Inhibition of *Enterococcus faecalis* by Calcium Peroxide

Yong Liang SU[1], Xiao Yan WANG[2]

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 mycoderm 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*
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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-

1 First Clinical Division, Peking University School and Hospital of Stomatology, National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing Key Laboratory of Digital Stomatology, Beijing, P.R. China.
2 Department of Cariology and Endodontology, Peking University School and Hospital of Stomatology, National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing Key Laboratory of Digital Stomatology, Beijing, P.R. China.

Corresponding author: Dr Xiao Yan Wang, Department of Cariology and Endodontology, Peking University School and Hospital of Stomatology, 22# Zhongguancun South Avenue, HaiDian District, Beijing 100081, P.R. China. Tel: 86-10-82195525; Fax: 86-10-82195525; Email: wangxiaoyan@pkuss.bjmu.edu.cn

ment because of its antimicrobial properties, especially its action on Gram-negative bacteria[11,12]. However, some authors have suspected it is effective in eliminating *E. faecalis*[13,14]. The search for a better alternative has led to the use of newer antimicrobial agents such as CaO_2.

CaO_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_2 also possesses capacities for bleaching, disinfection and deodorsing[15]. Hence, CaO_2 has been widely used in agriculture, aquiculture and medicine[15]. In its natural state, CaO_2 dissolves in water to form hydrogen peroxide (H_2O_2) and $Ca(OH)_2$ and thus is considered a 'solid form' of H_2O_2[15]. The antibacterial properties of $Ca(OH)_2$ stems from its ability to increase the pH of a solution[16]. However, Mura et al presumed that reactive oxygen species (ROS) are released from CaO_2 and suggested that the species of ROS were H_2O_2 and the superoxide anion ($\bullet O_2^-$)[17]. Thus, high pH and/or ROS may be the sterilisation mechanism of CaO_2, but no research has been published on this mechanism.

As a bactericidal alkali and oxygen-releasing agent, CaO_2 may be effective in controlling *E. faecalis* infections in the root canal. The purpose of this study was to evaluate the antibacterial effects of CaO_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 *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_2 at 37°C). After 24 h, the microbial cells were resuspended in BHI to achieve a final concentration of 3×10^8 cells/ml. Analytical grade $Ca(OH)_2$ and CaO_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 $Ca(OH)_2$ or CaO_2 was added. The concentrations of $Ca(OH)_2$ 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_2 were 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/ml, respectively. Then, *E. faecalis* was added into the tubes (0.1 ml of 3×10^8 cells/ml). After 24 h, the MIC and MBC of $Ca(OH)_2$ and CaO_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

Enterococcus faecalis suspensions (0.1 ml) were spread on BHI agar and incubated (5% CO_2 at 37°C). After the *E. faecalis* mycoderm formed on the BHI agar, CaO_2 paste (mixed with distilled water; 53% w/w) or $Ca(OH)_2$ paste (mixed with distilled water; 53% w/w) were put on the mycoderm. After cultivation for 24 h (5% CO_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 *E. faecalis* (3×10^8 cells/ml) for biofilm formation. The BHI culture medium containing *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_2 at 37°C). (ii) PBS group: the hydroxyapatite sheets were immersed in 2 ml PBS and incubated for 48 h. (iii) $Ca(OH)_2$ group: the hydroxyapatite sheets were immersed in 2 ml PBS containing 30 mg $Ca(OH)_2$ and incubated for 48 h. (iv) CaO_2 group: the hydroxyapatite sheets were immersed in 2 ml PBS containing 30 mg CaO_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 CaO_2 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 CaO_2 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 CaO_2 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 CaO_2 (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 CaO_2 solution.

Figure 2 showed typical inhibition zones of *E. faecalis* by $Ca(OH)_2$ and CaO_2 on BHI agar plates. No inhibition zone was observed for $Ca(OH)_2$, while the inhibition zone of CaO_2 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). CaO_2 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 CaO_2 group < $Ca(OH)_2$ group < PBS group < BHI group ($P < 0.05$).

Discussion

CaO_2 showed a lower MIC and MBC towards *E. faecalis* than $Ca(OH)_2$. CaO_2 forms H_2O_2 and $Ca(OH)_2$ when it dissolves in water and is thus considered a 'solid form' of H_2O_2[15]. 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[18]. However, in this study, the pH of CaO_2 solution

Table 1 MIC and MBC of CaO_2 and $Ca(OH)_2$ towards *Enterococcus faecalis*.

	MIC (mg/ml)	MBC (mg/ml)
CaO_2	2.0-2.5	3.0-3.5
$Ca(OH)_2$	4.5-5.5	14.5-15.5

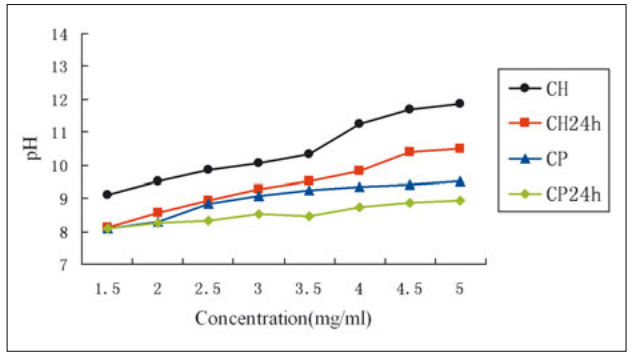

Fig 1 The pH of $Ca(OH)_2$ and CaO_2 suspensions of different concentrations before and after bacteriostatic action. CH indicates $Ca(OH)_2$, CP indicates CaO_2, 24 h means that pHs were measured after incubation of the sample with *Enterococcus faecalis* for 24 h; pHs of other samples were measured after 0 h.

