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An in-vitro evaluation of anti microbial efficacy of chlorhexidine gel and double antibiotic paste as an intracanal medicament against E. faecalis by modified direct contact test

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Abstract

Background: Micro-organisms are the etiological factors for the pulp and periapical diseases; therefore the chief aim of endodontic treatment is to eliminate microorganisms from the root canal space. Of these Enterococcus Faecalis is one of the most resistant microbial species that remains within the root canal.

Materials method: Extracted 40 human mandibular first premolars were taken and were divided into 3 groups; GROUP 1(n=10) -2% Chlorhexidine gel, GROUP 2(n=10) - double antibiotic paste and GROUP 3(n=10) - combination of 2% CHX+ double antibiotic paste and1control group. The E. faecalis was incubated in in-vitro conditions for 7 days at 37°C. After which the samples were subjected to the antibiotic assay.

Modified Direct contact test was done by placing a standardized suspension of Enterococcus faecalis on the test materials in a 96 well microtiter plate. The bacterial growth was measured spectrophotometrically using ELISA reader at intervals.

Results: The analysis of the antimicrobial activity showed promising results. However, the combination of two medicaments has better activity against E. faecalis.

Conclusion: Out of four groups examined, best results in terms of eradication of E.faecalis from the root canal samples were observed by the combination with chlorhexidine gel and double antibiotic paste.

Keyword: CHX, E. faecalis, Double antibiotic paste

Introduction

Endodontic infection is considered as a polymicrobial disease.[1] Successful endodontic therapy of the teeth affected with dental caries consists of thorough disinfections of the root canals which cannot be attained by standard treatment alone. Hence, the use of endodontic medicaments for sterilization of root canals especially resistant microbes like Enterococcus faecalis has become a necessity. Complete debridement and reduction of the bacterial infection from the root canal space seems to be necessary for long-term success of endodontic treatment.[2, 3] The eradication of microorganisms from the infected root canal system is a complicated task including instrumentation, irrigation and application of intracanal medicaments. It is noticeable that many researchers have shown that significant portions of the root canal walls remain untouched after mechanical instrumentation. Consequently, chemical irrigators and intracanal medicaments seem necessary for eradication of infected tissues and microorganisms in addition to mechanical debridement. [4]

Enterococcus Faecalis (E. Faecalis) has been found in 38% of the failed root canal-treated teeth.[5] The ability to tolerate the rough environmental changes which is believed to be due to its high alkali tolerance [6] and tubular invasion ability of this cocci which protects it from intracanal endodontic medicaments,has made E. Faecalis a treatment-resistant microorganism.

Another alternative root canal medication other than calcium hydroxide is chlorhexidine (CHX) gluconate with well-known broad-spectrum antimicrobial effects.[8] CHX molecule consists of two symmetric 4-cholorophenyl rings and two biguanide groups connected by a central hexamethylene chain. Due to the positively charged molecule, CHX interacts with negatively charged phosphate groups on the microbial cell wall and causes its leakage. Mechanism of action for CHX is its absorption onto the cell wall which causes cellular leakage and allows the CHX molecule to penetrate into the bacteria.[8-10] CHX has been shown to be more effective in eliminating microorganisms like E. Faecalis which resisted against CH inside the dentinal tubules.[10-12]Several researchers have pointed to the potential advantage of chlorhexidine gluconate (CHX) as an antimicrobial medicament in endodontic therapy.

Recently a mixture of metronidazole, ciprofloxacin and minocycline, also known as the triple antibiotic paste (TAP), has been used as an intracanal medicament for disinfecting the root canal during tissue regeneration and retreatment.[13] Many case reports and studies have shown that minocycline causes visible crown discolouration.[14] Hence it is eliminated in a double antibiotic paste (DAP) that consist of only ciprofloxacin and metronidazole. Metronidazole is a wide spectrum bactericidal antibiotic.[15] In vitro experiments have shown that 10 µg/ml metronidazole can eliminate more than 99% of bacteria found in infected root canals. On the other hand, increasing the concentration of metronidazole could not kill all the bacteria. Therefore to sterilize the infected root

canal, other antibiotics such as ciprofloxacin is added.[14] The synthetic fluoroquinolone ciprofloxacin inhibits DNA gyrase in bacterial nuclei, degrading the DNA by exonucleases and resulting in a bactericidal effect. Ciprofloxacin has very potent activity against Gram-negative pathogens, but its activity is limited against Gram-positive bacteria and most anaerobic bacteria are resistant to ciprofloxacin. Consequently, ciprofloxacin is combined with metronidazole in the treatment of mixed infections .[16]

