In vitro evaluation of antifungal activity of calcium hydroxide paste mixed to different drugs, against *Candida albicans*

Letícia Vendrametto **FORCIN**[1]
Rafaela Pignatti de **FREITAS**[2,3]
Raquel Zanin **MIDENA**[2,4]
Ana Carolina Villas Bôas **WECKWERTH**[5,6]
Guilherme Ferreira da **SILVA**[2,7]
Paulo Henrique **WECKWERTH**[2,8]

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ABSTRACT

Introduction: the frequency of invasive mycoses caused by opportunistic fungal pathogens has increased significantly in the last decades. Among the main etiological agents of opportunistic mycoses is *Candida albicans*. This yeast has several important virulence factors for producing disease. Some studies have demonstrated that C. albicans colonized root canals and dentinal tubules. The spectrum of antimicrobial activity of calcium hydroxide dressing should include this yeast. **Objectives:** the aim of the present study was to determine the in vitro susceptibility of four C. albicans strains collected from the oral cavity and a standard strain ATCC 10231 to calcium hydroxide paste associated with antifungal, antibiotic and anti-inflammatory drugs. The efficiency of the Ca(OH)2 paste associated with the drugs on the yeasts was analyzed by the radial diffusion method and also by the direct contact method. Data were analyzed by Kruskal Wallis test and the Dunn post-test were used to indicate the differences between the groups with a significance level of 5%. **Results:** all antifungal drugs increased the action of the calcium hydroxide pastes against *Candida albicans*. **Conclusions:** the association of antifungals with $Ca(OH)_2$ may be considered for use as intracanal medicaments.

Keywords: Endodontics. Calcium Hydroxide. *Candida albicans*. Anti-Inflammatory Agents.

[1]Universidade do Sagrado Coração, Centro de Ciências da Saúde (Bauru/SP, Brazil).

[2]Universidade do Sagrado Coração, Pró-Reitoria de Pesquisa e Pós-Graduação (Bauru/SP, Brazil).

[3]Master's degree in Oral Biology, Universidade do Sagrado Coração (Bauru/SP, Brazil).

[4]Doctorate degree in Applied Dental Sciences, Endodontics area, Universidade de São Paulo, Faculdade de Odontologia de Bauru (Bauru/SP, Brazil).

[5]Instituto Lauro de Souza Lima, Pesquisador Científico da Secretária da Saúde do Estado de São Paulo (Bauru/SP, Brazil).

[6]Specialist in Clinical Analysis, Universidade do Sagrado Coração (Bauru/SP, Brazil).

[7]Doctorate degree in Dentistry, Universidade Estadual Paulista, Faculdade de Odontologia de Araraquara (Araraquara/SP, Brazil).

[8]Doctorate degree in Tropical Diseases, Universidade Estadual Paulista (Botucatu/SP, Brazil).

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Contact address: Paulo Henrique Weckwerth
Rua José Quaggio, 1-63, Jd. Marambá – Bauru/SP – CEP: 17.030-590
E-mail: phweck@terra.com.br - phweck@usc.br

Introduction

Candida albicans, one of the main etiological agents of opportunist mycoses,[1] is isolated most frequently from clinical samples of nails, skin, oral mucosa, vagina and ocular mucosa. An estimated 46% of healthy individuals are carriers of C. albicans in the oral microbiota.[2]

C. albicans is known to be involved in cases of endodontic infections, especially in teeth with pulp necrosis and periapical lesions.[3,4,5] Studies have demonstrated that this yeast is capable of colonizing dentin[6] and invading dentinal tubules.[7] Therefore, during disinfection of root canals, it is necessary to use substances, such as intracanal medications, with the purpose of eliminating C. albicans as well.

