Abstract: An ideal pulp-capping agent needs to have good biocompatibility and promote reparative dentinogenesis. Although the effects of capping agents on healthy pulp are known, limited data regarding their effects on bacterial contaminated pulp are available. This study aimed to evaluate the reaction of contaminated pulps to various capping agents to assist clinicians in making informed decisions. Human dental pulp (HDP) cell cultures were developed from extracted human molars. The cells were exposed to a bacterial cocktail comprising Porphyromonas gingivalis, Prevotella intermedia, and Streptococcus gordonii before being cocultured with capping agents such as mineral trioxide aggregate (MTA) Portland cement (PC), and Dycal. HDP cell proliferation was assayed by MTS colorimetric cell proliferation assay, and its differentiation was evaluated by real-time PCR for detecting alkaline phosphatase, dentin sialophosphoprotein, and osteocalcin expressions. MTA and PC had no apparent effect, whereas Dycal inhibited HDP cell proliferation. PC stimulated HDP cell differentiation, particularly when they were exposed to bacteria. MTA and Dycal inhibited differentiation, regardless of bacterial infection. In conclusion, PC was the most favorable agent, followed by MTA, and Dycal was the least favorable agent for supporting the functions of bacterial compromised pulp cells.

Keywords: dental pulp; capping agent; bacteria; proliferation; differentiation.

Introduction

During pulpotomy and pulp-capping procedures, the dental pulp may be damaged because of infection, mechanical trauma, and chemical exposure. Dentin bridge formation and continuous root development are possible during vital pulp therapy. Some evidence suggests that a dentin bridge is formed as a response to pulp irritation. Pulp cells affected by trauma or carious lesions undergo an inflammatory process, ultimately stimulating osteo/orthodentin bridge formation that partially or completely occludes pulpal exposure (1,2). The cellular response driving such repair may be the proliferation and differentiation of odontoblast-like cells that are derived from dental pulp progenitors (3).

It can be anticipated that the choice of pulp-capping materials such as mineral trioxide aggregate (MTA), Portland cement (PC), or Dycal (a hard-setting calcium hydroxide paste) could influence the bioactivities of human dental pulp (HDP) cells and the repair and regeneration process of the dentin-pulp complex. MTA induces the proliferation of mouse odontoblast-like cells in undifferentiated pulp cells in vitro, and set MTA is not irritating and shows good biocompatibility in vivo (4-6). MTA facilitates faster HDP cell proliferation and
induces more dentin bridge formation than Dycal (7-9). Studies have also compared the effects of MTA and PC. PC shares many components with and macroscopically has almost identical properties as MTA (10,11). Both MTA and PC initiate dentin bridge formation after pulpotomy in dog teeth (12) and are considered to be good endodontic filling materials that facilitate tissue healing with minimal inflammatory response as demonstrated in a guinea pig study (11).

For most previous studies, healthy and non-compromised pulp cells were used to evaluate the reactions to pulp-capping agents. In a clinical setting, pulps treated with direct pulp-capping agents or pulpotomy are commonly compromised by bacterial exposure because of caries or trauma. To better simulate a clinical setting, this study aimed to evaluate the proliferation and differentiation of HDP cells that were exposed to different pulp-capping agents in the presence or absence of bacterial infection, for which relevant oral bacterial species were used. This study is expected to help clinicians make educated choices for pulp-capping materials in relevant clinical situations.

Materials and Methods

HDP cell culture

HDP samples were collected from extracted human third molars. Teeth were collected from adults (age, 18-25 years) at the Oral Surgery Clinic of The University of Texas School of Dentistry, Houston. The study was approved by the Institutional Review Board of the University of Texas Health Sciences Center, Houston (HSC-DB-06-0618). All third molars were extracted because of impaction with no carious lesions. The surfaces of the extracted teeth were cleaned and cut around the cementoenamel junction using a sterilized fissure bur to reveal the pulp chambers. The pulp tissue was gently separated from the crown and root and digested in 3 mg/mL collagenase type I and 4 mg/mL dispase for 1 h at 37°C. A single-cell suspension was obtained by passing cells through a 70-μm strainer. The cells were washed, and HDP cells were cultured with a bacterial cocktail for 2 h. Bacteria were then removed, and the cells were then cultured in 1 mL of antibiotic-free medium with 0.5% glucose as the carbon source. All bacteria were cultured overnight to a final OD_{600} of >1.0. P. gingivalis, P. intermedia, and S. gordonii cells were added to prewarmed tissue culture media at 37°C without antibiotics to obtain a final concentration of 3.75 × 10^7 colony-forming units/mL for each bacterial strain. An aliquot of this polymicrobial cocktail was added to each culture well, resulting in a 750:1 (bacteria:HDP) ratio. To maintain the vitality of anaerobes, HDP cells were incubated in the presence of the bacterial mixture for 2 h.

