Cysteamine Enhances Biofilm Eradication Efficacy of Calcium Hydroxide

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Abstract

Introduction: Calcium hydroxide ($Ca[OH]_2$) is a widely used interappointment dressing, but its antibacterial property is compromised by dentin. Hence, the addition of chlorhexidine (CHX) with $Ca(OH)_2$ has been proposed. However, the antimicrobial efficacy of this mixture compared with $Ca(OH)_2$ alone is currently still debatable. Cysteamine is a mucolytic agent used to reduce the viscosity of mucus through the disruption of proteins, which are also important components of the extracellular matrix of biofilms. The aims of this study were to determine the efficacy of cysteamine alone and in combination with $Ca(OH)_2$ to eradicate *Enterococcus faecalis* biofilm compared with CHX with $Ca(OH)_2$, and to determine if this effect is affected by dentin. **Methods:** The biofilm eradication efficacies of $Ca(OH)_2$ alone and with cysteamine were determined using 7-day *E. faecalis* biofilm cultured on dentin discs and compared with $Ca(OH)_2$ with 2% CHX. The effects of dentin on the efficacies of $Ca(OH)_2$ alone and with either cysteamine or CHX were examined. **Results:** Cysteamine alone completely abolished *E. faecalis* biofilm at 200 mg/mL. The combination of $Ca(OH)_2$ with either cysteamine at 10 mg/mL or 2% CHX completely obliterated *E. faecalis* biofilm. Cysteamine with $Ca(OH)_2$ completely eradicated *E. faecalis* biofilm despite preincubation with dentin, whereas CHX with $Ca(OH)_2$ was less effective. **Conclusions:** Cysteamine effectively eliminated *E. faecalis* biofilm and showed synergistic effects in combination with $Ca(OH)_2$, which were unaffected by dentin. Hence, our findings support the use of cysteamine as a potential adjunct to $Ca(OH)_2$ as an interappointment dressing. *(J Endod 2016;42:742–746)*

Key Words

Calcium hydroxide, endodontic biofilm, *Enterococcus faecalis*, intracanal dressing, mucolytics

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Calcium hydroxide ($Ca[OH]_2$) is one of the most widely used interappointment intracanal dressings in endodontic therapy. Although the use of $Ca(OH)_2$ significantly reduces microbial loads in infected root canals, culture-negative root canals cannot be consistently obtained. In fact, a meta-analysis of 8 clinical trials concluded that $Ca(OH)_2$ has limited efficacy in the elimination of bacteria from root canals (1). This lack of efficacy is attributed to dentin buffering, which reduces the alkalinity of $Ca(OH)_2$, which is essential for its antimicrobial action (2). Because reducing the bacteria load in an infected root canal impacts treatment outcomes (3), it is important to identify new agents that are capable of maintaining their bactericidal properties in the root canal environment.

Bacteria exist in infected root canals mainly as biofilms where the microbial communities are embedded in an extracellular polymeric substance (EPS). The major components of EPS are polysaccharides and proteins and, to a lesser extent, lipids, nucleic acids, and other organic matter (4). In proteins, disulfide bonds are formed between cysteine residues in which a thiol (-SH) side chain participates in enzymatic reactions as an electron donor (5).

Chlorhexidine (CHX) has been proposed as a viable alternative to $Ca(OH)_2$ as an intracanal medicament because of its broad-range antimicrobial properties and substantivity (6). CHX has been shown to be effective against *Enterococcus faecalis*, one of the frequently isolated bacterial species from teeth with failed root canal treatment (7), whereas $Ca(OH)_2$ was less effective (8). However, CHX has poor tissue-dissolving properties and has limited effects against endotoxins (9, 10). Therefore, formulations containing CHX and $Ca(OH)_2$ have been introduced, but studies so far have not shown CHX to significantly improve the antimicrobial efficacy of $Ca(OH)_2$ (11).

