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Microbial Susceptibility to Calcium Hydroxide Pastes and Their Vehicles

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The aim of this study was to investigate the susceptibility of some microorganisms commonly isolated from root canals to calcium hydroxide in combination with several vehicles by the agar diffusion method. Stainless-steel cylinders were placed on each inoculated agar medium. The test medications and their controls were placed inside the cylinders. The zones of growth inhibition were measured and recorded after the incubation period for each plate, and the results were analyzed statistically. *Enterococcus faecalis* was most resistant, whereas the anaerobic *Porphyromonas endodontalis* was more susceptible to all medications, followed by *P. gingivalis* and *Prevotella intermedia/intermedia*. $Ca(OH)_2$ + CMCP + glycerin showed significantly larger mean zones of inhibition when compared with the other medications. We conclude that anaerobic Gram-negative bacteria are more susceptible to calcium hydroxide pastes than facultative Gram-positive microorganisms.

Bacteria or their products are considered to be the primary etiologic agent of pulpal necrosis and periapical lesions. Therefore, their elimination is one of the most important steps in endodontic therapy. The majority of the infecting bacteria, together with their principal substrate of necrotic pulp debris, may be removed by routine endodontic procedures such as instrumentation and irrigation of the pulp space and the use of an intracanal medication with antimicrobial activity. However, this is not always fully achieved in clinical practice due to the anatomical complexities of many root canals, and consequent limitations of access by instruments, irrigants, and intracanal medications (1). Moreover, the efficacy of these measures may also depend on the vulnerability of the involved species, which may not be uniform (2).

Anaerobic bacteria, especially black-pigmented Gram-negative ones, have been linked to the signs and symptoms of endodontic disease (3, 4). Facultative bacteria such as *Enterococcus faecalis*, have also been isolated from pathologically involved root canals and may be related to failure of endodontic therapy (2, 5–7).

Calcium hydroxide plays an important role in endodontics by its ability to induce hard tissue formation, its moderate antibacterial action, and its tissue dissolving capability (8). In addition to acting as a physical barrier, the calcium hydroxide dressing may both prevent root canal reinfection and interrupt the nutrient supply to the remaining bacteria. Its high pH (approximately 12.5) has a destructive effect on cell membranes and protein structure (9).

The agar diffusion method has been widely used to test the antimicrobial activity of dental materials and medications (10–11). It allows direct comparisons of intracanal medications against the test microorganisms, indicating which medication has the potential to eliminate bacteria in the local microenvironment of the root canal system.

The purpose of this study was to investigate variations in the susceptibility of some aerobes and facultatively and strictly anaerobic microorganisms commonly isolated from the root canals to calcium hydroxide associated with several vehicles.

MATERIALS AND METHODS

The microorganisms used in this experiment were two aerobes, six facultatively anaerobic bacteria, and four black-pigmented Gram-negative anaerobes commonly isolated from infected root canals, as follows: *Candida albicans* (NTCC 3736), *Bacillus subtilis* (ATCC 19659), *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Streptococcus sanguis* ATCC 10556, *Streptococcus sobrinus* 6715, *Streptococcus mutans* OMZ 175, *Actinomyces naeslundii* M104, *Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Prevotella intermedia/nigrescens*, and *Prevotella denticola*. The last four microorganisms were isolated from clinical trials. The aerobes and facultative anaerobes were kindly donated by the Center of Oral Biology, University of Rochester, NY.

