Antibacterial Efficacy of Nisin and Calcium Hydroxide with a Proton Pump Inhibitor as an Intracanal Medicament

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ABSTRACT

Aim: To evaluate the antimicrobial efficacy of nisin and calcium hydroxide with and without pantoprazole against *Enterococcus faecalis* in comparison with chlorhexidine (CHX) 2% solution.

Materials and methods: The antibacterial effect of the following experimental groups as intracanal medicaments (group I nisin, group II Ca(OH)₂ powder 29% conc., group III Ca(OH)₂ with pantoprazole 20 mg, group IV Ca(OH)₂ with pantoprazole 40 mg, group V CHX 2% solution, and group VI saline) was evaluated using the agar diffusion test for a time period of 24 hours. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) against *E. faecalis* were also determined. Statistical analysis was performed using Kruskal–Wallis test and chi-square test.

Results: The agar diffusion test showed zones of inhibition for groups I, II, and V. Nisin and CHX groups showed the maximum zone of inhibition compared with other experimental groups. The MIC values for the experimental groups I, II, III, and V were 0.2 mg/mL, 0.45%, 0.45% + 0.03 mg/mL, and ≤ 0.01% respectively. The MBC values were tabulated.

Conclusion: The antimicrobial efficacy of nisin as an intracanal medicament was similar to CHX 2%; Ca(OH)₂ with pantoprazole was not effective against *E. faecalis*. The MIC value of nisin is 0.2 mg/mL against *E. faecalis*.

Clinical significance: Nisin, when used as an intracanal medicament, is effective in eliminating *E. faecalis* when compared with the combination of Ca(OH)₂ with pantoprazole, a proton pump inhibitor (PPIs).

Keywords: *Enterococcus faecalis*, Intracanal medicament, Nisin, Pantoprazole.

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INTRODUCTION

Enterococcus faecalis is the most common facultative anaerobic bacteria isolated from both secondary and persistent root canal infections. Studies conducted by Love,¹ Stuart et al,² and Sirén et al³ have shown that apart from the contributing factors, such as complex root canal anatomy and ineffective chemomechanical instrumentation, *E. faecalis* possesses certain virulence factors (lytic enzymes, cytolysin, aggregation substance, pheromones, and lipoteichoic acid), invades and adheres to the dentinal tubules with a depth of penetration ranging from 500 to 1000 µm, and has the ability to survive in harsh environmental conditions due to its potential to transform into the viable but noncultivable (VBNC) state.

Calcium hydroxide, commonly used as an intracanal medicament, has an effective antibacterial action against most endodontic microflora. But *E. faecalis* is resistant to the antimicrobial activity of Ca(OH)₂ due to its PPI action and its potential to withstand high alkalinity. The pH in the canal reaches neutral levels (in the presence of *E. faecalis*) on the use of Ca(OH)₂, leading to bacterial growth and survival in the root canal.³ According to Tang et al,⁴ there are three major reasons for bacterial survival and growth despite the use of calcium hydroxide dressing: (1) The capacity of some bacteria to survive in dentinal tubules and ramifications, (2) pH in the canal reaches neutral levels after a rapid use of all the Ca(OH)₂, and (3) the microleakage of the temporary filling. However, studies conducted by various authors have shown that 2% CHX gel is effective in completely eliminating *E. faecalis* from the dentinal tubules up to a period of 15 days, attributed to its substantive antimicrobial activity.^{5,6}

The search for an effective intracanal medicament aims to achieve superior disinfection of the root canal system, long-term clinical success of endodontic therapy, and to increase the strength and stability of the radicular dentin collagen. This led to the recent advances in the development of various materials that can be used as intracanal medicaments, such as Propolis, bioactive glass, ozonated water, corticosteroids, grape seed extract, octenidine, Nisin,⁷ and PPIs.

Nisin, a chemical commonly used as a food preservative (meat and dairy products), is recently recommended for use as an intracanal medicament. Discovered in 1928, it is a naturally occurring antimicrobial cationic peptide, produced by *Streptococcus lactis* subspecies *lactis*. Chemically, it is a polycyclic antimicrobial peptide with 34 amino acid residues which includes uncommon amino acids, such as lanthionine, methyllanthionine, didehydroalanine, and didehydroaminobutyric acid. It has antimicrobial activity against a wide range of Gram-positive bacteria and their spores, even against drug-resistant *E. faecalis* isolates.⁸⁻¹¹

