In vitro determination of direct antimicrobial effect of calcium hydroxide associated with different substances against *Enterococcus faecalis* strains

Paulo Henrique **WECKWERTH**, DDS, MS, PhD[1]
Natália Bernecoli **SIQUINELLI**, PharmD[2]
Ana Carolina Villasbôas **WECKWERTH**, DDS[3]
Rodrigo Ricci **VIVAN**, DDS, MS[4]
Marco Antonio Hungaro **DUARTE**, DDS, MS, PhD[5]

ABSTRACT

Objective: To determinate the direct antimicrobial effects of *Casearia sylvestris Swart* (guaçatonga), propylene glycol, and of chlorhexidine associated to calcium hydroxide paste against 40 *Enterococcus faecalis* strains isolated from the oral cavity when direct contact. **Methods:** After activation, the bacterial strains were suspended in sterile saline to 1.0 McFarland standard. The suspension was placed in direct contact with calcium hydroxide paste [$Ca(OH)_2$] + pure propylene glycol, $Ca(OH)_2$ + chlorhexidine 1% in propylene glycol, and $Ca(OH)_2$ + guaçatonga extract in propylene glycol by covering paper points, previously contaminated for 3 minutes, with the different pastes. Antimicrobial activity was evaluated at 6, 24, 48, 72 hours, and at 7 days. After the incubation period, the points were removed from the pastes and incubated in Letheen broth at 37°C for 48 hours. Following that, 0.1ml of the Letheen broth was transferred to tubes containing brain heart infusion (BHI) broth and incubated again at 37°C for 48 hours. Turbidity was observed in the medium. After that, *M-Enterococcus* agar plates were seeded with BHI broth from each tube and colony growth was assessed. **Results:** All the bacterial strains were inhibited by all pastes at the evaluated periods. **Conclusions:** It was concluded that the addition of these substances to calcium hydroxide did not interfere with its direct antimicrobial effect.

Keywords: Environmental Microbiology. *Enterococcus faecalis*. Calcium hydroxide. Products with antimicrobial action.

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[1]Microbiology Professor, Sagrado Coração University, Bauru, SP, Brazil.
[2]Graduate of the Sagrado Coração University, Bauru, SP, Brazil.
[3]Microbiologist at the Lauro de Souza Lima Institute, Bauru, SP, Brazil.
[4]Endodontics Professor, Sagrado Coração University, Bauru, SP, Brazil.
[5]Endodontics Professor, Bauru Dental School, University of São Paulo, Bauru, SP, Brazil.

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Correspondence address: Marco Antonio Hungaro Duarte
Rua Anna Pietro Forte, 3-18 (lote A12), Residencial Villagio 1 - Bauru, SP, Brazil
Zip code: 17.018-820. E-mail: mhungaro@fob.usp.br

Introduction

The Enterococcus genus includes members previously classified as Group D Streptococci due to the presence of Group D cell wall antigen, a glycerol teichoic acid associated with the cytoplasmic membrane. Enterococci are normal inhabitants of the gastrointestinal tract and are found in lesser amounts in the vagina and male urethra.[1]

These have become important pathogenic microorganisms in humans, mainly due to their resistance to antimicrobial agents and to recently studied virulence factors.[2]

These Gram-positive cocci are arranged as pairs or in small chains, and are very hard to differentiate from streptococci. They are facultative anaerobes that thrive at 35°C, typically growing on the surface of blood agar plates as gamma-hemolytic cultures and on M-Enterococcus agar medium as deep-red or purplish colonies. Enterococci are tolerant to bile at 40% and can hydrolyze esculin. Moreover, they are able to grow in the presence of 6.5% sodium chloride, and can be distinguished from bacteria in genus *Staphylococcus* by their inability to produce catalase.[3]

Enterococcus faecalis are frequently found in root canals after failed endodontic therapy.[4,5,6]

Being highly resistant to several medications, they are also among the few microorganisms that display *in vitro* resistance to calcium hydroxide. This resistance is related to a proton pump[7] or to biofilm formation.[8] In an attempt to overcome this resistance, the addition of different substances to calcium hydroxide has been proposed.

One of the additives suggested is chlorhexidine, a biguanide. Calcium hydroxide-chlorhexidine paste has shown better antimicrobial action *in vitro*, compared with calcium hydroxide paste with pure water.[9] Despite its positive antimicrobial effect, this association has shown greater peroxide ion release, resulting in greater tissue irritation.[10]

New alternatives have been proposed in endodontic therapy, including natural substances such as propolis and phytotherapeutic agents. One of these phytotherapeutic agents is *Casearia sylvestris Sw* infusion or alcoholic extract.