Each microbial strain was evaluated against calcium hydroxide pastes prepared with the following vehicles: (a) sterile distilled water (aqueous vehicle); (b) sterile saline (aqueous vehicle); (c) anesthetic solution (aqueous vehicle, 3% Carbocaine (mepivacaine) with no vasoconstrictor, Sterling Drug, New York, NY); (d) glycerin (viscous vehicle); (e) polyethyleneglycol (viscous vehicle,

proprietary brand: Calen™, S.S. White Artigos Dentários, Rio de Janeiro, RJ, Brazil); (f) camphorated parachlorophenol (CMCP, oily vehicle-Frank's paste, 2:1); and (g) CMCP + glycerin (2:1:1). Distilled water, a physiologic solution of 0.85% sterile saline, anesthetic solution, glycerin, polyethyleneglycol, CMCP and CMCP + glycerin (1:1) were used as controls. The calcium hydroxide pastes were prepared using calcium hydroxide P.A. (Quimis Mallinckrodt, Inc., USA). The consistency of the pastes was similar to that of toothpaste, with a viscosity of 3501 cP at 0.1 rpm (Brookfield Digital Reometer, model DV-III-IV), and pH 12.5 determined with a pH meter (Analion, pH digital PM 605, USA). Calen is the proprietary brand of Leonardo & Leal's paste, with the following formulation: calcium hydroxide (2.5 g), zinc oxide (0.5 g), hydrogenized colophony (0.05 g), and polyethyleneglycol 400 (1.75 ml). This is the only proprietary brand of a calcium hydroxide paste containing this viscous vehicle (12).

All microorganisms were previously subcultured in appropriate culture media and under gaseous conditions to confirm their purity.

Facultative strains were individually inoculated into tubes containing 5 ml of a sterile 0.85% saline solution. The suspension was adjusted spectrophotometrically to match the turbidity of a McFarland 0.5 scale. Five hundred microliters of each test microorganism suspension were inoculated into glass bottles containing 50 ml of brain-heart infusion agar at 46°C, vortexed, and poured onto 130-mm plates containing a previously set layer of Mueller Hinton (MH) agar (Oxoid, Unipath Ltd, Basingstoke, UK).

For the anaerobes, isolated colonies were suspended to reach 1.0 on the McFarland scale. Sterile swabs were dipped into the bacterial suspension and inoculated onto prereduced 70-mm plates containing 5% sheep-blood-fastidious anaerobe agar (FAA, Lab-M, Bury, UK). Appropriate inoculum procedures were used to provide a semiconfluent growth of the tested microorganisms.

Sterilized stainless-steel tubes of 8.0 × 1.0 × 10 mm (inner diameter, 6 mm) were added to the surfaces of the media and filled with 40 μL of each test substance and its control. The plates were kept for 2 h at room temperature to allow the diffusion of the agents through the agar and then incubated at 37°C under appropriate gaseous conditions and for an appropriate period of time: aerobes, 24 h; facultatives, 24 to 48 h in a CO_2 incubator (Jouan, Saint Herblain, France), in an atmosphere of 10% CO_2; and anaerobes in the anaerobic work station (Don Whitley Scientific, Bradford, UK) in an atmosphere of 5% to 10% H_2, 10% CO_2, 80% to 85% N_2 for 7 days. Zones of inhibition of microbial growth around the cylinder containing the tested substances were measured and recorded after the incubation period. The inhibitory zone was considered to be the shortest distance (mm) from the outer margin of the cylinder to the initial point of the microbial growth. Six replicates were made for each microorganism. Analysis of variance (ANOVA) was used to determine the differences in susceptibility to intracanal medication between microbial species.

RESULTS

Figure 1 shows the average values of the microbial susceptibility to all calcium hydroxide pastes. Figure 2 shows the average values of the growth inhibition zones created by all calcium hydroxide pastes. The mean zones of microbial inhibition for each medication (including the active controls) are presented in Table 1.

The susceptibility of individual microorganisms to the calcium hydroxide pastes was varied. *E. faecalis* (1.13 mm) was most resistant, followed by *A. naeslundii* M104 (1.6 mm) and *Streptococcus sobrinus* (1.67 mm), whereas the anaerobic *P. endodontalis* (4.46 mm) was more susceptible to all pastes, followed by *P. gingivalis* (3.93 mm) and *P. intermedia/nigrescens* (3.43 mm) (Fig. 1).

