Antibacterial Efficacy of Pastes Against *E Faecalis* in Primary Root Dentin: A Confocal Microscope Study

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Objectives. *Management of abscessed primary teeth often present endodontic failure owing to questioned efficiency of dressings or obturating pastes to eliminate Enterococcus faecalis, a resistant bacterium, residing in depth of dentinal tubules. The present study evaluates the antimicrobial efficacy of two antibacterial and two obturating pastes in dentinal tubules of primary teeth infected with Enterococcus faecalis using viability stain and confocal laser scanning microscope (CLSM).* ***Study design.*** *Total 28 samples were prepared. Four groups with 6 samples each were made according to antibacterial pastes i.e. 1% or 2%Chlorhexidine (CHX) + calcium hydroxide (CH), CH + iodoform (Metapex) and Zinc Oxide Eugenol (ZOE). Dentinal tubules from the root canal side were infected with E. faecalis by centrifugation of the bacterial suspension. Two specimens from each group were subjected to 1, 7 and 15 days antibacterial pastes exposure. Viability staining followed by CLSM were used to quantitatively analyze the dead cell count directly inside dentin.* ***Result:*** *Univariate analysis showed that all medicaments were significantly effective ($p<.05$). Kruskal wallis ANOVA test did not show significant difference among four medicaments at day 1 while it was significantly different at day 7 & 15. Paired sample student's t-test revealed significant difference in efficacy between 1& 15 days for 1%CHX+CH; between 1&15,and 7&15 days; between all days for ZOE. Ranking of antimicrobial efficacy of tested medicament was (most effective to the least): 1%CHX+CH(15)> ZOE(15)> Metapex(15)> 2%CHX+CH(15)> 2%CHX+CH(7)> 2%CHX+CH(1)> 1%CHX+CH(7)> 2%CHX+CH(15)> Metapex(1)> ZOE(1)> ZOE(7).* ***Conclusions:*** *All medicaments were effective against E. faecalis in dentine of primary teeth and their efficacy increased with longer contact with 1%CHX+CH being most effective at day 15. Inclusion of 1% CHX in dressings or obturating pastes might minimize the endodontic relapse and maximize the tooth retention in functional state in pediatric dentistry.*

Key Words: *Root canals, children, Enterococcus faecalis, live/dead fluorescent stain Confocal laser scanning microscopy.*

INTRODUCTION

Control of infection particularly in primary teeth is fundamental because the ample medullary bone spaces favor dissemination of infection and also because the developing permanent tooth germ is very close to the roots of the primary teeth.[1] However, the complex morphology of the root canal system in deciduous teeth, present accentuated curves and a large number of accessory canals which makes it difficult to achieve proper cleansing by mechanical instrumentation and irrigation of canals.[2]

One of the major factors associated with endodontic failure is the persistence of polymicrobial infection disseminated within the root canal system and periradicular area of primary teeth.[3] Facultative anaerobic bacteria, such as *Enterococcus faecalis* (*E. faecalis*), have been considered one of the most resistant species and a possible cause of failure of root canal treatment.[3,4] Viable microorganisms may remain in the dentinal tubules, uninstrumented areas of the canals and in other irregular spaces after root canal preparation. These microorganisms, in supporting environment can proliferate and reinfect the root canal system leading to root canal failure.[5, 6]

Therefore, the goal of root canal treatment is to eliminate the bacteria from the root canal and the infected dentin. Eradication of the infectious and resistant microorganism, *E. faecalis* in particular, has been the focus of numerous *in vivo* and in vitro studies in endodontics.[7, 8] It is therefore important that root canal medicaments and obturating paste used in primary teeth not only have antimicrobial activity against these bacteria seated deeply and diffused throughout the root dentine but also be biocompatible to periradicular tissue.

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Obturation of primary root canal with zinc oxide eugenol (ZOE) has a long history with a success rate of 65% to 88.5% and is still practiced.[9, 10] Commercially available mixture of calcium hydroxide and iodoform in oil base (Metapex), is being used as an alternative to ZOE as a root canal filling material. Not only almost complete reduction in pre-op signs and symptoms was observed with Metapex but also negative cultures were obtained from infected primary tooth with its use.[11]Inspite of its effectiveness against a variety of microorganisms of the root canal flora, it has clearly been demonstrated to be ineffective against *E. faecalis*.[12]

