Inhibition of *Enterococcus faecalis* by Calcium Peroxide

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**Objective:** To investigate the inhibition of *Enterococcus faecalis* by calcium peroxide (CaO\(_2\)).

**Methods:** The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Enterococcus faecalis* by CaO\(_2\) and calcium hydroxide (Ca(OH)\(_2\)) were determined by direct exposure tests \(n = 10\). The inhibition zone of *E. faecalis* mycobacterium treated with CaO\(_2\) and Ca(OH)\(_2\) paste (53% w/w) was observed using agar diffusion tests \(n = 20\). The inhibition of *E. faecalis* biofilms by CaO\(_2\)/phosphate-buffered saline (PBS) and Ca(OH)\(_2\)/PBS suspensions were observed using confocal laser scanning microscopy and the percentages of live bacteria in the biofilms calculated.

**Results:** The MIC of Ca(OH)\(_2\) (4.5 to 5.5 mg/ml) was higher than the MIC of CaO\(_2\) (2.0 to 2.5 mg/ml) \((P < 0.05)\), and the MBC of Ca(OH)\(_2\) (14.5 to 15.5 mg/ml) was higher than that of CaO\(_2\) (3.0 to 3.5 mg/ml) \((P < 0.05)\). No inhibition zone was observed for Ca(OH)\(_2\) in agar diffusion tests, while the diameter of the inhibition zone around CaO\(_2\) was 8.6 ± 0.4 mm. There were significant differences between groups in the percentages of surviving bacteria in *E. faecalis* biofilms after treatment \((P < 0.05): \) group CaO\(_2\) < group Ca(OH)\(_2\) < group PBS < group BHI.

**Conclusion:** The inhibition of *E. faecalis* by CaO\(_2\) was greater than that by Ca(OH)\(_2\).

**Key words:** biofilm, calcium hydroxide, calcium peroxide, *Enterococcus faecalis*


The main objective of endodontic therapy is to eliminate bacteria from the root canal and to prevent the regrowth of residual microorganisms\(^1\). Infected tissue removal is achieved by thorough biomechanical cleaning, including shaping and irrigating the canal with proteolytic disinfecting solution. However, thorough irrigation of root canals with antimicrobial solution might not be sufficient to eliminate all microorganisms from the root canal\(^2\). Inability to completely eradicate microorganisms and/or provide a bacterial seal can result in the failure of root canal therapy. Hence, the use of intracanal medicaments has been advocated to further reduce the number of microorganisms, for example, in teeth with chronic sinus infection, and in teeth that are undergoing revision of failed root canal procedures.

*Enterococcus faecalis* is a Gram-positive bacterium often isolated from persistent root canal infections. It can penetrate deeply into dentinal tubules and resist bactericidal substances commonly used in endodontic procedures\(^3,4\). Its prevalence in asymptomatic, persistent endodontic infection is about 77%\(^5\). Siren et al\(^6\) found that *E. faecalis* was the most common enteric bacterium isolated from the root canal in both primary treatment and retreatment groups, and it appears as a monoinfection. *E. faecalis* has the capacity to live in dentinal tubules enduring prolonged periods of starvation\(^7,8\), and *E. faecalis* biofilms have greater magnitude more resistant to antimicrobials than planktonic bacteria\(^9,10\).

Ca(OH)\(_2\) was first introduced to dentistry in 1920 and has been widely accepted as an intracanal medica-
ment because of its antimicrobial properties, especially its action on Gram-negative bacteria\textsuperscript{11,12}. However, some authors have suspected it is effective in eliminating \textit{E. faecalis}\textsuperscript{13,14}. The search for a better alternative has led to the use of newer antimicrobial agents such as CaO\textsubscript{2}.

