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IN VITRO STUDY OF ANTIMICROBIAL ACTIVITY OF CALCIUM HYDROXIDE MIXED WITH DIFFERENT VEHICLES AGAINST E.FAECALIS AND CANDIDA ALBICANS

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ABSTRACT

OBJECTIVE. : The purpose of this in vitro study was to investigate antimicrobial activity of calcium hydroxide (CH) in combination with chlorhexidine gluconate (CHX),glycerine, chitosan and saline against Enterococcus faecalis and Candida albicans.

STUDY DESIGN: Antimicrobial activity was determined using agar diffusion test. Standard well in the cultivated agar plates were filled with one of the calcium hydroxide preparations and control agents. The zones of microbial inhibition were measured after incubation period.

RESULTS. : The combination of Calcium hydroxide with chlorhexidine demonstrated most antibacterial activity of all other preparations. All the tested groups had significant antimicrobial action against E faecalis and C albicans.

CONCLUSION : Antimicrobial activity of Calcium hydroxide may change with the type of the vehicle.

KEYWORDS : Calcium Hydroxide, Chitosan, Chlorhexidine, Glycerine, Antimicrobial Activity, Vehicles

INTRODUCTION

Microorganisms which persists and multiply inside the root canal system is the main causative factor in pulpal and peri radicular lesions .Because endodontic infections are polymicrobial, many microorganisms take part in constitution ofbiofilms and become more resistant to endodontic procedures. *Candida albicans* and *Enterococcus faecalis* are considered the most resistant species microorganisms which are responsible for root canal treatment failures(Hancock, Sigurdsson, Trope, & Moiseiwitsch, 2001) .Consequently, antimicrobial agents can be combined to be effective against these polymic robialflora. Using this idea, different vehicles used to carry the medicaments can have adirect influence on the release, time of onset of action of the medicament, penetration of the intracanal medicaments into dentinal tubules, and also the dissociation of drugs(Athanassiadis, Abbott, George, & Walsh, 2009).

Calcium hydroxide(CH) is the most commonly used medicament in endodontics with significant antibacterial effects on intracanal microorganisms. The efficacy of this material depends on the penetration ofhydroxyl ions into the dental tubules and accessory canals, where bacteria and their products accumulate. (Siqueira & Lopes, 1999), CH can prevent reinfection of the canal and impair nutritional supply of the residual microorganisms in the root canal system by forming a physical barrier.

In order to have optimal efficacy in the root canal system, CH should be spread all over the canal walls to be in close contact with them(Haapasalo, Shen, Wang, & Gao, 2014). So calcium hydroxide powder is mixed with different vehicle for the continuous release of hydroxyl ions. So in this study we have combined CH with chlorhexidine, chitosan, glycerin which have proven antimicrobial action.

Different studies have shown controversial results over the antimicrobial efficacy of calcium hydroxide mixed with CHX. Therefore, thepurpose of the present study was to assess antimicrobial activity of CH when mixed with different vehicles against E.faecalis and C. albicans by agar diffusion method and to examine the invitrosusceptibilityof these microorganisms to amixture of CH and CHX.

MATERIALS AND METHODS

This in vitro study was conducted in the department of Microbiology, Kannur medical college, Anjarakandy, Kannur. Calcium hydroxide(CH) powder was mixed with 2% chlorhexidine digluconate CHX (RC prep), glycerin (100%), 0.5% chitosan and saline to form a slurryat 1.5:1(vol/wt). Freshly prepared pastes of CH and a vehicle were used for each test. Standard E.faecalis (ATCC 29212) and C. albicans (ATCC 60193) strains were used for this study.

In the present study CH+CHX(group1),CH+Glycerine (group2),CH+chitosan(group3) andCH+Saline(group 4)as the positive control group.