Proton pump inhibitors are a group of drugs with the mechanism of action of pronounced and long-lasting reduction of gastric acid secretion, most commonly used for the treatment of peptic ulcer.¹² Previous studies conducted have shown that the association of omeprazole with calcium hydroxide displayed selective antimicrobial activity against Endodontic microbes.¹³ Therefore, in this study, a more potent PPI, pantoprazole, commercially available in a tablet form 20 and 40 mg, was used. In this study, the synergistic effect of pantoprazole with $\text{Ca}(\text{OH})_2$ against *E. faecalis* was investigated. The PPIs not only reduce acid secretion but also increase the sensitivity to antimicrobials, maintaining the alkaline pH.¹⁴ Hence, this study aimed at evaluating the antimicrobial efficacy of nisin and calcium hydroxide with and without pantoprazole against *E. faecalis* in comparison with CHX 2% solution.

MATERIALS AND METHODS

Bacterial Strain used in the Study

Enterococcus faecalis ATCC 29212 (American Type Culture Collection) was maintained in the Microbiology laboratory of our institution and was revived in Mueller Hinton Broth (MHB, HiMedia, India) and stored at 4°C. Fresh subcultures were made on MacConkey agar plates (HiMedia, India).

The experimental groups used in the study are tabulated in Table 1.

Preparation of the Stock Solutions

Group I—Nisin: Nisin (Bimal Pharma Pvt Ltd, Mumbai, India) dissolved in sterile injectable water at a concentration of 10 mg/mL.

Group II— $\text{Ca}(\text{OH})_2$: $\text{Ca}(\text{OH})_2$ (ProDent, Rathanagiri, India) was prepared in sterile distilled water at a concentration of 29%.

Group III— $\text{Ca}(\text{OH})_2$ + Pantoprazole (20 mg): $\text{Ca}(\text{OH})_2$ (2.9 gm) + Pantoprazole (ALKEM laboratories, India) (20 mg) were dissolved in 10 mL of sterile injectable water.

Group IV— $\text{Ca}(\text{OH})_2$ + Pantoprazole (40 mg): $\text{Ca}(\text{OH})_2$ (2.9 gm) + Pantoprazole (40 mg) were dissolved in 10 mL of sterile injectable water.

Group V—CHX 2% solution commercially available as Asep RC (Anabond Stedman Pharma Research Ltd, Chennai) was used.

Group VI—Saline was used (Nirlife, Nirma Ltd, India).

AGAR DIFFUSION ASSAY

Agar diffusion assay was done according to Clinical Laboratories Standards Institute (CLSI) guidelines.¹⁵ Using the well diffusion susceptibility test, the antibacterial efficacy was detected by challenging bacterial isolates with antibacterial agents on the wells that were created on the surface of an agar plate seeded with a lawn culture of *E. faecalis* for 24 hours (Fig. 1).

Figs 1A to F: Agar diffusion assay. (A) Nisin, (B) $\text{Ca}(\text{OH})_2$, (C) pantoprazole 20 mg + $\text{Ca}(\text{OH})_2$, (D) pantoprazole 40 mg + $\text{Ca}(\text{OH})_2$, (E) CHX 2%, (F) saline

Table 1: Experimental groups

Groups	Experimental groups
I	Nisin
II	$\text{Ca}(\text{OH})_2$ powder 29% concentration
III	Pantoprazole 20 mg with $\text{Ca}(\text{OH})_2$ powder
IV	Pantoprazole 40 mg with $\text{Ca}(\text{OH})_2$ powder
V	CHX 2% solution (positive control)
VI	Saline (negative control)

Fig. 2: Microbroth dilution assay to determine MIC

Minimum Inhibitory Concentration

Microbroth dilution assay was done to determine the MIC value of the test solutions (groups I–III & V) as per CLSI Institute guidelines.¹⁵ The analysis was performed using doubling dilutions of the test solutions. The test solutions were double serially diluted from wells 1 to 11 of each row. The last well of each row served as the culture control (no test solution was added). The assay was performed in triplicates for all the test solutions. The MIC is the lowest concentration of the test solution that completely inhibited the growth of *E. faecalis* (Fig. 2).

Minimum Bactericidal Concentration

The MBC was determined by spot inoculating onto Mueller Hilton agar plates. Plates were incubated at 37°C overnight. The absence of growth was scored as a bactericidal activity (Fig. 3).

Statistical Analysis

Statistical analysis was done using Kruskal–Wallis Test and chi-square test. There was statistically significant difference when the probability value was $p < 0.05\%$.

