

ORIGINAL ARTICLE

Antimicrobial activity of calcium hydroxide and chlorhexidine on intratubular *Candida albicans*

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This study investigated the efficacy of calcium hydroxide and chlorhexidine gel for the elimination of intratubular *Candida albicans* (*C. albicans*). Human single-rooted teeth contaminated with *C. albicans* were treated with calcium hydroxide, 2% chlorhexidine gel, calcium hydroxide plus 2% chlorhexidine gel, or saline (0.9% sodium chloride) as a positive control. The samples obtained at depths of 0–100 and 100–200 μm from the root canal system were analyzed for *C. albicans* load by counting the number of colony forming units and for the percentage of viable *C. albicans* using fluorescence microscopy. First, the antimicrobial activity of calcium hydroxide and the 2% chlorhexidine gel was evaluated by counting the number of colony forming units. After 14 days of intracanal medication, there was a significant decrease in the number of *C. albicans* colony forming units at a depth of 0–100 μm with chlorhexidine treatment either with or without calcium hydroxide compared with the calcium hydroxide only treatment. However, there were no differences in the number of colony forming units at the 100–200 μm depth for any of the medications investigated. *C. albicans* viability was also evaluated by vital staining techniques and fluorescence microscopy analysis. Antifungal activity against *C. albicans* significantly increased at both depths in the chlorhexidine groups with and without calcium hydroxide compared with the groups treated with calcium hydroxide only. Treatments with only chlorhexidine or chlorhexidine in combination with calcium hydroxide were effective for elimination of *C. albicans*.

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INTRODUCTION

The main objectives of endodontic treatment are elimination of microorganisms and prevention of reinfection within the root canal system. Resistant microorganisms can remain in the root canal system even after mechanical instrumentation and irrigation procedures because of its complex anatomy.¹ Moreover, microbial persistence and growth in dentinal tubules, lateral canals and apical ramifications have also been shown.^{1–2} Many studies^{3–4} demonstrate the effectiveness of intracanal medications against antimicrobial resistant microorganisms. Additionally, these treatments control the persistent exudation and destructive action of osteoclasts during external root resorption.^{5–6} Polymicrobial endodontic infections are mainly composed of anaerobic species.⁷ *Candida albicans* (*C. albicans*) is the most commonly isolated yeast in root canal system infections, and the presence of *C. albicans* has been reported using culture, molecular and electron microscopy methods.⁸

Endodontic treatment may induce bacteria to enter a ‘viable but non-culturable’ (VBNC) state during which growth ceases, but the

microorganisms remain viable. Yeasts are also capable to enter a VBNC state.^{9–10} When favorable conditions are reestablished, the microorganisms can return to a culturable state.¹¹ The VBNC state could be evaluated by techniques that assess respiration, enzymatic activity and cellular activity by different methods.^{12–13} Among the various methods, fluorescence microscopy has been used to examine VBNC state. A fluorescence analysis is used commonly with vital staining techniques to determine the viability profile, architecture and spatial distribution in microbial biofilms.¹⁴

Due to the associated antimicrobial properties, calcium hydroxide [$\text{Ca}(\text{OH})_2$] has been widely used as an intracanal medication,¹⁵ despite the limited action against *Enterococcus faecalis* (*E. faecalis*)¹ and *C. albicans*.¹⁵ Comparatively, chlorhexidine emerged as an intracanal medication because of persistent antimicrobial action when adhered to anionic substrates.¹⁶ The combination of $\text{Ca}(\text{OH})_2$ and chlorhexidine resulted in antimicrobial properties that were more effective than treatment with $\text{Ca}(\text{OH})_2$ only and did not affect the sealing ability of root canal obturation.¹⁷ However, a recently study shows

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that chlorhexidine reduced the success of root canal treatment.¹⁸ Understanding the antimicrobial chlorhexidine efficacy toward endodontic infections should enhance interpretation of clinical results. Although some clinical studies have supported the efficacy of calcium hydroxide as an intracanal medicament, other studies have questioned its efficacy and indicated chlorhexidine instead of calcium hydroxide.¹⁹ Therefore, this study used both culture and fluorescence microscopy methods to investigate whether treatment with chlorhexidine only or in combination with Ca(OH)₂ could eliminate *C. albicans*.