CaO\textsubscript{2} is a white or yellowish solid peroxide, which can slowly decompose to release oxygen at a ‘controlled’ rate when in contact with hydrous media. Besides its stable oxygen releasing capability, CaO\textsubscript{2} also possesses capacities for bleaching, disinfection and deodorizing\textsuperscript{15}. Hence, CaO\textsubscript{2} has been widely used in agriculture, aquiculture and medicine\textsuperscript{15}. In its natural state, CaO\textsubscript{2} dissolves in water to form hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and Ca(OH)\textsubscript{2} and thus is considered a ‘solid form’ of H\textsubscript{2}O\textsubscript{2}\textsuperscript{15}. The antibacterial properties of Ca(OH)\textsubscript{2} stems from its ability to increase the pH of a solution\textsuperscript{16}. However, Mura et al presumed that reactive oxygen species (ROS) are released from CaO\textsubscript{2} and suggested that the species of ROS were H\textsubscript{2}O\textsubscript{2} and the superoxide anion (O\textsubscript{2}\textsuperscript{-})\textsuperscript{17}. Thus, high pH and/or ROS may be the sterilisation mechanism of CaO\textsubscript{2}, but no research has been published on this mechanism.

As a bactericidal alkali and oxygen-releasing agent, CaO\textsubscript{2} may be effective in controlling \textit{E. faecalis} infections in the root canal. The purpose of this study was to evaluate the antibacterial effects of CaO\textsubscript{2} through a direct exposure method, agar diffusion method and by confocal laser scanning microscopy.

Materials and methods

Bacterial strain and medications

For this in vitro study, a single standard strain of \textit{Enterococcus faecalis} (ATCC 29212; Lab of Microbiology, Beijing Stomatological Hospital, Capital Medical University) was selected. The strain was incubated on brain heart infusion broth (BHI) agar solid culture medium for 24 h. Single colonies were taken and incubated into BHI liquid medium (5% CO\textsubscript{2} at 37°C). After 24 h, the microbial cells were resuspended in BHI to achieve a final concentration of 3 × 10\textsuperscript{8} cells/ml. Analytical grade Ca(OH)\textsubscript{2} and CaO\textsubscript{2} (China National Pharmaceutical Group Chemical Reagent, Shanghai, China) were used in this study.

Determination of MIC and MBC

BHI broth (4 ml) was placed in a centrifuge tube, then Ca(OH)\textsubscript{2} or CaO\textsubscript{2} was added. The concentrations of Ca(OH)\textsubscript{2} were 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 14.0, 14.5, 15.0 and 15.5 mg/ml, respectively. The concentrations of CaO\textsubscript{2} were 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/ml, respectively. Then, \textit{E. faecalis} was added into the tubes (0.1 ml of 3 × 10\textsuperscript{8} cells/ml). After 24 h, the MIC and MBC of Ca(OH)\textsubscript{2} and CaO\textsubscript{2} was determined. The MIC was defined as the minimum concentration without bacterial growth by macroscopic observation (without turbidity in the BHI broth). The MBC was defined as the minimum concentration where there was no bacterial growth on BHI agar plates. Samples were repeated ten times for each group. All data were analysed using SPSS 16.0 software (SPSS, Illinois, USA).

Observation of inhibition zone

\textit{Enterococcus faecalis} suspensions (0.1 ml) were spread on BHI agar and incubated (5% CO\textsubscript{2} at 37°C). After the \textit{E. faecalis} mycorm formed on the BHI agar, CaO\textsubscript{2} paste (mixed with distilled water; 53% w/w) or Ca(OH)\textsubscript{2} paste (mixed with distilled water; 53% w/w) were put on the mycorm. After cultivation for 24 h (5% CO\textsubscript{2} at 37°C), the inhibition zones were observed and measured (n = 20). All data were analysed using SPSS 16.0 software.