Chlorhexidine (CHX), emerged recently as an effective endodontic irrigant and medicament, has excellent anti-microbial properties. It can disinfect the dentinal tubules, adsorb onto the tooth and get released over a substantial period of time. Chlorhexidine is a broad spectrum antibacterial agent that is effective against *E. faecalis*.[13]Recent studies have suggested that CHX could be used in combination with CH to improve its antimicrobial efficacy against CH–resistant microorganisms.[14]

Microbiological sampling techniques can estimate the number of colony forming units of cultivable bacteria and the quantitative analysis of the dentin infection can be done. However, viable but non cultivable bacteria cannot be represented and this method does not give clear information about the spatial distribution of bacteria inside the dentin. Histologic sections show the distribution of the bacteria in infected dentin but do not give information about the viability of the bacteria. Despite the great advances of polymerase chain reaction technology to detect culture difficult species and uncultivable microorganisms, this method cannot distinguish between viable and dead cells.[15]

During the last decade, new fluorescent dyes and sensitive detection equipment have been developed to improve the methods of separating living cells from dead cells and have the potential for endodontic research.[15] A dentin block model, which has been adopted for the evaluation of the antibacterial effect in dentin for more than two decades, allowed bacterial penetration into dentinal tubules for up to 500 micron from the main root canal. More recent studies have visualized bacteria in dentinal tubules by Confocal Laser Scanning Microscopy (CLSM), using viability stain and which has been reported to be an appropriate way not just to visualize bacteria but also to identify live and dead bacteria in the infected dentin.[7, 15]

Therefore, the present study has been constituted to evaluate antimicrobial efficacy of two conventional obturating pastes (Metapex, ZOE) and two experimental viscous pastes (1% or 2% CHX mixed with CH) using live/dead viability staining and Confocal Laser Scanning Microscopy for in-situ identification of live and dead *E. faecalis* in infected dentinal tubule.

MATERIALS AND METHOD

Sample Preparation

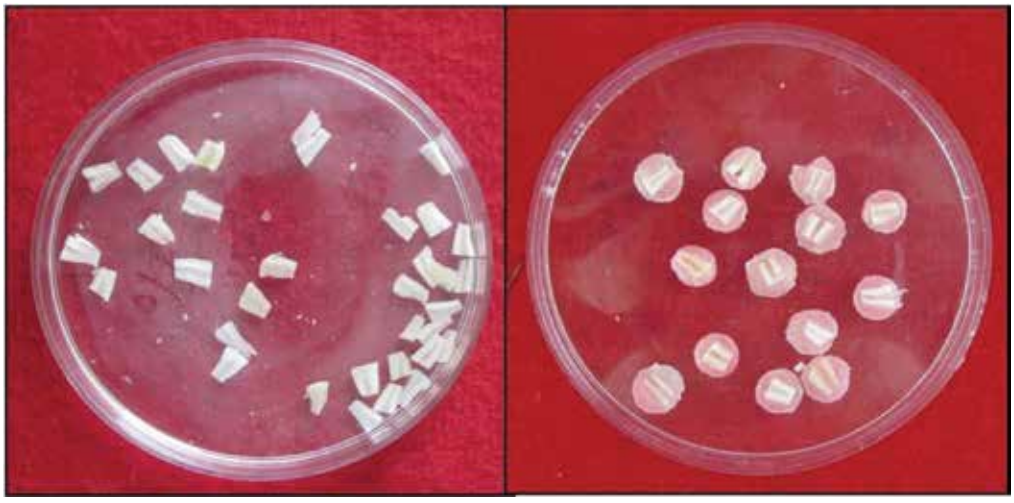
Approval for the research work was taken from ethical committee of institute Modern Dental College & Research Centre, Indore, India. Twenty eight, caries-free single rooted deciduous incisors and canines, freshly extracted for serial extraction purpose were collected from the department of Pedodontics and Preventive dentistry. Teeth were kept in 0.01% sodium hypochlorite solution until used. Root dentin block were prepared according to method described by Jingzhi *et al* [7] The size of the refined specimen was about 4 x 4 x 2 mm (fig-1 A).

Semicylindrical halves of dentin specimens were fixed in acrylic disc (Rapid Repair, Pyarax Polymers, Roorkee, India) of 2 mm thickness (fig-1, B). The acrylic disc with fixed dentin specimen had dimension to be fitted in middle of the Eppendorf tube. Upper and lower surface of prepared disc were polished to remove any acrylic from dentin specimen. Smear layer on both sides of the specimen was removed by immersion in 5.25% sodium hypochlorite and 6% citric acid each for 4 minutes in an ultrasonic bath (Model CD-4820, Huajin (China) Ltd., Tangshan, China). Specimens were rinsed in sterile water for 1 minute after smear layer removal. Two out of these 28 samples were kept as negative control. One out of these was checked for efficacy of sterilization in broth culture, while another was kept for CLSM observation.

