Efficacy of Contemporary and Novel Intracanal Medicaments against *Enterococcus Faecalis*

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Objectives. To compare the antibacterial activity of propolis (30% in methyl cellulose), curcumin (2.5mg/mL of methyl cellulose), 2% chlorhexidine gel (CHX), 2% metronidazole gel (MZ) and a mixture of 2% CHX and 2% MZ against Enterococcus faecalis in vitro. Calcium hydroxide served as the control. Study design. The inhibitory effect of the medicaments on E. faecalis was determined by the agar diffusion test and tube dilution test.The rate of bactericidal activity was evaluated by the time-kill assay. Zones of inhibition data and time to kill data were statistically analyzed by ANOVA and post hoc Tukey test (P=0.05). Results. CHX demonstrated the highest mean zone of inhibition (34 ± 3mm) which was not significantly different (P> 0.05) from curcumin (33 ± 2mm) and MZ (30 ± 2mm). Calcium hydroxide showed only contact inhibition. The time kill assay showed a time dependent action of each medicament. Conclusions. All tested agents except calcium hydroxide demonstrated significant reduction of viable bacteria at the time periods. The intracanal medicaments tested brought about a time dependent antibacterial effect on E.faecalis

Key words: Chlorhexidine, Curcumin, Calcium hydroxide, Metronidazole, Propolis, Intracanal medicament, Root canal failure

INTRODUCTION

Cleaning and shaping of the root canal system is crucial for the success of root canal treatment. While shaping is primarily achieved by the instrumentation process, cleaning is brought about by chemical adjuncts in the form of irrigants and intracanal medicaments [1].Routine intracanal treatment procedures (biomechanical preparation) may not adequately eliminate bacteria from the complex anatomical features of the root canal system [1].In order to ensure further reduction in microbiota, intracanal medicaments need to be used between appointments [2].

The persistence of microorganisms may be considered the primary cause of root canal failure. The ability of *Enterococcus faecalis* (*E.faecalis*) to penetrate into the dentinal tubules and resist bactericidal substances has been claimed to be the reason for this organism to be implicated in persistent root canal infections [3].

Calcium hydroxide is one of the most commonly used intracanal medicaments. Owing to the alkaline pH, this material has a wide anti microbial spectrum [4].However *E.faecalis* has been shown to be resistant to the actions of calcium hydroxide [5].This has let to widespread research in endodontics looking for an alternative intracanal medicament.

Chlorhexidine gluconate (2%) has been recommended as a potential alternative to calcium hydroxide. This broad spectrum antibacterial agent is able to destroy gram positive and gram negative microbes. Metronidazole (2%) has been shown to be superior to calcium hydroxide in inhibiting *E.faecalis* [6].Natural remedies are increasingly finding their way into endodontic treatment with agents like *Morinda citrofolia* [7], triphala [8], curcumin [9] and propolis [10] being evaluated as irrigants and intracanal medicaments. Curcumin (diferuloylmethane), the main yellow bioactive component of turmeric has a wide spectrum of biological actions, including antimicrobial, anti-inflammatory and antioxidant activities. Its antibacterial activity against *E.faecalis* has been documented [9]. Propolis is basically composed of flavonoids, phenolics and aromatic compounds [10]. The objective of this study was to determine the antimicrobial activity of propolis, curcumin, 2% chlorhexidine gel, 2% metronidazole gel and a combination of chlorhexidine and metronidazole. Calcium hydroxide served as the control. The purpose of this study is to show that the antimicrobial activity of the experimental agents was not significantly different from that of calcium hydroxide.

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MATERIALS AND METHOD

This *in vitro* study evaluated the antibacterial activity of the five test agents; group 1- propolis (30% in methyl cellulose), group 2-curcumin (2.5mg/mL in methyl cellulose), group 3- 2% chlorhexidine gel (CHX, Biodinamica, Ibipora, Parana, Brazil), group 4-2% metronidazole gel (in methyl cellulose, MZ), group 5-combination of 2% CHX and 2% MZ. Calcium hydroxide paste (Ultracal XS, Ultradent, South Jordan UT, USA) was used as the control.