Calcium hydroxide pastes [$Ca(OH)_2$] are the most frequently used delayed dressing in Endodontics, due to their biocompatibility and antimicrobial activity. However, C. albicans has been shown to be resistant to $Ca(OH)_2$,[8,9] because it is capable of surviving in environments with high pH values.[2] Furthermore, the release of Ca^{++} ions may favor the growth and morphogenesis of Candida.[10,11] With the aim of broadening the antimicrobial spectrum of calcium hydroxide pastes, the addition of other substances to these dressings has been proposed.[12]

Studies have demonstrated that the polysaccharide chitosan,[13,14] antibiotics of the beta-lactam class such as cefepime, meropenem, piperacillin/tazobactam and the glycopeptide Vancomycin,[15] derivatives of the fluoroquinolone levofloxacin,[16] have an effect on the growth of C. albicans in vitro and on the biofilm formed. However, there is a scarcity of studies that evaluate the action of calcium hydroxide pastes associated with anti-inflammatory and anti-fungal agents against this type of yeast.

As it is known that C. albicans may be present in the microbiota of teeth with pulp necrosis,[3,4,5] it is feasible and opportune to conduct a study with the aim of determining the in vitro susceptibility of strains of oral C. albicans and standard ATCC strains to the different calcium hydroxide pastes associated with antifungal, antibiotic and anti-inflammatory drugs.

Material and methods

To develop this research, four strains of *Candida albicans* were used, which were isolated from the oral cavity of patients attended at the Endodontic service of the Dental Clinic of the "Universidade Sagrado Coração" (USC), Bauru. These strains were stored in the fungal culture section of the microbiology laboratory of USC. A standard strain American Type Culture Collection (ATCC) 10231 was used as control in the study.

The stored yeasts and strain ATCC 10231 were activated on Sabouraud dextrose agar plates (Merck®) and incubated in a micological oven at 37°C, for 24 to 48 hours.

For the antifungal activity tests, 11 different $CaOH_2$ pastes were tested, as follows: $CaOH_2$+propylene glycol (Group A); $CaOH_2$+2% chlorhexidine (Group B); $CaOH_2$+clotrimazol (Group C); $CaOH_2$+ ketoconazol (Group D); $CaOH_2$+fluconazol (Group E); $CaOH_2$+itraconazole (Group F); $CaOH_2$+levofloxacin (Group G); $CaOH_2$+amoxicillin (Group H); $CaOH_2$+triple antibiotic powder (200 mg ciprofloxacin + 500 mg metronidazole + 100 mg minocycline) (Group I); $CaOH_2$+ibuprofen (Group J); $CaOH_2$+sodium diclofenac (Group K).

Evaluation of the antifungal action of pastes associated with drugs, by the radial diffusion method

To evaluate the antifungal activity of the $CaOH_2$ pastes studied, the radial diffusion technique was used on the surface of Sabouraud dextrose agar discs (Merck®)[17] (Fig 1).