Seeding HDP cells with capping agents in the presence or absence of bacterial infection

In this study, three capping agents, including ProRoot MTA (Dentsply, Johnson City, TN, USA), PC (Txi Operations Lp, Dallas, TX, USA), and Dycal (Dentsply) were evaluated. There were eight groups: 1) HDP alone; 2) HDP + bacteria; 3) HDP + MTA; 4) HDP + PC; 5) HDP + Dycal; 6) HDP + bacteria + MTA; 7) HDP + bacteria + PC; and 8) HDP + bacteria + Dycal. Each group had at least triplicate samples.

For groups that were exposed to bacteria, HDP cells were seeded onto 12-well culture plates at an initial density of 5 × 10^4 cells/well in 1 mL of culture medium for 24 h. The culture medium was removed, and the cells were washed to remove residual antibiotics. HDP cells were then cultured in 1 mL of antibiotic-free medium with bacterial cocktail for 2 h. Bacteria were then removed, cells were washed, and HDP cells were cultured with a fresh medium.

For groups that were treated with a capping agent, capping agents were first mixed according to the manufacturer’s instructions, after which they were poured onto cell culture inserts with 1-μm pore size (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and partially set, before placing them in cell culture dishes. The capping agents generated a capping layer of approximately 10-mm diameter and 0.5-mm thickness; this layer was not in direct contact with the cells because of the presence of the insert and culture medium. HDP cells were fed with fresh DMEM every other day. When the culture reached 70-80% confluence, the cells were harvested for further experiments. HDP cells up to passage 3 were used in the study.

Preparing bacterial cells

Porphyromonas gingivalis (P. gingivalis) strain W83 and Prevotella intermedia (P. intermedia) strain 17 were anaerobically grown at 37°C in trypticase soy broth that was supplemented per liter with 1 g yeast extract, 5 mg hemin, and 1 mg menadione. Streptococcus gordonii (S. gordonii) strain DL1 was cultured under static conditions in the brain heart infusion broth that was supplemented per liter with 5 g yeast extract and 0.5% glucose as the carbon source. All bacteria were cultured overnight to a final OD_{600} of >1.0. P. gingivalis, P. intermedia, and S. gordonii cells were added to prewarmed tissue culture media at 37°C without antibiotics to obtain a final concentration of 3.75 × 10^7 colony-forming units/mL for each bacterial strain. An aliquot of this polymicrobial cocktail was added to each culture well, resulting in a 750:1 (bacteria:HDP) ratio. To maintain the vitality of anaerobes, HDP cells were incubated in the presence of the bacterial mixture for 2 h.
were exposed to the capping agents for 1, 7, and/or 14 days during the experimentation.

For groups exposed to both the bacteria and capping agents, HDP cells were first infected with the bacteria for 2 h, after which the bacteria were removed and the cells were cultured with the capping inserts for 1, 7, and/or 14 days during the experimentation.

**MTS colorimetric cell proliferation assay**

Cell proliferation was measured using the CellTiter 96 Aqueous One Solution assay kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. In brief, on days 1 and 7, the medium was removed and replaced with 0.3 mL fresh medium. Then, 20 µL of the CellTiter 96 Aqueous One Solution reagent was added to each well. The plates were incubated in the dark at 37°C for 1 h, after which 200 µL medium from each well was added to 96-well plates, and the absorbance was read at 490 nm using the SPECTRAmax 190 multiplate reader with SOFTmax PRO version 3.0 software (Molecular Devices, Sunnyvale, CA, USA). The mean absorbance of the wells that contained the cell-free medium was used as the baseline and was deducted from the absorbance of cell-containing wells. Five individual wells per group were analyzed for each time point.

**Real-time PCR**

Total RNA was extracted from cultured HDP cells using the RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA) according to the manufacturer’s instructions. DNase I was added to remove remaining genomic DNA. Then, 0.5 µg total RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions.

