Original Article

In Vitro Evaluation of Antimicrobial Activity of Calcium Hydroxide with Aqueous Vehicles in Dental Treatment

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ABSTRACT

Objective: The purpose of this study was to assess the in vitro antimicrobial activity of calcium hydroxide associated with aqueous vehicles against aerobes and facultative anaerobic microorganisms commonly isolated from infected root canals.

Study Design: Experimental Study.

Place and Duration of Study: This study was conducted in the Department of Pharmacology, University of Karachi from Sept. 2007 to March 2009.

Materials and Methods: The microbial strains were evaluated against calcium hydroxide pastes prepared with calcium hydroxide powder mixed with aqueous vehicles. Antimicrobial activity of the vehicles was also evaluated. For such purpose agar diffusion and broth dilution method was used.

Results: The results showed that calcium hydroxide mixed with aqueous vehicles was inhibitory against all the microbial strain tested. Calcium hydroxide pastes also eliminated the Enterococcus faecalis (the most resistant organisms in infected root canals) effectively. The results are statistically significant when calcium hydroxide was mixed with aqueous vehicles ($p<0.05$).

Conclusion: We concluded from our study that aqueous vehicles play a very important role in eliminating the endodontic bacteria particularly Enterococcus faecalis which is very challenging for the endodontists while treating the patient.

Key Words: Calcium Hydroxide, Aqueous Vehicles, Route Canal Treatment

INTRODUCTION

Root canal therapy plays a vital role in the line of dental treatment. Root canal therapy consists of proper cleaning, shaping, irrigation and obturation of root canals which results in the reduction or elimination of bacteria.[1] However, complete elimination of bacteria is very difficult to achieve in clinical practice due to the anatomical complexities of root canals such as accessory canals, canal ramifications, apical deltas, fins and transverse anasmastos.[2, 3] The inter-appointment intra-canal medication is common during root canal treatment procedures to eliminate the bacteria from infected root canals.[4, 5, 6] The application of the medicament is usually common in those cases where there is pain or continuing exudates. Calcium hydroxide was originally introduced in dentistry by Hermann in 1920 and since then it is used widely in dental treatment throughout the world particularly in root canal treatment. The antibacterial effects of calcium hydroxide are due to the damage to the microbial cytoplasmic membrane by the direct action of hydroxyl ions. The hydroxyl ions induce lipid per oxidation that results in the destruction of phospholipids and structural components of the cellular membrane. The alkaline pH of calcium hydroxide is also responsible for the breakdown of ionic bond which maintains the protein structure of the bacterial cell membrane. Hydroxyl ions also react with the bacterial DNA results in the inhibition of DNA replication by splitting the strands of DNA. Availability of calcium ions at the site of action also exerts therapeutic effects through ion channels. Calcium hydroxide also absorbs carbon dioxide which is responsible for its antimicrobial activity. It impedes the carbon dioxide supply to CO_2-dependent bacteria in the infected canals.[7, 8]Although calcium hydroxide has been used for over 80 years there are still many questions to be answered regarding its inhibitory activity against pathogens.[9]

MATERIALS AND METHODS

The microbial strains were evaluated against calcium hydroxide pastes prepared with calcium hydroxide powder mixed with aqueous vehicles by agar diffusion method[10, 11]and broth dilution method.[9] The antimicrobial activity of vehicles was also evaluated by using the above mentioned methods.

Vehicles: The vehicles include:

- Distilled water
- Saline
- Anesthetic solution (3%Mepivacaine hydrochloride, used in dentistry)

The pastes were prepared on a sterile glass slab with a sterile spatula. The consistencies of the pastes were similar to that of the tooth paste.

Microbial strains: The following microbial strains were used in this study, commonly isolated from infected root canals.

Aerobic strains:
- Staphylococcus aureus
- Bacillus subtalis
- Streptococcus mutans
- Escherichia coli

Fungi/ Yeast:
- Candida albicans

Facultative anaerobe
- Enterococcus faecalis

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

Agar Diffusion Method: The agar diffusion method has been widely used to test the antimicrobial activities of endodontic medicaments.[12, 13]

Preparation of Mueller-Hinton Agar:
- Suspend 38 g of the medium in one liter of distilled water.
- Heat with frequent agitation and boil to completely dissolve the medium.
- Autoclave at 121°C (15 lbs pressure) for 15 minutes. Cool to room temperature.
- Pour cold Mueller Hinton agar into sterile petri dishes on a level, horizontal surface to give uniform depth. Allow to solidify at room temperature.

Check prepared Mueller Hinton agar to ensure the final pH is 7.3 ± 0.1 at 25°C.