In this study a modified direct contact test (MDCT) which measured the effect of direct and close contact between the test microorganism and the tested material on microbial viability, regardless of the solubility and diffusability of the antimicrobial components, is described. The antimicrobial properties of two endodontic medicaments and their combination is evaluated by the MDCT.

To our knowledge, there are no microbiological studies comparing the efficacy of 2%DAP and 2%CHX gel alone and in combination. Hence, the aim of this invitro study is to evaluate the antimicrobial efficacy of chlorhexidine and its combination with DAP as an intracanal medicament in response to E.faecalis through modified direct contact test.

Materials and Method

Forty freshly extracted, single rooted and single canaled human mandibular first premolars with fully formed apices, without curvatures that had been extracted for periodontal/prosthetic reasons were included in the study. The extracted teeth for the current study was collected from the Department of Oral and Maxillofacial surgery, The Oxford Dental College, Bangalore. The present study was conducted in the Department of Conservative Dentistry and Endodontics, The Oxford Dental College, Bangalore. Modified direct contact test was conducted in the Dextrose laboratories, Bangalore. The experimental strain used is ATCC29212, and was obtained from the Department of Microbiology, The Oxford Medical College, Bangalore.

Tested Materials

Two available root canal medicaments, 2%Chlorhexidie digluconate gel (cerkamed polland) and DAP {ciprofloxacin and metronidazole (1:1) (cipla)} were used in this study. To obtain the antibiotics from their commercially available formulations, capsules were opened and the powder contained therein was collected, whereas tablets were crushed with mortar and pestle and were compounded using equal portions of each component antibiotic, weighed on a precision balance. Briefly, antibiotic powders were mixed with saline solution 0.9 % at a 1:1 powder/solution ratio.[17] To obtain gel consistency, 0.2gm of methyl cellulose was added, and was standardized to 2%.

After extracting the teeth, Bone, calculus and soft tissues on the root surfaces was slightly removed by means of a periodontal curette. These teeth were placed in 5.25% NaOCl for 1 hr in order to disinfect the root surfaces. The samples were then be stored in 0.9% physiological saline. The crowns were cut at the CEJ with a diamond disc in conjugation with the physiological saline irrigation and were standardized to 16mm. Apical patency of the canal was evaluated using # 15k file. The roots were instrumented 0.5 mm beyond the apex using the ProTaper Rotary system with 5.25% NaOCl irrigation, to size F3. The samples were placed in 17% EDTA ultrasonic bath for 4 mins to remove smear layer and flushing was done with 5.25 % NaOCl. To remove the remnants of both EDTA and NaOCl each tooth was rinsed in 10ml of physiological saline. Each tooth specimen was then placed in a microtube containing 2ml of Tryptic soy broth (TSB) and was autoclaved twice at 121°C, 15lb for 30 mins.

After this, samples were stored in an incubator at 37°C for 24hrs. Sterile TSB was inoculated with 1ml of E.faecalis suspension. The tubes were then closed and incubated at 37°C for 48 hrs. Bacterial viability and purity was checked in randomly picked sample tubes. After that, samples were inoculated with 1x 10^8 cells/ml concentration of E. faecalis and were incubated for 48 hrs at 37°C.

Each sample was randomly divided into 3 groups and 1 control group, n=10 and each prepared medicament was injected into the infected sample roots from coronal to apical till it gets extruded from the apex. The experimental design employed the following experimental groups.

Group I - 2% CHX gel (Cerkamed Polland)

Group II - 2% DAP (cipla)

Group III - 2% CHX gel and 2% DAP

Group IV - Control group (saline).

