Original Article

Antimicrobial effect of an oxazolidinone, lantibiotic and calcium hydroxide against *Enterococcus faecalis* biofilm: An *in vitro* study

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Abstract

Aims: The aim was to evaluate and compare the antimicrobial efficacy of an oxazolidinone (linezolid [LZ]), lantibiotic (nisin), and calcium hydroxide against *Enterococcus faecalis* biofilm formed on tooth substrate after 2 and 7 days. **Methods:** Single rooted human mandibular premolars were decoronated, biomechanically prepared, and vertically sectioned along the midsagittal plane to obtain a standardized tooth substrate. Standardized suspension of *E. faecalis* and tooth substrate was incubated for 3 weeks to allow growth of biofilm. At the end of 3 weeks, the grouping was done according to the medicament used – Group I - LZ, Group II - nisin, Group III - calcium hydroxide, Group IV - negative treatment. Disk of the medicaments used were prepared and placed upon Petri dishes along with bacterial emulsion on Mueller-Hinton agar. The zones of inhibition were checked after 2 and 7 days. **Statistical Analysis Used:** The scores were statistically analyzed using Tukey honest significant difference test and one-way analysis of variance. **Results:** Zone of inhibition obtained with LZ was widest followed by nisin and calcium hydroxide after a period of 2 days ($P < 0.001$). The size of the zone of inhibition remain unchanged for LZ and nisin group after 7 days ($P > 0.001$) unlike calcium hydroxide group where the zone decreased ($P < 0.001$). **Conclusion:** LZ showed maximum antimicrobial potential against *E. faecalis* biofilm followed by nisin and calcium hydroxide after 2 and 7 days. The antimicrobial effect of LZ and nisin was not affected with the lapse of time, but that of calcium hydroxide significantly decreased.

Key words: Biofilm, *Enterococcus faecalis*, linezolid, nisin

INTRODUCTION

Bacterial invasion of the root canal system is crucial for the onset and persistence of periapical disease. Thus, the main objective of endodontic treatment is to kill microorganisms in the root canal system. *Enterococcus faecalis* is a nonspore-forming, fermentative, facultative anaerobe, Gram-positive coccus, recovered in a high proportion of endodontic failure cases and in approximately one-third of root canal treated teeth with persistent lesions.[1,2]

The survival and virulence factors that make *E. faecalis* a very resistant species is its ability to invade dentinal tubules, survive as a monoculture without the support of other bacteria and ability to endure prolonged periods of nutritional deprivation. *E. faecalis* grows by forming biofilms which is an adaptive process.[3] Bacteria sequestrated in biofilms are shielded and are often harder to kill than their free-floating or planktonic counterparts.[4]

During root canal therapy biomechanical preparation and root canal shaping effectively reduce microbiota, but do not completely eliminate bacteria in the lateral and accessory root canals, isthmi, and apical deltas.[5] Studies have reported that intracanal medication between appointments reduce bacteria from the complexities of root canal system.[6]

Over the years, calcium hydroxide has been the most widely used intracanal medicament in endodontic practice. Various studies have shown that *E. faecalis*

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resists the highly alkaline environment produced by the calcium hydroxide dressing.[7,8] Hence, an alternative medicament for eradicating *E. faecalis* from complex root canals is required to achieve successful endodontic treatment.

Nisin and linezolid (LZ) are the two new intracanal medicaments which have shown promising results in previously conducted studies against *E. faecalis* biofilm.

Linezolid has gained popularity on the basis of its wide spectrum of activity against Gram-positive organisms, including vancomycin-resistant *E. faecalis*. LZ is a synthetic antibiotic belonging to a new class of antimicrobials called the oxazolidinones. LZ disrupts bacterial growth by inhibiting the initiation process in protein synthesis.[9]

Nisin, a naturally occurring antimicrobial peptide, is produced by Streptococcus lactis subspecies lactis. It has antimicrobial activity against a wide range of Gram-positive bacteria and their spores,[10] even against drug-resistant *E. faecalis* isolates.[11] Its use in dentistry has so far been limited.