Cysteamine is the simplest aminothiol derived from cysteine and has a pKa of 9.42. Under alkaline conditions produced by $Ca(OH)_2$ (pH > 12.5), it deprotonates and forms thiolate anions ($-S^-$), which disrupt both intramolecular and intermolecular disulfide bonds of bacterial proteins (12). Collectively, destruction of disulfide bonds of proteins not only denatures key bacteria enzymes important for their metabolism and survival but also weakens the structural integrity of the EPS of biofilms. By the same mechanism, cysteamine is used to reduce the viscosity of purulent sputum in patients with chronic airway inflammation to facilitate mucus clearance through depolymerization of mucin glycoproteins (13). In addition, cysteamine is bactericidal and is used to disrupt and prevent the formation of pathogenic biofilms associated with cystic fibrosis (14, 15). Therefore, we hypothesize that the combination of the mucolytic and antimicrobial properties of cysteamine could potentially enhance the properties of $Ca(OH)_2$ in disrupting and eradicating endodontic biofilms. To date, the efficacy of cysteamine to eliminate oral biofilms has not been tested. Thus, the aims of this study were as follows:

1. To determine the efficacy of cysteamine alone and in combination with $Ca(OH)_2$ to eradicate *E. faecalis* biofilm compared with CHX with $Ca(OH)_2$
2. To determine if the biofilm eradication efficacy of cysteamine with $Ca(OH)_2$ is affected by dentin

Materials and Methods

Dentin Disc Preparation

Dentin discs were prepared from the root segments of extracted premolars by sectioning 5 mm of the root tip and removing the crown. Cross sections of 500-μm

thickness were prepared by sectioning the root segments with an Isomet Low Speed Saw (Buehler, Lake Bluff, IL). Discs were treated with 1% sodium hypochlorite for 15 minutes to remove remnant organic matter followed by rinsing with H_2O. The discs were treated with 6% citric acid (pH = 4.0) for 4 minutes in an ultrasonic bath (Techspan, Auckland, New Zealand) to remove the smear layer followed by rinsing with distilled H_2O for 1 minute. Dentin discs were sterilized by autoclaving.

E. faecalis Biofilm Culture

E. faecalis ATCC 29212 was purchased from the American Type Culture Collection (Manassas, VA). *E. faecalis* biofilms were cultured by inoculating dentin discs placed in 24-well plates (Nunc, Rochester, NY) with 1 mL brain-heart infusion broth (Acumedia, Lansing, MI) containing 10^6 colony-forming units of *E. faecalis*. This setup was incubated anaerobically at 37°C for 7 days with media changed every 2 days.

Effect of Cysteamine on *E. faecalis* Biofilm

A stock solution of 200 mg/mL cysteamine (Sigma-Aldrich, St Louis, MO) was prepared in H_2O. The solution was adjusted to a pH of 7 using 6 mol/L NaOH (Sigma-Aldrich). The solution was filter sterilized by using a 0.2-μm syringe filter (Pall, Port Washington, NY). The 7-day-old *E. faecalis* biofilm on dentin discs was gently washed with sterile 1× phosphate-buffered saline (PBS) twice to remove planktonic bacteria. Dentin discs were transferred to sterile 1.5-mL tubes and exposed to either sterile H_2O or cysteamine (50–200 mg/mL) for 7 days and incubated at 37°C anaerobically. The biofilm was dislodged from the dentin discs by vortexing for 5 minutes followed by serial dilution and plating on brain-heart infusion agar plates. The number of bacterial colonies was enumerated after 24 hours of incubation at 37°C anaerobically.

Effect of Cysteamine, CHX, and/or $Ca(OH)_2$ on *E. faecalis* Biofilm

Saturated $Ca(OH)_2$ solution was prepared fresh by dissolving $Ca(OH)_2$ powder (Sigma-Aldrich) in H_2O. Cysteamine was dissolved in $Ca(OH)_2$ solution at a concentration of 10 mg/mL. This concentration was determined through serial titrations from our pilot studies. The 7-day old *E. faecalis* biofilm on dentin discs was gently washed with sterile PBS twice to remove planktonic bacteria. Dentin discs were exposed to either sterile H_2O, $Ca(OH)_2$, 2% CHX (Sigma-Aldrich), or cysteamine with $Ca(OH)_2$ and incubated at 37°C anaerobically for 7 days. The amount of remaining viable bacteria was determined as described previously.

