



## In Vitro Evaluation of Antibacterial Efficacy of Nisin Calcium Hydroxide and Triple Antibiotic Paste in Three Different Vehicle

Authors

**Dr Supratim Tripathi<sup>1</sup>, Dr Pallavi Mittal<sup>2\*</sup>, Dr Sharmistha Deb<sup>3</sup>, Dr Sheetal Verma<sup>4</sup>**

<sup>1</sup>Reader, Department of Conservative Dentistry and Endodontics, Career Post Graduate Institute of Dental Sciences and Hospital, Lucknow

<sup>2,3</sup>PG Final Year Student, Department of Conservative Dentistry and Endodontics, Career Post Graduate Institute of Dental Sciences and Hospital, Lucknow

<sup>4</sup>Assistant Professor, Department of Microbiology, King George's Medical University, Lucknow

\*Corresponding Author

**Dr Pallavi Mittal**

PG Final Year Student, Department of Conservative Dentistry and Endodontics, Career Post Graduate Institute of Dental Sciences and Hospital, Lucknow, UP, India

### Abstract

*Enterococcus Faecalis* is the most common facultative anaerobic bacteria isolated from both secondary and persistent root canal infections. *E. faecalis* invades and adheres to the dentinal tubules with a depth of penetration ranging from 500 to 1000  $\mu\text{m}$ , and has the ability to survive in harsh environmental conditions due to its potential to transform into the viable but noncultivable (VBNC) state. This Paper highlights the use of Nisin, Calcium Hydroxide and Triple Antibiotic Paste each as an intracanal medicaments combined with propylene glycol, CHX and saline used as a vehicle. 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*. Propylene glycol which was suggested by Laws in 1962 has been evaluated for its possible use as a vehicle in the field of endodontics. It permits the release of calcium and hydroxyl ions essential for the therapeutic action. It has been found to be antibacterial, nonirritating to the periapical tissue. 2% CHX is effective in completely eliminating *E. Faecalis* from the dentinal tubules up to a period of 15 days attributed to its substantive antimicrobial activity. This study aims to see the combined effect of Nisin, Calcium Hydroxide and Triple Antibiotic Paste when mixed with different vehicle.

### Introduction

Bacteria remaining within the root canal are a significant factor in endodontic failures. Hence retreatment of endodontically treated teeth is a major challenge to clinicians. *Enterococcus Faecalis* is the most common facultative anaerobic bacteria isolated from both secondary and persistent root canal infections.<sup>[2]</sup> Therefore for the

achievement of sterilization and healing of infected root dentin the use of antimicrobial medicaments in combination with mechanical cleansing enhances the success of treatment.

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)<sub>2</sub> due to its PPI action and its potential to withstand high alkalinity. Studies have demonstrated that 2% Nisin, a chemical commonly used as a food preservative (meat and dairy products) is recently recommended for use as an intracanal medicament. Chemically, it is a polycyclic antimicrobial peptide with 34 amino acid residues which include uncommon amino acids, such as Ianthionine, methylanthionine, didehydro and didehydroaminobutyric acid. It has an antimicrobial activity against a wide range of gram positive bacteria and their spores, even against drug resistant *E. faecalis* isolates.<sup>[2]</sup> Triple Antibiotic paste a combination of ciprofloxacin, metronidazole and minocycline has been suggested for root canal disinfection as it contains polymicrobial infections consisting of both aerobic and anaerobic bacteria species which may not be effective when single antibiotics are used.

To facilitate its application in the field of endodontics, it is generally mixed with vehicles. The type of vehicle used has a direct relationship with the concentration and velocity of ionic liberation as well as with the antibacterial action when the paste is carried in to the contaminated area.<sup>[4]</sup>

Recently, a newly introduced vehicle, Propylene Glycol, has been evaluated for its possible use as a vehicle in the field of endodontics. It permits the release of calcium and hydroxyl ions essential for the therapeutic action. It has been found to be antibacterial, non irritating to the periapical tissue.<sup>[3]</sup>

Chlorhexidine (CHX) has been widely used in Endodontics. It has an antibacterial activity against Gram positive and Gram negative microorganisms.<sup>[1]</sup> It can be used as a vehicle because of its substantivity property, its adsorption capacity and slow liberation of active molecules by dental tissue. Chlorhexidine is effective in completely eliminating *E. faecalis* from the dentinal tubules upto a period of 15 days, attributed to its substantive antimicrobial activity.

Saline is a neutral vehicle with no antimicrobial property, only aids in easy placement of medicaments.