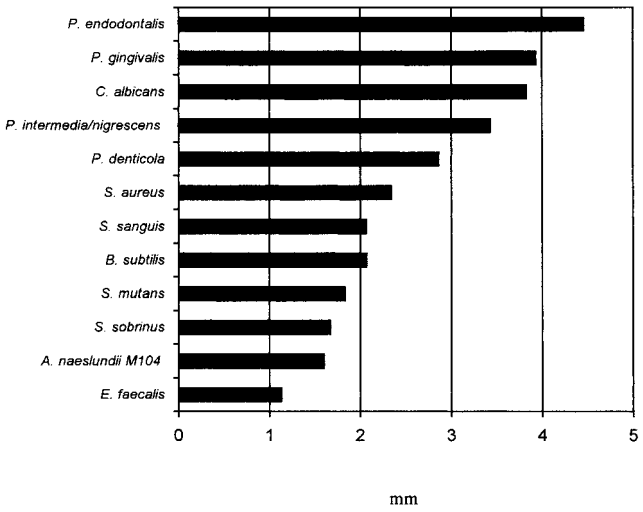

FIG 1. Average (in mm) of the zones of inhibition of each microorganism against all $Ca(OH)_2$ pastes tested.

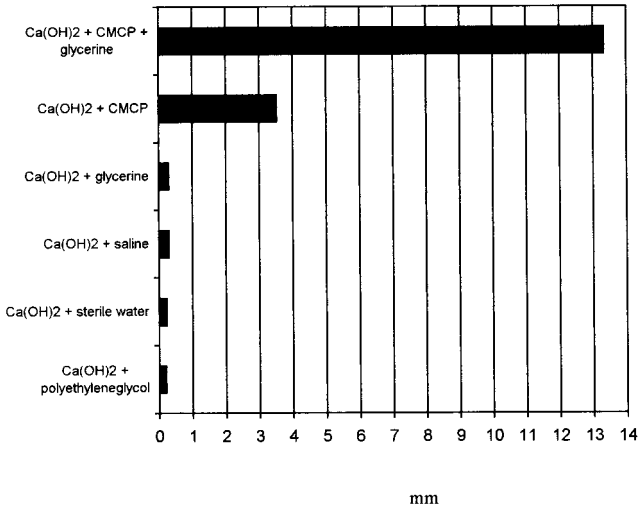

FIG 2. Average (in mm) of zones of inhibition produced by each calcium hydroxide paste against all microorganisms tested.

Based on the average diameters of the zones of microbial growth inhibition, the antimicrobial effects of the calcium hydroxide pastes could be ranked from strongest to weakest as follows: $Ca(OH)_2$ + CMCP + glycerin, $Ca(OH)_2$ + CMCP, $Ca(OH)_2$ + glycerin, $Ca(OH)_2$ + anesthetic, $Ca(OH)_2$ + saline, $Ca(OH)_2$ + H_2O, $Ca(OH)_2$ + polyethyleneglycol (Calen™) (Fig. 1).

All calcium hydroxide pastes were inhibitory at least by direct contact against all microbial strains tested. $Ca(OH)_2$ + CMCP + glycerin showed significantly larger mean zones of inhibition (13.33 mm) when compared with the other medications ($p < 0.05$).

Calen™, the proprietary brand tested (viscous vehicle, polyethyleneglycol), produced the smallest inhibition zone (0.22 mm), followed by the pastes prepared with aqueous (water, saline, or anesthetic) and viscous vehicles (glycerin). $Ca(OH)_2$ associated with CMCP demonstrated weak activity (3.52 mm) compared with

CMCP alone (6.19 mm) or CMCP + glycerin (8.75 mm). The controls (water, saline, anesthetic, glycerin, and polyethyleneglycol) were inert, forming no inhibitory zone with any of the microorganisms tested (Table 1).