Each prepared dentin specimen was placed at middle of the Eppendorf tube with the canal (pulpal) side up. Any gaps between

Figure 1: Sample preparation

A. Semi-cylindrical specimens B. Acrylic disc with embedded samples



the dentin specimen and the inner wall of tube were carefully sealed with composite (Filtek™, 3M ESPE, Bangalore, India) and light-cured for 20 seconds.

Dentin infection with E. faecalis using ultracentrifugation machine

Clinical isolate of E. faecalis, obtained from department of pathology, SAIMS, Indore, India, was grown in air at 370C overnight on brain heart infusion agar plates for the experiments. The bacteria were suspended in brain heart infusion (BHI)) broth (HiMedia laboratories Pvt. Ltd., Mumbai, India) and standardized spectrophotometrically to 3x106 colony-forming units (cfu)/mL(OD405=0.05).

Five hundred micro liters of E. faecalis suspension in BHI broth was added to each filter tube with the dentin specimen inside. Tubes were centrifuged (Thermoscientific, Sorvall Legend Micro21r, USA)at 1400 g, 2000 g, 3600 g, and 5600 g in a sequence twice each for 5 minutes. A fresh solution of bacteria was added between every centrifugation. The solution that had penetrated through the dentin piece was discarded. In order to facilitate bacterial recovery, all tubes were then incubated at 37oC in BHI broth for 24 hours after centrifugation.

Disinfection of dentin

The dentin specimens were taken out of each tube, and the surrounding composite with the acrylic disc was removed, followed by rinsing the dentin specimen in sterile water for 1 minute and air drying. Out of 26 specimens, 24 were divided into 4 groups according to the four test medicament used (Group 1: 1%CHX+CH; Group 2: 2%CHX + CH; Group 3: Metapex; Group 4: ZOE). Thus each group had six samples while positive group had two samples.

Pastes preparation

Medicaments taken for the study were; 2% and 1% Chlorhexidine (Neelkanth Health Care Pvt. Ltd., Jodhpur, India), Calcium hydroxide powder (Neelkanth Health Care Pvt. Ltd., Jodhpur, India), Zinc Oxide powder (Prime Dental Products Pvt. Ltd., Mumbai, India) and Metapex (Metabiomed Co. Ltd., Chungbuk, Korea). For 1% CHX + CH paste, 2 scoops (20 mg) of CH was mixed homogeneously with 0.3 ml of 1%CHX solution and 4 -5 drops of glycerin with cement spatula on a glass slab to have paste like consistency. For 2% CHX + CH paste 1% CHX was replaced with 2% CHX and was mixed in same manner. For Zinc Oxide Eugenol paste 2 scoops of Zinc Oxide powder was mixed homogeneously with 2-3 drops of eugenol to have paste like consistency.

Medicaments were applied with the help of cement carrier on the root canal surface of the dentin specimen. Cemental sides of the specimens were painted with nail varnish. From two positive controls, one dentin specimen was taken for scanning electron microscope (SEM) and another for CLSM.

SEM Examination

One semicylindrical dentin specimens without disinfection treatment were washed with phosphate-buffered saline (PBS) for 5 minutes after centrifugation of the bacterial suspension. Fixation was done by 2.5% glutaraldehyde for 30 minutes and 1% osmium tetroxide (OsO4) for 1 hour. Two specimens were prepared to observe dentin canals from longitudinally sectioned one specimen. The specimens were dehydrated by increasing concentrations of ethanol, dried by using a critical point drier, and sputter-coated with gold-palladium in a vacuum evaporator. The dentinal tubules were observed for presence of bacteria using SEM (Jeol JSM-5400, Labexchange, Tokyo, Japan) at a magnification of 3000 x operating at 10 kV.

Fluorescent staining

For fluorescent staining, samples were washed with sterile water and PBS for 1 min and vertically fractured through the root canal into 2 halves to expose a fresh surface of longitudinally visible dentin canals for CLSM examination. The fractured dentin pieces were stained with 2 to 3 drops of fluorescent LIVE/DEAD BacLight Bacterial Viability stain (Invitrogen, Molecular Probes, Oregon, USA) containing SYTO 9 and propidium iodide. Stained Samples were incubated in the dark at room temperature for 20 or 25 min.