This plant is native to Latin America, from Mexico to Argentina. It is found throughout Brazil, being particularly common in the state of São Paulo. It is popularly known as guaçatonga, erva de lagarto ("lizard's herb"), vassitonga, bugre branco, among other names. The word "guaçatonga" originated from the Tupi-Guarani (indigenous language), showing that this species was known by the native populations of Brazil.[11]

Guaçatonga extract has shown antiinflamatory[13] and antimicrobial action.[14] However, no studies showing whether the addition of phytotherapeutic agents to calcium hydroxide paste interferes with its antimicrobial action can be found in the scientific literature.

With this in mind, the objective of the present study was to evaluate the sensitivity of several *Enterococcus faecalis* strains isolated from the oral cavity to direct contact with calcium hydroxide pastes associated with *Casearia sylvestris Sw* (guaçatonga) in propylene glycol, calcium hydroxide with pure propylene glycol, or calcium hydroxide and 1% chlorhexidine in propylene glycol.

Metodology

Preparation of the extract

The *Casearia sylvestris Sw* leaves used in this study were collected at the Lageado farm, School of Agronomical Sciences - Unesp, in Botucatu, state of São Paulo, and identified at the herbarium of the Sagrado Coração University (USC) - Bauru, São Paulo, Brazil.

After harvesting and desiccation, the material was further dehydrated in an air-circulating oven under controlled temperature until constant weight was achieved. Following that, the leaves were triturated in a knife mill and used to prepare the extract. The dehydrated material was macerated in propylene glycol (extracting solution) following a ratio of 25 grams of powder to 200 ml of extracting solution. The plant powder remained in the extracting solution for eight days, with sporadic agitation during that period. The entire extraction process took place in an amber colored container (in order to prevent possible interference by light) and at room temperature (25°C).

Enterococccus Strains

Forty *E. faecalis* strains from the USC Microbiology laboratory bacterial library were used in the present work. These strains were cultured from bacterial samples obtained from the oral cavity of patients seen at the USC School of Dentistry Endodontics clinic in Bauru, Brazil.

All strains had been frozen at -20°C and were isolated in M-Enterococcus agar medium (Difco®). Strains were then identified following a standard identification routine described by Koneman et al.[1]

Activation of the strains was carried out on M-Enterococcus agar plates (Difco®) in an oven set at 36°C for 18-24 hours. Subsequently, colonies were suspended in BHI broth (Oxoid®) until complete turbidity of the medium was observed.

Antimicrobial substances tested

All bacterial substances tested in this study were based on calcium hydroxide P.A. paste (Table 1).

The pastes were prepared by mixing 2 grams of powder to 70 drops of each corresponding vehicle, resulting in a mixture with toothpaste-like consistency after spatulation. For each material tested, approximately 12 grams of calcium hydroxide paste were manipulated.

Table 1. Pastes used in the experiment.

$Ca(OH)_2$ + guaçatonga extract in propylene glycol
$Ca(OH)_2$ + 1% guaçatonga solution in propylene glycol
$Ca(OH)_2$ + pure propylene glycol

Assessment of the antimicrobial activity

The inoculum suspensions in BHI broth (Oxoid®), were diluted in 5 ml sterile saline to reach turbidity corresponding to 1 McFarland standard ($3x10^8$ cells/ml).

For the antimicrobial activity test, 1,200 paper points (Tanari®, Tanariman Ltda), previously sterilized by autoclaving, were immersed in the experimental bacterial suspensions for 3 minutes in order to achieve contamination. Following that, the paper points were aseptically removed from the bacterial suspension and distributed on the surface of sterile Petri dishes. The paper points were then covered by the different pastes being evaluated. The Petri dishes were covered and kept in an oven at 37°C.

At 6, 24, 48, 72 hours, and at 7 days, the paper points were removed from direct contact with the pastes and placed in test tubes containing 4 ml sterile Letheen Broth (Difco®). The broth was incubated at 37°C for 48 hours and visually assessed for macroscopic turbidity.

An inoculum containing 0.1 ml of Letheen broth was transferred to a test tube with 4 ml BHI broth that had been incubated under the same conditions. The BHI broth test tubes with no evidence of turbidity were considered as negative, and the ones displaying turbidity of the broth were seeded on M-Enterococcus agar in order to determine whether the bacterial strains remained viable.

All the experimental procedures were conducted under aseptic conditions with the aid of a laminar flow hood, and assays were performed in duplicate. One experiment was carried out with a standard *Enterococcus faecalis* ATCC 29212 strain.

The pH of each paste was measured after manipulation and placement in deionized water, with the aid of a pH meter.

Results

The pH values for the pastes were: 12.67 for the calcium hydroxide + 1% chlorhexidine, 12.62 for the calcium hydroxide + propylene glycol, and 12.60 for the calcium hydroxide + *Casearia sylvestris Sw* extract.

The assessment of antimicrobial activity for the three different pastes at 6, 24, 48, 72 hours, and at 7 days post-incubation showed that all strains were inhibited in all periods of evaluation (Table 2).