The Gold standard that has been documented till date for *E. faecalis* is Calcium Hydroxide + Chlorhexidine combination. Keeping in mind the biocompatibility issues this study aims to the use of three different antimicrobial agents, Nisin, Chlorhexidine and Triple Antibiotic paste which strongly condom the propogation of *E. faecalis* in association of three different vehicle, Propylene Glycol, Chlorhexidine and saline with increased substantivity and sustained release of the medicaments.

### **Aim and Objectives**

The aim of the study is to evaluate the antibacterial efficacy of Nisin, Calcium Hydroxide & Triple Antibiotic Paste in three different vehicles, Chlorhexidine, Propylene Glycol and Saline. Chlorhexidine + Calcium Hydroxide being the positive control.

### **Materials and Methods**

The study will be conducted in the Department of Conservative Dentistry and Endodontics, Career Post Graduate Institute of Dental Sciences and Hospital, Lucknow.

One hundred and eighty extracted fully mature human permanent maxillary central incisors teeth with single canal were included in this study. They were cleaned and debrided for the removal of calculus. After debridement, teeth will be stored in 0.1% thymol solution until use.

Samples were decoronated upto length of 7 mm. The working length determination was done by 10K file until it was just visible at the apical foramen and keeping it short by 1 mm. Chemomechanical debridement of root canal was done for all samples. Prepared samples will be divided into two groups comprising of eight experimental group and one control group. Experimental groups were further subdivided in to following groups:

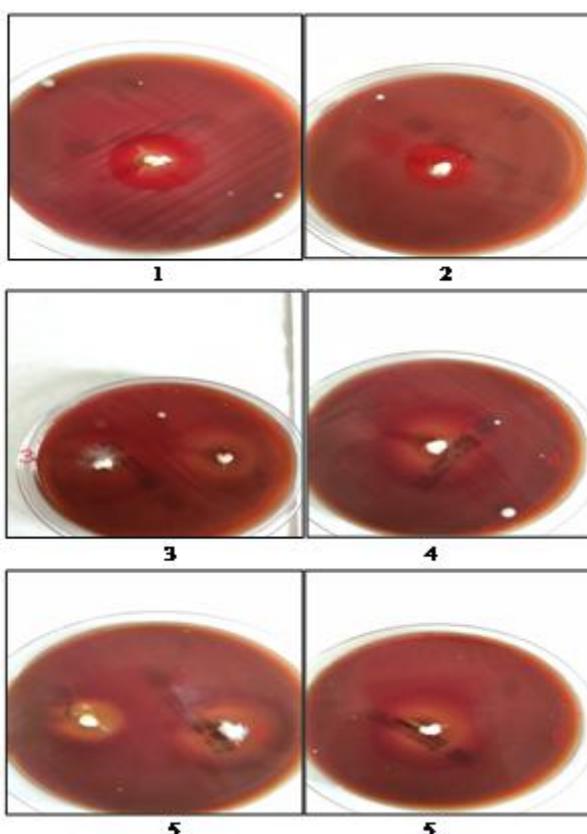
| Experimental Subgroups | Medicaments                                |
|------------------------|--|
| Subgroup A             | Calcium Hydroxide + Propylene Glycol       |
| Subgroup B             | Calcium Hydroxide + Saline                 |
| Subgroup C             | Triple Antibiotic Paste + Propylene Glycol |
| Subgroup D             | Triple Antibiotic Paste + Chlorhexidine    |
| Subgroup E             | Triple Antibiotic Paste + Saline           |
| Subgroup F             | Nisin + Propylene Glycol                   |
| Subgroup G             | Nisin+ Chlorhexidine                       |
| Subgroup H             | Nisin + Saline                             |

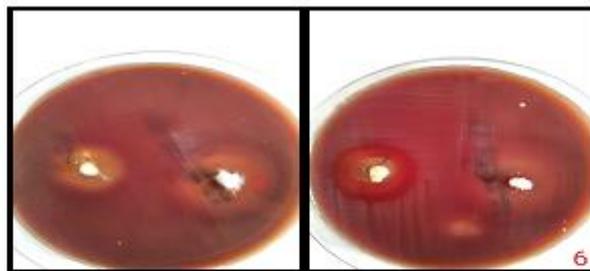
| Positive Control group | Medicament                        |
|------------------------|-----------------------------------|
| Subgroup I             | Calcium Hydroxide + Chlorhexidine |

All the prepared samples were sterilized and incubated. Bacterial lysis was done in Blood sheep agar broth. The reaction to the medicaments was done for a time period of 24hrs, 48 hrs, 72 hrs

and 168 hrs. After every time slot the extent of lysis has administered and the readings were subjected to statistical analysis.