Primers were designed using LightCycler Probe Design Software version 1.0 (Roche Diagnostics Corporation, Indianapolis, IN, USA). The specificity of the primers was confirmed by BLAST analysis. The sequences for the primers were as follows: alkaline phosphatase (ALP), forward 5’-GGACATCGCCTACCAG, reverse 5’-CCGTCACGTTGTTCCT; dentin sialophosphoprotein (DSPP), forward 5’-GGAATGGAGAGAGGACTGCT, reverse 5’-AGGTGTTGTCTCCGTCAGT; osteocalcin (OCN), forward 5’-GCTTTTGGCGTTTGTG, reverse 5’-GGAAGCGGGGATCAGA; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; internal control), forward 5’-TCGGAGTCAACGGATT, reverse 5’-CCACGACGTACTCAG. Real-time PCR was performed using a BioRad MJ MiniOpticon Personal Thermal Cycler Detection System (BioRad Laboratories). Reactions were performed with a total volume of 20 µL, with 10 µL of 2´ SYBR Green PCR Master Mix, 5 µL of primer (20 mM concentration), and 5 µL of cDNA template. The amplification condition was 95/5, 55/7, and 72/20 [temperature (°C)/time (s)] for 40 cycles. All reactions were conducted in triplicate for each sample. The negative control (no template cDNA) was run to reveal any potential contamination. Melting curve analysis was performed for each completed reaction to ensure single product amplification. After calibrator normalization and amplification efficiency correction, PCR products were quantified by comparing the amplification of the target gene to that of GAPDH using the relative quantification software version 1.0 (BioRad Laboratories).

**Statistical analysis**

All experiments were repeated thrice with a minimal of three replicates. The normality of the data was tested by Kolmogorov-Smirnov test. Normally distributed data was analyzed with Tukey-Kramer multiple comparisons test of one-way analysis of variance using the SigmaPlot 11.0 software (Systat Software, Inc., San Jose, CA, USA). Multiple comparisons were performed between HDP cells alone and HDP cells with MTA, PC, or Dycal in the presence or absence of bacterial infection. Statistical significance was set at a P value of <0.05.

**Results**

**Proliferation of HDP cells**

The MTA and PC groups demonstrated similar growth patterns to HDP cells alone; i.e., all three groups had a significant increase in cell numbers from day 1 to day 7 regardless of bacterial infection. There was no significant difference in the number of cells among the three groups at the assay time points, indicating that neither MTA nor PC affected HDP cell proliferation (Fig. 1). The Dycal group had significantly less number of cells than HDP cell alone for under both the non-compromised and compromised conditions on day 7 (Fig. 1), indicating that Dycal inhibited HDP cell proliferation.

**Differentiation of HDP cells**

The mRNA expression of odontoblastic differentiation genes was quantified with real-time PCR on days 1 and 14. Because no cells from the Dycal group (regardless of bacterial infection) survived on day 14, samples from the two Dycal groups with the presence and absence of bacteria on day 14 were not included in the PCR assay.

**Day 1**

For DSPP, PC stimulated but Dycal inhibited its expres-
sion compared with HDP cells alone under compromised conditions (Fig. 2). For OCN, both PC and MTA stimulated its expression compared with HDP cells alone under compromised conditions. Dycal inhibited the expression under both non-compromised and compromised conditions (Fig. 3). For ALP, PC significantly stimulated but Dycal inhibited its expression compared with HDP cells alone under compromised conditions (Fig. 4).

Day 14

For DSPP, PC significantly stimulated its expression compared with HDP cells alone under both non-compromised and compromised conditions. MTA inhibited DSPP expression compared with HDP cells alone under compromised conditions (Fig. 2). For OCN, PC had no significant effect on its expression compared with HDP cells alone under both non-compromised and compromised conditions. MTA significantly inhibited OCN
expression compared with HDP cells alone under both non-compromised and compromised conditions (Fig. 3). For ALP, both PC and MTA negatively regulated its expression compared with HDP cells alone under both non-compromised and compromised conditions (Fig. 4).

It appears that bacteria had some stimulatory effect on pulp cell differentiation. Bacterial infection transiently increased DSPP expression on day 1 in the HDP cells alone, MTA, and PC groups (Fig. 2) and increased OCN expression on day 1 in the MTA group (Fig. 3). In addition, bacterial infection demonstrated a more persistent stimulation of OCN expression till day 14 in the HDP cells alone and PC groups (Fig. 3).

Discussion
This study found that in general, MTA and PC had no effect on HDP cell proliferation, whereas Dycal significantly inhibited the proliferation. PC stimulated HDP cell differentiation, particularly when cells were exposed to bacteria. MTA and Dycal inhibited HDP cell differentiation, regardless of bacterial infection.

The human oral cavity harbors a complex polymicrobial community that comprises diverse microbial species (13). For teeth with deep caries lesions, large restorations, or open fractures, oral bacteria may come in direct contact with pulp cells. In this study, HDP cell cultures were exposed to a bacterial cocktail comprising S. gordonii, P. gingivalis, and P. intermedia. S. gordonii, a gram-positive bacteria, is considered to be an initial colonizer that is present in high numbers on supragingival tooth surfaces and has a tendency to invade deep dentinal tubules (14-16). It is cariologically significant, particularly in cases that are refractory to routine endodontic procedures (16,17). P. gingivalis and P. intermedia are black-pigmented gram-negative bacteria that colonize the subgingival crevice (18). P. gingivalis is consistently encountered in endodontic infections and is suspected to play a role in the etiology of acute abscesses (19). P. intermedia is among the most frequently detected species in primary caries infections (16). Because of the polymicrobial nature of dental caries, HDP cell cultures were exposed to the bacterial cocktail to mimic the clinical situation.

