Antimicrobial Efficacy of Triple Antibiotic Paste & Calcium Hydroxide mixed with 2% Chlorhexidine or Saline against Enterococcus Faecalis : An in Vitro Study.

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Abstract

Introduction: Intracanal medicament with antimicrobial property is essential for complete removal of microorganism from the root canal system during the retreatment cases of root canal.

Aim: To evaluate antimicrobial efficacy of triple antibiotic paste and calcium hydroxide when mixed with either 2% chlorhexidine or saline against Enterococcus faecalis (E. Faecalis).

Material and Methods: An agar well diffusion assay method was used to determine the efficacy of the experimental medicaments against E. faecalis(ATCC 292122). Medicaments were divided into 4 groups; triple antibiotic powder with saline or chlorhexidine, and $Ca(OH)_2$ (plus normal saline or 2% chlorhexidine). The diameters of the growth inhibition zones against E. faecalis for each group were recorded and compared. The differences between groups were analyzed by one-way ANOVA and Tukey's post hoc tests for intergroup analysis.

Results: The triple antibiotic paste groups exhibited significantly higher zones of inhibition. The highest was exhibited by the combination of TAP with 2% chlorhexidene followed by saline ($p<0.001$). The $Ca(OH)_2$ combined with saline exhibited the least zone of inhibition in all the groups ($p>0.0001$).

Conclusion: The combination of triple antibiotic paste in combination with 2% chlorhexidine exhibits better antibacterial action against E. faecalis.

Keywords: Agar diffusion test, Brain heart infusion broth, Inhibition zone

Introduction

Removal of microorganisms from the root canal system during endodontic treatment is important since it plays an integral role in the access and maintenance of pulp and periapical disease[1]. Long standing success of endodontic treatment depends on complete reduction of the bacterial infection from the root canal space[2,3]. Many researchers have found that despite instrumentation, irrigation and application of intracanal medicaments a noteable portions of the root canal walls remain untouched[4]. As result of which chemical irrigators and intracanal medicaments plays a major role in removal of infected tissue and microorganism[5]. Failure of root canal treatment is mainly due to presence of microorganisms in apical third of the root canals[4].

Since E. faecalis is an enteric facultative gram positive bacterium, it grows without the attendant of other bacteria in the root canals[6-14]. Its high alkali tolerance is believed to be responsible for its ability to withstand rough environmental changes[15] and tubular invasion ability of this cocci which protects it from intracanal endodontic medicaments, has made E. Faecalis a treatment-resistant microorganism[16].

The use of calcium hydroxide (CH) as an intracanal medicament has approved to destroy bacteria due to its high alkalinity[17]. There are evidences to prove that E. faecalis is resistant to CH into deeper layers of dentinal tubules as the pH is compensated by the buffering action of dentin[18-19]. So considering the shortcomings of CH, finding an alternative intracanal medicament would be beneficial.

In the recent times, during tissue regeneration , disinfection of the root canal has been brought about by the use of triple antibiotic paste (a mixture of metronidazole, ciprofloxacin and minocycline)[20]. Owing to their antibacterial properties calcium hydroxide and triple antibiotic paste have proven to assist the growth of pulp dentin complex in immature necrotic teeth[21]. Sato et al. has proven that use of triple antibiotic paste can eliminate bacteria in deep areas of the root canal system[22]. Use of this triple antibiotic paste in dogs have shown positive result in disinfection of immature teeth with apical periodontitis[23].

To date, there is insufficient documentation regarding the antimicrobial effect of triple antibiotic paste in eliminating E. faecalis. For this purpose, this investigation compared the in vitro antimicrobial efficacy of triple antibiotic paste and calcium hydroxide in combination with either 2% chlorhexidine or saline against E. Faecalis.