MATERIALS AND METHODS

Teeth preparation

The experimental protocol was approved by the local Institutional Ethical Committee (28/2006). Sixty-four extracted single-rooted teeth were cleaned with periodontal curettes to remove periodontal tissue and stored in 10% formaldehyde for 2 weeks, then they were washed under running water for 48 h, blot dried and stored in 0.9% sterile saline at 4 °C for no longer than 7 days.^{20–21} Fifteen teeth served as the positive control, fifteen teeth were in each of the three experimental groups, two teeth were prepared to analyze the effectiveness of smear layer removal and two teeth were used to confirm *C. albicans* contamination by scanning electron microscopy. Specimens were prepared as previously described.^{22–23} Briefly, the crowns (2–3 mm from the cement–enamel junction) and apical portion of the roots (3–5 mm) were removed. Biomechanical preparations were performed using #15–#40 K-files (Dentsply Maillefer, Ballaigues, Switzerland). All teeth were submitted to an ultrasonic bath for 10 min in 17% ethylene diaminetetraacetic acid (EDTA) (Farmácia Flor & Ser Manipulação, São Paulo, Brazil) followed by 10 min in 5.25% NaClO (Farmácia Flor & Ser Manipulação, São Paulo, Brazil) to eliminate the smear layer.²⁴ Then, the specimens were stored in 0.9% sterile saline until the experimental procedures were performed.

C. albicans growth

C. albicans (ATCC 10231) was cultured in Sabouraud broth. Yeast cell morphology was confirmed using the Gram method and a stereomicroscope (MS 23358; Wild Heerbrugg, Romanshorn, Switzerland). Cell counts and concentrations were determined using a Neubauer chamber (Propper Manufacturing, Long Island City, NY, USA).

Experimental root canal infection

The roots were placed in test tubes containing 5 mL of Sabouraud broth, and 1.5×10^8 *C. albicans* cells were diluted in 5 mL of medium, which maintained contact between the yeast suspension and root canal walls.^{22–23} Fresh Sabouraud broth was supplemented weekly to ensure *C. albicans* viability. All specimens were incubated aerobically at 37 °C for 21 days. These incubations were examined daily, and the turbidity for each sample was recorded. *C. albicans* contamination of dentinal tubules was confirmed using scanning electron microscopy. After this period, each root canal was irrigated with 5 mL of 0.9% sterile saline, dried with sterile paper points, and divided into four groups ($n=15$).

Intracanal dressings

Teeth samples were submitted to the following intracanal dressings:

Group I (15 samples): Ca(OH)₂ paste (Calen; S.S.WHITE Artigos dentários Ltd, Rio de Janeiro, RJ, Brazil).

Group II (15 samples): 2% chlorhexidine gel (chlorhexidine gluconate plus 1% natrosol gel) (Flor & Ser- Farmácia de Manipulação-Bauru, São Paulo, Brazil).

Group III (15 samples): Ca(OH)₂ paste combined with 2% chlorhexidine gel (1:1).

Group IV (15 samples): 0.9% sterile saline (NaCl) (positive control).

Each dressing was applied under sterile conditions using a syringe and needle to completely fill each root canal. After the excess dressing was removed, coronal and apical orifices were sealed with temporary restorative cement (Saint Maur des Fosses, Paris, France). The specimens were then placed into Petri dishes, covered with damp sterile gauze and incubated at 37 °C for 14 days.

Dentinal fragment samples

After the incubation period, the restorative cement was removed, and the experimental samples were irrigated with 5 mL of sterile saline. Subsequently, root canals were irrigated with 1 mL of neutralizing solution for each antimicrobial agent: 0.5% citric acid for Ca(OH)₂ and 0.5% Tween 80 in 0.07% soy lecithin for 2% chlorhexidine gel and for 2% chlorhexidine gel combined with Ca(OH)₂. A final irrigation with 1 mL of sterile saline was then performed, and all root canals were dried with sterile paper points.

