Susceptibility of Microorganisms Isolated from Necrotic Pulps to Calcium Hydroxide

Susceptibilidade de microrganismos isolados de polpa necrótica ao hidróxido de cálcio

Susceptibilidad de microorganismos aislados de pulpas necróticas al hidróxido de cálcio

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Calcium hydroxide has been used as pulp-capping agent and canal dressing due to its antimicrobial and anti-inflammatory properties besides its ability to induce formation of mineralized tissues. The aim of this study was to evaluate the susceptibility to calcium hydroxide of 146 bacterial strains isolated from endodontic infections. MIC was determined by using an agar dilution method, while contact bactericide activity was performed through in broth. All the isolates were sensitive to calcium hydroxide in concentrations that varied from 0.5mg/ml to 128 mg/ml, and the genera *Enterococcus*, *Pseudomonas*, *Staphylococcus* and *Actinomyces* were the most resistant. Gram-negative anaerobes proved to be the most sensitive isolates. All the isolates were inhibited after 60 minutes of contact with the alkali in concentration of 100mg/ml.

Keywords: Calcium Hydroxide; Microbial Sensitivity Tests; Infection; Dental Pulp**;** Bacteria.

INTRODUCTION

Periapicopathies are commonly related to the presence of organic materials and microorganisms and their toxic products inside canal systems and immunologic reactions to these components[1,2]. The microorganisms may reach pulpal tissues through different means of access, such as carious lesions, dental fractures, exposed dentinary tubules, accessory canals, apical foramen and hematogenic routes. Thus, during endodontic treatment, several techniques are used to eliminate or, at least, control this microflora, creating conditions for canal filling. However, a percentage of cases result in failure and bacterial infection plays essential part in this phenomenon[1, 2,3,4,5].

Despite the fact that some studies in vitro have given evidence that calcium hydroxide presents inhibitory activity with oral microorganisms or involved in endodontic infections[6,7,8,9,10], other studies have reported less favorable results[11,12,13] However, in most of the cases reported in literature, the number of tested strains is small and, at times, this account for just a small parcel of microflora isolated from teeth with necrotic pulp.

Thus, this study aimed to evaluate, *in vitro*, the susceptibility of 146 microorganisms isolated from teeth with necrotic pulp to calcium hydroxide and the bactericide activity of this alkali with regard to these isolated microorganisms.

MATERIAL AND METHODS

1. MICROORGANISMS

It was tested 146 strains of microorganisms isolated from 58 teeth with necrotic pulp, obtained from 52 patients (aged from 14 to 59 years). Table 1 shows the identity of tested strains. The microorganisms were identified through biochemical tests and cellular and colonial morphology, as described anywhere.

2. PREPARATION OF BACTERIA

The obligate and facultative anaerobes were grown for 24-48 at 37ºC in 5 ml of brain heart infusion broth (Difco) supplemented with hemin (0,5 μg/ml), menadione (5μg/ml) and yeast extract (0.5%), under anaerobiosis (90% N_2 + 10%, CO_2). The inocula were standardized to contain 10^8 CFU/ml, determined in spectrophotometer.

3. DETERMINATION OF MINIMAL INHIBITORY CONCENTRATIONS OF CALCIUM HYDROXIDE: METHOD, CULTURE MEDIUM AND INCUBATION

An agar dilution method was used and the culture medium employed was Wilkins Chalgren agar enriched with horse blood (5%) and supplemented with hemin (0.5 μg/ml), menadione (5 μg/ml) and yeast extract (0.5%). Calcium hydroxide was added into culture medium in concentrations that varied from 0.25 mg/ml to 256 mg/ml.

Table 1- Microorganisms submitted to susceptibility tests to calcium hydroxide.