AGAR DIFFUSION TEST

Hundred microliters of test organisms E.faecalis and C albicans suspensions were obtained from prepared culture and inoculated in a culture plate with previously set layers of Muller Hinton Agar and Sabouraud dextrose agar

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respectively for each organism. The strains were inoculated in brain heart infusion and incubated at 37°C for 24 hours. Microbial cells were resuspended in saline to give a final concentration of 1.5 10^8 cells/ml, similar to that of tube #0.5 of the McFarland scale. For agar diffusion test, Petri plates with 20 ml Muller Hinton Agar (Merck) were inoculated with 0.1 ml of one of the microbial suspensions. Holes (4 mm in depth, 6 mm in diameter) were punched in the cultivated agar plates and filled with one of the CH preparations or control agents. Each agar plate contained only one medicament. Nystatin (antifungal; Mycostatin; Bristol-Myers Squibb, NJ) and cefotaxime (antibacterial; Fortum; GlaxoSmith Kline) were the control agents. The plates were re incubated aerobically at 37°C for 24 hours. Then the diameter of microbial inhibition zones around each well was measured and recorded in millimeters. Statistical analysis was performed with one way anova and post hoctukey test.

RESULT

Analysis of variance (one way anova) was performed as parametric test to compare different groups for both E faecalis and C albicans.

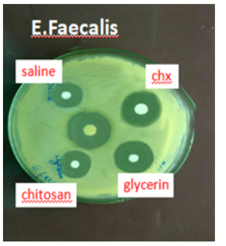


Fig 1:Zone of inhibition formed against E faecalis

Fig 2:Zone of inhibition formed against C albicans

TABLE 1: one way anova with post hoc tukey test

	Number of values	Minimum	25% Percentile	Median	75% Percentile	Maximum	Mean	Std. Deviation	Std. Error
CHLORHEXIDINE-CH	5	16	16.25	17	17.75	18	17	0.7906	0.3536
GLYCERINE-CH	5	12.5	13.25	14.5	15	15	14.2	1.037	0.4637
CHITOSAN-CH	5	11	11.25	12	12.75	13	12	0.7906	0.3536
SALINE-CH	5	9	9.25	10	10.75	11	10	0.7906	0.3536
F	61.2								
p value	<0.0001								

Graph 1: INHIBITON OF EACH AGENT AGAISNT E FAECALIS

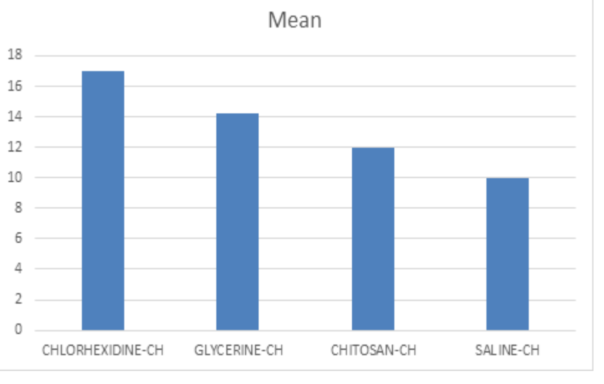
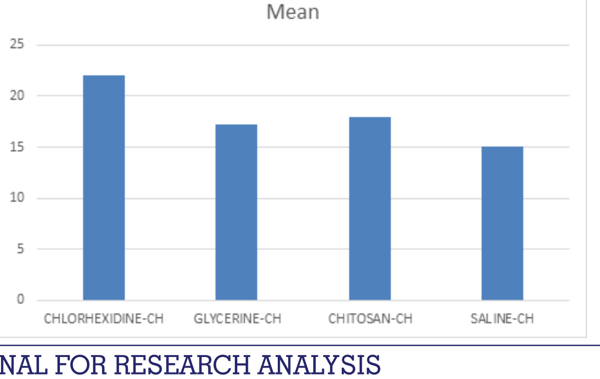


TABLE 2:ONE WAY ANOVA WITH POSTHOC TUKEY TEST AGAISNT CANDIDA ALBICANS

	Number of values	Minimum	25% Percentile	Median	75% Percentile	Maximum	Mean	Std. Deviation	Std. Error
CHLORHEXIDINE-CH	5	20	20.75	22	23.25	23.5	22	1.369	0.6124
GLYCERINE-CH	5	16.5	16.5	17	18	19	17.2	1.037	0.4637
CHITOSAN-CH	5	17	17.25	18	18.75	19	18	0.7906	0.3536
SALINE-CH	5	14	14.25	15	15.75	16	15	0.7906	0.3536
F	40.68								
p value	<0.0001								