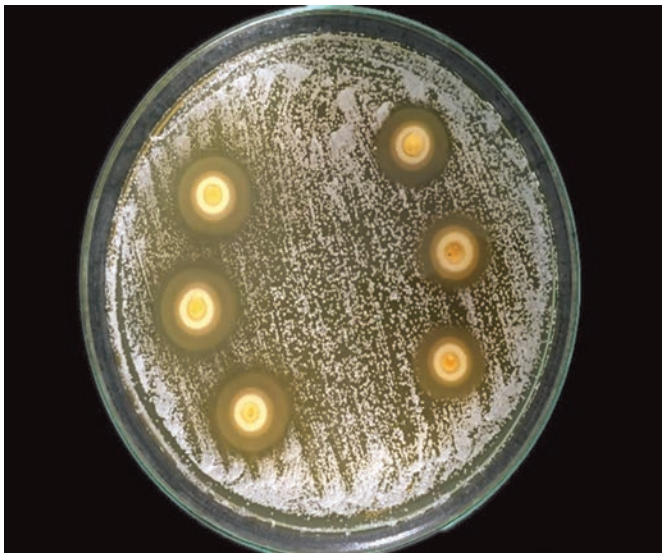

Figure 1. Agar Diffusion Test

From the yeast activation discs, four colonies were transferred to a tube containing 5 mL of Sabouraud dextrose (Merck®) broth that was incubated at 37°C overnight. From the growth, the adjustment for the optical density of the turbidity to the McFarland standard scale 1.0 ($3x10^8$ UFC mL^{-1}) was prepared in sterile saline solution. Petri dishes measuring 150 x 10 mm, previously prepared with Sabouraud dextrose agar (Merck®) with a thickness of 6 mm were excavated in wells measuring 5 mm in diameter by 3 mm deep. Seeding was done through sterile cotton swab on the surface of the plates, taking care not to seed the internal parts of the excavations The plates were placed in an oven for 30 minutes to dry the surface of the culture medium before placement of the pastes. The pure $Ca(OH)_2$ paste was obtained by manipulating the powder with propylene glycol, used as vehicle, until the consistency of toothpaste was obtained. The pastes associated with the drugs were prepared by adding 5% by weight of the drug in proportion to the total weight of calcium hydroxide, also using propylene glycol as vehicle; After spatulation, the wells were filled with the pastes, by means of syringes, and the plates were left for pre-incubation, at ambient temperature for 2 hours. After this, they were incubated in an oven at 37°C, under adequate atmospheric conditions, for 24 hours. The inhibition haloes were measured with the aid of a digital pachymeter under reflected light. The test was performed in triplicate.

Evaluation of the antifungal action of pastes associated with drugs, by the direct contact method

To evaluate the antifungal activity of the $CaOH_2$ pastes studied, the direct contact technique was used, with the pastes in direct contact with the paper contaminated with the strains of C. albicans.[17]

From the yeast activation discs, 5 colonies were transferred to a tube containing 5 mL of Sabouraud dextrose (Merck®) broth that was incubated at 37°C overnight. From the growth, the adjustment for the optical density of the turbidity to the McFarland standard scale 1.0 ($3x10^8$ UFC mL^{-1}) was prepared in sterile saline solution. For each strain tested, 13 sterile, absorbent paper cones (Tanari, Tanariman Indústria Ltda., Manacaru, Brazil) were immersed in the fungal suspension for contamination for 5 minutes. After this, these cones were distributed among sterile Petri dishes and were covered with the 11 different $CaOH_2$ pastes. The cone was covered with sterile saline, and served as control. The plates were stored closed in an oven. In the time intervals of 6, 24 and 72 hours, the cones were removed from contact with the pastes, and were immersed in tubes containing 5mL of sterile Letheen (Difco) broth, and were incubated at 37°C for 48 hours and evaluated relative to macroscopic turbidity. Subsequently, 100 µL of the Letheen broth was transferred to tubes containing 5mL of Sabouraud dextrose broth (Merck®) and were incubated in the same conditions as those for Letheen broth. After 48 hours, aliquots of 100 µL were removed from all the tubes, and these were seeded on the surface of Sabouraud dextrose (Merck®) agar, for the purpose of determining fungal viability. All the experimental procedures were conducted under aseptic conditions, and in triplicate.

Statistical analysis

The data were statistically analyzed by the Kruskal-Wallis test for overall comparison, and by the Dunn test for individual comparison between the groups, with a level of significance of 5%. Statistical analysis was performed with the GraphPad Prism 5 software program (GraphPad Software, Inc. La Jolla, USA).

Results

The results obtained with the radial diffusion test showed that the association of the different antifungal agents potentiated the action of calcium hydroxide paste against *Candida albicans* (Fig 2). This was because the Groups with clotrimazole (C), Ketoconazol (D), fluconazol (E) and itraconazole (F) exhibited significantly larger inhibition haloes when compared with the pure $Ca(OH)_2$ paste (Group A). On the other hand, the pastes containing antibiotic or anti-inflammatory medications (Groups G to K) exhibited the lowest antimicrobial effects against the yeast evaluated ($p=0.05$). With regard to the data collected by means of the direct contact method, analysis showed that there was no fungal viability in any of the studied $CaOH_2$ pastes (Fig 3).

