Original Article

Antimicrobial efficacy of clindamycin, linezolid, and calcium hydroxide as root canal medicaments on tubular infection against *Enterococcus faecalis* biofilm: An *in vitro* study

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ABSTRACT

Aim: The aim of the study was to evaluate and compare the antimicrobial efficacy of linezolid, clindamycin, and calcium hydroxide ($Ca(OH)_2$) as an intracanal medicament on *Enterococcus faecalis* biofilm.

Methods: Fifty-six root blocks obtained from extracted single-rooted human teeth were decoronated, and the apical part of the root was removed to obtain a 6 mm cylinder of radicular dentin. The specimens were standardized for diameter, infected with microorganisms, and randomly divided into four groups. linezolid, clindamycin, $Ca(OH)_2$, and methylcellulose (control) were placed in the root canal for 7 days. Dentin shavings were collected from 200 to 400 μm depth, and bacterial load was assessed by counting colony-forming units. Scores were statistically analyzed using the Kruskal–Wallis ANOVA and the Mann–Whitney test.

Results: Linezolid and clindamycin had better antibacterial effects than control at both 200 μm and 400 μm depth after 7 days.

Conclusion: Linezolid and clindamycin outperformed $Ca(OH)_2$ in reducing bacteria and were equally efficient against *E. faecalis* but showed no significant differences in antimicrobial efficacy.

Keywords: Calcium hydroxide, clindamycin, *Enterococcus faecalis*, linezolid

INTRODUCTION

As root canal infections are biofilm mediated, the treatments are focused on making canals radiographically perfect, desiring for debridement of complex root canal systems. Microorganisms in the oral cavity could spread throughout the body and cause systemic disease and also colonize the root canal system, causing inflammation and lysis of periradicular tissues. *Enterococcus faecalis* has a significant pathogenic role in secondary infections by surviving even in the unfavorable conditions of treated root canal and being a treatment-resistant microorganism, against intracanal endodontic medicament by its traits such as its capacity to compete with other microorganisms, penetrate dentinal tubules, and withstand nutritional deprivation.[1]

Intracanal medicaments are used to treat endodontic conditions such as bacteria resistant to conventional therapy, and treatment cannot be completed due to the presence of pain or persistent exudate.[2] Calcium hydroxide ($Ca(OH)_2$) is one of the most commonly used first-line intracanal medicaments for necrotic cases with established infections, having a pH value ranging between 12.5 and 12.8. Due to its highly alkaline, tissue-dissolving, and antimicrobial properties, many studies have demonstrated its ability to reduce bacterial load.[3,4]

In addition to endodontic therapy, antibiotics can be administered systemically, locally, and prophylactically.

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Antibiotics can be used for prophylactically in specific cases such as systemic complications, rapid infection spread, and prevention of secondary infections. Areas of the root canal system that are inaccessible to irrigants and the mechanical cleaning procedures within the canal may contain pathogens. To decrease the number of viable bacteria, an antibiotic contained in an intracanal medicament must be able to disperse into these areas allowing the improved periapical healing response.[5]

Clindamycin and linezolid have shown promising antibacterial efficacy against *E. faecalis*. Clindamycin is used as a systemic antibiotic to treat acute infections and flare-ups and is effective against a variety of endodontic pathogens.[6] It has a bacteriostatic effect and serves as a temporary dressing.[7]

Linezolid is a synthetic antibiotic of the oxazolidinone group which has shown good activity against Gram-positive organisms, including vancomycin-resistant *E. faecalis*.[8] Since Clindamycin and linezolid have antibacterial efficacy against *E. faecalis*, this study evaluated its viability in root canal dentine. Null hypothesis of the study assumed no statistically significant difference in the antimicrobial efficacy of linezolid, clindamycin, and $Ca(OH)_2$ against *E. faecalis*.

Sample size estimation was done using G*Power sample size estimation software (version 3.1.9.7;Heinrich-Heine-Universitat Dusseldorf, Dusseldorf, Germany).

Formula for sample size estimation:

$$n = 2\ Sp^2\ [Z_{1-\alpha/2} + Z_{1-\beta}]^2/\mu_d^2$$

Sp = Pooled standard deviation, μd = Mean difference.

Since there are 4 groups in the study, the final minimum sample size is 56 (14 per group).