|                                   | 24 Hrs | 48 hrs | 72 hrs | 1 week |    | 24 hrs | 48 hrs | 72 hrs | 1 week |    | 24 hrs | 48 hrs | 72 hrs | 1 week |
|-----------------------------------|--------|--------|--------|--------|----|--------|--------|--------|--------|----|--------|--------|--------|--------|
| NISIN+PG (A1)                     | 22     | 25     | 30     | 30     | A2 | 20     | 22     | 25     | 26     | A3 | 20     | 20     | 25     | 25     |
| TAP + CHX (B1)                    | 20     | 18     | 18     | 18     | B2 | 18     | 18     | 18     | 18     | B3 | 18     | 20     | 20     | 20     |
| NISIN+ SALINE (C1)                | 15     | 16     | 20     | 20     | C2 | 15     | 16     | 20     | 20     | C3 | 15     | 16     | 20     | 20     |
| CA(OH) <sub>2</sub> + PG (D1)     | 19     | 19     | 19     | 19     | D2 | 18     | 19     | 19     | 19     | D3 | 19     | 19     | 19     | 19     |
| CA(OH) <sub>2</sub> + CHX (E1)    | 20     | 20     | 22     | 22     | E2 | 20     | 20     | 25     | 25     | E3 | 20     | 20     | 25     | 25     |
| CA(OH) <sub>2</sub> + SALINE (F1) | 20     | 22     | 25     | 25     | F2 | 22     | 25     | 22     | 22     | F3 | 25     | 25     | 25     | 25     |
| NISIN + CHX (G1)                  | 18     | 18     | 18     | 18     | G2 | 21     | 22     | 22     | 22     | G3 | 20     | 22     | 22     | 22     |
| TAP + PG (H1)                     | 25     | 25     | 25     | 25     | H2 | 21     | 23     | 22     | 22     | H3 | 20     | 22     | 22     | 22     |
| TAP + SALINE (I1)                 | 18     | 18     | 20     | 20     | I2 | 18     | 20     | 22     | 22     | I3 | 15     | 15     | 22     | 22     |





After 1 Week Inhibitory Zones of Different Group

1-NISIN + PG, 2- TAP + CHX, 3- NISIN + SALINE, 4- Ca(OH)<sub>2</sub> + PG, 5-Ca(OH)<sub>2</sub> + CHX, 6-Ca(OH)<sub>2</sub> + SALINE, 7- NISIN + CHX, 8- TAP+ PG, 9- TAP+ SALINE.

**Results**

Statistical software IBM SPSS statistics 20.0 was used for the analysis of the data and Microsoft word and Excel were used to generate graphs, tables.etc. Statistical analysis was done using Kruskal–Wallis Test and Shapiro Wilk’s test. There was statistically significant difference when the probability value was  $p < 0.05$ .

wells inoculated with the experimental groups. All the groups I, II, III, IV,V,VI,VII,VIII,IX showed inhibitory zones. The maximum diameter of 30 mm (1week) was obtained with Nisin when used with propylene glycol as a vehicle. The inhibitory zones of nisin at 24 hrs (22 mm), 48 hrs (25 mm), and 72 hrs (25 mm) were comparable to the positive control.

**Agar Well Diffusion Assay**

The antibacterial efficacy was detected by the formation of the zone of inhibition around the

