formation**
The cells were plated in 24-well tissue culture plates at a density of 1.25×10^5 cells/well and cultured in ROS17/2.8 with 50 mM β-glycerophosphate (β-GP) and 50 µg/mL ascorbic acid (AA) for up to 11 days to assess mineralized nodule formation as a function of osteogenic behavior.
The culture medium was changed every second or third day. The condition of the cells and nodule formation were checked routinely by phase-contrast microscopy (Nikon DIAPHOT, Nikon Co., Tokyo, Japan). Mineralized nodules were detected by staining with Alizarin Red S (Wako Pure Chemical Industries Ltd.), as described previously.

**Determination of calcium and protein content**
The cells were plated in 24-well tissue culture plates at a density of 1.25×10^5 cells/well and cultured in ROS17/2.8 with 50 mM β-GP and 50 µg/mL AA for 11 days, at which time the medium was discarded, 300 µL of 0.5 M HCl was added to each well, and the cells were incubated overnight to decalcify the mineralized nodules. The calcium content was determined quantitatively using the Calcium E-Test Kit (Wako Pure Chemical Industries Ltd., Osaka, Japan). The protein content was determined quantitatively using the protein assay solution (Bio-Rad Laboratories, Hercules, CA, USA) after evaporation of the HCl from the samples.

**Statistical analysis**
All experiments were performed in triplicate. Each value represents the mean±standard deviation (SD). In this study, the SD is considered to be the standard uncertainty of the measurements. The significance (p<0.05) of differences among the groups was determined using ANOVA test followed by Student-Newman-Keuls Test.

**RESULTS**

**Cell proliferation**
Figure 2 shows cell proliferation of ROS17/2.8 after exposure to premixed-CPC, MTA and SuperEBA for 3, 5, 7, and 9 days. Premixed-CPC, MTA and the control indicated significantly higher cell proliferation than SuperEBA (p<0.05). There were no significant difference among premixed-CPC, MTA and the control.

**ALPase activity**
Figure 3 shows the ALPase activity at 5, 7, and 9 days. There were no significant difference among premixed-CPC, MTA, and the control at each experimental point except for 3 days culture. SuperEBA showed significantly (p<0.05) lower ALPase activity at all time points.

**Mineralized nodule formation**
Figure 4 shows the mineralized nodule formation, as confirmed by Alizarin Red S staining, of test materials for up to 11 days of culture. In the control, premixed-CPC, and MTA groups, mineral deposits were detectable with the naked eye in the 7-day culture. The mineralized nodules enlarged between 9 days and 11 days.

In contrast, in the presence of SuperEBA, the appearance of staining of mineralized nodules by Alizarin Red S did not occur throughout the experimental period. The intensity of Alizarin Red S staining was clearly lower than those in the other three groups.

**Calcium and protein content**
Figure 5 shows the Ca/protein ratios of the mineralized nodules of the 11 days culture in the presence or absence of the test materials. The ratios in control, premixed-CPC...
DISCUSSION

The aim of this study was to evaluate osteoblast-like cell response to premixed-CPC on proliferation, mineralization and ALPase activity, as compared with other endodontic materials. Our results showed that premixed-CPC has the same characteristics as those of the control and MTA. Previous studies using various cell culture systems have shown that MTA is one of the least cytotoxic dental materials. Similarly, premixed-CPC has been reported to have excellent biocompatibility due to the fact that premixed-CPC was nearly completely converted to hydroxyapatite (HA) within 24 h in an aqueous environment. The present results are consistent with these previous findings, showing that there were no significant differences among premixed-CPC, MTA and control in nearly all tests. In contrast, the results showed that SuperEBA exhibited cytotoxicity. SuperEBA cement consists of a powder containing zinc oxide (60–75%), fused quartz or alumina (20–35%) and hydrogenated resin (6%), and a liquid containing of 63% ethoxybenzoic acid (EBA) and 37% eugenol. The EBA encourages the formation of a crystalline structure that improves the strength of the material. Eugenol has been reported to be an antimicrobial and anti-inflammatory agent; however, previous in vivo and in vitro studies have demonstrated its toxic affects. It has been reported that eugenol inhibits mitochondrial activity and alters the cell membrane. ALPase activity, which is closely related to new bone formation, was used as a measure of osteoconductivity of the test material. Sugawara et al. showed that CPC exhibited a much higher ALPase activity than the control. However, our results showed that premixed-CPC, MTA and control cells indicated no significant difference at each experimental point except for the 3-day culture. One possible reason for the different...
results is that we used a premixed-CPC whereas a
conventional powder-and-liquid CPC was used in
Sugawara et al.15. Other possible reasons include the
differences in the type of cells used, the conditions for
preparing the test materials, and the test material-to-
culture medium ratio. It is noted that in the present
study the test specimens were allowed to set for 24 h at
37°C in 100% humidity, and then immersed for 3 days in
α-MEM prior to cell culture. The previous tissue culture
studies have focused on the cytotoxicity of endodontic
materials in the freshly mixed state before setting15,16.
When freshly mixed, these materials release a host of
chemical by-products that are cytotoxic to the cells in the
culture. Under clinical conditions, however, these
by-products are diluted in the interstitial tissue fluids
and are eliminated through the vasculature. Therefore,
recent studies have examined the effect of preset, washed
materials on cells. Thus, any cytotoxic effects observed
on these pre-conditioned samples would suggest
persisting tissue incompatibility.