METHODOLOGY

Dentin specimen preparation

Fifty-six extracted single-rooted human permanent teeth with mature apices were selected. Using a diamond disc (Dentsply Sirona Inc.) attached to a slow-speed micromotor handpiece (NSK Ltd Japan) under water cooling, the crown portion of the tooth beneath the cementoenamel junction and the apical region of the root was cut. Using cylindrical diamond burs (Mani. Inc.) attached to a high-speed handpiece (NSK Ltd Japan), cementum was removed from the root surface to standardize the external diameter of the sample to approximately 4 mm. Using this technique, it was possible to achieve 6 mm of radicular dentin cylinders from the middle third of the root. A round ISO no 014 bur (Mani Inc.,) was used to standardize the internal diameter of the root canal space.

The dentin specimens were immersed in an ultrasonic bath (Ultrasonic Bath, Vector 55, Jeltraft, Jelenko) of 17% ethylenediaminetetraacetic acid (EDTA) (Dent Wash; Prime Dental Products PVT. Ltd) (pH = 7.8) for 5 min followed by 5.25% sodium hypochlorite (NaOCl) for 5 min and then in diluted water for 10 min, to eliminate both organic and inorganic debris. Inoculation of *E. faecalis* biofilm specimens was transferred to individual microcentrifuge tubes containing 1 mL of tryptone soya broth (TSB) (Himedia Mumbai, India), and then they were placed in an autoclave for 20 min at 121°C temperature and 15 psi pressure for sterilization of the specimens. To ensure sterilization, all specimens were incubated at 37°C for 24 h. To achieve dentinal tubule contamination and biofilm formation, 0.5 mL of *E. faecalis* (ATCC 21292) inoculum, which is equivalent to 0.5 McFarland standard, was transferred aseptically to previously sterilized individual microcentrifuge tubes that contained 1 mL of fresh TSB (Himedia, Mumbai, India) and sterile dentin blocks. Each procedure was completed in a laminar flow environment. For 21 days, the samples were incubated at 37°C. Every 2 days, the previous media was replaced with fresh media.

The purity of the cultures was evaluated using the Gram test. Scanning electron microscopy was used to assess the development of bacterial biofilm into dentinal tubules in two specimens after the contamination period.[1]

Preparation of intracanal medicament

A homogeneous 20 mg/mL of clindamycin was obtained by combining 20 mg of clindamycin powder with 1 mL of distilled water, 40 mg of methylcellulose, and mixing for 2 h using a magnetic stir bar 0.33 g of linezolid (Himedia Mumbai, India) powder was mixed with 1 mL of distilled water. $Ca(OH)_2$ was mixed with sterile saline in a 1:1 ratio by volume, and methylcellulose (SDFCL Mumbai, India) (40 mg/mL) was used as a control group.

Treatment of contaminated specimens

The specimens were rinsed with 1 mL of sterile saline for 10 min after the incubation period to eliminate the incubation broth.

Following that, two layers of nail varnish were applied to the outer surface of the specimens. The specimens were divided into four groups randomly and provided with

intracanal medications in each group (14 specimens per group).

Group 1: Control (methylcellulose), Group 2: $Ca(OH)_2$ (1:1), Group 3: linezolid 0.3%, and Group 4: clindamycin 2%.

The procedure was conducted at room temperature under strict aseptic conditions in the biological safety cabinet (Bioclean Air Devices, Chennai, Tamil Nadu, India), to avoid contamination from environmental microbes. Lentulospiral No. 25 was used to carry the intracanal medicaments into the canals and to simulate the clinical condition, paraffin wax was used to seal both ends of dentinal blocks. The specimens were then incubated for 1 week at 37°C and 100% humidity. After 7 days, the paraffin wax was removed, and sterile normal saline was used to irrigate the root canals. With Gates Glidden drills No. 4 and No. 5, dentin shavings were collected at two depths that are at 200 μm and 400 μm, respectively.

The dentin shavings obtained by each Gates Glidden drill were immediately and separately collected in microtubes filled with 1 mL of TSB and vortexed for maximum leaching of bacteria from the samples which were then incubated for 24 h at 37°C. After 24 h, the contents of each tube were serially diluted for 10 times with 1 mL of broth in 9 mL of sterile saline. On brain–heart infusion agar plates, 50 μL of the dilution was spread, and these plates were then incubated for 24 h at 37°C. The colony-forming units (CFUs) were counted using a digital colony counter. The purity of cultures was checked by Gram staining and subculturing on the agar plate.

Statistical analysis was done by the Kruskal–Wallis ANOVA test and the Mann–Whitney test.

RESULTS

There was a statistically significant reduction in the mean number of CFUs of *E. faecalis* per milligram of dentin shavings after 1 week of using medicaments at both 200 μm and 400 μm ($P < 0.005$). All the medicaments used significantly had better antibacterial effects than the control group at both 200 μm and 400 μm depth [$P < 0.005$, Tables 1 and 2].