Before examination, samples were washed with PBS for 1 min and air dried completely. One negative and two positive specimens were stained under the same protocol Samples with the medicament were kept in incubator at 370 C for 1, 7, 15 days. Two samples from each group were taken out at 1,7,15 days respectively and stained with viable fluorescent stain to be observed under CLSM.

CLSM Analysis

The excitation/emission wavelengths were 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide. Fluorescence from the stained cell was viewed by using CLSM (Carl Zeiss Meta510, North ryde, Australia). The mounted specimens were observed by using a 20x lens with an additional zoom of 20x. CLSM images were acquired by the software EZ-C1 version, 3.40 build. 691(Nikon corporation, Tokyo, Japan) at a resolution of 512 x 512 pixels. The border of the root canal and the freshly fractured dentin surface was first located with the microscope, and at least two randomly selected places were scanned with the CLSM. Three or four images (0.5 mm step size) from each scan were obtained. Out of these eight images, four were randomly selected for further analysis.

Image acquisition was done in CLSM, associated computer. Images for each experimental medicament (4 readings at different depth) were analyzed using Image J software (Public domain, developed by NIH, dept. of Health and Human Sciences, USA) for counting red (dead) cell count. Dead cells, counted with the software were tabulated and univariate analysis of variance, paired student's t-test, Kruskall Wallis ANOVA and Mann Whiteney post hoc comparison using Bonferroni correction were performed to analyze the difference between efficacy (dead cell count) of 4 medicament at day 1, 7 and 15 using SPSS 16.0 software (SPSS Inc, Chicago, IL, USA).

RESULTS

Four medicaments were evaluated (viscous mix of 1% CHX + CH and 2% CHX + CH, Metapex and ZOE) for their antimicrobial efficacy against *E. faecalis* in dentinal tubules of root dentin of primary teeth.

Images obtained from negative control showed weak green fluorescence suggestive of non specific signals. Absences of bacteria in dentinal tubules of sample were further confirmed by broth culture of another negative control. No turbidity was observed after 24-48 hrs, suggestive of effective sterilization of samples (fig. 2 A). Another negative control sample observed under CLSM showed weak live (green) and predominant dead (red) bacterial cells (fig.-2, C) showing effective sterilization. SEM observation of positive control showed presence of coccoidal bacteria (E. faecalis) on the split surface of dentinal tubules (fig.-2, B).

Univariate analysis of dead cell volume obtained from experimental groups showed that all medicament were significantly effective ($p< 0.05$) (table-1). Paired sample student's t-test showed significant difference between 1&15 days for 1%CHX+CH and Metapex; between 1&7, 1&15, 7&15 for ZOE (table-1). Kruskall Wallis ANOVA (at 95% confidence interval for mean) showed no significant difference among groups at day 1($p=0.113$), while it was significant at day 7 and 15 ($p=0.008$ and 0.016 respectively). Therefore it was subjected to Mann Whitney post hoc comparison using Bonferroni correction for intergroup comparison, which revealed that there was no inter-group significant difference except between Metapex and ZOE at day 7 (table-2). Ranking of antimicrobial efficacy of tested medicament, based on means was (most effective to the least): 1%CHX+CH(15)> ZOE(15)> Metapex(15)> 2%CHX+CH(15)>2%CHX+CH(7)>2%CHX+CH(1)> 1%CHX+CH(7)>2%CHX+CH(15)>Metapex(1)>ZOE(1)> ZOE(7) (table-1, fig-3).

Figure 2: Control groups in SEM and CLSM

Broth culture of negative control, no turbidity seen

SEM image of positive control showing dentinal tubules with coccoidal bacteria

CLSM image of negative control showing predominant red colored dead cell within dentinal tubules

