
Effect of radiopaque Portland cement on mineralization in human dental pulp cells

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Objective. The aim of this study was to investigate whether radiopaque Portland cement (RPC) facilitates the mineralization process in human dental pulp cells (HDPCs) compared with pure Portland cement (PC).

Study design. Under a scanning electron microscope (SEM), cellular morphology was evaluated. Alkaline phosphatase (ALP) activity was analyzed, and nodule formation was assessed by performing Alizarin Red S staining. In addition, the mRNA expressions of mineralization-related proteins were evaluated by performing a real-time polymerase chain reaction.

Results. On SEM evaluation, healthy HDPCs were found adhering to the surfaces of PC and RPC. The ALP activity increased in the PC and RPC groups compared with the control group at 1 day. Alizarin Red stain increased in the PC and RPC groups compared with the control group at 2 and 3 weeks. The mRNA expression of dentin sialophosphoprotein increased at 14 days in the PC and RPC groups.

Conclusions. These results show that PC and RPC have similar effects in terms of mineralization and suggest that RPC also has the potential to be used as a clinically suitable pulp-capping material. (*Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2009;108:e82-e86)

Portland cement (PC) is the base material of mineral trioxide aggregate (MTA), and it has shown favorable results in both in vivo and in vitro tests. Several studies have suggested that PC is biocompatible and can be used as a safe pulp-capping material.¹⁻³ However, pure PC does not contain radiopaque agents; therefore, it is not considered to be clinically suitable.⁴ Therefore, a radiopacifier, such as bismuth oxide, should be added to PC to obtain acceptable radiopacity. We previously reported that this radiopaque Portland cement (RPC) had a cytotoxicity similar to that of PC in human dental pulp cells (HDPCs)⁵; however, the effects of RPC on odontoblastic differentiation in HDPCs are not completely understood at present.

The mineral formation process during dentinogenesis appears to be a highly controlled event that involves noncollagenous proteins, such as osteonectin (ON) and dentin sialophosphoprotein (DSPP).⁶ DSPP has been known to play an important role in tooth formation and odontoblastic differentiation.⁷ ON is another major noncollagenous protein of bone and dentin, and it is responsible for the mineralization properties of these

tissues.⁸ We previously evaluated ON and DSPP as mineralization markers in HDPCs.³

Although the biocompatibility of RPC has been previously reported, little is known about the detailed events regarding mineralization in HDPCs. The aim of the present study was to investigate whether RPC facilitates mineralization and odontoblastic differentiation in HDPCs compared with PC.

MATERIALS AND METHODS

Primary human dental pulp cell culture

The human dental pulp tissues obtained from sectioned teeth were removed aseptically, rinsed with Hanks buffered saline solution, and placed in a 100-mm Petri dish. The dental pulp tissues were minced into small fragments with a blade and cultured in Dulbecco modified Eagle medium (DMEM; Biofluid, Rockville, MD) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA) with 100 U/mL penicillin and 100 U/mL streptomycin (Life Technologies, Rockville, MD). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cell cultures between the fifth and sixth passages were used in this study. For mineralization experiments, HDPCs were cultured in 50 µg/mL ascorbic acid and 10 mmol/L β-glycerophosphate-containing medium as described previously.^{9,10} All experimental procedures were approved by the Institutional Review Board of Wonkwang University Dental Hospital (Iksan, Korea).

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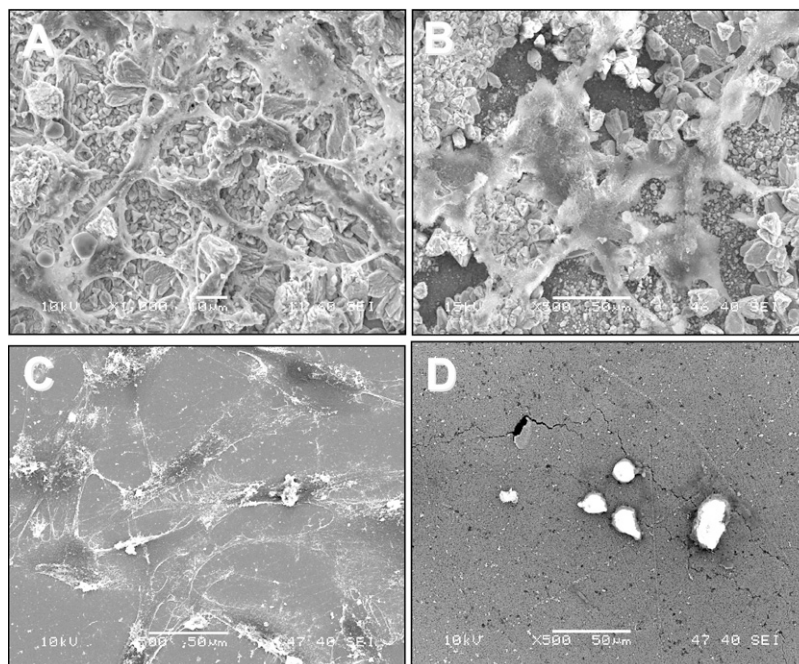