Inoculation of the test plates:
- Tubes containing 5 ml of sterile saline were individually inoculated with aerobes and facultative anaerobic strains.
- The suspension was adjusted spectrophotometrically to match the turbidity of 0.5 McFarland scale.
- Glass flasks containing 50 ml of BHI agar at 46^0C were inoculated with 500 microlitre of each microbial suspension, mixed and poured on to 130-mm plates containing a previously set layer of Mueller Hinton (MH) agar.[14,15]

Formation of the wells in the test plates:
- Three wells of 6mm were made for six microorganisms each time on Mueller Hinton agar.
- Wells were formed by removing the agar.
- A total of 36 wells were used, compromising 18 wells for the tested pastes and 18 for control groups.

Addition of calcium hydroxide pastes and controls:
- Each well was filled with test substance and its control.

Incubation of the test plates:
- The plates were kept for 2 hours at room temperature to allow the diffusion of the agents through the agar and then incubated at 37^0C under appropriate period of time for 24 hours in an incubator. The complete antimicrobial effect was observed after 24 hours on all microbial indicators.[16]

Measurement of zones of microbial growth inhibition:
- Zones of inhibition of microbial growth around the well containing the tested substances and controls were measured and recorded after the incubation period.
- The inhibitory zone was considered the shortest distance (mm) from the outer margin to the initial point of the microbial growth. The measurement was done by vernier calliper.

Analysis of variance (ANOVA) was used to determine the differences in susceptibility to intra-canal medication between microbial species after 24 hours and by calculating the p- values using Newman-Keuls test.

Broth Dilution Method: In broth dilution method[9], 18 test tubes were prepared for the tested pastes and another18 for the control groups.

Inoculation of the broth: The microorganisms were individually inoculated in to tubes containing 5 ml (Brain Heart Infusion) BHI sterile 0.85% saline solution. The suspension was adjusted spectrophoto-metrically to match the turbidity of 0.5 McFarland scale.

Addition of calcium hydroxide pastes and controls:
- Calcium hydroxide pastes and controls were added to the prepared tubes respectively.

Incubation of the test tubes:
- The tubes were kept for 2 hours at room temperature to allow the diffusion of the agents through the broth and then incubated at 37^0C under appropriate period of time for 24 hours in an incubator.

Antimicrobial activity was visually determined either by growth or no growth of bacteria.

RESULTS

Table 1 shows the area of zones of microbial inhibition in mm by calcium hydroxide associated with aqueous vehicles. Based on the diameters of the zones of

microbial growth inhibition, the antimicrobial effects of calcium hydroxide pastes could be ranked from strongest to weakest according to the vehicle: calcium hydroxide + distilled water (21.666mm), calcium hydroxide + anesthetic solution (21mm), calcium hydroxide + saline (20.833mm). Data analyzed by one-way ANOVA (df = 8, 45) showed that calcium hydroxide combined with vehicles had a significant effect on tested microorganisms ($p < 0.05$).

Table 2 shows that the aqueous vehicles such as distilled water, saline, anesthetic solution, had no antimicrobial action except on *Escherichia coli* on which anesthetic solution showed smaller inhibition zones of microbial growth of 3.333 mm.

Table No.1: Zones of microbial growth inhibition (in mm) produced by calcium hydroxide associated with aqueous vehicles.

Ca (OH) 2 + Vehicles	Candida albicans	Bacillus subtalis	Staphlyococcus aureus	Enterococcus faecalis	Sterptococcus mutans	Escherichia coli	Mean
Distilled Water	23	22	21	22	18	24	21.666
Saline	21	20	21	21	19	23	20.833
Anesthetic Solution	22	22	22	22	16	22	21

Ca (OH) 2: Calcium Hydroxide

Table No. 2: Zones of growth inhibition (in mm) produced by aqueous vehicles used as control.

Vehicles	Candida albicans	Bacillus subtalis	Staphlyococcus aureus	Enterococcus faecalis	Sterptococcus mutans	Escherichia coli	Mean
Distilled Water	0	0	0	0	0	0	0
Saline	0	0	0	0	0	0	0
Anesthetic Solution	0	0	0	0	0	20	3.333

Table No.3: Comparison of calcium hydroxide + aqueous vehicles against aqueous vehicles alone

	Distilled water			Saline			Anesthetic solution		
	Mean (mm)	St.dev	p	Mean (mm)	St.dev	p	Mean (mm)	St.dev	p
Calcium hydroxide + aqueous vehicles	21.67	2.07	0.00	20.83	1.33	0.00	21.00	2.45	0.004
Aqueous Vehicles	0.167	0.408		0.167	0.408		3.33	8.16	

Table No.4: Growth inhibition provided by calcium hydroxide associated with aqueous vehicles.