Noticeably, at 200 μm, $Ca(OH)_2$ showed higher mean CFU than 400 μm. However, at 200 μm, $Ca(OH)_2$ showed a better antibacterial effect than the control group. The antimicrobial effect of linezolid and clindamycin 2% was statistically similar to each other at both 200 μm and 400 μm. At 200 μm, $Ca(OH)_2$ showed reduction by 25%, whereas linezolid and clindamycin showed an overall reduction of 75%. At 400 μm, $Ca(OH)_2$, linezolid, and clindamycin showed an almost complete reduction of microorganisms.

Table 1: Comparison of four groups with colony forming unit ($\times 10^2$) in 200 μm by Kruskal–Wallis ANOVA

Groups	Mean	SD	Median	Quartile range	Mean rank
Group 1	42.5	25.2	51.0	33.0	13.50
Group 2	26.0	19.5	28.5	26.0	10.25
Group 3	0.3	0.5	0.0	0.5	5.13
Group 4	0.3	0.5	0.0	0.5	5.13
H			9.7933		
P			0.0204*		

*$P<0.05$. SD: Standard deviation

Table 2: Comparison of four groups with colony-forming unit ($\times 10^2$) in 400 μm by Kruskal–Wallis ANOVA

Groups	Mean	SD	Median	Quartile range	Mean rank
Group 1	2.5	2.1	2.5	3.0	13.00
Group 2	0.0	0.0	0.0	0.0	7.00
Group 3	0.0	0.0	0.0	0.0	7.00
Group 4	0.0	0.0	0.0	0.0	7.00
H			10.2531		
P			0.0165*		

*$P<0.05$. SD: Standard deviation

DISCUSSION

Root canal therapy depends on root canal disinfection with coronal and apical seals along with the significant role of intracanal medicaments.[9] In the current study, the methodology for assessing the efficacy of endodontic medicaments in the disinfection of dentinal tubules was adopted from the study done by Zargar *et al*.[10]

In vitro model used in this study with some modifications was similar to the one developed by Haapasalo and Orstavik. Human dentin blocks were preferred to accurately mimic clinical situations.[11] The mid-root dentin blocks were utilized as an invasion of the coronal and mid-root dentin occurs more readily at this level.[12] *E. faecalis* is the most resistant microorganism in root canal microbiota, present in 63% of teeth with posttreatment deterioration.[13]

In the present study, *E. faecalis* biofilm was cultured for 21 days since it has been hypothesized that it is less responsive to antibiotics and more closely resembles the clinical environment. An incubation period of 3 weeks allowed the formation of bacterial clusters surrounded by a carbohydrate matrix. In addition, it is more resistant to disinfectants as compared to younger biofilm.[13,14]

The cementum was removed to prevent *E. faecalis* from infecting dentinal tubules.[15] Valderhaug[16] found that if the

cementum layer is damaged, microorganisms can enter the tooth through dentin. In contrast, studies suggested that eliminating cementum is essential for controlled infection. Teeth were placed in an ultrasonic bath containing EDTA and NaOCl to remove the smear layer. According to the literature, lower concentrations of NaOCl are ineffective against *E. faecalis*. Therefore, a concentration of 5.25% NaOCl was employed in this study. Nail varnish was applied to seal tubule openings. The results of the present study revealed that new intracanal medicaments are effective against *E. faecalis*. To compare the effectiveness of diluted medications in creating a sterile root canal system, clindamycin was tested against linezolid and $Ca(OH)_2$. The medicaments used were standardized by weight as they were commercially available.

After data analysis, all three medicaments significantly reduced *E. faecalis* colony count. However, clindamycin and linezolid could eliminate *E. faecalis* in 1 week. Some studies claim that compared to other intracanal medications including tetracycline, doxycycline, chlorhexidine, and propolis, clindamycin has a more potent antibiofilm action.[17]

Methylcellulose was employed as a carrier, enabling the controlled release of medicaments to prolong their therapeutic effects and half-lives.[18]

The present study indicated that at 200 and 400 μm depth, 20 mg/mL of clindamycin demonstrated a substantial antibiofilm effect (reduced viable bacteria by over 75% and 100% at 200 μm and 400 μm, respectively). Clindamycin has been used for decades as a broad-spectrum antibiotic.[19] In certain studies, clindamycin was suggested as a first-line treatment for odontogenic infections and local infections such as adult periodontitis and periapical infection since the therapeutic concentration is typically maintained at the infection site.[20]

