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EVALUATION OF IRRIGANT- INTRACANAL MEDICAMENT REGIMEN AGAINST ENTEROCOCCUS FAECALIS BIOFILM COUNT

Running title: Antimicrobial activity of irrigant- intracanal medicament combination.

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ABSTRACT

Background: The major factor for endodontic failure is the inability to control and prevent persistent microbial infections. Irrigants and untracanla medicaments are used to reduce microorganisms. A good combination has to be studied

Aim: To compare the antibacterial efficacy of 3 irrigants- 3% Sodium Hypochlorite, 3% Calcium Hypochlorite and 2% Chlorhexidine against E.faecalis biofilm and then to assess if any further reduction in microbial load after the placement of 2 intracanal medicaments - Calcium hydroxide and 1% Metronidazole + 0.25% Chlorhexidine.

Materials and Methods: Sixty- three mandibular incisors were used to form a 6-week E. faecalis dentinal biofilm. Canals were instrumented up to Protaper F2. The tooth samples were divided into 4 groups as per irrigation regimen as Group A- Sodium Hypochlorite (n=20), Group B- Calcium Hypochlorite (n=20), Group C- Chlorhexidine (n= 20) and Group Control (n =3). The Groups A, B and C were further divided into 2 subgroups each for the placement of intracanal medicament. After 7 days, the specimens were analysed for presence of colonies of *E.faecalis*.

Results: When compared to control group, significant difference was observed between all test groups. But statistically no significant difference was found between the three irrigants. Both Calcium hydroxide and Metronidazole +Chlorhexidine have effectively destroyed the remaining E.faecalis culture, hence exhibiting their bacteriocidal nature without any significant difference between them.

Conclusion: Irrigation alone did not eliminate E.faecalis, combining Irrigant with medicament eliminated all E.faecalis and different combinations of irrigants and medicaments were equally effective.

Keywords: Antimicrobial, Dentin biofilm, irrigants, intracanal medicaments.

MAIN TEXT

ORGINIAL RESEARCH: IN-VITRO STUDY

INTRODUCTION

The complete eradication of microorganisms from the root canal system before obturation establishes a favourable outcome for endodontic treatment. [(1)] Undoubtedly, the major factor for endodontic failure is the inability to control and prevent persistent microbial infections.[(2)] Therefore, infected root canals need to be subjected to combined chemo-mechanical treatment involving instrumentation and copious irrigation with disinfectants. Still some bacteria and their byproducts could persist in the root canal system. The placement of intracanal medication is to destroy these residual microorganisms and their toxins and any residual bacteria that have not been removed during canal preparation.

Enterococcus faecalis is one such persistent organism that, despite making uponly a small proportion of the flora in untreated canals, plays a major role in the etiology of persistent periradicular lesions after root canal treatment. It is a common isolatein persistent endodontic infection and known to survive as a sole organism in such cases. Hence a combination of irrigantand medicament may be useful to eliminate *E,faecalis*from the root canals.

Thus, the present *in vitro* study was undertaken to compare the antibacterial efficacy of 3 irrigants-3% Sodium Hypochlorite, 3% Calcium Hypochlorite and 2% Chlorhexidine gluconateagainst *E.faecalis* biofilm and then to assess ifanyfurther reduction in microbial load after the placement of 2 intracanal medicaments - Calcium hydroxide and 1% Metronidazole + 0.25% Chlorohexidine. i.e) to compare the antibacterial efficacy of a combination of these irrigants and intracanal medicaments.

MATERIALS & METHODS

Preparation of the samples:

Sixty- three freshly extracted intact, non-carious, single rooted human mandibular incisors (confirmed by radiograph) with fully formed apices were chosen for the study. An informed consent was obtained from patients undergoing extraction for orthodontic reasons. The teeth were initially stored in hydrogen peroxide solution, cleaned of superficial debris, calculus, tissue tags and stored in normal saline to prevent dehydration. The tooth specimens were sectioned below the cementoenamel junction (CEJ) with a diamond disc to obtain a standard tooth length of 16 mm.