Graph 2: INHIBITON OF EACH AGENT AGAISNT E FAECALIS



DISCUSSION

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
CHLORHEXIDINE-CH vs GLYCERINE-CH	4.800	10.47	Yes	***	2.946 to 6.654
CHLORHEXIDINE-CH vs CHITOSAN-CH	4.000	8.729	Yes	***	2.146 to 5.854
CHLORHEXIDINE-CH vs SALINE-CH	7.000	15.28	Yes	***	5.146 to 8.854
GLYCERINE-CH vs CHITOSAN-CH	-0.8000	1.746	No	ns	-2.654 to 1.054
GLYCERINE-CH vs SALINE-CH	2.200	4.801	Yes	*	0.3459 to 4.054
CHITOSAN-CH vs SALINE-CH	3.000	6.547	Yes	**	1.146 to 4.854

Utilizing abiocompatible intracanal medicament between appointments,help to diminish or eradicate bacteria in the root canal system and it enhance the success of root canal therapy(El Karim, Kennedy, & Hussey, 2007). As *E. faecalis* is the commonly seen organism in infected root canals ,it was chosen as the test organism. Virulency of E. faecalis in failed endodontically treated teeth may be related to its ability maintain the capability to invade dentinal tubules and adhere to collagen in the presence of human serum(Love, 2001).In addition to this, different fungal speciesare also given special attention in failure of endodontic treatments. Almost all the isolated species of fungi belong to the Candida clade, predominantly Candida albicans(Mozayeni, Hadian, Bakhshaei, & Dianat, 2015); which is the culprit responsible for development of pulpal and periapical infections.*C.albicans* promotes colonization the root canal by its collagenolytic activity and also it uses dentin as a nutrient source leading to its high virulence(Yadav, Chaudhary, Saxena, Talwar, & Yadav, 2017).

Calcium hydroxide is the most commonly used intracanal medicament and its antibacterial effect is mainly due to thehigh PH by release of hydroxyl ions (Sjogren, Figdor, Spangberg, & Sundqvist, 1991).This hydroxyl ions diffuse through the dentin and reach sufficient levels to be lethal against the bacteria located inside the dentinal tubules[3]. Because of the buffering property of dentin, pH value of $Ca(OH)_2$ may be insufficient to kill some bacterial strains, particularly *E. faecalis*, which can survive at a pH value of 11.5(Kim & Kim, 2014).

Although calcium hydroxide can effectively eliminate most of the root canal pathogens, E. faecalis and C. albicans are resistant to calcium hydroxide according to TurkBT and BallalV et al(Turk, Sen, & Ozturk, 2009).Its resistance is due to the basic pH of calcium hydroxide,which in a basic medium ,proton pump activity occurs inside the microorg anismwhich acidifies the environment and forms a biofilm(Maekawa et al., 2013).

Candida albicans is also resistant to calcium hydroxide. According to Ballal V et al CH showed higher efficacy against *Candida albicans* at the first 24 hours and its effect was reduced after 72 hours. Its resistance mechanism has yet to be fully understood but it seems that C. albicans due to biofilm formation has a strikingly biphasic killing pattern in response to antibacterial agent(Delattin, Cammue, & Thevissen, 2014).
In this study all experimental groups were found to be effective against E faecalis and C albicans. Group1 CHX-CH combination showed the largest inhibition zone against E. faecalis and C.albicans compared to all other group. Chlorhexidine has a broad spectrum antimicrobial activity and substantivity effect, and its optimal antimicrobial activity is achieved at pH range of 5.5-7.0.(Athanassiadis et al) . Therefore it is likely that alkalinizing the pH by adding CH to CHX will lead toprecipitation of CHX molecules and thereby decreases its effectiveness (Mohammadi & Abbott, 2009).But it has been demonstratedthat ,there is no change in alkalinity of CH when mixed with CHX. Nevertheless, CHX alone does not act as a physical barrier in solution or gel form, but combination of CHX with CH paste act as a barrier in the root canal system long enough to eliminate existing microorganisms and to stop recontamination(Ballal, Kundabala, Acharya, & Ballal, 2007)Another factor which explain antimicrobial synergism consist of production of more reactive oxygen species (ROS), by combination of CHX and CH.(Turk et al., 2009)