Observation of biofilm inhibition

Hydroxyapatite sheets (Clarkson Chromatography Company, Pennsylvania, USA) were placed in 24-well culture plates and immersed in 2 ml of BHI culture medium containing \textit{E. faecalis} (3 × 10\textsuperscript{8} cells/ml) for biofilm formation. The BHI culture medium containing \textit{E. faecalis} was replaced every 2 days. After 14 days, the hydroxyapatite sheets were washed twice in phosphate-buffered saline (PBS) and divided into four groups: (i) BHI group: the hydroxyapatite sheets were immersed in 2 ml BHI medium and incubated for 48 h (5% CO\textsubscript{2} at 37°C). (ii) PBS group: the hydroxyapatite sheets were immersed in 2 ml PBS and incubated for 48 h. (iii) Ca(OH)\textsubscript{2} group: the hydroxyapatite sheets were immersed in 2 ml PBS containing 30 mg Ca(OH)\textsubscript{2} and incubated for 48 h. (iv) CaO\textsubscript{2} group: the hydroxyapatite sheets were immersed in 2 ml PBS containing 30 mg CaO\textsubscript{2} and incubated for 48 h. The hydroxyapatite sheets with biofilm were stained with LIVE/DEAD reagent (SYT09: PI: PBS = 1.5 µl: 1.5 µl: 1.0 ml) for 30 min. Then they were washed twice in PBS, treated with 1.5 µl anti-fluorescent quencher and observed after 24 h. All samples were examined under a confocal laser scanning microscope (Leica TCS-SPE; Leica Microsystems GmbH, Mannheim, Germany) at 63× magnification.
The viable, live bacteria were green, while the dead were red, and overlapping alive and dead bacteria produced either orange or yellow samples. Each sample was scanned from the outside of the biofilm (away from the hydroxyapatite sheet) to the inside along the Z-axis. The biofilm observed was divided into three layers: outer one-third, middle one-third and inner one-third. The percentage of viable cells was calculated for each layer using IpWin32 software and the data was analysed using SPSS 16.0.

Results

Table 1 showed that the MIC of CaO₂ towards *E. faecalis* was 2.0 to 2.5 mg/ml, while the MIC of Ca(OH)₂ was 4.5 to 5.5 mg/ml. The MBC of CaO₂ was 3.0 to 3.5 mg/ml, while that of Ca(OH)₂ was 14.5 to 15.5 mg/ml. There were statistically significant differences between the two treatments (*P* < 0.05).

Figure 1 showed the pH of CaO₂ and Ca(OH)₂ solutions before and after incubation with *E. faecalis*. The pH increased along with an increasing concentration of Ca(OH)₂. When the concentration of Ca(OH)₂ was 5 mg/ml, the pH reached 11.87. However, the pH ranged from 8.10 to 9.50 in a solution of CaO₂ (1.5 to 5.0 mg/ml). After incubation with bacteria for 24 h, the pH of the Ca(OH)₂ solution decreased. A much slighter decrease in pH was observed for the CaO₂ solution.

Figure 2 showed typical inhibition zones of *E. faecalis* by Ca(OH)₂ and CaO₂ on BHI agar plates. No inhibition zone was observed for Ca(OH)₂, while the inhibition zone of CaO₂ was 8.6 ± 0.4 mm in diameter.

Figures 3 and 4 showed the results of biofilm inhibition. The percentage of live bacteria in the inner layer of the biofilms was higher than that in the outer layer for all groups. Ca(OH)₂ killed some *E. faecalis* in biofilms (Fig 3). CaO₂ treatment resulted in the lowest percentage of live bacteria in the whole of the biofilm, as well as in each of the inner, middle and outer layers. The percentage of live bacteria in the CaO₂ group < Ca(OH)₂ group < PBS group < BHI group (*P* < 0.05).

Discussion

CaO₂ showed a lower MIC and MBC towards *E. faecalis* than Ca(OH)₂. CaO₂ forms H₂O₂ and Ca(OH)₂ when it dissolves in water and is thus considered a ‘solid form’ of H₂O₂. The sterilisation mechanism of Ca(OH)₂ mainly depends on a high pH value caused by dissociation of hydroxyl ions; the high pH may destroy cell membranes, denature structural proteins and enzymes, and damage DNA. However, in this study, the pH of CaO₂ solution

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<td>Ca(OH)₂</td>
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Fig 1  The pH of Ca(OH)₂ and CaO₂ suspensions of different concentrations before and after bacteriostatic action. CH indicates Ca(OH)₂, CP indicates CaO₂, 24 h means that pHs were measured after incubation of the sample with *Enterococcus faecalis* for 24 h; pHs of other samples were measured after 0 h.