Materials and Method

In the current study the medicaments tested were in powdered form. They were as follows: the antibiotic mixture, the individual components of the triple antibiotic mixture-metronidazole (Flagyl, Winthrop Pharmaceuticals, UK), ciprofloxacin (Bayer plc, UK) and minocycline (Expanscience Laboratories, Paris, France), $Ca(OH)_2$ powder. The triple antibiotic mixture and $Ca(OH)_2$ powder were either mixed normal saline (NaCl 0.9%, Darupakhsh, Tehran,Iran) or 2% chlorhexidine (Consepsis, Ultradent Inc., South Jordan, UT. USA). $Ca(OH)_2$ powder mixed with normal saline served as the control group. The triple antibiotic paste was prepared for each group with identical amount of the three antibiotic powders (mg) and then mixed with 1mL normal saline or 2% chlorhexidine.

Preparation of the Medium for Enterococcus faecalis:

The strains of microorganisms used for the study were standard strains of Enterococcus faecalis ATCC 29212 (Department of Bacteriology, Haffkine Institute, Mumbai, India) and were subcultured in blood agar plate and were incubated at 37ºC for 24 hours. A pure, single Enterococcus faecalis colony was isolated from the same cultured plate and Gram's staining was done to confirm its growth, which was observed under oil immersion microscope and then inoculated with a brain heart infusion broth (BHI- Broth). The BHI-broth was incubated at 37ºC for 24 hours period and checked for bacterial growth by

changes in turbidity.

A drop of BHI(brain heart infusion) broth containing Enterococcus faecalis was placed into saline solution and checked for correct bacterial concentration with a spectrophotometer. The density of the bacterial suspension is standardized by comparing the broth at a density equivalent to the barium sulfate standard of 0.5 McFarland units, which was equivalent to 1.5 x 10^8 colony forming units per milliliter (CFU/ml).

Antimicrobial activity by Agar Well-Diffusion Method:

Plates were prepared with Mueller Hinton Agar. The sterility of the plates were checked and the fresh inoculums of Enterococcus faecalis of 0.5 McFarland standard suspensions were prepared. A sterile non-toxic cotton swab was dipped on a wooden applicator into standardized inoculums (turbidity so adjusted, as to obtain confluent growth on the Petri-plate) and the soaked swab was rotated firmly against the upper inside wall of the tube to express excess fluid. The entire agar surface of the plate was streaked with the swab three times, and the plate was turned at 60^0angles between each streaking. The inoculums was allowed to dry for 5-15 minutes with lid in place.

Four wells were created using 8mm sterile cork borer. The desired amount of the medicament to be tested were mixed and were placed in the well against E. faecalis. 10-15 minutes were allowed for diffussion of the medicament in agar and then were incubated immediately at 35± 2^0C for 24 hours. The whole experiment was carried out under aseptic conditions and was be repeated twenty times to ensure reproducibility.

Measurement of Inhibition Zones

Zones of bacterial growth inhibition will be measured at the end of 24 hours. The zone of inhibition will be measured in milimeters using inhibition zone measuring scale.

Statistical Analysis

After data collection, data entry was done in Microsoft Excel and analyzed using the SPSS software v20 (IBM Corp., Armonk, NY) . Descriptive statistics were shown as Mean and standard deviation. To evaluate the differences between the antimicrobial efficacy of triple antibiotic paste and calcium hydroxide in combination with either normal saline or 2% chlorhexidine one-way ANOVA test was used and for comparison within the groups Tukey's multiple comparison test(table 2) was used. P values less than 0.05 were considered as significant.

Results

The means of the diameters of the growth inhibition zones for each group of medicament are presented in (Figure 1,2).The range of inhibitory values between experimental groups varied broadly. One way ANOVA was used to calculate P-value and showed significant differences (P<0.0001)(Table 1).Overall, triple antibiotic paste/saline, triple antibiotic paste/2% chlorhexidine (groups 1, 2) had the largest zones of growth inhibition in the well diffusion assay (figure 1).Ca(OH)/2% chlorhexidine showed minimal range of inhibitory effect against E. faecalis. $Ca(OH)_2$/normal saline showed least antimicrobial efficacy against E. faecalis.