Microorganisms	Number of strains
Streptococcus sp.[1]	3
S. milleri	5
S. mitis	3
S. sanguis	5
S. salivarius	4
S. oralis	2
S. defectives	1
S. mutans	5
S. pyogenes	2
A viscosus	2
A. naeslundii	3
A. israelii	3
A. odontolyticus	1
A. meyeri	1
Lactobacillus sp.[1]	8
Fusobacterium sp.[1]	1
F. nucleatum	17
F. necrophorum	1
F. periodonticum	1
Porphyromonas sp.[1]	4
P. gingivalis	2
Prevotella loescheii	2
P. denticola	3
P. intermedia-nigrescens	10
Eubacterium lentum	5
E. nodatum	3
E. alactolyticum	2
Bacteroides fragilis	4
Peptostreptococcus micros	6
P. anaeroius	4
Propionibacterium sp.[1]	5
Staphylococcus aureus	5
S. saprophyticus	3
S. epidermidis	10
S. lugdunensis	1
S. haemolyticus	1
Enterococcus faecalis	4
E. faecium	1
Pseudomonas aeruginosa	2
P. cepacia	1

[1] Strains not identified to species level

Plates with or without (contro1) calcium hydroxide were inoculated with 10^5 UFC/spot by using a Steers' replicator (Cefar Ltd., Brazil). Plates were incubated anaerobically, at 37ºC for 48h. The minimal inhibitory concentration was defined as the lowest concentration of the antimicrobial agent capable of completely inhibiting macroscopic bacterial growth.

4. TIME-KILL CURVE OF ANTIMICROBIAL ACTIVITY OF CALCIUM HYDROXIDE

The methodology used was described by Barnard

et al.[14]. The inoculum of 10^9 CFU of the tested strain was transferred to tubes containing 5ml of BHI broth supplemented with hemin (0.5μg/ml), menadione (5μg/ml), yeast extract (0.5%) and 100 mg/ml of calcium hydroxide. The tubes were incubated under anaerobiosis (90% N_2 +10% CO_2), at 37ºC, for 1, 5, 10, 20, 30 and 60 minutes, 1, 2, 7 and 14 days.

After incubation, the cells were pelleted, washed three times in PBS and plated on blood agar for up to 15 days, at 37ºC, under anaerobiosis, to check for growth of viable cells.

RESULTS

The MIC values are shown in Table 2. Growth of tested microorganisms was inhibited by concentrations of calcium hydroxide that varied from 0.5 mg/ml to 128 mg/ml. The isolates of the genera *Enterococcus*, *Actinomyces*, *Pseudomonas* and *Staphylococcus* were more resistant to the alkali. Obligate anaerobes, particularly Gram-negative bacteria were the most sensitive tested isolates (Table 2).

Table 2- Susceptibility to calcium hydroxide of 146 bacterial strains isolated from endodontic infections

Microorganism (n)	MIC[1] (mg/ml)		
	Range	MIC_{50}	MIC_{90}
Streptococcus sp. (30)	0.5 - 16.0	2.0	8.0
Actinomyces sp. (10)	1.0 - 16.0	4.0	16.0
Lactobacillus sp. (8)	0.5 - 4.0	1.0	4.0
Fusobacterium sp. (20)	1.0 - 4.0	1.0	4.0
Porphyromonas sp. (6)	0.5 - 4.0	1.0	4.0
Prevotella sp. (15)	1.0 - 8.0	1.0	4.0
Eubacterium sp. (10)	2.0 - 16.0	4.0	8.0
B. fragilis (4)	2.0 - 8.0	2.0	8.0
Peptostreptococcus sp. (10)	1.0 - 4.0	2.0	4.0
Propionibacterium sp. (5)	1.0 - 8.0	2.0	8.0
Staphylococcus sp. (20)	4.0 - 32.0	8.0	32.0
Enterococcus sp. (5)	16.0 - 64.0	64.0	64.0
Pseudomonas sp. (3)	8.0 - 64.0	32.0	64.0

[1] Minimal inhibitory concentration

Table 3- Bactericidal activity of calcium hydroxide on 146 bacterial strains recovered from necrotic pulps

Time of contact	Bacterial strains that lost viability N (%)
1 minute	2 (1.37)
5 minutes	36 (24,66)
10 minutes	87 (59.59)
20 minutes	112 (76.71)
30 minutes	138 (94.52)
60 minutes	146 (100.00)
1 day	146 (100.00)
2 days	146 (100.00)
7 days	146 (100.00)
14 days	146 (100.00)

Table 3 shows the results of bactericidal activity of calcium hydroxide. All the tested strains lost viability after 60 minutes of contact with the alkali.

