Original Article

In Vitro Evaluation of Antimicrobial Activity of Calcium Hydroxide with Oily 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 with oily 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 April 2009 to March 2011.

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

Results: The results showed that calcium hydroxide mixed with oily vehicles was inhibitory against all the microbial strain tested. Calcium hydroxide pastes eliminated the *Enterococcus faecalis* (the most resistant organism in infected root canals) as well as other microorganisms effectively and the oily vehicles have also inherent antibacterial properties.

Conclusion: We concluded from our study that although calcium hydroxide when mixed with oily vehicles can eliminate the endodontic bacteria but such oily vehicles may be irritant to the periapical tissue. Therefore further studies should be done on their role as an intracanal medicine in different combinations with calcium hydroxide.

Key Words: Calcium Hydroxide, Oily Vehicles, Root Canal Treatment.

INTRODUCTION

Calcium hydroxide is used widely in dental treatment throughout the world particularly in root canal treatment, since its introduction in dentistry by Hermann in 1920. However, its mechanisms of cell damage are still not well known. The most accepted explanation for Calcium hydroxide antimicrobial mechanism is its high pH. The release of hydroxyl ions in an aqueous environment causes damage to the bacterial cytoplasmic membrane and disrupts both protein denaturation and DNA of bacterial cells. 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.[1, 2]

One of the main goals of root canal treatment is to eliminate bacteria and their by-products before obturation. Although dentists try to clean and irrigate the root canal system properly, some bacteria still remain entrapped in the dentinal tubules which causes root canal infection or re-infection.[3] During root canal treatment, apart from the root canals bacteria can also exists in those areas which are inaccessible to mechanical instrumentation and irrigating solutions.[4, 5] It has been shown that, if the canal is not dressed with a disinfectant between two visits, microorganisms will multiply rapidly within days to near the original numbers. To eliminate as many bacteria as possible from the root canals it is necessary to maintain proper mechanical instrumentation, irrigation of the canals with the irrigating solutions which are used to remove or dissolve the organic and inorganic debris.[6, 7, 8]

Calcium hydroxide is considered to possess many of the properties of an ideal root canal dressing, acting as a physical barrier, preventing root canal re-infection and interrupting the nutrient supply to the remaining bacteria. However, for calcium hydroxide to exert its antimicrobial activity, directly or indirectly, an ideal timing is required for effective destruction of microorganisms.[6]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 oily 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:

- Camphorated paramonochlorophenol

- Camphorated paramonochlorophenol + glycerine
- Camphorated paramonochlorophenol + polyethyleneglycol

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 spectrophoto-metrically 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 spectrophotometrically 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 with oily 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 + CMCP (34.5mm), calcium hydroxide + CMCP + glycerine (25.5mm), calcium hydroxide + CMCP + polyethyleneglycol (23.833mm). Data analyzed by one-way ANOVA showed that calcium hydroxide combined with vehicles showed no significant effect on tested microorganisms ($p < 0.05$).

Table 2 shows that oily vehicles such as CMCP, CMCP + glycerine and CMCP + polyethyleneglycol showed larger inhibition zones of microbial growth of 31.166mm, 33.166mm and 21.166mm respectively.

Table 3 shows the comparison of calcium hydroxide pastes with oily vehicles against oily vehicles alone. According to Newman-Keuls test the results are not statistically significant when calcium hydroxide mixed with oily vehicles: ($Ca(OH)_2$ + CMCP, $p = 0.53$), ($Ca(OH)_2$ + CMCP + glycerine , $p = 0.11$), ($Ca(OH)_2$ + CMCP + polyethyleneglycol, $p = 0.62$) ($p < 0.05$).

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

Ca (OH) 2 + Vehicles	**Candida albicans**	**Bacillus subtalis**	**Staphlyococcus aureus**	**Enterococcus faecalis**	**Sterptococcus mutans**	**Escherichia coli**	**Mean**
CMCP	30	21	30	41	48	37	34.5
CMCP + Glycerin	24	21	24	29	32	23	25.5
CMCP + Polyethyleneglycol	18	21	22	23	34	25	23.833

Ca (OH) 2: Calcium Hydroxide, CMCP: Camphorated paramonochlorophenol

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

Vehicles	**Candida albicans**	**Bacillus subtalis**	**Staphlyococcus aureus**	**Enterococcus faecalis**	**Sterptococcus mutans**	**Escherichia coli**	**Mean**
CMCP	22	22	30	40	40	33	31.166
CMCP + Glycerin	30	23	30	40	48	28	33.166
CMCP + Polyethyleneglycol	18	0	20	29	30	30	21.166