These medicated samples were incubated at 37°C for 7 days. After 7 days, the intracanal medicament was removed from canals by irrigation with 10ml of normal saline. Bacterial samples were collected by drilling the apical ⅓ of the roots using #4 Gates Glidden drill

Antimirobial efficacy

Precultures of Enterococcus faecalis in Brain heart infusion broth were incubated at 37ºC.After an overnight of incubation the cultures were diluted, and then the colony-forming units of bacterial cells in sample was determined . Wells were punched onto the agar medium with a sterilized cork borer. Spread plate technique was carried on Brain heart infusion agar medium plates with pre-cultured Enterococcus faecalis strain of suspension (100µL) and the three samples were loaded into the wells. Then the plates were incubated at 37ºC for 24- 48 hrs and after the incubation, the zones of inhibition were measured.

Statistical Analysis

Statistical Package for Social Sciences [SPSS] for Windows, Version 22.0. Released in 2013. Armonk, NY: IBM Corp., was used to perform statistical analyses.

Descriptive Statistics: Descriptive analysis includes expression of ZOI and CFUs of E. Faecalis in terms of Mean & SD for each study group.

Inferential Statistics: One-way ANOVA test followed by Tukey's post hoc test was used to compare the mean ZOI for E. Faecalis between different groups. Kruskal Wallis test followed by Mann Whitney post hoc test was used to compare the mean CFUs / ml (in log 10) between different groups. The level of significance [P-Value] was set at $P<0.05$.

Results

The test results demonstrated the comparison of mean zone of inhibition for E. Faecalis between groups. The mean zone of inhibition for Group 1 was 45.67 ± 4.04, for group 2 was 29.67 ± 4.51, for group 3 was 50.00 ± 3.00 and for group 4 was 0.00 ± 0.00. These differences in the mean scores between 4 study groups was statistically significant at $p<0.001$. [Refer graph no. 1]

Multiple comparison of mean differences between groups showed that group 3 showed significantly higher mean zone of inhibition compared to group 2 & group 4 at $P<0.001$. This was then followed next with group 1 showing significantly higher mean zone of inhibition as compared to group 2 & group 4 at $P=0.002$ and $P<0.001$ respectively. Finally, group 2 also showed significantly higher mean zone of inhibition as compared to group 4 at $P<0.001$. However, no significant difference was noted between group 1 and group 3 [$P=0.44$]. [Refer graph no. 2].

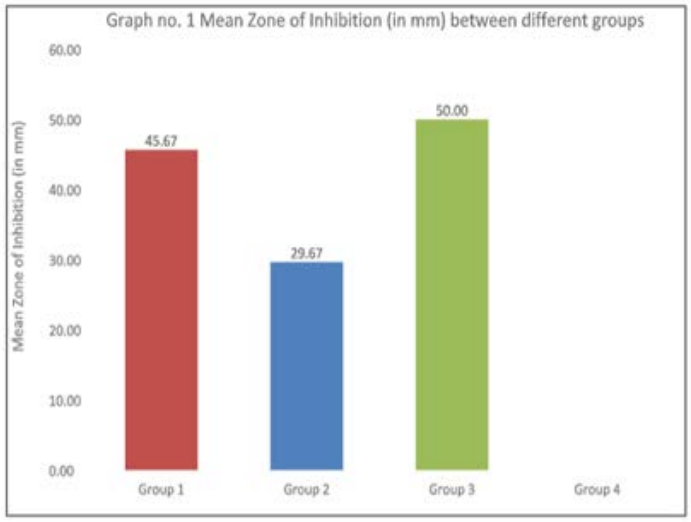
Note: Group 1 – Double Antibiotic Paste, Group 2 - 2% Chlorhexidine gel, Group 3 – combination of 2% CHX+ double antibiotic paste, Group 4 – Negative Control