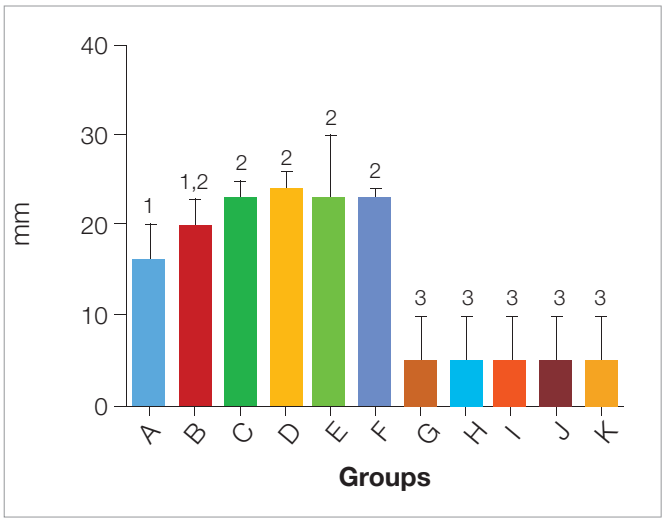

Figure 2. Mean values of inhibition haloes against Candida albicans for the different experimental groups (A-K).
*Different numbers indicate statistically significant difference (p=≤.)5)
A) $CaOH_2$+propylene glycol; **B**) $CaOH_2$+2% chlorhexidine; **C**) $CaOH_2$+clotrimazol; **D**) $CaOH_2$+ ketoconazol; **E**) $CaOH_2$+fluconazol; **F**) $CaOH_2$+itraconazole; **G**) $CaOH_2$+levofloxacin; **H**) $CaOH_2$+amoxicillin; **I**) $CaOH_2$+triple antibiotic powder (200 mg ciprofloxacin + 500 mg metronidazole + 100 mg minocycline); **J**) $CaOH_2$+ibuprofen; **K**) $CaOH_2$+sodium diclofenac.

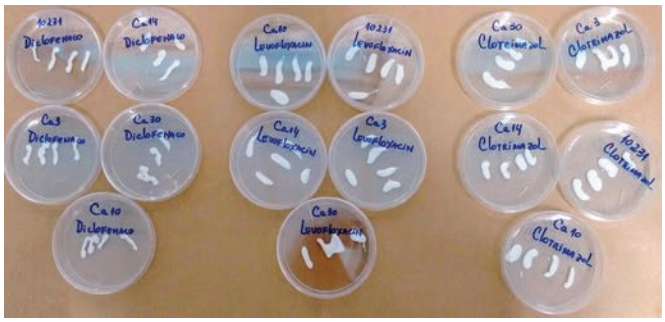

Figure 3. Petri dishes demonstrating the paper cones in direct contact with the different pastes.

Discussion

Candida albicans is the species most frequently isolated from clinical samples, and it is estimated that 46% of healthy individuals are carriers of C. albicans in the oral microbiota.[2] Studies with the association of calcium hydroxide combined with different drugs and vehicles have been conducted to evaluate the antifungal activity against the yeast *Candida albicans*.[18,19,20,21]

In the present study, radial diffusion and direct contact tests were performed to analyze the antifungal activity. The methodology of the radial diffusion test has been widely used for establishing the antimicrobial spectrum of calcium hydroxide.[17,22,23] However, this test is characterized as being qualitative, because it only reveals microbial susceptibility by means of measuring the inhibition haloes, therefore it does not distinguish the bactericidal and bacteriostatic properties of the tested materials, and does not provide any information about the viability of the microorganism tested.[24] Whereas, the direct contact test has been used in many studies, because it is directly correlated to the efficacy of the pastes; moreover it is independent of many variables such as those of the method of diffusion, and it is easy and practical to perform.[22]