DISCUSSION

The antimicrobial activity of calcium hydroxide, given by the high pH, is related to the release of hydroxyl ions in aqueous environment, which requires an ideal time for effective destruction of microorganisms, acting in direct or indirect contact in dentinal tubules (13). The lethal effects of hydroxyl ions on bacterial cells are probably due to the following mechanisms: (a) damage to the bacterial cytoplasmic membrane; (b) protein denaturation; and (c) damage to the DNA (14). To be effective against bacteria located inside the dentinal tubules, the hydroxyl ions from calcium hydroxide should diffuse into dentin at sufficient concentrations and should exceed the dentin buffering ability, reaching pH levels sufficient to destroy bacteria. Another mechanism that explains its antimicrobial activity is the ability of calcium hydroxide to absorb carbon dioxide in the root canals (15), which is essential for bacteria such as *Capnocytophaga*, *Eikenella*, and *Actinomyces* spp. (16) and could be supplied by bacteria such as *Fusobacterium*, *Bacteroides*, *Porphyromonas*, and *Streptococcus* spp. If calcium hydroxide absorbs carbon dioxide, CO_2-dependent bacteria will not survive. Therefore, the use of an intracanal medication will disturb established nutritional interrelationships, eliminating some bacteria that might be essential to the growth of others or leaving some bacteria whose presence will prevent the growth of others.

The time needed for calcium hydroxide to optimally disinfect the root canal system is still unknown and might be related to the presence or absence of root canal exudation, type of microorganism involved, location of the microorganism in the root canal system, and presence or absence of the smear layer.

In this study, all calcium hydroxide pastes mixed with inert vehicles had an antimicrobial action, but just by direct contact, in agreement with previous studies (10, 11). Other studies have also reported the failure of calcium hydroxide to eliminate enterococci effectively (2, 5–7) because they tolerate very high pH values, varying from 9 to 11. In this study, *E. faecalis* was the microorganism showing the smallest inhibition zone against all tested intracanal medications, whereas strict anaerobes such as *P. endodontalis*, *P. gingivalis*, and *P. intermedia/nigrescens* showed the largest inhibition zones. It could be extrapolated that even though the anaerobic Gram-negative bacteria, such as *Prevotella* or *Porphyromonas* spp. are associated with painful infectious exacerbations due to the presence of the endotoxin, which can stimulate production of bradykinin, a potent pain mediator, they are more susceptible to calcium hydroxide pastes. On the other hand, facultative Gram-positive bacteria such as *E. faecalis*, *A. naeslundii*, and *S. sobrinus*, which have been associated with asymptomatic root canals (2–4), seems to be more resistant to root canal therapy and are therefore related to endodontic failures.

This study has shown that $Ca(OH)_2$ pastes kill bacteria by direct contact, which means that the remaining microorganisms in contact with this medication in the root canal will be eradicated if they are not tolerant to it.

The superior in vitro antimicrobial activity of calcium hydroxide associated with camphorated paramonochlorophenol (CMCP) has long been recognized. Kaiser (17) and Frank (18) recommended this association in treating nonvital teeth with wide open apices.

TABLE 1. Mean area of the zones of microbial growth inhibition in mm (n = 6) provided by calcium hydroxide associated with several vehicles, CMCP and CMCP + glycerine against all microorganisms tested