**Table 1:** Intra group & Intergroup Comparisons

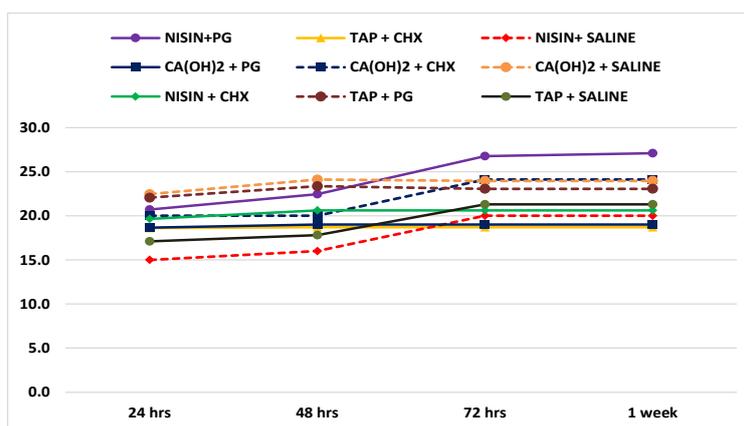
| Material                  |      | 24 hrs | 48 hrs | 72 hrs | 1 week | Friedman Test |         |
|---------------------------|------|--------|--------|--------|--------|---------------|---------|
|                           |      |        |        |        |        | chi sq        | p-value |
| NISIN+PG                  | Mean | 20.70  | 22.45  | 26.75  | 27.10  | 57.10         | <0.001  |
|                           | SD   | 0.98   | 2.09   | 2.45   | 2.22   |               |         |
| TAP + CHX                 | Mean | 18.60  | 18.70  | 18.70  | 18.70  | 0.230         | 0.972   |
|                           | SD   | 0.94   | 0.98   | 0.98   | 0.98   |               |         |
| NISIN+ SALINE             | Mean | 15.00  | 16.00  | 20.00  | 20.00  | 60.00         | <0.001  |
|                           | SD   | 0.00   | 0.00   | 0.00   | 0.00   |               |         |
| CA(OH) <sub>2</sub> + PG  | Mean | 18.65  | 19.00  | 19.00  | 19.00  | 21.00         | <0.001  |
|                           | SD   | 0.49   | 0.00   | 0.00   | 0.00   |               |         |
| CA(OH) <sub>2</sub> + CHX | Mean | 20.00  | 20.00  | 24.10  | 24.10  | 60.00         | <0.001  |
|                           | SD   | 0.00   | 0.00   | 1.41   | 1.41   |               |         |
| CA(OH) <sub>2</sub> +     | Mean | 22.45  | 24.10  | 23.95  | 23.95  | 14.06         | 0.003   |

|                     |         |        |        |        |        |       |        |
|---------------------|---------|--------|--------|--------|--------|-------|--------|
| SALINE              | SD      | 2.09   | 1.41   | 1.47   | 1.47   |       |        |
| NISIN + CHX         | Mean    | 19.65  | 20.60  | 20.60  | 20.60  | 39.00 | <0.001 |
|                     | SD      | 1.31   | 1.96   | 1.96   | 1.96   |       |        |
| TAP + PG            | Mean    | 22.05  | 23.35  | 23.05  | 23.05  | 35.06 | <0.001 |
|                     | SD      | 2.26   | 1.31   | 1.47   | 1.47   |       |        |
| TAP + SALINE        | Mean    | 17.10  | 17.80  | 21.30  | 21.30  | 58.37 | <0.001 |
|                     | SD      | 1.41   | 2.07   | 0.98   | 0.98   |       |        |
| Kruskal Wallis Test | Chi-Sq. | 140.79 | 139.55 | 143.58 | 145.58 |       |        |
|                     | p-value | <0.001 | <0.001 | <0.001 | <0.001 |       |        |

The Kruskal Wallis test of intergroup comparison shows significant differences among various groups at 24 hrs, 48 hrs, 72 hrs and 1 week (p<0.001 for each). At 1 week the maximum mean score was 27.10±2.22 seen for the NISIN+PG while minimum 18.70±0.98 for the TAP+CHX.

According to Friedman Test, the intragroup comparison revealed that with time maximum

changes occurred in NISIN+Saline and Ca(OH)<sub>2</sub>+CHX (chi sq=60.0, p<0.001) which just followed by TAP+Saline (chi sq=58.37, p<0.001) and NISIN+PG (chi sq=57.10, p<0.001) while no significant change with time was found in TAP+CHX (chi sq=0.230, p=0.972)



The Line Graph: Intergroup comparison [Graph 1]

**Discussion**

*E. faecalis* is among the most antibiotic-resistant bacteria known at present. *E. faecalis* has the ability to quickly acquire and disseminate antibiotic resistance genes by pheromone signals produced within the genus and species as well as by other bacterial genera.<sup>[14]</sup> The primary aim of endodontic treatment is to minimize the surgical intervention and maintain health and function of infected teeth by biomechanical preparation of the root canal (cleaning, shaping and disinfection) and to hermetically seal it with no discomfort to the patient, and provide conditions for the periradicular tissues to heal. In the current study, Nisin exhibited superior antimicrobial activity

compared with Ca(OH)<sub>2</sub> and TAP when used along with Propylene Glycol as a vehicle. 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. Experiments conducted by Severina et al proved that nisin is less toxic, odorless, colorless, tasteless, and has low drug resistance rates compared with other similar antimicrobial peptides.<sup>[2]</sup> Nisin exhibits its antibacterial effect by the following mechanisms: According to Jack et al, 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 al.<sup>[2]</sup> 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 al showed that it disrupts the cellular mechanism, inducing leakage of small intracellular contents from the cell.