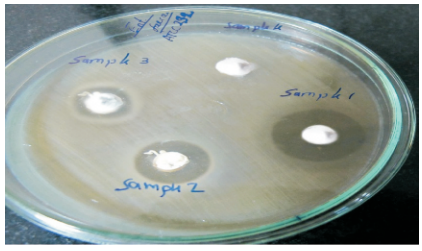

Figure 1. The zones of inhibition against E. faecalis, for the groups

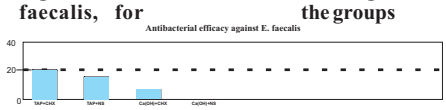

Figure 2. Meanwise presentation of the antibacterial efficacy by means of zones of inhibition.

Source	Sum of Square	Degree of Free -dom	Mean Square MS	F Statis -tics	P Value
Treatment	3157.6854	3	1052.5618	117.08	<.0001
Error	683.2545	76	8.9902		
Total	3840.9399	79			

Table 1:Statistical analysis (ANOVA) against Enterococcus Faecalis.

Group	P Value	Conclusion
TAP+CHX vs. TAP+ Saline	<0.05	Significant
TAP+CHX vs. Ca(OH)+CHX & Ca(OH)+Saline	<0.01	Significant
TAP+Saline vs. Ca(OH)+CHX & Ca(OH)+Saline	<0.01	Significant
Ca(OH)+CHX vs. Ca(OH)+Saline	<0.01	Significant

Table 2: Statistical analysis Tukey's post hoc multiple comparisons against Enterococcus Faecalis

Discussion

Traditionally, agar diffusion method and agar dilution method are commonly applied for detecting antimicrobial susceptibility. In our study, Kirby-Bauer method (Agar Disc Diffusion method) was chosen instead of the Agar Dilution method. The agar diffusion test we used in this study is useful for evaluating the in vitro antimicrobial activities of medicaments; many studies have used this method for evaluations of antibacterial effects of various endodontic materials.. The disadvantage of the Agar Dilution method is that this technique can alter some of the properties of the material being tested.

Hence, we chose the Agar Disc Diffusion method, as in this method the chemical properties of the medicaments are not changed and the antimicrobial resistance can be detected by challenging bacterial isolates with antimicrobial discs. Moreover, this is an easy and less technique sensitive method.

The bacterial species E. faecalis was selected as representing an organism. Multiple studies have used E. faecalis as a target microorganism to determine the result of antibacterial agents[24-26]. E. faecalis are more susceptible to infect teeth with failed endodontic treatment than the ones with primary infection[27]. Predominantly in cases where pH is not preserved, E. faecalis is also impervious to calcium hydroxide which is commonly used[28-30]. Chlorhexidine in a 2% gel or liquid has shown favourable results in complete eradication of E. faecalis from the dentinal tubules and root canal space[31-33]. Turk et al. observed better results when calcium hydroxide mixed with 2% chlorhexidine digluconate than calcium hydroxide with other medicaments against E. faecalis[34]. Therefore we utilised chlorhexidine as one of the vehicles for this study. To evaluate which substance had the highest antibacterial effect, each component was independently mixed with normal saline. The results showed the triple antibiotic powder, either mixed with normal saline or 2% chlorhexidine, produced the largest zone of inhibition against E. faecalis. Antibacterial activity of chlorhexidine may be attributed to its chemical characteristics.

In the present study, the antimicrobial effect of the triple antibiotic paste against E. faecalis were compared to calcium hydroxide, either mixed with normal saline or 2% chlorhexidine. Considering the low antibacterial capability of $Ca(OH)_2$/chlorhexidine and the high ability of triple antibiotic powder/normal saline in this study, we conclude the ability of the former is related to its mixed antibiotic contents, not to the vehicle's properties.

The zones of inhibition generated by groups containing calcium hydroxide were much smaller than the zones of the antibiotic-containing groups, concurring with previous literature that calcium hydroxide is not an effective intracanal medicament when root canals are infected by E. faecalis. E. faecalis is the most common cause of failed root canal therapy[11-13]; thereby use of triple antibiotic paste as an intracanal medicament is advisable. It is also noted that triple antibiotic paste has shown better treatment results when used in primary infections of open apex where E. faecalis is not present[35-38].

Conclusion

Under the limitations of this study, the triple antibiotic paste is very effective against E. faecalis and can be considered as a more powerful root canal medicament compared to calcium hydroxide pastes.

References

References are available on request at editor@healtalkht.com