The data obtained clearly showed that the associations of the calcium hydroxide pastes with the antifungal medications had significantly more efficient antifungal activity when compared with the association of pastes with antibiotic and anti-inflammatory agents, and pure $Ca(OH)_2$ paste. Previous studies have demonstrated that $Ca(OH)_2$ is not totally efficient against C. albicans, so that it is important to associate it with other substances.[9] The antifungal agents evaluated acted on the cell membrane of C. albicans, potentiating the action of the calcium hydroxide paste.

On the other hand, the association of antibiotic and anti-inflammatory medications caused an antagonist effect on the intracanal medication, seeing that the antifungal effect provided by these pastes was lower, including the $Ca(OH)_2$ with propylene glycol. A previous study observed that the pastes with antibiotic and anti-inflammatory agents were more effective against bacteria, because they present a specific mechanism of action against these microorganisms.[12] Furthermore, for the calcium hydroxide to produce adequate activity against C. albicans, a longer time of contact with this fungus is required,[2] which may have interfered in our results.

In the evaluation of antifungal activity by the direct contact technique, all the pastes were shown to be efficient for rendering the yeasts inviable in all the variables of time. These data obtained in our study corroborated the findings of Estrela et al., 2001[22] using the same direct contact technique, in which the yeast C. albicans was inhibited after 48h of contact,

irrespective of association with the drugs used. The hypothesis for this fact is supported by the probable enzymatic inactivation and damaged caused to the cytoplasmic membrane of yeasts by calcium hydroxide, favoring their destruction, irrespective of its association with the studied drugs. This is probably due to the extreme pH conditions (12.6) obtained at the time of spatulation of the pastes, and is maintained for a long period of time, during which there is a total loss of biological activity of the yeasts.[25]

Conclusions:

Considering the results of this study, we could conclude that the association of antifungal agents with calcium hydroxide pastes could be an alternative for potentiating the antimicrobial effect of intracanal medications.