The amount of dentin scrapings was weighed, and the concentration was adjusted per mg of scrapings. Dentinal fragment samples were collected using Gates Glidden Drills: #5 for a depth of 0–100 µm and #6 for a depth of 100–200 µm (500 r·min⁻¹, 1 N, electric motor K Driller Endo Plus). The roots were positioned in microcentrifuge tubes containing 1 mL of BHI (brain heart infusion) broth to collect scrapings at a depth of 0–100 µm or at a depth of 100–200 µm. For all of the samples, bur #5 and also #6 were used each one for three times for the entire length of the root canal. After use, the burs were discarded, and new sterilized burs were used for another tooth. To prevent contamination between 0–100 µm and 100–200 µm samples, the burs were rotated in seventy alcohol solution, followed by distilled water and finally rotated in phosphate-buffered solution (PBS).

Scanning electron microscopy

Four similarly prepared tooth samples were used for scanning electron microscopy evaluation. Two teeth were prepared to analyze the effectiveness of smear layer removal, and two teeth were used to confirm *C. albicans* contamination. The teeth were bisected longitudinally, irrigated with EDTA+NaClO or PBS as control, and incubated as described above. After fixation, dehydration and gold coating, the samples were examined under scanning electron microscopy (Phillips XL30 FEG; Philips, Eindhoven, The Netherlands).²¹

Assessment of antimicrobial activity

Immediately after collection, dentine samples were mixed for 1 min. Aliquots of 25 µL were seeded on Sabouraud agar and incubated at 37 °C for 48 h. After incubation, colony forming units (CFUs) were counted using a CP600 colony counter (Quimis Aparelhos Científicos, Diadema, SP, Brazil). To determine the CFU·mL⁻¹, the number of microorganisms growing in the plate was multiplied by the dilution factor and by the volume used to seed the plate.²⁵ The procedure was performed in triplicate. *C. albicans* purity was confirmed by colony morphology and Gram staining.

For fluorescence microscopy analysis, the dentin suspensions were mixed for 30 s, centrifuged at 600g for 5 min and washed with PBS. Each pellet was suspended in 25 µL of PBS by vigorous agitation. The samples were stained with 25 µL of fluorescein diacetate (viable yeast cells were stained green) and 25 µL of ethidium bromide (nonviable yeast cells were stained red) at 37 °C for 15 min and then analyzed using

confocal microscopy (TCS model, SPE; Leica, Mannheim, Germany). Yeast viability was expressed as the mean percentage of viable *C. albicans* over the total number of microorganisms by randomly counting three fields in three different microscope slides ($\times 40\times 100$ magnification).²⁶ The wavelength used to visualize fluorescein diacetate was 488 and 568 nm was used for ethidium bromide.

Statistical analysis

The results are expressed as the means \pm s.d. Two-way analysis of variance followed by Bonferroni's test was employed, and significant differences were considered as $P < 0.05$. GraphPad PRISM (version 5) was used for graphic representations.

RESULTS

Antimicrobial activity of chlorhexidine and Ca(OH)₂ against *C. albicans*

The effectiveness of smear layer removal was analyzed (Figure 1a). After a twenty-one-day period, *C. albicans* contamination of dentinal tubules was confirmed by scanning electron microscopy (Figure 1b).

The antimicrobial activity of Ca(OH)₂ and 2% chlorhexidine gel was evaluated by counting the number of CFUs. The use of Ca(OH)₂ and 2% chlorhexidine in combination and separately significantly reduced the number of *C. albicans* CFUs recovered at depths of 0–100 and 100–200 μ m compared with the control (Figure 2). A significant decrease was observed in *C. albicans* CFUs from a 0–100 μ m

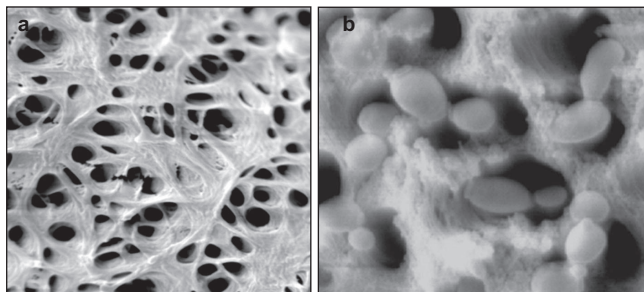


Figure 1 Scanning electron microscopy image. (a) Showing smear layer removal. (b) Showing *C. albicans* in human dentinal tubules 21 days after infection.