Sterilization of tooth samples:

After instrumentation, the tooth samples (n=63) were immersed in sterile distilled water and autoclaved at 121º C at 15 lbs pressure for 20 mins. The tooth samples were transferred into clean glass test tubes (5 tooth / test tube) containing 5ml of sterile Mueller-Hinton broth (Himedia, India) and autoclaving was repeated. The tubes were checked for sterility at 48 hours.

E.*faecalis* Biofilm formation:

Fresh overnight cultures of *E. faecalis* ATCC 29212 was prepared in sterile MHB and the turbidity was adjusted to MacFarlands standard 0.5 (HiMedia Laboratories Pvt Ltd, Mumbai, India) such that it corresponds to a cell density of 1.5 x 10^8 cells/ml. The test tubes containing the tooth samples were inoculated with *E. faecalis* ATCC 29212, followed by incubation in an orbital shaker at 37^{o}C for 6 weeks to form *E. faecalis* dentinal biofilm.

Instrumentation of the tooth samples:

After 6 weeks of incubation, the tooth samples were thoroughly rinsed with sterile saline to remove the planktonic cells of *E. faecalis* leaving behind the biofilm formed on the dentin. The root canal orifice was enlarged using Sx followed by which instrumentation was carried out using Dentsply ProTaper system S1, S2, F1 till F2 using EDTA gel.

Irrigation of the tooth samples:

The tooth samples were divided into 4 groups, Group A- Sodium Hypochlorite (n=30), Group B- Calcium Hypochlorite (n=30), Group C- Chlorhexidine (n= 30) and Control (n =3). Copious irrigation with saline and the respective irrigant was done after subsequent instrumentation.

Placement of Intracanal Medicament:

The Groups A, B and C were further divided into 2 subgroups each for the placement of intracanal medicament. In group A, 10 teeth were packed with Calcium Hydroxide and labelled (D1-10), 10 with Metronidazole and Chlorhexidine combination (M+C) and labelled (D11-20) using lentinospirals. Groups B and C were divided in similar manneras E1 and E2 and F1 and F3 respectively. The intracanal medicament was placed for a period of 7days and incubated aerobically at 37°C.A sterile F2 paper point sample was placed for 30 seconds in each tooth following which the dentin shavings from each tooth was removed using peeso-reamer size 2. The paper point and the shavings were transferred aseptically in the respectively labelled eppendorf tubes containing 1 mL of sterile saline solution.About 10 µl of the samples were inoculated onto sterile Muller Hinton Agar plates by spread plate method. The inoculated plate was incubated at 37^0 C for 24 hours. After 24 hours the plates were observed for the presence of colonies of *E.Faecalis.*

RESULTS

A one-way ANOVA was used to test the difference in percentage kill of E. faecalis biofilm. A post-hoc analysis was employed using a multiple range test to determine homogenous subsets among the test agents. The level of significance was set at ($p \leq 0.05$).When compared to control group, significant difference was observed between all test groups.Group A (3% Sodium hypochlorite) showed minimal mean reduction, and Group B (3% Calcium hypochlorite) showed moderate reduction. Group C (2% CHX irrigation) showed most reduction of *E. faecalis.* But statistically no significant difference was found between the three groups [Table 1].Both Calcium hydroxide and M+C have effectively destroyed the remaining *E.faecalis* culture, hence exhibiting their cidal nature without any significant difference between them [Table 2].