DISCUSSION

The action mechanisms of calcium hydroxide are extensive and varied. Hydroxyl ions would act inhibiting microbial enzymes and would create favorable conditions for periapical repair, besides causing peroxidation of the phospholipides of the microbial membrane structures. This alkali can also interfere in the substantivity of nutrients, since it affects ionization of the cellular membranes and of the nutrients themselves, as discussed by Estrela et al.[15], besides breaking up the bacterial LPS[16,17], which would reduce the inflammatory response induced by the endotoxin released by the cell wall of Gram-negatives bacteria.

As many organisms gain their energy from redox reactions, which depend on the creation of a proton or electric gradient between the two sides of the citoplasmatic membrane, the alkaline environment produced by calcium hydroxide besides producing alterations in the permeability of the citoplasmatic membrane by creating new means of entrance for the protons, which would no longer cross the bacterial ATPases, would also retain the protons on the external side, preventing the same, through action of hydroxyls, from crossing the ATPases and leading to the synthesis of high energy phosphated compounds used in cellular anabolism. Some bacterial species, such as *E. faecalis*, have physiologic peculiarities in the proton pump, which may avoid the activity of strong alkalis when associated to the buffer capacity of dentine.[18] However, the present study showed that all bacterial groups were inhibited by calcium hydroxide after a direct exposure.

The clinical efficiency of calcium hydroxide dressings is fully admitted[19-23]. However, studies concerning antimicrobial activity of the alkali on microorganisms, *in vitro*, present extremely conflicting results. Results reported by Gencoglu, Külekçi[24]; Alaçam et al.[25], Georgopoulou et al.[6], Iordanoglou et

al.[26], Suzuki et al.[8], Han et al.[9] and Haenni et al.[10], have given evidence that calcium hydroxide presents antimicrobial activity *in vitro* on most microorganisms, including *Enterococcus faecalis, Streptococcus mutans, S. anginosus, Actinomyces viscosus, Porphyromonas gingivalis, Bacteroides fragilis, Prevotella melaninogenica, P. oralis, Veillonella alcalescens, Peptostreptococcus anaerobius, P. micros, Capnocytophaga* sp., *Pseudomonas aeruginosa, Escherichia coli* and *Fusobacterium nucleatum*, whereas studies by Haapasalo, Orstavik[27], Siqueira Jr. et al.[11] and Siqueira Jr, Uzeda[12] and Seabra et al.[13] suggest that the alkali only presents reduced antimicrobial activity on the more sensitive microorganisms, such as the obligate anaerobes and is deprived of inhibitory activity on microorganisms more tolerant to alkaline pH, such as strains of *E. faecalis* isolated by Byström et al.[20], which tolerated pH 11.5 . However, a significant part of these negative results with this alkali was obtained by using methodology of diffusion in agar, which has limitations.

This method is not recommended by the NCCLS (National Committee for Clinical Laboratory Standards) for drugs susceptibility testing of obligate anaerobes and other fastidious microorganisms, as these microorganisms hardly ever produce confluent growth, which limits the possibility and accuracy of defining a halo of bacterial growth inhibition.

In the test of antimicrobial activity of calcium hydroxide on anaerobes and other bacteria, it is necessary to perform a incubation of the alkali into the culture medium, 48-72 h before bacterial inoculation, in a atmosphere of nitrogen, since the association N_2+ CO_2 frequently employed in tests with anaerobes, could neutralize the pH of the agar by formation of calcium carbonate[28]. The formation of calcium carbonate due to reaction of carbon dioxide with calcium hydroxide, which does not present the alkali's antimicrobial and biological activities, may also account for reduction of microbial susceptibility *in vitro*. Hence, antimicrobial activity of the alkali should be higher in carbon dioxide concentrations found in human tissues, several times smaller the concentrations used in the antimicrobial tests *in vitro*.

The use of the diffusion in agar methodology is of scarce values in view of poor solubility of calcium hydroxide in water (1.2g/l), thus limiting the diffusibility of alkali. In this method, the presence of ample haloes of bacterial growth inhibition may indicate greater toxicity of the drug on the target microorganism, greater diffusibility of the drug or both. Thus, evaluation of haloes of bacterial growth inhibition should take into consideration the limitations of the method, as larger haloes of growth inhibition do not, necessarily, mean greater antimicrobial activity of the active principle over the microorganisms tested *in vitro.*

Data shown in Table 1 shows that *Enterococcus faecalis* was the microorganism most resistant to the alkali, whereas obligate anaerobes behaved as the most sensitive isolates, as also described by Suzuki et al.[8] and discussed by Chávez de Paz[29]. However, our results show a higher sensibility to calcium hydroxide out of all the microbial groups tested than previously reported.