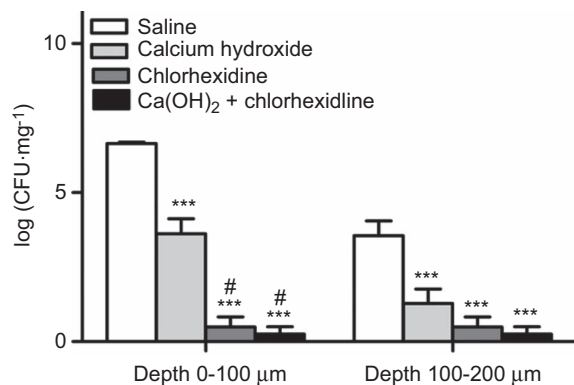


Figure 2 Antimicrobial activity of intracanal dressings against intratubular *C. albicans*. The bars show the number of CFUs of *C. albicans* recovered after Ca(OH)₂ and chlorhexidine treatment. The results are represented as the mean \pm s.d. The data were analyzed by two-way ANOVA followed by Bonferroni's test. *** $P < 0.01$ compared with the control; # $P < 0.01$ compared with Ca(OH)₂. ANOVA, analysis of variance; CFU, colony forming unit.

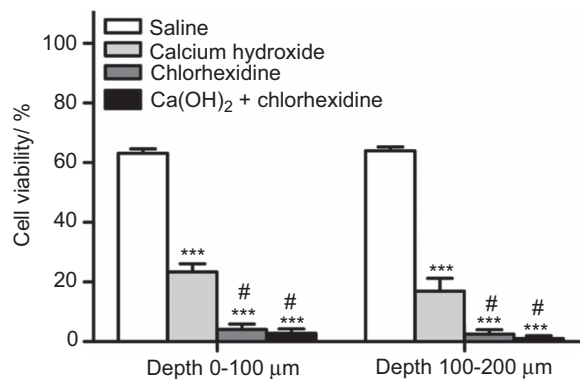


Figure 3 Viability of *C. albicans*. The antimicrobial activity of Ca(OH)₂ and the 2% chlorhexidine gel was analyzed by fluorescence microscopy. The results are represented as the mean \pm s.d. The data were analyzed by two-way ANOVA followed by Bonferroni's test. *** $P < 0.01$ compared with the control; # $P < 0.01$ compared with Ca(OH)₂. ANOVA, analysis of variance.

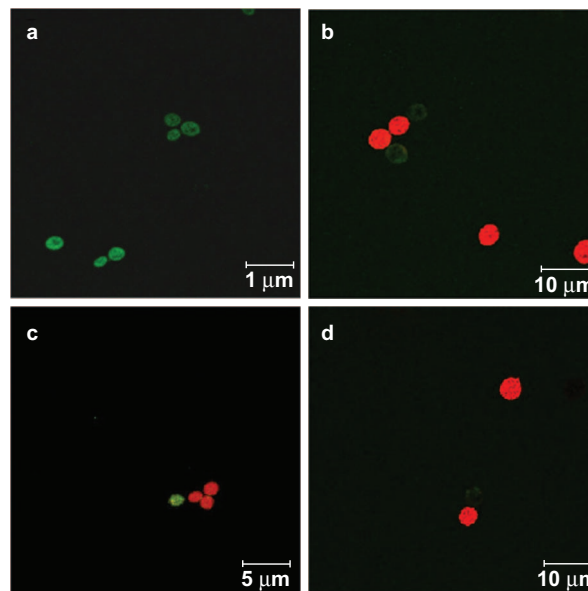


Figure 4 *C. albicans* viability after treatment. (a) With saline solution. (b) With calcium hydroxide. (c) With 2% chlorhexidine gel. (d) With Ca(OH)₂ combined with 2% chlorhexidine gel. Images were obtained at $\times 40$ magnification (a and c) and $\times 100$ magnification (b and d).

depth after treatment with chlorhexidine with or without Ca(OH)₂ compared with treatment with Ca(OH)₂ only (Figure 2). However, no differences were observed at a depth of 100–200 μ m for any of the antimicrobial medications (Figure 2).