Contrary to local administration, systemic use of clindamycin is linked to some gastrointestinal side effects, including diarrhea and pseudomembranous colitis.[10] The result of the present study was similar to the study of Zargar *et al*. on the comparison of the antimicrobial activity of clindamycin with $Ca(OH)_2$; clindamycin was effective in eliminating *E. faecalis*. Contrary to the findings of the present study, Molander *et al*. found that clindamycin has no advantage over conventional root canal medications in terms of antibacterial activity.[21]

According to the present study, $Ca(OH)_2$ has an antibiofilm effect at 200 μm, without complete elimination of the biofilm, whereas it was significantly different from the control group at a depth of 400 μm. $Ca(OH)_2$ has a poor antimicrobial effect on *E. faecalis* due to the buffering mechanism of dentin which might reduce its pH by making it ineffective against *E. faecalis,* whereas *E. faecalis* maintains pH homeostasis by transporting protons to the inner side of the cell, acidifying its cytoplasm in elevated alkalinity. Along with that it has limited solubility and diffusibility, making it difficult to enter dentinal tubules.[22]

Supporting past findings about the resistance of *E. faecalis* to $Ca(OH)_2$, this investigation showed that it had little to no effect on the microorganism. The results obtained from the present study are in line with those of investigations by Kandaswamy *et al*.[13],Saber and El-Hady *et al*.[17], Vasudeva *et al*.[15], and Gomes *et al*.[23]

Linezolid demonstrated stronger antibacterial activity against *E. faecalis* than $Ca(OH)_2$ which may be a result of the different mechanisms of action of the two medicaments. To exert its effect, linezolid binds to the 23S subunit of the 50S subunit, which prevents the development of the 70S ribosome complex, which is necessary for the initiation of protein synthesis. However, mutation of the ribosome-binding site leads to enterococcal resistance to the linezolid. It also has side effects such as nausea, tongue discoloration, oral moniliasis, taste perversion, diarrhea, headaches, and myelosuppression.[8,24,25]

The findings from this study showed that the linezolid group and the clindamycin group had no significant variance in CFU after 7 days at 200 μm depth. The CFU generated in the $Ca(OH)_2$ group after 7 days, had higher mean values than other medicament groups at 200 μm but no significant difference at 400 μm depth. All three medicament groups showed better antibacterial efficacy at 400 μm depth, with no significant difference in CFU.

In the present study, clindamycin provided 75% inhibition of *E. faecalis* at depths of 200–400 μm after 1 week. The possible reason could be the bactericidal dosage of 2% and 0.3% increased diffusion of the medicament into the dentinal tubules.

Pavaskar *et al*.[8] reported that linezolid has 14-day antimicrobial efficacy against *E. faecalis*, while $Ca(OH)_2$ wanes after 72 h. On average, complete inhibition of *E. faecalis* at both depths (200 and 400 μm) was observed with linezolid, followed by 75% reduction with clindamycin at 200 μm and almost complete inhibition at 400 μm. However, $Ca(OH)_2$ had an overall reduction of only 25% at 200 μm.

From this study, we found linezolid and clindamycin to be promising in eliminating Enterococcus faecalis in comparison with $Ca(OH)_2$. The intraoral environment of a diseased root canal could not be replicated in an *in vitro* study. During the procedure, it was impossible to standardize the quantity of dentinal shavings examined, drill time, and heat produced. The intracanal medicament that is effective against one microorganism *in vitro* may not be necessarily effective against the same microorganism *in vivo* due to necrotic tissues and tissue fluids which may reduce the activity. The longest period a drug was left in the canal throughout this experiment was only 7 days. To comprehend how drugs function as an antibacterial against *E. faecalis,* further research in this area is necessary. However, caution should be taken when using antibiotics to prevent the emergence of resistance. This study provides a basis for future investigations to assess the implications of linezolid and clindamycin formulations on biofilm organisms.

Limitations of the study

The intraoral environment inside a diseased root canal could not be replicated in this *in vitro* study. Despite all efforts to deliver quantified amounts of microorganisms and intracanal medications, it was not possible to standardize some factors, including the quantity of dentinal shavings examined per sample, the time required to drill a punch hole, and the amount of heat produced during the procedure. A single microorganism was used to infect the root canal. Endodontic infections are multimicrobial; therefore, interactions between different organisms may have different dynamics than those seen in this study and also the intracanal medicament that is effective against one microorganism *in vitro* may not be necessarily effective against the same microorganism *in vivo*.

CONCLUSION

Linezolid and Clindamycin demonstrated a significant reduction of bacterial count against *E. faecalis* followed by $Ca(OH)_2$. Linezolid was equally efficient as compared to clindamycin against *E. faecalis*.

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Conflicts of interest

There are no conflicts of interest.

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