Fig 2  Inhibition zones of *Enterococcus faecalis* by Ca(OH)₂ and CaO₂ paste (53% w/w) on BHI agar plates after cultivation for 24 h. CH indicates Ca(OH)₂; CP indicates CaO₂; red arrows indicate inhibition zones.
free radicals generated by hydrogen peroxide led to a low bacterial inhibition effect. However, in the present study, the CaO₂ was in contact with the *E. faecalis* for 24 h, and produced oxygen free radicals continuously via a slow interaction with water. Thus we were able to observe an obvious inhibitory effect on planktonic *E. faecalis*.

In the agar diffusion test used for observation of inhibition zones, the test results depend on the solubility and diffusivity of medicaments in the agar, rather than just their efficacy against the organism²²,²³. In this study, there was no inhibition zone of *E. faecalis* around Ca(OH)₂ paste, while there was a clear inhibition zone around CaO₂ paste. This indicates that the solubility and diffusivity of CaO₂ in agar appears to be better than that of Ca(OH)₂.

A previous study reported that *E. faecalis* biofilms grew well on the hydroxyapatite sheet and the biofilm entered a relatively stable state after 14 days, after which the total number of bacteria did not change significantly²⁴. Therefore, we selected the hydroxyapatite sheet as a biofilm substrate in this study. Based on the MIC, MBC and inhibition zone results, it was not a surprise that CaO₂ showed stronger inhibition effects towards *E. faecalis* biofilms than Ca(OH)₂. However, it should be noted that the *E. faecalis* biofilm was not completely killed by CaO₂ in this study, indicating that the biofilm has a stronger capacity to resist killing than the planktonic state. The extracellular matrix (a gel polymer) of biofilms has a buffer action against antibacterial agents²⁵. In addition, as bacteria in the inner layer

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**Fig 3** Biofilm images obtained by confocal laser scanning microscopy followed by 3D reconstruction; with a 63× magnification. Green indicates live bacteria; red indicates dead bacteria. BHI - brain heart infusion; PBS - phosphate-buffered saline; CH - Ca(OH)₂; CP - CaO₂.

**Fig 4** The percentage of viable cells in different layers of *Enterococcus faecalis* biofilms treated with antimicrobials: CH - Ca(OH)₂; CP - CaO₂; BHI - brain heart infusion; PBS - phosphate-buffered saline.

was around 8.10 to 9.50, which is much lower than the antibacterial pH (around 12)¹⁹. This indicates that the antibacterial mechanism of CaO₂ may arise from the oxygen free radicals released from hydrogen peroxide. Block et al confirmed that hydrogen peroxide generated oxygen free radicals and the reaction with macromolecules (such as membrane lipids and DNA) lead to bacterial death²⁰. However, a previous study showed that 3% H₂O₂ solution, which also generated oxygen free radicals, had no significant inhibitory effect on planktonic *E. faecalis*²¹. In that study, there was not enough contact time (with the method of irrigating immediately) for H₂O₂ to kill the bacteria; the short lifetime of the oxygen
relatively lack nutrition, their starvation state means they could be resistant to antibacterial agents.

Within the limitations of this study, CaO₂ showed greater inhibitory effects against planktonic and biofilm forms of Enterococcus faecalis than Ca(OH)₂.

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Conflicts of interest

The authors reported no conflicts of interest related to this study.

Author contributions

• Dr Yong Liang Su for carrying out the research and preparing the paper.
• Dr Xiao Yan Wang for the design of the study and directing the research.

References