DISCUSSION

A 6-week biofilm was used in this study, because the microorganism has the ability to form biomineralised or calcified biofilm on root canal dentine which may be a factor that contributes to the persistence of this bacteria. [3]

Although NaOCl irrigation is considered Gold standard, it is not regarded as optimum for E.faecalis, because of its penetration ability of dentinal tubules. [4] Other drawbacks of sodium hypochlorite include its inability to remove smear and its high tissue irritability that can cause inflammation or necrosis and also its adverse effect on bond strength.[5]

Calcium hypochlorite (Ca(OCl)2) normally used for industrial sterilization.[6]$Ca(OCl_2)$ has also shown to eliminate E. faecalis and is comparable to NaOCl. [7]It is expected to have more antimicrobial efficacy than NaOCl because of the higher generation of hypochlorous acid when mixed with water. [8] As Hypochlorous acid is responsible for the antibacterial activity by disruption of several vital functions of the microbial cell.[9] This is in accordance with the present study, where (Ca(OCl)2) was found to be more effective than NaOCl but was statiscally insignificant.

The most effective irrigant in this study was found to be Chlorohexidine. This could be due to the lower contact angle in preparations containing chlorhexidine, enabling better diffusion into the tubules. [10] In another study, a 10-minute irrigation with 2% chlorhexidine before obturation of the root canal resulted in complete elimination of *E. Faecalis*. [11]But none of the irrigants showed complete eradication of *E.faecalis*.

In addition to comparing the antibacterial effectiveness of these three irrigants, combination of these irrigants with two medicaments was also assessed in this study. Because in clinical situations, instrumentation and irrigation alone does not reliably eliminate bacteria,the need of using different intracanal medicaments becomes logical.[12]It was found that both intracanal medicaments used in this study, calcium hydroxide ($Ca(OH)_2$), and a combination of metronidazole and CHX gel, was able to completely eliminate any residual *E.faecalis*.

Calcium hydroxide is a common and effective intracanal medicament known todisinfect in a period of seven days.[13] However, E. faecalis has been reported to be resistant to Ca(OH) as a result of its ability to penetrate the dentinal tubules and adapt to changing environment. [3]

The initial bacterial load in endodontic infections consists of facultative bacteria, but as time progresses, there is increase in obligate anaerobic. [14] The obligate anaerobes are fairly easy to eliminate, in contrast to the facultative anaerobes which are capable of surviving chemo-mechanical.[15]*E. faecalis* is facultative anaerobe that is highly resistant to commonly used intracanal medicament including calcium hydroxide. Hence, metronidazole having bactericidal action against facultative anaerobes can also be considered as an adjunct to endodontic treatment.

Though Metronidazole is known to be more effective against obligate anaerobic bacteria than on aerobic and facultative anaerobic bacteria [16], a study [17]showed it to have antibacterial effect even on E. faecalis, a facultative anaerobic bacterium. A combination of metronidazole and chlorohexidine medicaments was hypothesized to act in synergistic manner with better efficacy.

In our study, a combination of metronidaozle and chlorhexidine proved as effective as calcium hydroxide against *E.faecalis*.Similar study by [18]showed that this combination was better than calcium

hydroxide as an intracanal medicament against *E.faecalis*.This could be because both CHX and metronidazole have been shown to exhibit free radical mediated mechanisms of bacterial killing.[17]

CONCLUSION

Within the limitations of the study, it was found that irrigation alone did not eliminate *E.faecalis* and there was no significant difference between NaOCl and chlorhexidine. Combining irrigant with medicament eliminated all *E.faecalis* and different combinations of irrigant and medicament were equally effective.

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ETHICAL CLEARANCE: Not required for an in-vitro research.

CONFLICT OF INTEREST: Nil

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TABLES

Table 1 –CFU/ml for irrigant groups

Control CFU/ml ≥1,00,000			
Groups	Mean CFU	± SD	P Value
A	91	16.32	<0.05
B	87	12.86	<0.05
C	83	10.74	<0.05

Table 2–CFU/ml for irrigant+Intra-canal Medicamentgroups

Control			
Groups	Mean CFU	± SD	P Value
D 1	24	5.23	<0.05
D 2	27	5.86	
E 1	27	3.32	<0.05
E 2	23	3.83	
F 1	19	4.16	<0.05
F 2	22	3.92	