Ca (OH) 2 + Vehicles	Candida albicans	Bacillus subtalis	Staphlyococcus aureus	Enterococcus faecalis	Sterptococcus mutans	Escherichia coli
Distilled Water	N.G	N.G	N.G	N.G	N.G	N.G
Saline	N.G	N.G	N.G	N.G	N.G	N.G
Anesthetic Solution	N.G	N.G	N.G	N.G	N.G	N.G

Ca (OH) 2: Calcium Hydroxide

Table No.5: Growth inhibition produced by aqueous vehicles used as control.

Vehicles	Candida albicans	Bacillus subtalis	Staphlyococcus aureus	Enterococcus faecalis	Sterptococcus mutans	Escherichia coli
Distilled Water	Gr	Gr	Gr	Gr	Gr	Gr
Saline	Gr	Gr	Gr	Gr	Gr	Gr
Anesthetic Solution	N.G	N.G	N.G	N.G	N.G	N.G

Gr: Growth, N.G: No growth

Table 3 shows the comparison of calcium hydroxide pastes with aqueous vehicles against aqueous vehicles alone. According to Newman-Keuls test the results are statistically significant when calcium hydroxide mixed with aqueous vehicles: ($Ca(OH)_2$ + distilled water, $p = 0.00$), ($Ca(OH)_2$ + saline, $p = 0.00$), ($Ca(OH)_2$ + anesthetic solution, $p = 0.004$) ($p < 0.05$).

Table 4 shows that when calcium hydroxide mixed with vehicle it showed no growth of bacteria and the broth appeared transparent as compared to the broth that was turbid containing bacteria. The above table

proved that calcium hydroxide is an excellent antibacterial agent against all microorganisms tested.

Table 5 shows that when only vehicles were mixed in to the test tubes containing bacteria it showed growth in case of distilled water, saline, and the broth was turbid due to the presence of bacteria where as in anesthetic solution the broth appeared transparent.

DISCUSSION

Intra-canal medicaments are indicated if there are clinical signs such as exudation, hemorrhage, perforation, root resorption, trauma or incomplete root formation. One of the intra canal medicines is calcium hydroxide and it has to be used with a vehicle. The type of vehicle used to prepare calcium hydroxide pastes produces differences in the velocity of ionic dissociation. Depending on the vehicle used, the medicament can have a different viscosity, which plays an important role.[17] Vehicle also plays a very important role in the overall disinfection process because it determines the velocity of ionic dissociation causing the paste to be solubilized and resorbed at various rates by the periapical tissues and from within the root canal[17, 18]

Calcium hydroxide should be combined with a liquid because the delivery of dry calcium hydroxide powder in narrow curved canal is difficult and a vehicle is required also for the release of hydroxyl ions. When calcium hydroxide is mixed with the vehicle, $Ca^{++}$and OH^{-} are rapidly released.[17, 18]

The aqueous vehicles promote a high degree of solubility when the paste remains in direct contact with the tissue and tissue fluids, rendering it solubilized and resorbed by macrophages. Hence from clinical point of view the root canal must be redressed several times until the desired effect is achieved.[17]

The results of this study suggests that among different vehicles when calcium hydroxide was mixed with distilled water then we observed the largest mean values against all microorganisms tested followed by anesthetic solution and saline. Thus, our study verifies that the use of calcium hydroxide with water is effective in eliminating the bacteria from the infected root canal in endodontic treatment in the first 24 hours as observed also by Ballal et al[19] in 2007. Whereas when vehicles were used as controls, only anesthetic solution showed some antimicrobial activity against *Escherichia coli* as indicted by Pelz et al.[20] and Gocmen et al.[21] in 2008. When calcium hydroxide was mixed with aqueous vehicles then the result was statistically significant ($p < 0.05$).

CONCLUSION

The present study confirmed the in vitro antimicrobial activity of calcium hydroxide associated with aqueous vehicles, and have shown to be effective ones ($p < 0.05$). The significant finding of our study is that Enterococcus faecalis the most resistant microorganism in infected root canals as observed by Ferrari et al.[22] and Pirani et al.[23] also showed good zones of inhibition against the tested paste of calcium hydroxide.

The first step in a study of the effectiveness of intra-canal medicament is the laboratory test. In vitro research to determine the antimicrobial activity depends on the sensitivity of the drug, bacterial source (wild strains or collection species), number of bacteria inoculated, pH of the substrates in plates or tubes, agar viscosity, storage conditions of the agar plates, incubation time and the metabolic activity of the microorganisms. However, in vitro results must be analyzed carefully before their extrapolation to clinical conditions.

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