Microorganisms	$Ca(OH)_2$ + Sterile water	$Ca(OH)_2$ + Saline	$Ca(OH)_2$ + Anesthetic Solution	$Ca(OH)_2$ + Glycerine	$Ca(OH)_2$ + Polyethyleneglycol (Calen®)	$Ca(OH)_2$ + CMCP	$Ca(OH)_2$ + CMCP + Glycerine	CMCP	CMCP + Glycerine	Average values of the microbial susceptibility to all medications tested
C. albicans (NTCC 3736)	2.3	2.6	2.6	2.8	1.7	3.5	11.3	4.0	4.5	3.92
B. subtilis (ATCC 19659)	0.5	1.0	1.0	1.0	0.7	3.8	6.5	3.0	3.5	2.33
S. aureus (ATCC 25923)	0*	0*	0*	0*	0*	4.7	11.7	5.3	7.0	3.19
E. faecalis (ATCC 29212)	0*	0*	0*	0*	0*	0.7	7.2	3.0	5.0	1.77
S. sanguis (ATCC 10556)	0*	0*	0*	0*	0*	2.5	12.0	6.0	13.5	3.47
S. sobrinus 6715	0*	0*	0*	0*	0*	1.5	10.2	6.0	13.5	3.78
S. mutans OMZ 175	0*	0*	0*	0*	0*	1.5	11.3	2.5	5.0	2.25
A. naeslundii M104	0*	0*	0*	0*	0*	2.5	8.7	5.0	5.0	2.35
P. gingivalis	0*	0*	0*	0*	0*	5.5	22.0	11.0	13.0	5.72
P. endodontalis	0*	0*	0*	0*	0*	6.2	25.0	12.5	15.0	6.52
P. intermedia/nigrescens	0*	0*	0*	0*	0*	5.0	19.0	10.0	12.0	5.11
P. denticola	0*	0*	0*	0*	0*	5.0	15.0	6.0	8.0	3.78
Average values of each medication against all microorganisms tested	0.23	0.3	0.3	0.32	0.2	3.52	13.33	6.19	8.75	

* Direct contact inhibition only.
CMCP = camphorated parachlorophenol.

Moreover, the addition of glycerin to Frank's paste seems to enhance its antimicrobial action because glycerin helps the diffusibility of $Ca(OH)_2$ + CMCP (19), a fact also observed in this study. However, many authors have shown that CMCP is a tissue irritant (9), a property that limits its clinical use.

In conclusion, calcium hydroxide, although suitable as an intracanal medication, cannot be considered a universal intracanal medication, because it is not equally effective against all bacteria found in the root canal. However, the association of antimicrobial agents with calcium hydroxide should be avoided, especially those that have been shown to be irritating to periapical tissues. Another medication, such as chlorhexidine gel (especially at 2%), which has a wide spectrum of antimicrobial activity with prolonged action, is biocompatible with periapical tissues, stays longer in contact with the microorganisms, and diffuses through the dentin tubules, should be considered (20).

Finally, laboratory tests of any kind are only the first steps in a study of the effectiveness of intracanal medications. The results of the agar diffusion method, as the other in vitro tests, depend upon the molecular size, solubility, and diffusion of the materials through the aqueous agar medium, the sensitivity of the drug, bacterial source (wild strains or collection species), the number of bacteria inoculated, pH of the substrates in plates, agar viscosity, storage conditions of the agar plates, incubation time, and the metabolic activity of the microorganisms. Therefore, the inhibition zones may be more related to the materials solubility and diffusibility in agar than to their actual efficacy against the microorganisms. However, great care was taken to keep the plates for 2 h at room temperature to allow the diffusion of the agents through the agar and then incubated at 37°C under appropriate gaseous conditions. Also, in this study a clear difference could be observed between the zones of microbial growth inhibition and the materials diffusion zone. In the inhibition zone, no bacteria were found inside the halo (e.g. the zone created by $Ca(OH)_2$ + CMCP + glycerin). On the other hand, in the material diffusion zone (e.g. the zone created by $Ca(OH)_2$ + glycerin), bacteria were present inside the halo.

On the basis of the results obtained and of the experimental conditions used in this study, we conclude that the anaerobic Gram-negative bacteria are more susceptible to calcium hydroxide pastes than the facultative Gram-positive microorganisms, and the diffusion ability and the antimicrobial activity of calcium hydroxide are affected by the type of vehicle utilized. However, in vitro results should be carefully analyzed before their extrapolation to the clinical conditions.

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