Till date to our knowledge, no study in the literature has been reported to compare the antimicrobial efficacy of LZ, nisin and calcium hydroxide against *E. faecalis* biofilm.

Thus, the aim of this study was to evaluate and compare the antimicrobial efficacy of LZ, nisin, and calcium hydroxide against *E. faecalis* biofilm formed on tooth substrate after 2 and 7 days.

METHODS

Specimen preparation

Forty extracted single-rooted human mandibular premolars free from cracks and caries were selected for the study. The teeth were gently scraped externally with 5.25% sodium hypochlorite by using the sterile gauge to remove debris. The teeth were then gently washed with distilled water and stored in 0.1% thymol until used. The crowns of all the teeth were sectioned with diamond disk (Carbodent; Gysi S.A, Buenos Aires, Argentina) below cementoenamel junction to obtain a standardized working length of 10 mm. Biomechanical preparation was done using Hand ProTaper with apical preparation till size F3 file (Dentsply-Maillefer, Ballaigues, Switzerland). The irrigation after each change of instrument was done using 2 ml of 3% sodium hypochlorite. The prepared samples were vertically sectioned along the midsagittal plane into two halves. The concave surface of the samples was minimally grounded to achieve a flat surface to enable *E. faecalis* to form a biofilm on the exposed root canal surfaces. The samples were then kept in an autoclave (Indfos Laboratory Autoclave) at 121°C for 20 min at 20 psi pressure to ensure adequate sterilization.

Biofilm preparation

Enterococcus faecalis (ATCC 29212) suspension was prepared by adding pure culture of *E. faecalis* grown in Mueller-Hinton broth. The suspension was standardized to achieve an optical density (OD) of 0.78 with the help of spectrophotometer (ultraviolet-visible [UV-Vis] Spectrophotometer 117, Systronics) at a wavelength of 580 nm. *E. faecalis* suspensions along with tooth samples were incubated at 37°C for a period of 3 weeks to allow the formation of biofilm. The culture medium was replaced every alternate day to avoid nutrient depletion and accumulation of toxic end products. The bacterial samples, incubated in Mueller Hinton agar, were taken every alternate day with a sterile paper point at 37°C for 24 h to check for cell viability and purity of the culture. At the end of 3 weeks, two samples were processed to confirm the presence of *E. faecalis* biofilm by scanning electron microscopy.

Preparation of bacterial emulsion

At the end of 3 weeks, the biofilm was scraped from the tooth substrate. Bacterial emulsion was prepared by adding the scraped biofilm to 0.9% NaCl solution and was standardized using OD method (OD - 0.78) with the help of spectrophotometer (UV-Vis Spectrophotometer 117, Systronics) at wavelength of 580 nm.

Application of medicaments

Grouping was done according to the type of medicament used against the biofilm. Group I (*n* - 10) - LZ (Linospan, Cipla Ltd., Mumbai central, Mumbai, India), Group II (*n* - 10) - nisin (Bimal Pharma Pvt., Ltd., Malad (E), Mumbai, India), Group III (*n* - 10) - calcium hydroxide (Prevest Denpro Ltd., 38 Industrial Estate, Digiana, Jammu, India), and Group IV (*n* - 10) - negative treatment.

Disk of the medicaments used were prepared by dipping discs of Whatman Filter paper no. 1 into a solution of a medicament with concentration of 0.33 g of medicament per ml of distilled water. The disks of medicaments were placed upon Petri dishes with prepared bacterial emulsion and were incubated at 37°C on Mueller-Hinton agar.

After a period of 2 days, Petri dishes of five samples of each group were checked for the zone of inhibition.

The procedure was repeated after 7 days for remaining five samples of each group. The scores for zone of inhibition were recorded, tabulated and subjected to statistical analysis using one-way analysis of variance and Tukey honest significant difference test. The software used was SPSS version 15.0 (Statistical Package for Social Sciences, Chicago).