Tong et al.<sup>[14]</sup> showed by adding Nisin to MTAD (mixture of Doxycycline, citric acid and detergent) shows increased effectiveness against *E. Faecalis* biofilm. Nisin exerts its antibacterial action by disrupting cell wall synthesis, forms pores in cell membrane and causes rapid reflux of essential cytoplasmic small molecules.

The successful use of Ca(OH)<sub>2</sub> paste as a root canal dressing is related to its dissociation into two ions, Ca<sup>+</sup> and Hydroxyl ions in which hydroxyl ion is responsible for alkalinizing the environment. The high PH of Ca(OH)<sub>2</sub> attenuate the inflammatory process, and its release of hydroxyl ions makes it an excellent antibacterial agent.<sup>[37]</sup> Lage-Marques et al.<sup>[28]</sup> concluded that calcium hydroxide pastes in aqueous and viscous vehicles are more effective than those in oily vehicles because the former reach higher pH levels more quickly and remain stable for a longer period of time. Other authors have also reported<sup>[21]</sup> the greater effectiveness of aqueous and viscous vehicles. Similarly to previous reports, this study evidenced that saline- and propylene glycol-containing pastes diffuse well through dentin, as can be seen in Tables 1, 2 and

The concern of the antibiotic paste is that it may cause bacterial resistance. TAP was proved to be biocompatible and contains both bactericidal (metronidazole, ciprofloxacin) and bacteriostatic (minocycline) agents to allow for successful revascularization. Metronidazole is a nitroimidazole compound that exhibits a broad spectrum of activity against protozoa and anaerobic bacteria. Minocycline is a semisynthetic derivative of tetracycline with a similar spectrum of activity. Ciprofloxacin, a synthetic fluoroquinolone, has a bactericidal mode of

action. Metronidazole and ciprofloxacin can generate fibroblasts. TAP can help promote functional development of the pulp-dentin complex.<sup>[25]</sup>

The delivery of calcium hydroxide powder is alone is difficult or impossible so it must be mixed with a liquid to facilitate its placement within the canal. Calcium hydroxide when mixed with vehicles has shown the potential to release calcium and hydroxyl ions through cementum. The rate of dissociation into ions and diffusion through dentinal tubules is determined by the vehicles used. Thus, vehicles play an important role in the ionic dissociation.

Propylene glycol (1,2-propanediol), is a dihydric alcohol. It was suggested by Laws in 1962 for its possible use as a vehicle in endodontics. Propylene glycol is a colorless liquid with mild acrid smell and sweet taste. Olitzky (1965) stated that concentrated solution of propylene glycol showed marked germicidal efficiency. Hence, it can be used as a vehicle as it has the potential for preventing and treating microbial infections. Bhat and Walkevar, Thomas et al.<sup>[39,40]</sup> stated that as compared to other commonly used vehicles for intracanal medicaments propylene glycol has been found to be less cytotoxic and also possess antibacterial properties that are highly beneficial in the endodontic treatment. Fava and Saunders said that propylene glycol possesses hygroscopic properties which allow absorption of water. This resulted in a sustained release of the intracanal medicament for prolonged period of time.<sup>[41]</sup>

Chlorhexidine has been used extensively in endodontics for irrigation and as an intracanal medicament and is considered as the gold standard.<sup>[5]</sup> It is a cationic biguanide and an antiseptic which has substantive properties and even at higher concentrations has very low toxicity. At low concentration, it is bacteriostatic, whereas at high concentration, it is bactericidal and shows antimicrobial activity from concentrations as low as 0.1%; and shows bactericidal activity at 2% and is biocompatible. The MIC of chlorhexidine started from 0.019%

against *P. gingivalis* to 0.078% against other facultative anaerobes with the highest concentration against *E. faecalis*, i.e., 0.156%.

All the above selected vehicles do possess antimicrobial activity along with different intracanal medicaments. No significant difference with time was found in TAP + CHX, whereas Nisin + PG shows maximum mean score at 1 week as compared to other groups.

The agar well diffusion method or well plate method or the agar diffusion method was used in this study as it is the most commonly used method of antimicrobial activity determination especially of newer substances like plant extracts, new drug formulations, etc. This technique is a well-accepted way of comparing the antibacterial effect of different dental materials, medicaments, etc.<sup>[45]</sup> Agar well diffusion was used for the combination of antimicrobial drugs with vehicles as though E-test being the latest method, it is not feasible for combination of drugs.

### Conclusion

Within the limits of this invitro study, it can be concluded that Nisin was effective at eradicating *E. faecalis* cells in pure culture and was comparable with calcium hydroxide, triple antibiotic paste in elimination of *E. faecalis* from within the root canal system when used with propylene glycol, chlorhexidine and saline as a vehicle.

### Clinical Significance

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

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