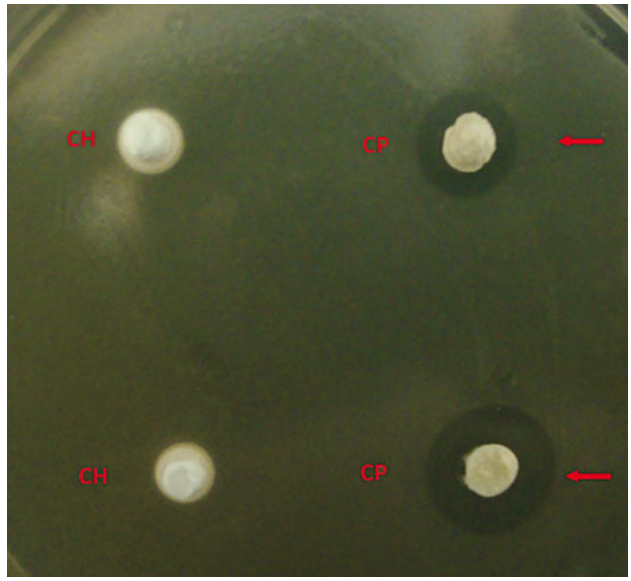

Fig 2 Inhibition zones of *Enterococcus faecalis* by $Ca(OH)_2$ and CaO_2 paste (53% w/w) on BHI agar plates after cultivation for 24 h. CH indicates $Ca(OH)_2$; CP indicates CaO_2; red arrows indicate inhibition zones.

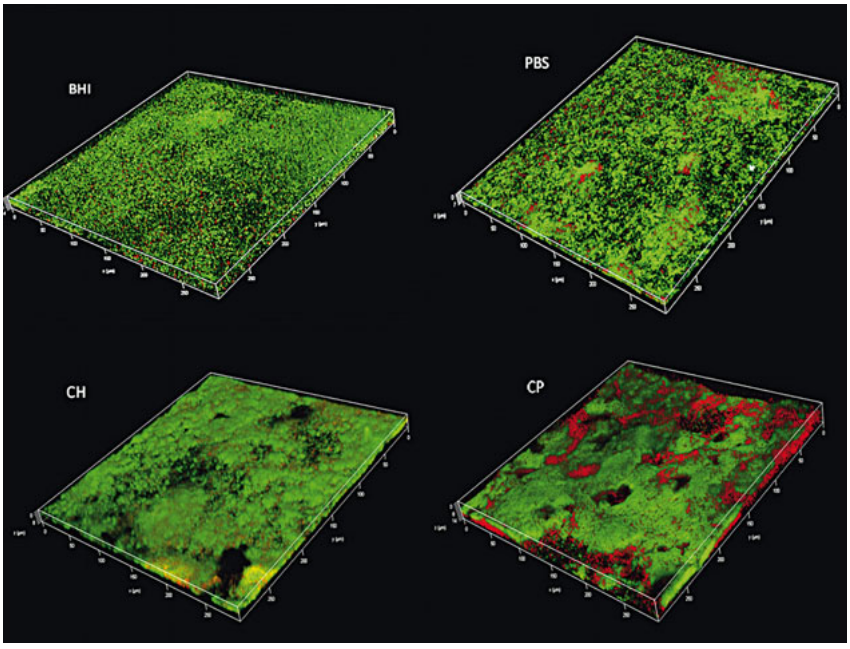

Fig 3 Biofilm images obtained by confocal laser scanning microscopy followed by 3D reconstruction; with a 63× magnification. Green indicates live bacteria; red indicates dead bacteria. BHI - brain heart infusion; PBS - phosphate-buffered saline; CH - $Ca(OH)_2$; CP - CaO_2.

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 CaO_2 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 death[20]. However, a previous study showed that 3% H_2O_2 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 H_2O_2 to kill the bacteria; the short lifetime of the oxygen free radicals generated by hydrogen peroxide led to a low bacterial inhibition effect. However, in the present study, the CaO_2 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*.

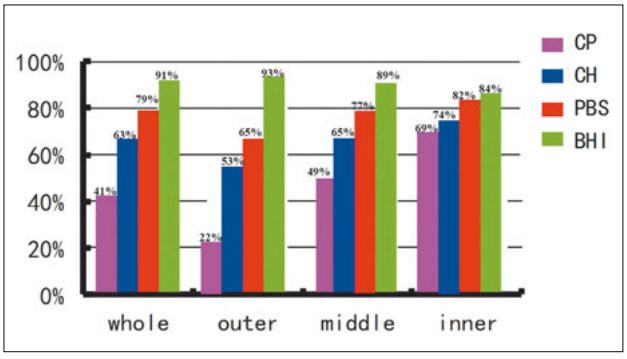

Fig 4 The percentage of viable cells in different layers of *Enterococcus faecalis* biofilms treated with antimicrobials: CH - $Ca(OH)_2$; CP - CaO_2; BHI - brain heart infusion; PBS - phosphate-buffered saline.

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 organism[22,23]. In this study, there was no inhibition zone of *E. faecalis* around $Ca(OH)_2$ paste, while there was a clear inhibition zone around CaO_2 paste. This indicates that the solubility and diffusivity of CaO_2 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 significantly[24]. 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 CaO_2 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 CaO_2 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 agents[25]. In addition, as bacteria in the inner layer

relatively lack nutrition, their starvation state means they could be resistant to antibacterial agents.

Within the limitations of this study, CaO_2 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.

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