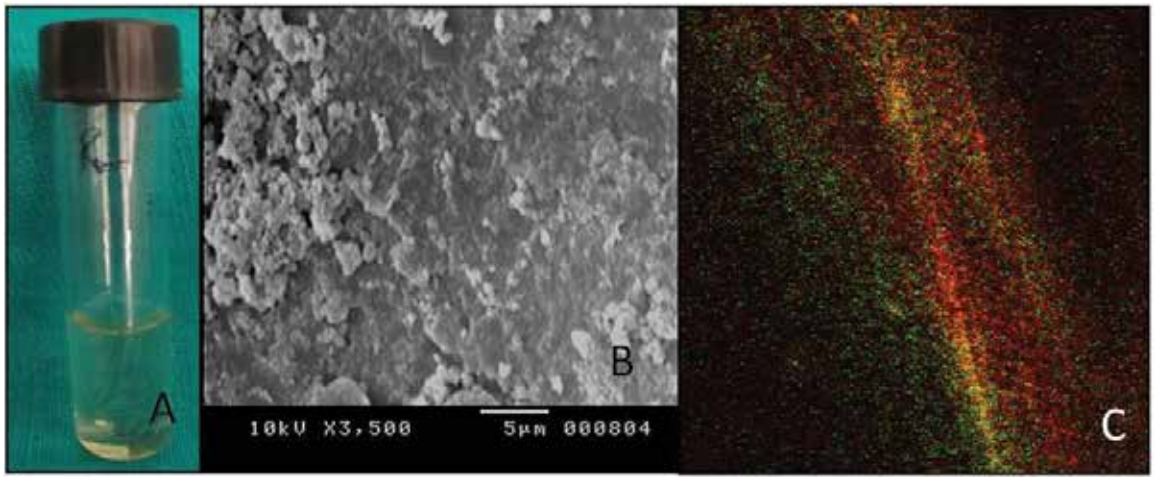
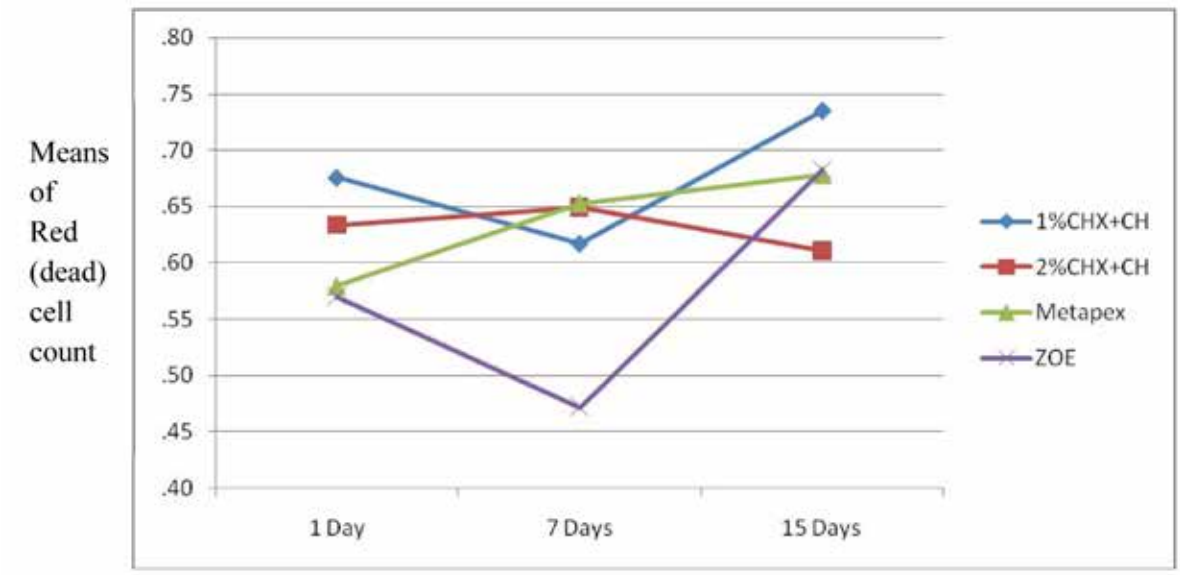


Table 1. Mean and variance of dead cell counts of *E.faecalis* in dentinal tubules treated by different medicaments and intra-group comparison with 2 tailed paired sample student's t-test.