Fig. 1. Scanning electron microscope observation of cells incubated for 48 hours on: **A**, Portland cement ($\times 1,000$); **B**, radiopaque Portland cement ($\times 500$); **C**, glass coverslip (negative control, $\times 500$); and **D**, IRM (zinc oxide–eugenol, positive control, $\times 500$).

Cell morphology analysis by scanning electron microscope

Under aseptic conditions, PC (Ssangyong, Seoul, Korea) and RPC (PC:Bi₂O₃ [Duksan Pure Chemical, Ansan, Korea] = 4:1 by wt%) (0.5 g) were mixed with sterile distilled water (0.2 mL) on a glass slab. Glass coverslips served as negative control and reinforced zinc oxide–eugenol (IRM; Dentsply-Caulk, Milford, DE) as positive control for these studies. The IRM was mixed according to the manufacturer's recommendations. Materials were then condensed into 1.5 \times 20 mm round paraffin wax molds. Materials were allowed to set for 24 hours in a humidified incubator at 37°C. The disks were all placed at the bottom of 12-well tissue culture plates.

The HDPCs were seeded at 1.0×10^5 cells per well on the prepared materials or glass coverslips. After a 48-hour incubation period, the dishes were fixed with 2.5% glutaraldehyde for 2 hours. Samples were then dehydrated in increasing concentrations of ethyl alcohol (70%, 80%, 90%, 95%, and 100%) for 20 minutes at each concentration, immersed in tert-butanol for 20 minutes, and dried with a CPD 030 critical point dryer (Bal-Tec, Balzers, Liechtenstein). Scanning electron microscopy (SEM) was performed using a JSM-6360 system (Jeol, Tokyo, Japan) operated at 10 kV.

Alkaline phosphatase activity assay

The HDPCs (1×10^5) in DMEM containing 10% FBS were seeded in 6-well tissue culture plates and incubated. After an initial attachment period of 24 hours, the PC and RPC were mixed and placed at the bottom of an insert well (Millicell; Millipore, Bedford, MA) with a membrane pore diameter of 0.4 μ m. After setting for 24 hours, the insert wells were placed inside the culture wells, and the cells were incubated for 1, 3, 7, and 14 days. The HDPCs were scraped into cold phosphate-buffered saline and then sonicated with a cell disruptor (Heat System; Ultrasonics, Plainview, NJ) in an ice-cold bath. The alkaline phosphatase (ALP) activity in the supernatant was determined by the method of Lowry et al.,¹¹ with *p*-nitrophenyl phosphate as a substrate. Absorbance was measured at 410 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Beckman DU-650; Beckman Coulter, Fullerton, CA).

Alizarin Red S staining

After culturing HDPCs in the presence of PC or RPC for 1, 2, and 3 weeks, Cells were fixed in 70% ice-cold ethanol for 1 hour and rinsed with distilled water. Cells were stained with 40 mmol/L Alizarin Red S, pH 4.2, for 10 minutes with gentle agitation. The pictures of