RESULTS

Linezolid group [Figure 1] gave the widest zone of inhibition followed by nisin [Figure 2] and calcium hydroxide [Figure 3] both after 2 and 7 days ($P < 0.001$). The antimicrobial efficacy against *E. faecalis* biofilm remain unchanged for LZ and nisin group after 7 days ($P > 0.001$) whereas in calcium hydroxide group, the zone of inhibition decreased after 7 days ($P < 0.001$).

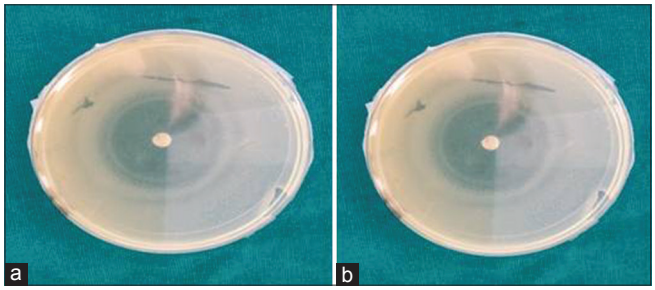

Figure 1: (a) Zone of inhibition produced by oxazolidinone (linezolid [LZ]) after 2nd day. (b) Zone of inhibition produced by oxazolidinone (LZ) after 7th day

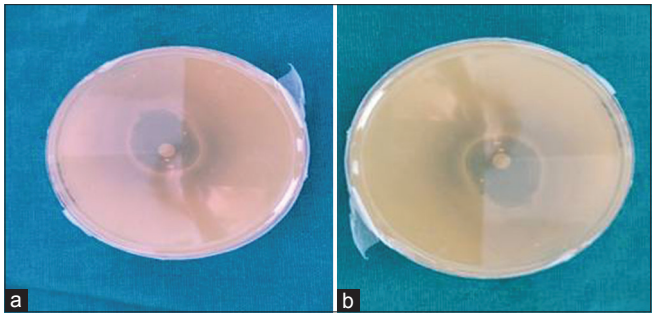

Figure 2: (a) Zone of inhibition produced by lantibiotic (nisin) after 2nd day. (b) Zone of inhibition produced by lantibiotic (nisin) after 7th day

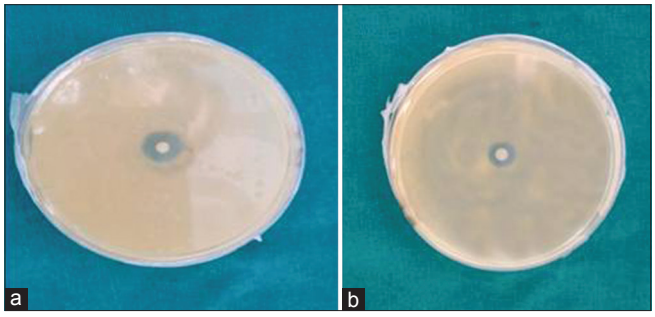

Figure 3: (a) Zone of inhibition produced by calcium hydroxide after 2nd day. (b) Zone of inhibition produced by calcium hydroxide after 7th day

DISCUSSION

Enterococcus faecalis is a pathogenic microorganism, frequently found in endodontic infections, especially in secondary and persistent lesions. It can deeply penetrate into dentinal tubules and has potential to form biofilms that resist intracanal medicaments commonly used in endodontic procedure.

In our study, *E. faecalis* biofilm was grown for 21 days as it has been reported that 21 days matured biofilm is less vulnerable to antimicrobials and better simulates the clinical scenario.[12] Biofilm was grown on the tooth substrate as biofilm-forming capacity and structural organization of *E. faecalis* are influenced by the chemical nature of the substrate.[13] McBain *et al*. reported that biofilm experiments conducted on polycarbonate or glass substrate does not provide a true indication of the bacteria-substrate interaction.[14]

The concentration of the medicaments used in this study was much higher than their minimum inhibitory concentration considering the fact that sessile bacteria on the surfaces or present within biofilm are much less readily inactivated than planktonic cells.[13]