Medicaments	1 Day		7 Days		15 Days		2 tailed paired sample student's t-test (α=0.05)		
	Mean±SD	Variance	Mean	Variance	Mean	Variance	1 vs 7 days	1 vs 15 days	7 vs 15 days
GR-1 (n=2) 1%CHX + CH	0.6756 ±0.0600	0.003601	0.6172 ±0.1429	0.020417	0.7352 ±0.0262	0.000689	0.457 (NS)*	0.018(S)#	0.446(NS)
GR-2 (n=2) 2%CHX + CH	0.6332 ±0.0640	0.004091	0.6490 ±0.0318	0.001008	0.6101 ±0.0001	9.6E-09	0.325(NS)	0.309(NS)	0.334(NS)
GR-3 (n=2) Metapex	0.5794 ±0.0667	0.004448	0.6527 ±0.0182	0.00033	0.6782 ±0.0178	0.000318	0.112(NS)	0.04(S)	0.024(S)
GR-4 (n=2) ZOE	0.5704 ±0.0561	0.003146	0.4720 ±0.0886	0.007845	0.6824 ±0.0193	0.000372	0.004(S)	0.028(S)	0.003(S)

(NS)*= Non significant, (S)#= Significant

FIGURE 3: Line diagram showing comparative antimicrobial efficacy of 4 tested medicaments at day 1, 7 and 15.



DISCUSSION

One of the major factors associated with endodontic failure is the persistence of microbial infection in the root canal system and periradicular area.[3] *E. faecalis*, which is an opportunistic, facultative anaerobe and is well recognized as a pathogen associated with persistent apical periodontitis in endodontically treated teeth and is highly prevalent in failed root filled teeth.[16]

Survival and virulence factors of *E. faecalis* endures prolonged periods of nutritional deprivation, binds to dentin and proficiently invades dentinal tubules, alters host responses, suppresses the action of lymphocytes, possesses lytic enzymes, cytolysin, aggregation substance, pheromones, and lipoteichoic acid.[17] Apart from this *E. faecalis* utilizes serum as a nutritional source, resists intracanal medicaments (i.e. CH), maintains pH homeostasis and competes with other cells. It is physically and ecologically strong. The bacteria do not die during centrifugation and so has been used in this study for infecting root dentin.[7] The 24-hour incubation period after centrifugation gave a possibility for the bacteria to recover from any damage they might have experienced. Moreover enterococci are round in shape and have a relatively small cell diameter, which make it easier to force them into dentinal tubules.[7]

The CLSM technique has application in the comparative assessment of antimicrobial endodontic medicaments. It facilitates the determination of bacteria both laterally within the tubules and longitudinally throughout the length of the root.[18] Viability staining better reflected true viability. The CLSM analysis used has advantage over the conventional fluorescence microscopy such as better image resolution and ability to eliminate scattered or out of focus light and to show individual bacterial cells inside the dentinal tubules.[7]

The present study used an alternative way to fill the dentinal tubules with *E. faecalis*, by using the power of centrifugation to force the bacteria into dentin. We used coronal parts of the root to prepare the dentin specimens because of the varied position of resorption in apical root of primary teeth. Also wider dentinal tubule openings allowed bacteria to easily find the opening during centrifugation. The time and gradually increasing force selected for centrifugation was according to suggestion by Jingzhi [7] as shorter times for centrifugation did not allow the bacteria enough time to move deep into the dentin.

This method resulted in homogenous and dense presence of bacteria in specimens deep in dentin (fig.2 B). The outer side (cement side) of the semi cylindrical dentin pieces was closed again by nail varnish before exposure to the test materials to simulate the difficulty these pastes have *in vivo* when penetrating dentin. After exposure to the antibacterial pastes for predetermined duration, the dentin specimens were fractured to reveal a fresh dentin surface followed by fluorescent staining that was used to start scanning for living and dead bacteria.

Though calcium hydroxide has been the most effective intracanal medicament against a variety of microorganisms of the root canal flora, it has clearly been demonstrated to be ineffective against *E. faecalis*.[12] Therefore the goal of associating chlorhexidine gluconate was to enhance the antimicrobial effectiveness of calcium hydroxide, particularly against resistant microorganisms such as *E. faecalis*.[19, 20] To be effective, CH should provide the environment with a minimum pH of 12.5 as *E. faecalis*, a resistant endodontic pathogen, can survive at pH 11.5. The efficiency of calcium hydroxide as an intracanal dressing is due to its ionic effect based on chemical dissociation into calcium and hydroxyl ions in aqueous solution which are responsible for alkalization of the medium.[20, 21] Alkalinity of calcium hydroxide drops down after 7 days making it ineffective to kill *E. faecalis*.[21]