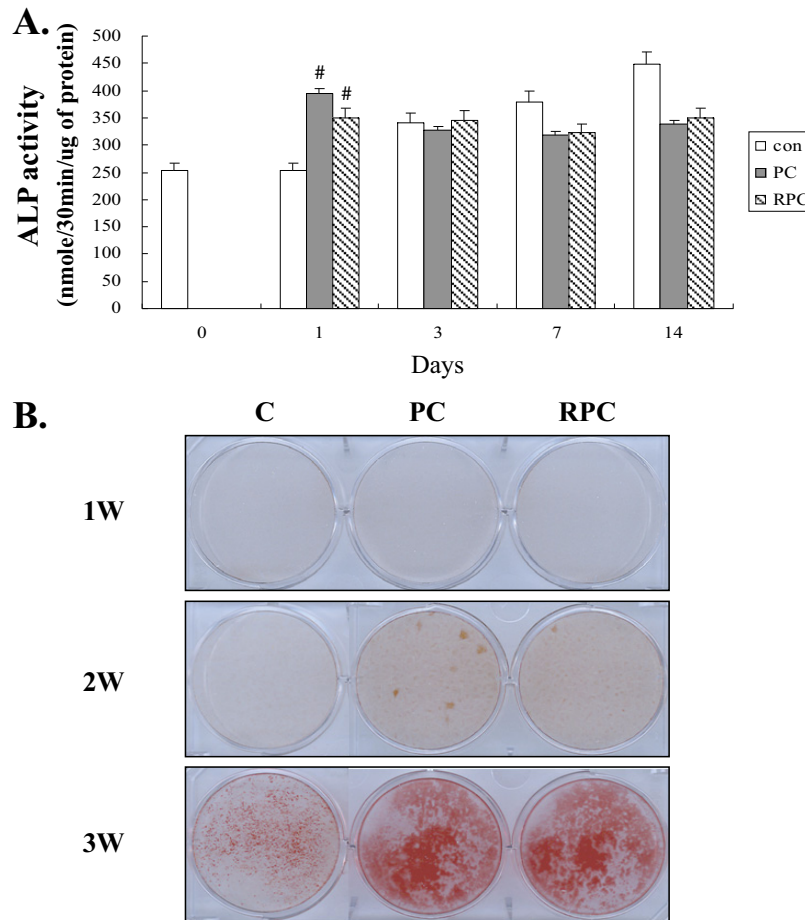


Fig. 2. Effects of Portland cement (PC) and radiopaque Portland cement (RPC) on: **A**, alkaline phosphatase (ALP) activity of human dental pulp cells (HDPCs); and **B**, Alizarin Red S staining in HDPCs [W, Week(s)]. [#]Significant difference ($P < .05$) compared with control (C).

Alizarin Red S staining were photographed under light microscopy.

Real-time polymerase chain reaction analysis

Cells were lysed to extract total RNA by using Trizol reagent (Life Technologies), according to the manufacturer's instructions. Quantifications of ON and DSPP mRNAs were performed by using Exicycler 96 thermal cycler (Bioneer, Daejeon, Korea). One microgram of each RNA sample was subjected to reverse transcription using the AccuPower RT PreMix (Bioneer). The sequences of the human DSPP and ON primers were as follows: DSPP (70 bp): forward 5'-GGG ATG TTG GCG ATG CA-3', reverse 5'-CCA GCT ACT TGA GGT CCA TCT TC-3', ON (127 bp): forward 5'-AGA AGC TGC GGG TGA AGA A-3', reverse 5'-TGC CAG TGT ACA GGG AAG ATG-3'. The amount of template was quantified by using the comparative cycle threshold method according to the manufacturer's pro-

ocol. Measured mRNA levels were normalized to the mRNA copies of β -actin. We performed these experiments from 3 different cell samples and quantification of mRNA was confirmed by using the same cell sample in triplicate.

Statistical analysis

A statistical analysis was conducted by a 1-way variance analysis. Tests for differences in the treatments were analyzed by Duncan multiple range tests, and any P value of $< .05$ was considered to be statistically significant.

RESULTS

Cell morphologic analysis

As shown in Fig. 1, well spread and flattened HDPCs were observed in close contact with the surfaces of PC and RPC. In the PC and RPC groups, many cellular extensions interacted with the cement's surface and

with the adjacent cells. The density and flattened characteristics of the HDPCs in both groups were very similar to what was observed on the glass coverslip control samples. In contrast, a few rounded cells were observed on the IRM surface, but no living cells were seen.

ALP activity assay

Fig. 2, A, shows the effect of PC or RPC on the ALP activity of the cells. The ALP activities in both groups showed maximal increase at 1 day, but decreased at 3, 7, and 14 days compared with the control group. However, there was no significant difference between the 2 groups ($P > .05$).

Alizarin Red S staining

To investigate the effect of PC and RPC on the mineralization of dental pulp cells, cells were stained with Alizarin Red S and examined under light microscopy for the formation of mineralized nodules. After the addition of PC and RPC, the formation of mineralized nodules in HDPCs increased at day 14 and 21 (Fig. 2, B).

Real-time polymerase chain reaction analysis

We analyzed mineralization markers ON and DSPP using cultured HDPCs grown in the presence of PC or RPC. The expression of ON and DSPP mRNAs in HDPCs in the induction medium increased in a time-dependent manner, with the maximum values on day 14 of induction. The expression of ON, as a percentage of housekeeping gene β -actin expression, showed no significant change from day 1 to day 14 of the cultivation period in the PC- and RPC-treated groups. But the expressions of DSPP mRNA in both groups were increased more than that of the control group at 14 days (Fig. 3).