In our study, the negative treatment group showed no zone of inhibition. In calcium hydroxide group zone of inhibition formed had significantly lower values in comparison to nisin and LZ group, both after 2 and 7 days [Table 1]. The antibacterial properties of calcium hydroxide are attributed to its high alkalinity and its ability to destroy the cytoplasmic membrane, denature bacterial proteins, and damage bacterial DNA.[15] *E. faecalis* passively maintains pH homeostasis through a proton pump that makes it resistant to calcium hydroxide. The proton pump of *E. faecalis* carries protons to the interior of the cell, acidifying its cytoplasm, in situations of increased alkalinity in its environment when treated with calcium hydroxide.[7]

Table 1: Mean, SD of zones of inhibition of different groups after 2 and 7 days

Groups	Zones of inhibition (mean±SD)	
	2 days	7 days
Group I	16±1.00$^{II, III}$	16±1.00$^{II, III}$
Group II	10±1.22$^{I, III}$	10±1.22$^{I, III}$
Group III	3.80±0.84$^{I, II,*}$	2.60±0.55$^{I, II,*}$

$^{I, II, III}$Significant difference with that group in that column, *Significant intragroup comparison (efficacy comparison at 2 and 7 days). $P<0.05$ considered as statistically significant. SD: Standard deviation

Moreover, in the clinical scenario, the dentine buffering effect might contribute toward reducing the pH of calcium hydroxide making it ineffective against *E. faecalis*. Furthermore, the low solubility and diffusibility of calcium hydroxide might further make it difficult to penetrate into dentinal tubules to exert any action.

In our study, nisin showed the greater bacterial inhibitory effect when compared to calcium hydroxide [Table 1]. This might be due to the different mode of action of nisin than that of calcium hydroxide. Nisin acts by inserting into the bacterial plasma membrane and triggering the activity of bacterial murein hydrolases which results in damage or degradation of the peptidoglycans and lysis of cells. This induces the leakage of small intracellular contents from the cell. This result is in accordance with results of a study by Hemadri *et al*. (2011),[16] although in their study planktonic suspensions of *E. faecalis* were used.

Linezolid showed greater antimicrobial efficacy against *E. faecalis* than calcium hydroxide [Table 1] which can be due to difference in the mechanism of action of the two medicaments.

Linezolid acts by preventing the formation of 70S ribosome complex which is responsible for the initiation of protein synthesis by binding to the 23S subunit of the 50S subunit.[17]

These results of our study are in accordance with results obtained by a study done by Pavaskar *et al*.[17] where planktonic suspension of *E. faecalis* was used.

When comparing LZ group with nisin group, LZ group showed better antibacterial effect both after 2 and 7 days. This might be because of difference in the mechanism of action of the two intracanal medicaments. However, to our knowledge no studies are present in current literature to corroborate and contradict the findings of our study.

The results of our study showed that there was no significant difference in the zone of inhibition formed in LZ group and nisin group after 2 and 7 days [Table 1].

Whereas the mean scores of the zone of inhibition formed in calcium hydroxide group after 7 days was of significantly lower values in comparison to 2 days [Table 1].

This can be explained by the fact that with time calcium hydroxide diffuses into agar media resulting in dissolution of calcium and hydroxyl ions which in turn decreases the pH of the media.[7] Pavaskar *et al*.[17] reported that the antimicrobial efficacy of LZ against *E. faecalis* planktonic suspension lasted for 14 days although that of calcium hydroxide declined after 72 h. Gangwar[18] reported that the inhibitory effect of calcium hydroxide preparation irrespective of the vehicle used was maximum at 24 h with slight decrease occurring at 96 h. However, at 168 h, some bacterial growth was observed.

CONCLUSION

Within the limitation of this study, it was concluded that LZ showed maximum antimicrobial potential against *E. faecalis* biofilm followed by nisin after 2 and 7 days.

Calcium hydroxide showed the least antimicrobial potential against *E. faecalis* biofilm after 2 and 7 days.

The antimicrobial effect of LZ and nisin was not affected with lapse of time, but that of calcium hydroxide decreased significantly with increasing time period.

Further studies need to be conducted to check the maximum duration for which LZ and nisin are effective against the *E. faecalis* biofilm.

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