CMCP: Camphorated paramonochlorophenol

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

	CMCP			CMCP+G			CMCP+P		
	Mean (mm)	St.dev	p	Mean (mm)	St.dev	p	Mean (mm)	St.dev	p
Calcium hydroxide + oily vehicles	34.50	9.52	0.530	25.50	4.14	0.11	23.83	5.49	0.62
Oily vehicles	31.17	8.11		33.17	9.13		21.2	11.6	

G = glycerin, P = polyethyleneglycol

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

Ca (OH) 2 + Vehicles	Candida albicans	Bacillus subtalis	Staphlyococcus aureus	Enterococcus faecalis	Sterptococcus mutans	Escherichia coli
CMCP	N.G	N.G	N.G	N.G	N.G	N.G
CMCP + Glycerin	N.G	N.G	N.G	N.G	N.G	N.G
CMCP + Polyethyleneglycol	N.G	N.G	N.G	N.G	N.G	N.G

Ca (OH) 2: Calcium Hydroxide, CMCP: Camphorated paramonochlorophenol, N.G: No Growth

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

Vehicles	Candida albicans	Bacillus subtalis	Staphlyococcus aureus	Enterococcus faecalis	Sterptococcus mutans	Escherichia coli
CMCP	N.G	N.G	N.G	N.G	N.G	N.G
CMCP + Glycerin	N.G	N.G	N.G	N.G	N.G	N.G
CMCP + Polyethyleneglycol	N.G	N.G	N.G	N.G	N.G	N.G

CMCP: Camphorated paramonochlorophenol, N.G: No Growth

Table 4 shows that when calcium hydroxide mixed with oily vehicles it showed no growth of bacteria and the broth appeared transparent as compared to the broth that was turbid containing bacteria. The above table proves that calcium hydroxide is an excellent antimicrobial agent against all microorganisms tested.

Table 5 shows that when only the oily vehicles were mixed in to the test tubes containing bacteria, it showed

no growth and the broth appeared transparent. Result shows that oily vehicles shows antimicrobial activity against all organisms tested.

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]

Oily vehicles are non-water-soluble substances that promote the lowest solubility and diffusion of the paste within the tissues. Pastes containing this kind of vehicle may remain within the root canal for longer period.[19]

The results of this study shows that when calcium hydroxide was mixed with camphorated paramonochlorophenol gives the largest mean values of growth inhibition against all microorganisms tested followed by camphorated paramonochlorophenol + glycerin and camphorated paramonochlorophenol + polyethyleneglycol respectively.

The antimicrobial activity is also confirmed when camphorated paramonochlorophenol alone was used. This control also destroyed the microorganisms by producing larger zones of microbial inhibition. Camphorated paramonochlorophenol with glycerin and camphorated paramonochlorophenol with polyethyleneglycol also showed larger zones of growth inhibition respectively.

Thus, this study showed that the oily vehicles gave the largest zones of microbial inhibition. However, without sufficient water available, camphorated paramonochlorophenol forms calcium parachlorphenolate thus preventing further hydrolysis as confirmed by Vianna et al.[20] in 2009. For this reason, pastes containing oily vehicles have restricted use and are only employed in those clinical situations that require a very slow ionic dissociation. Camphorated paramonochlorophenol has been shown to become ineffective in the root canal within 24-48 hours. Another fact that does not recommend the use of camphorated paramonochlorophenol as intra-canal medicament is that it does not act as a physicochemical barrier because it is commonly applied on sterile cotton pledgets placed in the pulp chamber or on paper points slightly moistened with this medicament. Such a barrier is important for preventing root canal recontamination due to growth of bacteria that are not removed during biomechanical preparation or to saliva and microorganisms infiltration caused by coronal micro leakage. The antimicrobial effect is delivered through vaporization of the medicament. Thus, the antimicrobial action of the medicament within the dentinal tubules and in the apical portion is therefore dependent on the volatility of the medicament. As the material does not persist for prolonged periods, hence some bacteria may survive and have the opportunity to multiply and persist in the root canal system.[17] Camphorated paramonochlorophenol is also irritant to the periapical tissues as confirmed by Vianna et al.[20] So it is better to use with a safe dental material such as calcium hydroxide as described by Magalhaes et al.[21] in2011.

CONCLUSION

Present study showed that calcium hydroxide combined with different oily vehicles produced larger zones of growth inhibition. These pastes alone show larger zones of growth inhibition, hence the results are not statistically significant because the oily vehicles themselves show the antimicrobial activity against the tested organisms. These oily vehicles may be irritant to the priapical tissues, therefore further studies should be done on their role as an intra-canal medicine in different combinations with calcium hydroxide.

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