The mineralized nodule formation of osteoblast
increased gradually between 7 days and 11 days in the
control, premixed-CPC and MTA groups (Fig. 4). Hence,
the determination of the amount of calcium and protein
content was focused on the maximum nodules formation
i.e. 11-day culture. This result showed that the control,
premixed-CPC, and MTA were not significant different
(Fig. 5). Therefore, it may indicate that both test
materials did not inhibit in vitro mineralization of
osteoblast, and are more biocompatible than SuperEBA.

Human mesenchymal stem cells (hMSC) showed
excellent attachment and viability on CPC13, and MTA
was able to assist hMSC adhesion, growth, and
migration34. Although there are no published reports
comparing the response of hMSC to the two materials, it
may be expected that CPC has the same levels of
biocompatibility as that of MTA to hMSC, besides
osteoblasts.

The cell culture system used in this study was
designed to evaluate the effect of test materials using
cell culture inserts to prevent direct physical interactions
between materials and cells. This system raises the
possibility of chemical reaction between the components
that diffuse from test materials and the elements of
α-MEM. However, all test materials were allowed to set
for 24 h in 100% humidity, and placed in α-MEM for 3
days before using them. Premixed-CPC may be converted
to stable HA in such condition, therefore, no component
may be released from this material to α-MEM. Whereas,
some investigators have reported that calcium ions were
released from MTA to medium17,20,21. Because α-MEM
already contains calcium ion, which is one of the
important elements for in vitro cell culture, calcium ions
released from MTA may not chemically affect other
elements of α-MEM.

The premixed-CPC exhibited extremely good
handling characteristics, which greatly facilitated
sample preparation. Under clinical conditions, premixed
pastes would shorten surgical time, avoid insufficient or
inhomogeneous mixing, and improve the implant
performance by mixing the paste in advance under well-
controlled conditions. The results obtained from this in
vitro study suggest that premixed-CPC could be used as
an endodontic cement not only because of its good
handling properties and shorter hardening time than
MTA but also because of its good mineralization effects.

ACKNOWLEDGMENTS

The authors gratefully thank Drs. Laurence Chung-Lung
Chow and Shozo Takagi, Paffenbarger Research Center,
ADAF at NIST, Maryland, USA for their valuable
support. This research work was financially supported by a
Grant-in-Aid for Scientific Research (C) from the
Japan Society for Promotion of Science (M.H.).

REFERENCES

1) Johnson BR, Witherspoon DE. In: Cohen S, Hargreaves K,
editors. Pathways of the pulp. 9th ed. St. Louis: Mosby
2) Gartner AH, Drone SO. Advances in endodonic surgery.
3) Xavier CB, Weismann R, de Oliveira MG, Demarco FF, Pozza
DH. Root-end filling materials: apical microleakage and
4) Chng HK, Islam I, Yap AU, Tong YW, Koh ET. Properties of
5) Torabinejad M, Watson TF, Pitt Ford TR. Sealing ability of a
mineral trioxide aggregate when used as a root end filling
6) Torabinejad M, Hong CU, McDonald F, Pitt Ford TR. Physical
and chemical properties of a new root-filling material. J
7) Albee FH. Studies in bone growth: Triple calcium phosphate
8) Brown WE, Chow LC. A new calcium phosphate water setting
9) Hong YC, Wang JT, Hong CY, Brown WE, Chow LC. The
periapical tissue reactions to a calcium phosphate cement in the
10) Sugawara A, Chow LC, Takagi S, Chohayeb H. In vitro
evaluation of the sealing ability of a calcium phosphate
cement when used as a root canal sealer-filler. J Endod 1990;
16: 162-165.
11) Cherung AM, Chow LC, Takagi S. In vitro evaluation of a
calcium phosphate cement root canal filler/sealer. J Endod
2001; 27: 613-615.
12) Sugawara A, Fujikawa K, Takagi S, Chow LC, Nishiyama M,
Murai S. Histopathological and cell enzyme studies of calcium
13) Takagi S, Chow LC, Hirayama S, Sugawara A. Premixed
Appl Biomater 2003; 67: 689-696.
14) Sugawara A, Fujikawa K, Hirayama S, Takagi S, Chow LC.
In vivo Characteristics of premixed calcium phosphate
cements when implanted in subcutaneous tissues and
periodontal bone defects. J Res Natl Inst Stand Technol 2010;
Effects of root-end filling materials on fibroblasts and
16) Saidon J, He J, Zhu Q, Safavi K, Spängberg LS. Cell and
tissue reactions to mineral trioxide aggregate and Portland