C. albicans viability was also evaluated by vital staining technique and fluorescence microscopy analysis (Figure 3). The intracanal dressings significantly reduced the percentage of viable yeast compared with the control ($P < 0.001$) (Figures 3 and 4a). Antifungal activity against *C. albicans* significantly increased at both depths in the chlorhexidine groups with or without Ca(OH)₂ ($P < 0.001$) compared with the groups treated with Ca(OH)₂ only (Figures 3 and 4).

DISCUSSION

Considered as one of the most resistant species in the oral cavity, *C. albicans* is a possible cause of root canal system treatment failure.^{3,27} Biofilms

containing *C. albicans* have important clinical relevance and exhibit increased resistance to antifungal therapy and host immune defenses.^{8,28}

In the present work, the impact of chlorhexidine and Ca(OH)₂ on the survival of intratubular *C. albicans* was evaluated by counting the number of CFUs and yeast viability using fluorescence microscopy. Different techniques are often used to determine the antibacterial activity of dental materials and for clinical research. CFU is a measure of viable bacterial or fungal cells. However, this method does not provide clear information about the spatial distribution of bacteria inside the dentin and the density of VBNC microbial population cannot be estimated. In contrast, a fluorescence analysis provides information regarding the proportion of responsive cells to a substrate within a population, showing a direct and precise estimation of live/dead cells. Fluorescent staining with ethidium bromide is a specific method to identify viable and nonviable cells because ethidium bromide only penetrates cells with damaged plasmalemmas.^{29–30} The results obtained in this study confirm the ability of these two techniques to determine the viability of *C. albicans* in the samples and allow for the recovery of microorganisms within dentinal tubules rather than planktonic yeast suspended in the lumen of root canal system.^{31–32}

Intracanal medication significantly reduced *C. albicans* CFUs at both depths investigated. Additionally, there was a significant difference at the 0–100 µm depth between Ca(OH)₂ treatment with or without chlorhexidine, but not at the 100–200 µm depth for these respective treatments. Even when using the same methodology, these results do not corroborate those observed with *E. faecalis*,³³ which may be due to the different abilities of *C. albicans* and *E. faecalis* to penetrate dentinal tubules.^{34–35} However, chlorhexidine with and without Ca(OH)₂ significantly increased antifungal activity against *C. albicans* compared with the groups treated with Ca(OH)₂ only. The effectiveness of chlorhexidine against *C. albicans* and *E. faecalis* has been shown previously.^{7,15} Chlorhexidine is a broad-spectrum bactericidal and fungicidal agent that prevents microbial colonization on the dentine surface for a prolonged period of time.^{7,9,16,36} It is important to note that there are several factors that could be influenced the results of an *in vitro* study, such as smear layer removal, the type of intracanal medication tested and the evaluation method, among others.³⁷

Studies have reported that the antimicrobial activity of chlorhexidine is reduced when used in combination with Ca(OH)₂,³⁸ however, the present study show similar antimicrobial activity for chlorhexidine combined with Ca(OH)₂ and chlorhexidine alone against *C. albicans*. These data are corroborated by a previous study by Zerella *et al.*¹⁷ who compared the effect of Ca(OH)₂, chlorhexidine and their combination on the disinfection of the pulp space of failed root-filled teeth during endodontic retreatment. The combination of chlorhexidine with Ca(OH)₂ paste could remain in the root canal system as a barrier for longer periods, which may eliminate a significant proportion of persistent microorganisms.³⁹

Ca(OH)₂ is an intracanal medication typically used in endodontics and has an antimicrobial property related to the release of hydroxyl ions.¹⁵ Although Ca(OH)₂ effectively eliminates most endodontic pathogens, studies have demonstrated that *E. faecalis* and *C. albicans* are resistant to Ca(OH)₂.^{1,15,36} In this work, the results corroborated previous study and indicate that treatment with Ca(OH)₂ alone killed *C. albicans*, however, with minor effectiveness than treatment with chlorhexidine alone.

CONCLUSION

Treatment with chlorhexidine or chlorhexidine in combination with Ca(OH)₂ was effective antimicrobial against *C. albicans* when used as

an intracanal medicament being more effective than treatment with Ca(OH)₂ alone.

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