Therefore some other mechanism of antibacterial medicament is needed not depending on hydroxyl ions. The antimicrobial property of CHX does not depend on the pH, but is related to the cationic molecule binding to negatively-charged bacterial cell walls, thereby altering the osmotic equilibrium of the cell.[22] CHX is a positively charged hydrophobic and lipophilic molecule that interacts with phospholipids and lipopolyssacrides on the cell membrane of bacteria and then enters the cell through some type of active or passive transport mechanism altering its integrity.[23] The

alteration of the cytoplasmic membrane permeability promotes precipitation of cytoplasmic proteins, alters cellular osmotic balance, interferes with metabolism, growth, cell division, inhibits the membrane ATPase and inhibits the anaerobic process.[23]

The change of dentinal pH caused by hydroxyl ions release from CH is slow and depends on several factors that can alter the rate of ionic dissociation and diffusion of CH, such as the level of hydrosolubility of the vehicle employed, difference in viscosity, acid-base characteristics, dentinal permeability, and level of existing calcification[4] Moreover a paste is considered chemically to be a colloid substance (a solid dispersed into a liquid). Therefore, the type of vehicle used may either facilitate or inhibit the ionic dispersion of the paste. Glycerine was added in the calcium hydroxide paste because this vehicle reduces the rapid dispersion of calcium hydroxide into the tissues and retains the paste physically and alkaline pH chemically in the desired area for longer intervals. This factor prolongs the action of the paste, and Ca^{2+} and OH^- ions are sustained released. It is via this mechanism and also that vehicle does not let the paste dry, these pastes remain in direct contact for extended time intervals.[21] Therefore we added aqueous CHX in viscous mix of CH making overall mix viscous only.

Metapex contains silicone oil as its vehicle and has a pH below that which is effective to kill *E. faeclis*.[24] This material has been used successfully as interim root canal dressing in permanent teeth and as obturating material in primary teeth. As it is used for longer days in primary teeth, this material has been used in this study to evaluate its efficacy against *E. faecalis* in primary teeth. ZOE, has been routinely used for obturation of root canals of primary teeth, so it has also been employed in the study for comparison.

Period of 15 days for experiment was selected on the basis of substantivity of CHX and according to the previous study performed by Souza M et al.25 , Rosenthal S et al.26 and Gomes BPFA et al.2 In the present study, all the medicaments showed antimicrobial activity against E. faecalis after 1,7,15 days (table-1, fig.-3). All tested medicaments differed significantly from each other (table 1). The combination of aqueous mix of 1% CHX + CH was found to kill 68%, 62% and 74% of *E. faecalis* after1, 7, 15 days respectively. This result agreed with the previous studies by Sassone [28] which found that 1% CHX was effective in killing *E. faecalis* both in contact test and agar diffusion test be it in presence of bovine serum albumin (BSA) or not. They had added BSA to simulate organic tissue present in the root canal. Our test also, being very close to as *in vivo,* support their findings.

However 1% CHX + CH showed the most antimicrobial activity after 15 days of contact. Result suggests the slow adsorption of the medicament (CHX) inside the dentinal tubules and substantially released in due course of time.[20] As adsorption of chlorhexidine to human teeth is slow, to predictably achieve antimicrobial substantivity, the dentin must be medicated with chlorhexidine for several days. Hence Nageshwar [20] suggested that chlorhexidine should be applied as an intacanal medicament between appointments using a suitable vehicle that would sustain its concentration for 7 days or longer. Accordingly 1%CHX and 2%CHX were mixed with viscous mix of CH. For initial 7 days, probably, antimicrobial activity was mainly due to hydroxyl ions and after the alkalinity drop at day 7 chlorhexidine started working, which increased substantially at day 15 (fig.-3).

2% CHX + CH pastes inhibited 63%, 65%,62%, after 1,7,15,days which is less than 1% CHX + CH paste. Ballal [29] in agar diffusion study found that 2% CHX + CH was more effective than CH alone but less effective than 2% CHX alone. Evans MD et al.[30] performed the study with infected dentin and agar diffusion test and found that 2% CHX + CH was more effective than CH + H2O. None of these studies had used 1% CHX and was done on agar diffusion plates. In this study 1% CHX as well as 2% CHX in combination with CH in viscous vehicle were compared as *in vivo* condition. Our results showed that 1% CHX + CH retain more substantivity of 1% CHX. Apart from this in this study aqueous solution of 1% and 2% CHX was mixed with viscous paste of CH, while previous studies had used 2% CHX gel in their experiments. Probably in this study aqueous 1% CHX was able to penetrate deeper in dentinal tubules and adsorbed on to the dentinal wall and get released after alkalinity drop at day 7. Our results support study by De Rossi A[31] in which healing of experimentally induced chronic periapical lesions in dogs were evaluated radiographicaly as well as histologically with or without CH + 1% CHX intracanal dressing. Significant radiological and histological reduction in periapical pathosis was found with this dressing. Similarly in a study by [32] *in vivo* effectiveness of CH + 1% CHX as intracanal medicaments in endodontic retreatment cases with periapical lesions was assessed. Their results suggested that CH + 1% CHX could be successfully used as intracanal medicament for disinfection in endodontic retreatment cases with periapical lesions. Overall antimicrobial efficacy of 2%CHX+CH was less than 1%CHX+CH, although not significantly (table-3, fig.-3). Higher concentration of hydroxyl ions from CH and cations from chlorhexidine, probably, competes with each other during initial days and