There was no occurrence of microorganisms able to withstand more than 60 minutes in direct contact with this alkali (Table 2). And, as also reported by Georgopoulou et al.[6] and Chávez de Paz[29], the Gram positive microorganisms were less sensitive and none of the tested microorganisms survived more than 30 minutes in contact with this compound, though bacteria such as *Enterococcus faecalis* were not submitted to the tests. These results are compatible to those of Byström et al.[20] who verified that on making direct contact between the aqueous solution saturated with calcium hydroxide and *Enterococcus faecalis*, these microorganisms were eliminated in minutes.

The small susceptibility of Gram-positive cocci and rods to antimicrobials possibly is the major factor related to the higher proportions of these bacteria after unsuccessful treatment and such resistance depends on their major physiologic and morphological features, such as the significant ability of *Enterococcus faecalis* to invade dentinal tubules and proliferate in

monomicrobial biofilms, whereas Gram negative anaerobic rods are completely dependent to complex ecological relationships[29].

In spite of *in vitro* antimicrobial properties, an adjunctive compound needs to be active against organized biofilms, which present several barriers to diffusion of the drug. Thus, the time of contact between the calcium hydroxide and the biofilms inside the root canals must be observed and clinicians should follow the correct clinical procedures properly. For calcium hydroxide, using water-soluble vehicle, the maintenance of the medicament inside root canals is a sensitive aspect, since 15-30 days of maintenance of calcium hydroxide dressing produce best histopathological results in dogs with periapical lesions. This necessity of long period of contact, in vivo, is related to the ability of dentine neutralize hydroxyls liberated from calcium hydroxide[30].

CONCLUSIONS

The results of the present study lead to the conclusion that all the isolated microorganisms thereby tested were inhibited by rising concentrations of calcium hydroxide and that the obligate anaerobes were more susceptible. All the isolated microorganisms being tested were inhibited by contact with 100 mg/ml of this alkali for a period of 60 minutes.

RESUMO

O hidróxido de cálcio tem sido utilizado como agente de capeamento pulpar e curativo de demora devido às suas propriedades antimicrobiana e anti-inflamatória, além de sua habilidade de induzir a formação de tecido mineralizado. O objetivo deste estudo foi avaliar a susceptibilidade de 146 isolados bacterianos de infecções endodônticas ao hidróxido de cálcio. A concentração inibitória mínima foi determinada pelo método da diluição em ágar, enquanto que a atividade bactericida de contato foi realizada em caldo. Todos os isolados foram sensíveis ao hidróxido de cálcio em concentrações que variaram de 0,5mg/ml a 128 mg/ml, e os gêneros Enterococcus, Pseudomonas, Staphylococcus e Actinomyces foram mais resistentes. Os anaeróbios Gram-negativos mostraram ser os isolados mais sensíveis. Todos os isolados foram inibidos após 60 minutos de contato com a concentração de 100mg/ml.

Palavras chave: Hidróxido de Cálcio; Testes de Sensibilidade Microbiana; Infecção; Polpa Dentária; Bactérias.

RESUMEN

El hidróxido de calcio se ha utilizado como agente de recubrimiento pulpar y apósito intracanal debido a sus propiedades antimicrobianas y anti-inflamatorias Además de su capacidad para inducir la formación de tejidos mineralizados. El objetivo de este estudio fue evaluar la susceptibilidad a hidróxido de calcio de 146 cepas bacterianas aisladas de infecciones endodónticas. Concentración mínima inhibitoria se determinó utilizando un método de dilución en agar, mientras que la actividad bactericida de contacto se realizó a través de caldo. Todos los aislamientos fueron sensibles a hidróxido de calcio en concentraciones que variaban de 0.5mg/ml a 128 mg/ml, y el Enterococcus, Pseudomonas, Staphylococcus y Actinomyces fueron los más resistentes. Gram-negativos anaerobios demostrado ser los aislamientos más sensibles. Todas las cepas fueron inhibidas después de 60 minutos de contacto con el álcali en la concentración de 100mg/ml.

Palabras clave: Hidróxido de Cálcio; Pruebas de Sensibilidad Microbiana; Infecció;n Pulpa Dental; Bacterias.

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