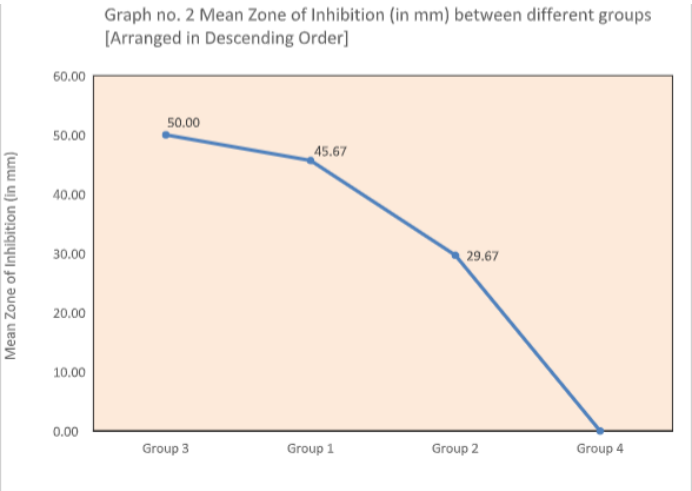
Multiple comparison of mean diff. Zone of Inhibition (in mm) b/w different groups using Tukey's Post hoc Test

(I) Groups	(J) Groups	Mean Diff.(I-J)	95% CI for the Diff.		P-Value
			Lower	Upper	
Group 1	Group 2	16.00	7.17	24.83	0.002*
	Group 3	-4.33	-13.17	4.50	0.44
	Group 4	45.67	36.83	54.50	<0.001*
Group 2	Group 3	-20.33	-29.17	-11.50	<0.001*
	Group 4	29.67	20.83	38.50	<0.001*
Group 3	Group 4	50.00	41.17	58.83	<0.001*

Comparison of mean Zone of Inhibition (in mm) between different groups using One-way ANOVA Test

Groups	N	Mean	SD	Min	Max	P-Value
Group 1	10	45.67	4.04	42	50	<0.001*
Group 2	10	29.67	4.51	25	34	
Group 3	10	50.00	3.00	47	53	
Group 4	10	0.00	0.00	0	0	





The test results demonstrated the comparison of mean CFUs of E. Faecalis [in log10] for E. Faecalis between different groups. The mean zone of inhibition for Group 1 was 0.259 ± 0.241, for group 2 was 0.593 ± 0.111, for group 3 was 0.000 ± 0.000 and for group 4 was 3.008 ± 0.093. These differences in the mean E. Faecalis CFUs / ml between different groups was statistically significant at P<0.001. [Refer graph no. 3]

Multiple comparison of mean differences between groups showed that group 3 showed significantly least mean CFUs / no growth compared to group 2 & group 4 at P=0.004 and P=0.001 respectively. This was then followed next with group 1 showing significantly lesser mean CFUs as compared to group 2 & group 4 at P=0.04 and P<0.001 respectively. Finally, group 2 also showed significantly lesser mean CFUs as compared to group 4 at P<0.001. However, no significant difference was noted between group 1 and group 3 [P=0.12]. [Refer graph no. 4]

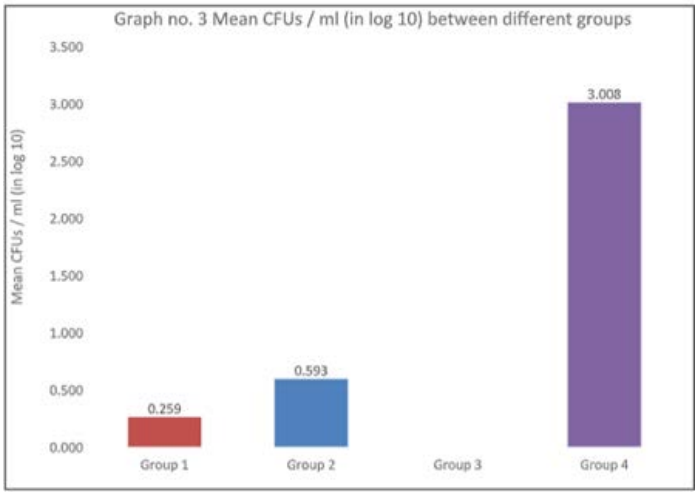
Comparison of mean CFUs / ml (in log 10) of E. Faecalis between different groups using Kruskal Wallis Test