Table 2:Inter-group comparison: Mann Whitney post hoc comparison using Bonferroni correction (α = 0.05 / 6 = 0.0083)

	1%CHX+CH	2%CHX+CH	Metapex	ZOE
Day 1				
1%CHX+CH		Not Sig	Not Sig	Not Sig
2%CHX+CH	p = 0.201		Not Sig	Not Sig
Metapex	p = 0.100	p = 0.221		Not Sig
ZOE	p = 0.045	p = 0.221	p = 0.754	
Day 7				
1%CHX+CH		Not Sig	Not Sig	Not Sig
2%CHX+CH	p = 0.317		Not Sig	Not Sig
Metapex	p = 0.199	p = 0.558		Sig
ZOE	p = 0.063	p = 0.040	p = 0.002	
Day 15				
1%CHX+CH		Not Sig	Not Sig	Not Sig
2%CHX+CH	p = 0.064		Not Sig	Not Sig
Metapex	p = 0.021	p = 0.064		Not Sig
ZOE	p = 0.021	p = 0.064	p = 0.773	

after exhaustion of hydroxyl ions at day 7, chlorhexidine activity dominated and efficacy of medicament increased then after.

Metapex, demonstrated the antimicrobial activity against *E. faecalis*, on 1,7,15 days, which is in favour of Gautam[24] and Gomes [27]. Our results suggested that antimicrobial effects of Metapex may be due to the combination with iodoform and to the oily vehicle which altogether synergize and prolong the action of the medicament. However, its effectiveness is significantly less as compared to 1% CHX + CH and 2% CHX + CH pastes (table-2).

Similar to our results Queiroz [3] also found significantly bigger diameter of inhibition zone with 1% CHX than ZOE against *E. faecalis*. On the contrary Piva [33] found no antimicrobial activity of ZOE against mixture of bacterial strains even after 72 hrs using direct contact test. The difference in results may be attributed to the use of mixture of strains, contact test and lesser time in their study. Nonetheless in our study also, its effectiveness was found to be least among antimicrobial pastes tested at day 1 and 7, which reached almost equal efficiency as that of 1%CHX+CH at day 15 (table-1, fig.-3). Kriplani R. et.al.34 had tested in vitro ZOE, CH and Metapex against E. faecalis using agar diffusion test and found ZOE and CH were less inhibitory while Metapex was non inhibitory at 24 hrs. Contrary to them our study showed efficacy ZOE and Metapex against E. faecalis at day 1, 7 and 15 with increasing efficiency at day 15. Efficiency of CH in our study was found maximum because it was combined with CHX. Therefore medicaments used for root canal dressing and obturation need to be tested in simulated in-vivo conditions and for longer durations.

Our study had tested medicaments for longer duration and almost simulated in-vivo conditions. Nonetheless smaller sample size is the limitation of this study. Future studies with larger sample size are recommended.

CONCLUSION

Within the limitations of this study following conclusions could be drawn:

1. All tested medicaments were effective against E. faecalis in root dentine of primary teeth at 1, 7 and 15 day but showed increasing efficiency at longer days of medicament contact.
2. 1%CHX+CH showed overall best antimicrobial efficacy against E. faecalis.
3. ZOE was least effective against E. faecalis during initial days but reached almost equal efficiency as other medicaments at day 15.
4. Lesser concentration of CHX when mixed with CH, retained more substantivity property of it.
5. Medicaments dispensed in oily vehicle take little longer time to be effective as compared to viscous vehicle.

Based on the results of this study CHX could be recommended to be incorporated in dressings and probably in future as well in obturating pastes especially for primary teeth with periradicular pathosis to effectively kill most resistant bacteria, *E. faecalis* within dentinal tubules. Future *in vivo* studies are recommended to validate the above statement.

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