References

1. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev. 2007;20(1):133-63.
2. Weckwerth PH, Carnietto C, Weckwerth ACVB, Duarte MAH, Kuga MC, Vivan RC. In vitro susceptibility of oral candida albicans strains to different ph levels and calcium hydroxide saturated aqueous solution. Braz Dent J. 2012;23(3):192-8.
3. Nair PNR, Sjögren U, Krey G, Kahnberg K-E, Sundqvist G. Intraradicular bacteria and fungi in root-filled, asymptomatic human teeth with therapy-resistant periapical lesions: a long-term light and electron microscopic follow-up study. J Endod. 1990;16(12):580-8.
4. Baumgartner JC, Watts CM, Xia T. Occurrence of Candida albicans in infections of endodontic origin. J Endod. 2000;26(12):695-8.
5. Mavor AL, Thewes S, Hube B. Systemic fungal infections caused by Candida species: epidemiology, infection process and virulence attributs. Curr Drug Targets. 2005;6(8):863-74.
6. Siqueira JF Jr, Rôças IN, Lopes HP, Elias CN, de Uzeda M. Fungal infection of the radicular dentin. J Endod. 2002;28(11):770-3.
7. Haapasalo HK, Sirén EK, Waltimo TM, Ørstavik D, Haapasalo MP. Inactivation of local root canal medicaments by dentine: an in vitro study. Int Endod J. 2000 Mar;33(2):126-31.
8. Waltimo TM, Sirén EK, Orstavik D, Haapasalo MP. Susceptibility of oral Candida species to calcium hydroxide in vitro. Int Endod J. 1999 Mar;32(2):94-8.
9. Waltimo TM, Orstavik D, Sirén EK, Haapasalo MP. In vitro susceptibility of Candida albicans to four disinfectants and their combinations. Int Endod J. 1999 Nov;32(6):421-9.
10. Holmes AR, Cannon RD, Shepherd MG. Effect of calcium uptake on Candida morphology. FEMS Microbiol Lett. 1991 Jan 15;61(2-3):187-93.
11. Klotz SA, Rutten MJ, Smith RL, Babcock SR, Cunningham MD. Adherence of Candida albicans to immobilized extracellular matrix proteins in mediated by calcium-dependent surface glycoproteins. Microb Pathog. 1993;14(2):133-47.
12. Freitas RP, Greatti VR, Alcalde MP, Cavenago BC, Vivan RR, Duarte MAH, et al. Effect of the association of nonsteroidal anti-inflammatory and antibiotic drugs on antibiofilm activity and ph of calcium hydroxide pastes. J Endod. 2017;43(1):131-4.
13. Pu Y, Liu A, Zheng Y, Ye B. In vitro damage of Candida albicans biofilms by chitosan. Exp Ther Med. 2014 Sept;8(3):929-34.
14. Costa E, Silva S, Tavaria F, Pintado M. Antimicrobial and antibiofilm activity of chitosan on the oral pathogen Candida albicans. Pathogens. 2014;3(4):908-19.
15. Sidrim JJC, Teixeira CEC, Cordeiro RA, Brilhante RSN, Castelo-Branco DSCM, Bandeira SP, et al. β-Lactam antibiotics and vancomycin inhibit the growth of planktonic and biofilm Candida spp.: an additional benefit of antibiotic-lock therapy? Int J Antimicrob Agents. 2015 Apr;45(4):420-3.
16. Mohamed BSR, Subramanian M, Shunmugiah KP. Inibitionos Candida albicans virulence factors by novel levofloxacin derivatives. Appl Microbiol Biotechnol. 2014;98(15):6775-85.
17. Weckwerth PH, Siquinelli NB, Weckwerth ACVB, Vivan RR, Duarte MAH. In vitro fetermination of direct antimicrobial effect of calcium hydroxide associated with different substances against Enterococcus faecalis strains. Dental Press Endod. 2011;1(1):46-51.
18. Delgado RJR, Gasparoto TH, Sipert CR, Pinheiro CR, Moraes IG, Garcia RB, et al. Antimicrobial activity of calcium hydroxide and chlorhexidine on intratubular Candida albicans. Inter J Oral Sci. 2013;5(1):32-6.
19. Ercan E, Dalli M, Dülgergil CT. In vitro assessment of the effectiveness of chlorhexidine gel and calcium hydroxide paste with chlorhexidine against Enterococcus faecalis and Candida albicans. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2006;102(2):e27-31.
20. Vianna ME, Gomes BPFA, Sena NT, Zaia AA, Ferraz CCR, Souza Filho FJ. In vitro evaluation of the susceptibility of endodontic ppathogens to calcium hydroxide combined with different vehicles. Braz Dent J. 2005;16(3):175-80.
21. Turk BT, Sen BH, Ozturk T. In vitro antimicrobial activity of calcium hydroxide mixed with different vehicles against Enterococcus faecalis and Candida albicans. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009;108(2):297-301.
22. Estrela C, Bammann LL, Pimenta FC, Pécora JD. Control of microorganisms in vitro by calcium hydroxide pastes. Int Endod. J. 2001;34(5):341-5.
23. Miyagak DC, Carvalho EM, Robazza CR, Chavasco JK, Levorato GL. In Vitro evaluation of the antimicrobial activity of endodontic sealers. Braz Oral Res. 2006;20(4):303-6.
24. Tobias RS. Antibacterial properties of dental restorative materials: a review. Int Endod J. 1988 Mar;21(2):155-60.
25. Estrela C, Pimenta FB, Ito IY, Bammann LL. In vitro determination of direct antimicrobial effect of calciaum hydroxide. J Endod. 1998 Jan;24(1):15-7.