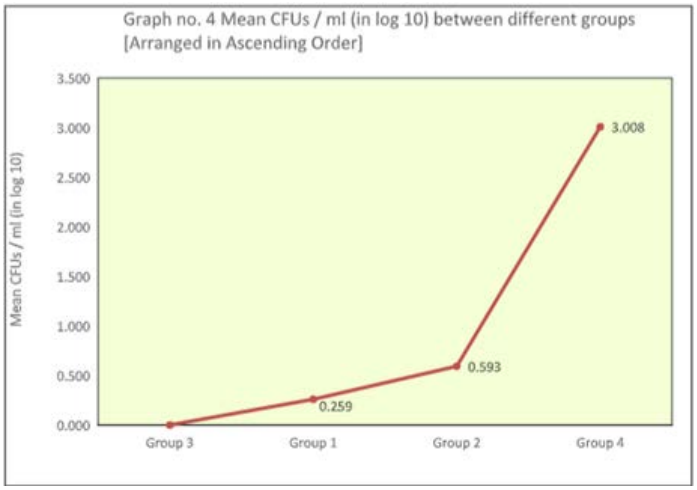
Groups	N	Mean	SD	Min	Max	P-Value
Group 1	10	0.259	0.241	0.00	0.48	<0.001*
Group 2	10	0.593	0.111	0.48	0.70	
Group 3	10	0.000	0.000	0.00	0.00	
Group 4	10	3.008	0.093	2.90	3.08	

Note: Group 1 – Double Antibiotic Paste, Group 2 - 2% Chlorhexidine gel, Group 3 - combination of 2% CHX+ double antibiotic paste, Group 4 – Negative Control

Multiple comparison of mean CFUs / ml (in log 10) b/w different groups using Mann Whitney's Post hoc Test

(I) Groups	(J) Groups	Mean Diff.(I-J)	95% CI for the Diff.		P-Value
			Lower	Upper	
Group 1	Group 2	-0.33	-0.70	0.03	0.04*
	Group 3	0.26	-0.11	0.63	0.12
	Group 4	-2.75	-3.12	-2.38	<0.001*
Group 2	Group 3	0.59	0.22	0.96	0.004*
	Group 4	-2.42	-2.78	-2.05	<0.001*
Group 3	Group 4	-3.01	-3.38	-2.64	<0.001*





Discussion

Endodontic failures can be attributable to inadequacies in shaping, cleaning and obturation, and also reinfection of the root canal system when the coronal seal is lost after completion of root canal treatment .[18] To increase the efficiency of instrumentation, root canal irrigating solutions and intracanal medicaments are used to eliminate the bacteria from the root canals. [19] This can be ascribed to the usual inability of instruments and irrigants in cleaning and disinfecting anatomical variables, which are common in the apical portion of the root canals.[20, 21]

E. Faecalis is associated with persistent apical periodontitis and resists elimination from root canals.[22] It has the capacity to proliferate in the deeper layers of dentine.[23] Thus the penetration of medicaments into dentinal tubules was evaluated by investigation of optical density of samples obtained by two different sizes of Gates Glidden drills. The results showed that the most effective medicament against E. Faecalis was the combination of DAP and CHX gel and after that, in descending order were DAP, CHX gel and saline. Studies have suggested that CHX gel is an effective intracanal medicament due to its broad antimicrobial spectrum, which is in agreement of the findings of the present study. Other studies also demonstrated that, 2% CHX gel was effective against E. Faecalis even 21 days after root dentine treatment.[23] In the study by Dametto et al.[24] 2% CHX gel and 2% CHX liquid significantly reduced the number of E. Faecalis colonies. The present results showed that the 2% CHX gel produced the mean inhibition zone and was effective against microorganism tested. Even at the highest concentrations, it has very low toxicity. Also, it absorbs onto dental tissues and mucous membranes resulting in its prolonged gradual release at therapeutic levels.[25] Since it is a positively charged hydrophobic and lipophilic molecule, it interacts with negatively

charged phospholipids and lipopolysaccharides on the cell membrane of microorganism and enters the cell through some type of active or passive transport mechanism, which alters the osmotic equilibrium of the cells. This increases the permeability of the cell wall, allowing the CHX molecule to penetrate into the micro-organism, followed by leakage of intracellular constituents, particularly phosphate entities such as adenosine triphosphate and nucleic acids.