Cultures of *E.faecalis* (ATCC 29212) were maintained at 37°C on nutrient agar (Hi –Media labs, Bangalore, India) under aerobic conditions. Colonies of bacteria were seeded into 10 mL Brain Heart Infusion (BHI) broth (Hi-Media labs) and grown over night at 37°C. Following incubation, the cultures were centrifuged and the cell pellet was washed in Phosphate Buffered Saline before re-suspending in fresh BHI. The final optical density was adjusted to 0.5 McFarland (10^8 CFU/mL).

The microbial suspension (0.1 mL) was inoculated on petri plates with 20 mL of Muller Hinton agar (Hi-Media labs).Wells of 5mm depth and 4mm in diameter were punched in the agar plates following which 30 µL of the test material was used to fill each of these wells. The plates were maintained at room temperature for 2 hours and then incubated for 48 hours at 37°C at atmosphere of 80% nitrogen, 10% hydrogen and 10% carbon dioxide. The zone of inhibition (mm) was measured by the shortest distance from the outer margin of the well to the initial point of microbial growth. Six replicates were made for each group. The data was analyzed using ANOVA and Tukey's test (P=0.05)

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the test agents against *E.faecalis* was evaluated using the tube dilution test. One mL aliquots of *E.faecalis* was taken from the stock culture and added to the sterile capped test tubes of the experimental groups. Following incubation at 37°C , turbidity was evaluated at 0, 1, 24, 48 and 72 hours time periods. The test tubes were examined for visible signs of bacterial growth. The highest dilution (lowest concentration) which showed a change in turbidity equal to or less than 0.050 was considered as MIC.

Bacterial cultures from the tubes with concentrations of the medicaments equal to or higher than the MIC were streaked on blood agar plates and incubated for 48 hours. The lowest concentration of agent that had no visible bacterial colonies in the agar plate at this time was recorded as the MBC [11].

The rate of bactericidal activity was assessed by the time kill test. *E. faecalis* was used to prepare suspensions of 10^6 CFU/ml in BHI broth. The agents at the concentrations of 2 x MBC and 4 x MBC were added to the suspensions. The aliquots were removed at three time periods (1,15,30,45 and 60 min) after the beginning of the experiment and plated onto BHI agar.CFU were counted after incubation for 24-48 hours. The broth without any agents was used as a control for bacterial growth at each time point. Testing was performed in triplicate. Differences in viable bacterial count at each time point were analyzed by one-way analysis of variance (ANOVA) and post hoc Tukey test (P=0.05)

RESULTS

Agar diffusion test

The mean zones of inhibition of the tested medicaments is presented in Table 1.The largest mean zone of inhibition (34 ± 3mm) was shown by group 3 (CHX), followed by group 2 (curcumin; 33 ± 2mm) and group 4 (MZ; 30 ± 2 mm).There was no significant difference in the mean zone of inhibition between group 2, 3, 4 (P>0.05). The zones of inhibition of these three groups was significantly higher than the control group (P<0.05).The mixture of chlorhexidine and metronidazole demonstrated a significantly smaller mean zone of inhibition than either chlorhexidine or metronidazole alone (P<0.05).Propolis had no significant difference when compared to the control group (P>0.05).

Table 1. Zones of inhibition (mean ± S.D) of the intracanal medicaments against *E.faecalis*

Group	Zone of inhibition (mm)
Propolis	8 ± 2 [a]
Curcumin	33 ± 2 [b]
Chlorhexidine gel	34 ± 3 [b]
Metronidazole gel	30 ± 2 [b]
CHX + MZ	18 ± 2 [c]
Calcium hydroxide	6 ± 2 [a]

Mean values labeled with different superscript letters were significantly different from the calcium hydroxide control (ANOVA and Tukey test) at the 5% level

Tube dilution test

The MIC and MBC values for the test groups have been shown in Figure 1.Calcium hydroxide was not inhibitory in the MIC and MBC assays against *E.faecalis*.