To evaluate pulp cell proliferation and differentiation, the cell number and transcript expression of odontoblast differentiation genes were monitored throughout the culture period. ALP is an early marker, whereas OCN is a later marker for odontoblast differentiation (20,21). DSPP is odontoblast specific and is expressed by terminally differentiated odontoblasts during the late stage of dentin matrix mineralization (22). Evaluating the temporal expression profile of differentiation markers will reveal how odontoblast differentiation is affected by capping agents and/or bacterial infection.

For the 7-day culture proliferation assay, HDP cells reached a maximal 90% confluence in the MTA and PC groups. Therefore, contact inhibition does not appear to affect the analysis. Although ALP is an early differentiation marker, its expression was monitored on day 14 of the cultures to be consistent with the analysis of other markers.

Mineralization was observed in HDP cell cultures toward the end of the 14-day culture period, particularly in the PC groups. Preliminary microscopic observations demonstrated sparse tubular structures in the mineralized matrix. Further studies, particularly those performed in in vivo animal models, would better mimic clinical scenarios and reveal more detailed data regarding the quality of the reparative dentin formed.

The major ingredients of PC include calcium phosphate, calcium oxide, and silica. MTA shares the same base materials with PC, with the addition of bismuth oxide, which increases its radiopacity (23,24). Dycal is a rigid, self-setting material that predominately comprises calcium hydroxide (25). PC has no cytotoxicity and facilitates pulp cell proliferation and differentiation (26-28). In our study, PC stimulates HDP cell differentiation, even when exposed to bacteria, although no major effect on HDP cell proliferation was identified. MTA has good biocompatibility and some stimulatory effect on pulp cell proliferation and differentiation (6,8,28-30). Our in vitro study reveals an inhibitory effect of MTA on pulp cell differentiation, and no appreciable effect on proliferation. The discrepancy between our study and previous studies could be because of the difference in the types of cells used, the setting stages of capping agents when applied to cells, and the evaluation methods. In our study, change in the medium pH owing to the setting phase of the capping agents may partially contribute to the observed inhibitory effects. Consistent with previous studies, Dycal has an overall negative effect on HDP cells in this study, exhibiting cytotoxic effects and impeding growth and differentiation of pulp cells (8,29,31).

Between PC and MTA, our study identified that PC had a favorable effect, whereas MTA had an inhibitory effect on HDP cell differentiation. It is speculated that some drawbacks of MTA, such as long setting time, alkaline pH, or presence of toxic elements (32,33), contribute to the observed inhibitory effect. However, the underlying mechanisms require further explorations.

An interesting finding of this study was that bacteria had some stimulatory effect on HDP cell differentiation,
as demonstrated by a transient increase in DSPP and more sustained upregulation of OCN expressions in the HDP cell alone and PC groups. The bacterial cocktail was directly added to the pulp cell culture; thus, the observed effect could have occurred because of a direct interaction between the bacteria and pulp cells and/or the influence of diffusible bacterial byproducts such as endotoxins, exotoxins, lytic enzymes, acids, and other virulence factors (34). In carious lesions without pulp exposure, bacterial byproducts can diffuse through dental tubules and irritate pulp cells (35). Our findings are supportive of previous observations; i.e., lipopolysaccharide, the major pathogenic factor of gram-negative bacteria, promoted odontoblastic differentiation (36) and sonicated extracts from \textit{P. gingivalis} facilitated early dentinogenic differentiation in HDP cell cultures (37).

During vital pulp therapy, the dentinogenic potential of pulp cells has major significance. The goals of vital pulp therapy include preservation of vitality and promotion of the dentinogenic ability of remaining pulp cells for continued root formation in undeveloped roots. Of the three pulp-capping agents tested in this study, PC was the most favorable, followed by MTA, and Dycal is the least favorable choice. PC demonstrates good biocompatibility and a general stimulation of pulp cell differentiation, regardless of bacterial infection. Together with its low cost and wide availability, PC appears to be an ideal material for endodontic procedures such as indirect and direct pulp capping, sealing of perforations, pulpotomy, and apicification. Further studies, particularly those performed with \textit{in vivo} animal models, would help validate our current findings and identify the most ideal capping agents for endodontic procedures.

**Conflict of interest**

The authors have no conflict of interest to declare.

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