Discusson

The efficacy of $Ca(OH)_2$ paste against *E. faecalis* and other microorganisms has been extensively discussed in the scientific literature.[15-19]

The addition of chlorhexidine has conferred greater antimicrobial efficacy to calcium hydroxide pastes used for disinfection of the dentin tubules.[7] However, Schäfer et al[17] observed no increase in efficacy against *E. faecalis* by associating $Ca(OH)_2$ with chlorexidine.

Ercan et al,[18] in an *in vitro* experiment involving extracted teeth, revealed that 2% chlorhexidine gel was more efficient against *E. faecalis* and Candida albicans compared to plain $Ca(OH)_2$ or to $Ca(OH)_2$ with 2% chlorexidine.

Enterococcus faecalis needs to be maintained in direct contact with calcium hydroxide in order to be killed[20,21]. In the present work, the least amount of time *Enterococcus faecalis* was kept in contact with the pastes was 6 hours, and none of the strains survived.

The results reported in this study for calcium hydroxide paste with chlorhexidine are in agreement with Estrela et al,[21] who used similar methodology.

Table 2. Antimicrobial action of the calcium hydroxide pastes against the different bacterial strains.

	$Ca(OH)_2$ + Propylene glycol					$Ca(OH)_2$ + 1% Chlorexidine					$Ca(OH)_2$ + Guaçatonga extract				
	6h	24h	48h	72h	7d	6h	24h	48h	72h	7d	6h	24h	48h	72h	7d
1	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
2	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
3	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
4	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
5	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
6	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
7	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
8	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
9	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
10	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
11	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
12	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
13	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
14	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
15	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
16	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
17	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
18	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
19	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
20	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
21	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
22	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
23	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
24	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
25	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
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28	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
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35	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
36	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
37	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
38	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
39	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
40	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -

The antimicrobial action of calcium hydroxide arises from the release of hydroxyl ions with consequent pH increase, reaching 11 to 12.5.[22] According to Siqueira-Júnior et al[23] the lethal effect of hydroxyl ions against bacterial cells is mainly due to the damage inflicted on their cytoplasmic membrane, protein denaturation, and direct damage to DNA, although it is now clear that one of the crucial factors for *E. faecalis* survival in high pH is the presence of a proton pump that enables cytoplasmic homeostasis, even in extremely alkaline environments.[7] *Enterococcus faecalis* strains have been found to survive in environments with pH as high as 10.5 to 11.0; pH values have to be greater than 11.5 in order to kill these strains.[24] In the present paper, all the pastes had pH greater than 12.5 and were able to kill all strains.

It is important to emphasize that the pH of calcium hydroxide pastes is generally higher than 11, and that the addition of several substances does not alter these values.[25,26] However, within the dentin tubules, the pH might not reach such high levels,[27,28] hence the suggested association of different substances to the pastes with the goal of enhancing the antimicrobial action, with positive results.[9]

In this paper, $Ca(OH)_2$ pastes in three different vehicles demonstrated great effectiveness against all *E. faecalis* strains after direct *in vitro* contact of the microorganism with the paste. The addition of guaçatonga did not interfere with the antimicrobial action of calcium hydroxide, confirming that the presence of this substance did not alter the pH of the paste. Further experiments should be carried out in order to demonstrate, both *in vivo* and *in vitro* (using extracted teeth) whether similar effect is observed. It is important to take into consideration that for calcium hydroxide to maintain its ability to raise the pH within the dentin tubules, the hydroxyl ions should diffuse throughout dentin in high enough concentrations to exert buffer effect and consequently induce a drastic increase in the local pH values.

The guaçatonga essential oil has shown effective action against Gram-positive bacteria such as *Enterococcus*, *Micrococcus*, *Staphylococcus aureus*, *S. epidermidis*[14] and *Bacillus cereus strains.*[29]

Methods in which the pastes are diffused on the agar surface, as described by Gomes et al,[15] or those involving direct contact with the paste, followed in the present work and others,[21] are susceptible to interference from several variables, namely differences in solubility and diffusion of the paste in the medium, the inoculum, pH of the agar components, agar viscosity, incubation times and temperature, and metabolic activity of the microorganism in the culture medium. All of these factors hinder the extrapolation of the results to a clinical setting, where other different factors may interfere with the antimicrobial action of the paste against microorganisms in the dentin tubules.

Therefore, it is unquestionable that future studies are needed in order to determine whether guaçatonga extract in propylene glycol is able to enhance the efficacy of calcium hydroxide pastes against *E. faecalis* in extracted teeth *in vitro* or *in vivo*, in actual clinical conditions. The discovery of antimicrobial biocomponents derived from this plant, with activity against bacteria found in the oral microbiota, may lead to new therapeutic alternatives in Dentistry.

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