Time kill assay

The time kill assay was performed at 5 time periods for two concentrations. At the 1 min and 15 min interval, all groups except calcium hydroxide, propolis brought about significant reduction in viable cell counts compared to the broth control. Groups treated with 2 x or 4x CHX or MZ brought about a significantly higher reduction of viable bacterial counts (P<0.05).The difference between curcumin (2x and 4x), CHX+MZ (2x and 4x) was not significantly different at 1 min (P>0.05), while at 15 min, curcumin (2x and 4x) was significantly better than the other groups (P<0.05) with the exception of 2x and 4x CHX (P>0.05).At the 15 minute interval, 2x and 4x curcumin and CHX brought about 100% bacterial reduction. With regards to MZ and CHX + MZ, after 15 min, the 2x or 4x concentration showed no significant improvement in reduction of bacterial counts (P>0.05). Propolis (2x and 4x) at 45 min and 60 min brought about significant reduction of bacterial cell counts as compared to the same material in the remaining time periods. Calcium hydroxide did not bring about 100% elimination of bacterial cells at any of the time periods tested and at all times, the cell count reduction brought about by calcium hydroxide was significantly lower than the test groups (P<0.05), with the exception of 1 and 15min where it was significantly different from propolis (P>0.05).

DISCUSSION

The present study evaluated the antibacterial activity of six medicaments. The results of this study showed a significant potential for natural remedies to be a part of root canal treatment. All experimental agents except propolis and CHX+MZ showed significantly better antibacterial activity than calcium hydroxide. Hence the null hypothesis must be partially rejected. Calcium hydroxide is ineffective against *E.faecalis*, primarily owing to the ability of the organism to serve as a proton pump inhibitor [12].In addition to this, in the clinical scenario, calcium hydroxide is unable to maintain its high pH inside the dentinal tubules allows *E.faecalis* to persist and replicate within the anatomical eccentricities of the root canal system [12].

Based on the results of the agar diffusion test, CHX and curcumin had the strongest antibacterial activity against *E. faecalis* followed by metronidazole, CHX/metronidazole mixture and propolis. Calcium hydroxide showed direct contact inhibition only. The poor performance of calcium hydroxide may also be explained by the fact that, owing to its high pH, the medicament may precipitate on the agar and thereby reduce its diffusion [13].Hence, the results of agar diffusion tests should be viewed with caution. To confirm the results of the same, a tube dilution test was performed. This was based on previous recommendations [14,15].Calcium hydroxide had no inhibitory effect on *E.faecalis* at any dilution. This is in accordance with previous reports [6,16,17].

Chlorhexidine gel (CHX) demonstrated the highest mean zone of inhibition in this study. The efficacy of CHX against *E.faecalis* is in accordance with other reports [6,18,19]. Chlorhexidine gluconate is a broad-spectrum antimicrobial agent and is effective against bacteria and fungi. Its action is related to the binding of the cationic molecule to the negatively charged bacterial cell walls, thereby altering the cell's osmotic equilibrium [13].A major advantage of chlorhexidine is its sustantivity, which allows prolonged residual antimicrobial effect. Another advantage is that it does not produce resistant microorganisms. Although several concentrations of CHX have been tested, 2% has been shown to be the most efficient [6].An interesting finding of this study was that, although CHX and metronidazole independently brought about considerable antibacterial effect, the combination (both with effective 2% concentration) failed to show a significant difference compared to the control. Both CHX and metronidazole have been shown to exhibit free radical mediated mechanisms of bacterial killing [6].It is possible that the actions of one compound is inhibited by the other when in combination. This needs further research.

The antibacterial activity of curcumin is similar to a recent report [9].The antibacterial activity of curcumin maybe attributed to its ability to eliminate the extracellular polysaccharide matrix of *E.faecalis*. The present study showed that curcumin was effective against *E. faecalis* at a relatively low concentration of 625 µg/mL. This appears to be the first study to compare curcumin and propolis with contemporary root canal medicaments. The antibacterial activity of propolis was significantly lesser than all other agents (except the control) used in this study. This is in accordance with previous reports [10,20,21].However, the aforementioned report demonstrated that propolis was significantly better than calcium hydroxide in its antibacterial activity, in contrast to the results of the present study .This could be attributed to methodological variables with the aforementioned work, which used dentin powder for reporting the antibacterial activity and hence it may not be possible to directly extrapolate the results to the present work. In addition to this, variables like anatomical factors of the root canal system can result in varied results in microbiological studies.

Further research is needed to evaluate the effects of these medicaments on root canal biofilms.

CONCLUSIONS

Chlorhexidine (2%) and curcumin demonstrated the maximum efficacy against *E.faecalis* followed by 2% metronidazole. The antibacterial activity of propolis and a mixture of chlorhexidine and metronidazole was comparable to calcium hydroxide. Curcumin may hold promise as an alternative to contemporary intracanal medicaments in pediatric endodontics.

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