Effect of Dentin Powder on Biofilm Eradication Efficacy of Cysteamine, CHX, and/or $Ca(OH)_2$

Dentin powder was prepared as described previously (16). Dentin powder (280 mg) was suspended in 1 mL $Ca(OH)_2$, 2% CHX, or cysteamine in $Ca(OH)_2$ for 2 hours. The suspension was filter sterilized before determination of their biofilm eradication efficacy to 7-day-old *E. faecalis* biofilm as described earlier.

Confocal Laser Scanning Electron Microscopy

E. faecalis biofilm was cultured on dentin discs for 7 days as described previously. The biofilms were exposed to H_2O, $Ca(OH)_2$, 2% CHX, or cysteamine with $Ca(OH)_2$ for 7 days. The discs were washed with PBS to remove planktonic bacteria, and the biofilm was stained with the FilmTracer LIVE/DEAD Biofilm Viability Kit (Invitrogen, Carlsbad, CA) and used according to the manufacturer's instructions. The discs were washed in sterile H_2O, mounted on a coverslip, and viewed using an Olympus FV1000 (Tokyo, Japan). Three-dimensional reconstructions of the biofilm image slices were performed with IMARIS (Bitplane, Zurich, Switzerland).

Statistical Analysis

All experiments were performed 3 independent times and each time in triplicate. Results were presented as the mean ± standard deviation. Statistical significance was determined by 1-way analysis of variance with the Tukey post hoc test. Differences were considered significant at $P < .05$.

Results

Efficacy of Cysteamine to Eradicate *E. faecalis* Biofilm

A significant reduction in viable bacterial counts was obtained when the 7-day *E. faecalis* biofilm was exposed to 100 mg/mL cysteamine at a pH of 7. Complete eradication of bacterial biofilm was obtained at 200 mg/mL cysteamine (Fig. 1).

Biofilm Eradication Efficacies of $Ca(OH)_2$ and in Combination with CHX or Cysteamine

After biofilm staining, confocal laser scanning electron microscopy showed untreated 7-day *E. faecalis* biofilm to be robust with most of the cells viable (Fig. 2*A*). Exposure of *E. faecalis* biofilm to $Ca(OH)_2$ revealed a drop in the number of viable cells, with a substantial amount of live cells present (Fig. 2*B*). In contrast, the treatment of *E. faecalis* biofilm with $Ca(OH)_2$ combined with 2% CHX led to a significant drop in viable cells, whereas together with 10 mg/mL cysteamine it resulted in eradication of the biofilm (Fig. 2*C* and *D*). The viability of *E. faecalis* in biofilms was further determined by culture. Exposure of *E. faecalis* biofilms to $Ca(OH)_2$ alone for 7 days led to a 40% reduction in viable cells (Fig. 2*E*). Complete eradication of viable bacteria in the biofilm was obtained with 7-day treatment with $Ca(OH)_2$ combined with either 2% CHX or 10 mg/mL cysteamine.

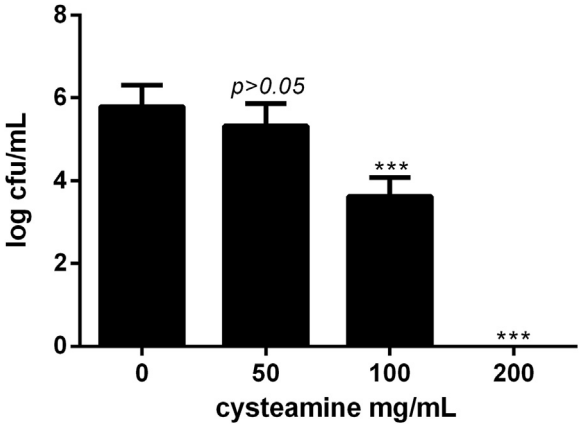

Figure 1. The antimicrobial effects of cysteamine on 7-day *E. faecalis* biofilm grown on dentin discs. *E. faecalis* biofilm was cultured anaerobically using dentin discs as the substrate. The biofilm was exposed to cysteamine at a pH of 7 for 7 days, after which the amount of viable bacteria was determined by serial dilution and plating on brain-heart infusion agar plates. ***$P < .001$ compared with the group without cysteamine.