It binds to hydroxyapatite and soft tissues, changing their electrical field to compete with microbial binding, thus decreasing microbial adherence. The result of the present study was similar to that of, Gomes et al.[26], Krithikadatta et al.[27], Basrani et al.[28] and Vaghela et al. [29], which

showed that 2% Chlorhexidine gel produced a better antimicrobial action as compared to 0.2% Chlorhexidine gel or Calcium hydroxide mixed with 0.2%chlorhexidine.

Antibiotic paste mixtures are used for the disinfection of the root canal system during regenerative endodontic procedures or as an intranasal medicament for further disinfection. However, previous studies led some concerns about their usage due to allergic reactions, bacterial resistance, tooth discoloration, decrease in root dentin micro hardness, and their detrimental effects on stem cells. Both systemic and topical antibiotics are used in dentistry. Systemic administration of antibiotics allows only negligible concentrations of drugs to reach the root canal, whereas the local administration allows the greater concentrations of drug to be used as intracanal medicaments, additionally decreases systemic consequences and complications. Use of intracanal medicaments eliminates higher proportions of bacteria from the root canals.[30] Complexity of root canal infections does not allows single irrigant or a medicament to be effective for sterilization of the root canal. Combination of medicaments decreases the chances of development of resistant bacterial strains and produces synergistic effect, and their antimicrobial action is long lasting with sustained medicament release.

Presently, the Double antibiotic mixture is composed of ciprofloxacin and metronidazole which is a nitroimidazole compound and is widely used for its broad-spectrum and strong antibacterial activity against anaerobic cocci, as well as Gram-negative and Gram-positive bacilli. Metronidazole permeates bacterial cell membranes, reaches the nuclei and binds to the DNA, disrupting its helical structure, and causing cell death. In the present study, consistent with the latest AAE recommendation of 1mg/ml as the minimal concentration, DAP in methyl cellulose showed a significant antibacterial effect compared with the saline group. It has also shown the mean inhibition zone and also the number of CFU are low compared to the other groups, which can be attributed to its anti-bacterial nature. Moreover, invitro studies have reported that 1mg/ml methylcellulose based DAP has significant antibacterial effect against E.faecalis and polymicrobial biofilms. Latham et all found that 1mg/ml of DAP showed minimal antibacterial effect on E.faecalis biofilm even after 4 weeks; this may be because the DAP used was in liquid form and methyl cellulose as a vehicle not only enhances the handling characteristics, but also the anti-bacterial properties which is consistent with the present study.[31]

The present study has used Modified Direct Contact Test, which is based on measuring the effect of close contact between test bacteria and the tested material on the kinetics of bacterial outgrowth using a temperature controlled microplate spectrophotometer. The modified DCT, by being quantitative and virtually independent of solubility and diffusion, was found more suitable to assay solid surfaces.[32] The results demonstrated the added value of modified DCT in the present study. Till date there is no sufficient data on the antimicrobial effect of the combination of these two medicaments i.e. DAP and CHX, and the present study has proved that the combination is much effective compared to the individual medicaments. However, further in vivo studies have to be done, to rule out the unwanted effects when placed inside the root canal.

Conclusion

The many hurdles in using antibiotics as intracanal medicaments are: Issues of resistance, limited spectrum of activity, lack of antifungal properties, and the hunt for the ideal intracanal medicament continues. In this regard, the combination of 2% chlorhexidine gluconate and DAP can

definitely be used as an intracanal medicament with chlorhexidine having an added advantage of bactericidal action, substantivity, biocompatibility, low toxicity, and lesser chances of developing resistance. However further studies have to be continued which will give us an appropriate understanding of the diffusion through dentinal tubules of these newer combinations of endodontic medicaments.

Within the limitations of the present study, it can be concluded that,

- Both the intracanal medicaments i.e., DAP and CHX gel were effective against the microorganism tested.
- The combination of the two medicaments has shown higher mean zone of inhibition and lesser colonies, compared the individual groups.
- However, research is warranted to compare the efficacy and biocompatibility of these combinations and to test the same in a mixed species biofilm as well as in a clinical scenario to obtain predictable outcomes and to establish a standardized treatment protocol.

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