DISCUSSION

Capping of the exposed pulp is designed to treat reversible pulpal injuries by sealing the pulp and stimulating the formation of reparative dentin.¹² Ultimately, the goal of treating the exposed pulp with an appropriate pulp-capping material is to promote the dentinogenic potential of dental pulp cells.¹³ Several studies have shown that PC is considered to be a less expensive biocompatible pulp-capping material that facilitates mineralization in HDPCs.^{3,14} However, at present, there is little information regarding the effect of RPC on odontoblastic differentiation in HDPCs. In this respect, we focused on whether RPC has effects similar to those of pure PC in terms of odontoblastic differentiation in HDPCs.

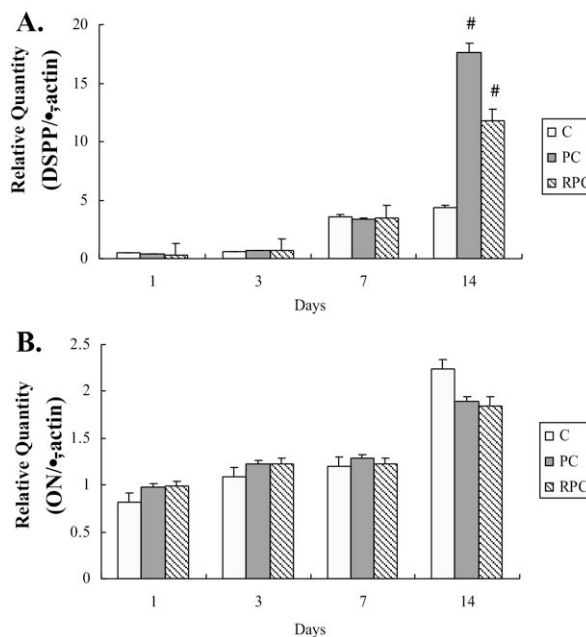


Fig. 3. Effect of PC and RPC on the expression of dentin sialophosphoprotein (DSPP) (A) and osteonectin (ON) (B) mRNAs in HDPCs. Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis of ON and DSPP mRNA expression in PC- and RPC-treated HDPCs. Total cellular RNA was isolated, and quantitative real-time RT-PCR analysis was performed. The relative mRNA levels were calculated by dividing the absolute levels of expression of ON and DSPP mRNAs with the absolute level of expression of β -actin mRNA. These data were calculated from 3 different cell samples with the same cell sample in triplicate. #Significant difference compared with control ($P < .05$). Abbreviations as in Fig. 2.

For reparative dentin to be formed on a substrate, DPCs should initially attach and proliferate. SEM revealed that the HDPCs adhering to PC or RPC appeared to have a normal cellular morphology, similar to the negative control group. These SEM results indicate that both PC and RPC are biocompatible in producing an extracellular mineralized matrix on a substrate for the mineralization process.

For osteoblastic or odontoblastic differentiation and mineralization, there should be an initial period of proliferation and biosynthesis for the extracellular matrix, followed by a period of cell differentiation.¹⁵ In early stages, the matrix matures and specific proteins associated with the pulp cell phenotype—such as ALP—are detected. In the present study, ALP activity achieved maximal increase at 1 day, suggesting that PC and RPC induce high levels of ALP activity in relatively early stages of mineralization.

In this odontoblastic differentiation study, we analyzed mineralization markers such as ON and DSPP by

using HDPCs grown in the presence of PC or RPC. These markers have been considered to play a regulatory role in the mineralization of reparative dentin.⁶ Because the DSPP gene is expressed mainly in odontoblasts, it has been considered to be a differentiation marker for odontoblasts in many studies.¹⁶ In the present study, DSPP mRNA up-regulation was observed in HDPCs in the presence of PC or RPC; it was remarkable at 14 days compared to the control group. These results suggest that odontoblast differentiation and reparative dentin formation in human dental pulp tissue may be facilitated by the presence of RPC as well as PC.

The differentiation of the cells to mature odontoblasts was evaluated under incubation and in the presence of PC or RPC, for 1, 2 and 3 weeks, by the time the calcification formed. The degree of Alizarin Red S staining showed a similar increase in PC or RPC compared with control (Fig. 2, B). These results suggest that both PC and RPC might induce hard tissue formation in HDPCs, under extended exposure to these materials.

Collectively, our results suggest that RPC is as biocompatible and able to facilitate odontoblastic differentiation in HDPCs in short- and long-term culture periods as PC. Finally, regarding the ability to promote mineralization, to our knowledge, this is the first study to report that RPC has the potential to be used as a clinically suitable pulp-capping material.

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