RESULTS

Agar Well Diffusion Assay

The antibacterial efficacy was detected by the formation of the zone of inhibition around the wells inoculated with the experimental groups. Groups I, II, and V showed inhibitory zones. The maximum diameter of 28 mm was obtained with CHX 2% at 60 μ L conc. The inhibitory zones of nisin at 40 μ L (20 mm), 50 μ L (21 mm), and 60 μ L (21 mm) were comparable to the positive control (Table 2).

Figs 3A to D: Minimum bactericidal concentration of (A) Nisin, (B) $\text{Ca}(\text{OH})_2$, (C) pantoprazole + $\text{Ca}(\text{OH})_2$, (D) CHX

Table 2: Agar well diffusion assay

Test solution	Diameter (mm) of the zone of inhibition		
	40 μ L	50 μ L	60 μ L
Nisin (10 mg/mL)	20	21	21
$\text{Ca}(\text{OH})_2$ (29%)	13	13	15
$\text{Ca}(\text{OH})_2$ (29%) + pantoprazole (20 mg)	No zone	No zone	No zone
$\text{Ca}(\text{OH})_2$ (29%) + pantoprazole (40 mg)	No zone	No zone	No zone
CHX (2%) (positive control)	24	25	28
Saline (negative control)	No zone	No zone	No zone

There was no statistically significant difference between nisin and CHX groups. Pantoprazole with $\text{Ca}(\text{OH})_2$ and saline groups showed no zone of inhibition. There was no statistically significant difference between groups III, IV, and VI (that expressed reduced antimicrobial efficacy) compared with the other experimental groups (Table 3 and Graph 1).

Minimum Inhibitory Concentration

The MIC values of the experimental groups against planktonic cells of *E. faecalis* ATCC 29212 according to this study are given in Table 4. There was no statisti-

Table 3: Statistical analysis—agar well diffusion assay

Groups	Nisin (10 mg/mL)	$\text{Ca}(\text{OH})_2$ (29%)	$\text{Ca}(\text{OH})_2$ (29%) + pantoprazole (20 mg)	$\text{Ca}(\text{OH})_2$ (29%) + pantoprazole (40 mg)	CHX (2%)	Saline
Chi-Square	2.000	2.000	0	0	2.000	0
Degree of freedom	2	2	2	2	2	2
p-value	0.368	0.368	1.000	1.000	0.368	1.000

Graph 1: Agar diffusion assay

Table 4: MIC values

Test solution	Nisin	Ca(OH) ₂	Ca(OH) ₂ + pantoprazole	CHX (2%)	Growth control
<i>E. faecalis</i> ATCC 29212	0.2 mg/mL	0.45%	0.45%, 0.03 mg/mL	≤0.01%	✓

Table 5: Statistical analysis table for MIC values

Test statistics	
MIC of experimental groups against planktonic cells of <i>E. faecalis</i> ATCC 29212	
Chi-square	0.600 ^a
Degree of freedom	3
p-value*	0.896

*p-value significant at <0.05 level; ^ap-value not considered

cally significant difference between nisin and the other experimental groups (Table 5).

DISCUSSION

The results showed that the experimental groups (I, nisin; II, calcium hydroxide; IV, CHX) exhibited antimicrobial action against *E. faecalis*. In the current study, nisin exhibited superior antimicrobial activity compared with Ca(OH)₂. This is because the antimicrobial mechanism of nisin is independent of the pH of the surrounding tissues. This provides a means to eradicate *E. faecalis* by a method to which it has no defense mechanism.⁸

In the present study, the antimicrobial effect of nisin was similar to CHX 2% solution, which was similar to the result obtained in the previous study conducted by Chinni et al.⁹ However, Turner et al¹⁶ showed that the antimicrobial activity of nisin was similar to calcium hydroxide in eliminating *E. faecalis* from radicular dentin walls. Nisin has a potent antimicrobial activity against a wide range of gram-positive microorganisms. Experiments conducted by Severina et al¹⁰ proved that nisin is less toxic, odorless, colorless, tasteless, and has low drug resistance rates compared with other similar antimicrobial

peptides. Nisin exhibits its antibacterial effect by the following mechanisms: According to Jack et al,¹⁷ it acts by inserting into the bacterial plasma membrane and triggering the activity of bacterial murein hydrolases, resulting in damage or degradation of the peptidoglycans and lysis of cells. Du Plessis et al¹⁸ reported that it is due to interaction with the phospholipid membrane of the target bacterial cell causing autolysis and irreparable damage to plasma membrane. Crandal et al¹⁹ showed that it disrupts the cellular mechanism, inducing leakage of small intracellular contents from the cell.^{7,11,16,20}

Tong et al^{20,21} evaluated the combined antimicrobial efficacy of Mixture of Doxycycline, citric acid and a detergent (Tween 80) with nisin as an intracanal medicament; the results indicated that nisin (in combination with doxycycline) improves the antimicrobial action of calcium hydroxide against pathogenic bacteria. Nisin exerts its antibacterial action by forming pores in cell membranes, disrupting cell wall synthesis, and causes rapid efflux of essential cytoplasmic small molecules. The pores made by nisin facilitate the penetration of hydroxyl ions of calcium hydroxide producing bactericidal action.