Valera et al stated that although 2% CHX gel significantly decreased the microbial count, intracanal medicaments including CH and CH+CHX completely eliminated the microorganisms from the canals(Pavaskar et al., 2012). Their findings confirmed the synergistic effects of CH and CHX. However, Delgado et al ,found no significant difference in terms of antibacterial properties of CHX gel with and without CH. Such controversy in the results may be attributed to the different culture techniques, root canal irrigating solutions, type of microorganisms and root canal anatomy.(Saatchi, Shokraneh, Navaei, Maracy, & Shojaei, 2014)Ina study by Lin et al. CHX alone and in combination withCH showed antibacterial efficacy greater than that of CH alone (Saatchi et al., 2014)

Study conducted by C. Maniglia-Ferreira et al on the evaluation of the antimicrobial effects of different intracanal medications in necrotic immature teeth showed that Ca(OH)2 paste in combination with 2 % CHX gel was devoid of any antimicrobial activity against E faecalis which is in contradiction to the present study(Maniglia-Ferreira et al., 2016).Systemic review and meta analysis by Saatchi et al showed thatCHX does not have synergetic antibacterial effect with CH. This may be due to deprotonation of CHX at high pH, which reduces its solubility and alters its interaction with bacterial surfaces as a result of the altered charge of the molecule (Saatchi et al., 2014)

Chitosan is a natural unbranched homopolymer obtained from chitin, an abundant by-product of seafood processing, via a de acetylation reaction with alkali.Positivelycharged -NH_3^+ groupof glucosamine present in chitosan, interact with negatively charged surface components of bacteria, resulting in extensive cell surface alterations, leakage of intracellular substances and ultimately causing damage of vital bacterial activities.(Raafat& Sahl, 2009) Chitosan binds to DNA and inhibits mRNAs ynthesis by penetrating toward the nuclei of microorganisms and interfering with the synthesisof mRNA andproteins .Consequently, it is possible that $Ca(OH)_2$ combined with chitosan inhibits the growth of E. faecalis and subsequently it may inhibit bacterial re-entry and recolonization(Elsaka& Elnaghy, 2012). A synergistic antibacterial effect was found in $Ca(OH)_2$ combined with chitosan against E. faecalis.

Another tested vehicle was 100% glycerin which is a sweet, syrupy liquid obtained from animal fats and oils or by the fermentation of glucose. Glycerol consists of a propane molecule attached to three hydroxyl (OH) groups. Recently glycerine has been recommended for use as vehicle as they possess better therapeutic and handling properties. According to this study, glycerin-CH combinations had potential antifungal effects and antibacterial effect. This activity can be due to continued dissociation of CH by imbibitions of water into paste, by hygroscopic nature of glycerine thus ensuring a continued therapeutic effect. Sylvia et al have reported this combination have extensive gradual and sustained release of Ca and hydroxyl lion.Rivera and Williams showed that glycerin help in easy placement of CH in the root canals. According to Sudeep et al CH- glycerine combination showed a gradual increase in the zone of inhibition upto 7 days of incubation. The negative results which is obtained in other studies with glycerine may be due to their inability to diffuse through agar due to their viscous nature more than absence of any antimicrobial activity.(Liu, Wei, Ling, Wang, & Huang, 2010)

Safavi and Nakayama concluded that higher concentrations of glycerin reduce the conductivity of CH that would decrease the antibacterial activity of CH which is in contrast to our study[30]. Because of the pH of glycerin-CH combination would not be high enough to eliminate E. faecalis, because it could tolerate very high pH values with the durability of cell membrane and by using specific proton pumps and enzymatic systems. (Portenier, Waltimo, Orstavik, & Haapasalo, 2005)Unlike other invitro tests, Agar diffusion method, mainly 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 diffusabilityin agar than to their actual efficacy against the microorganisms which might be a limitation in our study.

CONCLUSION

In summary the antimicrobial effect of $Ca(OH)_2$ is related to the hydroxyl ions released in an aqueous environment, which affects cytoplasmic membranes, proteins, and the DNA of microorganisms. Theaddition of vehicles or other agents might contribute to the antimicrobial effect of $Ca(OH)_2$. Although it remains controversial, the antimicrobial activity of $Ca(OH)_2$ can be increased with a mixture of $Ca(OH)_2$ with CHX.

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