The maximum diameter of 28 mm was observed at 60 µL conc. of 2% CHX solution. The MIC value for 2% CHX solution was 0.01%. Chlorhexidine is bacteriostatic at lower concentrations and bactericidal at higher concentrations (2%), and shows the property of substantivity. Various studies have shown the antimicrobial efficacy of 2% CHX used as an intracanal medicament against *E. faecalis*.^{22,23}

In group II, 29% concentration of Ca(OH)₂ showed 13 to 15 mm zone of inhibition. The antibacterial effect

of calcium hydroxide is related to the release and diffusion of hydroxyl radicals and the velocity of its release depends on the vehicle with which it is manipulated. The ion release is retarded with a viscous vehicle and is accelerated with an aqueous vehicle.^{22,24} Hence, distilled water was used as a vehicle in this study for the preparation of the stock solution of $\text{Ca}(\text{OH})_2$ with pantoprazole.

The reduced antibacterial efficacy of $\text{Ca}(\text{OH})_2$ against *E. faecalis* in this study may be due to the difference in the methodology when compared with other experimental studies.^{25,26} The antimicrobial activity of calcium hydroxide is attributed to its alkaline pH. The mechanism of action includes the following: Inhibition of bacterial proliferation, alteration of bacterial cell wall, denaturation of endotoxin, and lipopolysaccharide. However, *E. faecalis* neutralizes the alkaline pH of $\text{Ca}(\text{OH})_2$ by its PPI action.

In the present study, pantoprazole was combined with $\text{Ca}(\text{OH})_2$ to enhance the antibacterial efficacy by inhibiting the proton pump mechanism and maintaining the alkaline pH. The agar diffusion test showed no zone of inhibition for groups III (20 mg) and IV (40 mg) of pantoprazole with $\text{Ca}(\text{OH})_2$. This may be due to the interaction between pantoprazole and $\text{Ca}(\text{OH})_2$, and pantoprazole neutralized the antibacterial action of $\text{Ca}(\text{OH})_2$.¹⁴ In previous studies by Wagner et al,¹³ the combination of omeprazole, a PPI, with $\text{Ca}(\text{OH})_2$ as an intracanal medicament showed an increased antimicrobial efficacy against *E. faecalis* producing superior healing of periapical lesions with an increase in reparative bone areas in male Wistar rats. Another *in vivo* experimental study conducted by Gandhi et al,²⁷ in which 8.5% omeprazole + 5.2% NaOCl were used as the final irrigant, the microbial samples collected after 28 days of inducing periapical lesions showed a superior bactericidal activity against *E. faecalis* and the healing of the periradicular lesions with a decrease in the colony-forming units in comparison with other irrigants, namely CHX and MTAD.

The drawbacks of this study include the following: The antimicrobial efficacy of the experimental groups was not tested on root canal biofilm samples; the antimicrobial efficacy of pantoprazole may be inhibited by the agglomerate formation when combined with calcium hydroxide and the reduced diffusion ability of the experimental stock solution when the agar diffusion assay was used for testing; the pH of the experimental groups III and IV after the combination of calcium hydroxide with 20 and 40 mg of pantoprazole should have been evaluated before the testing the antimicrobial efficacy.

However, it remains clear that further studies are required to evaluate the chemical interaction between pantoprazole and $\text{Ca}(\text{OH})_2$ and its antibacterial efficacy against *E. faecalis* at various concentrations.

CONCLUSION

The results of the present study concluded that:

- The antimicrobial efficacy of nisin as an intracanal medicament was similar to CHX 2%.
- Pantoprazole with calcium hydroxide was not effective against *E. faecalis*.
- The MIC value of nisin is 0.2 mg/mL against *E. faecalis*.

CLINICAL SIGNIFICANCE

Nisin, when used as an intracanal medicament, is effective in eliminating *E. faecalis* when compared with the combination of $\text{Ca}(\text{OH})_2$ with pantoprazole, a PPI.

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