A Comparative Analysis of Novel Intracanal Medicaments & Chlorhexidine Against E Faecalis - An Invitro Study

Running title: Comparative Analysis of Novel Intracanal Medicaments & Chlorhexidine Against E Faecalis

Authors' full names and authors’ degrees and honorifics:

Dr. Ramesh Chandra MDS, Dr. Supratim Tripathi MDS, Dr Ankita Mehrotra MDS, Dr Mariyam Khan MDS, Dr. Nurez Anwar MDS, Dr. Aditya Bhagat BDS,

Authors’ institutional affiliations: Career post graduate institute of dental Sciences and hospital

Corresponding contributor: Aditya Bhagat, Department of Endodontics, Career post graduate Institute of Dental Sciences and hospital, lucknow, pin - 226013

No financial support was received.
No conflict of interest: Manuscript has been read and approved by all the authors, the requirements for authorship as stated earlier in this document have been met, and each author believes that the manuscript represents honest work.

Abstract:

Introduction: The success of root canal therapy heavily relies on the thorough disinfection of the root canal system, which necessitates the use of an intra-canal medicament. Persistent/secondary intracanal infection associated with endodontic treatment failure is often attributed to Enterococcus faecalis, the most found species. With the developing resistance among the microorganisms, new strategies for eradication are needed.

Aim: The present study was designed with an aim to evaluate whether a naturally occurring antibiotic “nisin” can be used as a potent intra canal medicament in combination with frequently used intra canal medicaments for eradication of E. faecalis from intra canal space

Objective: The present study compares the antibacterial effect of various combinations of Nisin with Metronidazole, with Calcium Hydroxide with Chlorhexidine and antibacterial effect of 2 % Chlorhexidine alone against E Faecalis.

Materials and Methods: In the present study, the effectiveness of four antibacterial materials, 10% Nisin with 5% Metronidazole (i.e Group I), 10% Nisin with Calcium Hydroxide (i.e Group II), 10% Nisin with 2% Chlorhexidine (i.e Group III), and 2% Chlorhexidine (i.e Group IV), were taken. The antibacterial efficacy of these medicaments against Enterococcus faecalis was assessed in vitro using disc diffusion method.

Results: Group IV 2% Chlorhexidine exhibited maximum inhibition of bacterial growth followed by Group III 10% Nisin with 2% Chlorhexidine, Group II 10% Nisin with Calcium Hydroxide and least by Group I 10% Nisin with 5% Metronidazole.

Conclusion: Group IV 2% Chlorhexidine was the most effective medicament against E. Faecalis compared to other combinations but nisin also holds a promising potential against Enterococcus faecalis."

1. Introduction

The development and continuation of dental diseases in the tooth pulp and surrounding tissues heavily rely on the activity of bacteria and their byproducts. Therefore, effectively removing the microorganisms is a crucial goal in endodontic treatment. Among different treatment approaches, chemo mechanical preparation is considered essential for reducing the bacterial load and facilitating the healing of periapical tissues. (1) Enterococcus faecalis is more frequently detected in cases of treatment failure compared to primary infections. (2) Starvation significantly enhances the resistance of E. faecalis, making it 1000 to 10,000 times more resilient.(3) It is likely that the physiological condition of cells, especially in retreatment cases, closely resembles the starvation phase.(4) One possible explanation for persistent endodontic infections could be the retention of microorganisms within the dentinal tubules of the root canal.(5) The ability of Enterococcus faecalis to penetrate these tubules and resist bactericidal substances is believed to contribute to its involvement in persistent root canal infections.

Proper shaping of the root canal system is crucial for successful root canal treatment, but it alone does not eliminate bacteria. While shaping is primarily achieved through instrumentation, cleaning is facilitated by chemical substances such as irrigants and intracanal medicaments. These adjuncts create a favorable environment for the repair of periapical tissues. (6) However, routine biomechanical preparation procedures may not effectively eliminate bacteria from the intricate anatomical features of the root canal system. (7) Calcium hydroxide is a commonly used intracanal medicament due to its broad antimicrobial spectrum resulting from its alkaline pH. (8) However, Enterococcus faecalis has shown resistance to the effects of calcium hydroxide. (9) As a result, extensive research in endodontics has focused on finding alternative intracanal medicaments. One potential alternative is chlorhexidine gluconate.
(2%), a broad-spectrum antibacterial agent capable of destroying both gram-positive and gram-negative microbes. Metronidazole (2%) has also demonstrated superiority over calcium hydroxide in inhibiting Enterococcus faecalis (10).

Natural remedies are increasingly being explored for their use in endodontic treatment. Agents such as Morinda citrifolia, triphala, curcumin, and propolis are being evaluated as irrigants and intracanal medicaments. Curcumin, the primary bioactive component of turmeric, possesses a wide range of biological actions, including antimicrobial, anti-inflammatory, and antioxidant activities. Nisin, a natural antimicrobial peptide produced by lactic acid bacteria, has received considerable attention as a bacteriocin. (11) It can effectively penetrate biofilms and create pores in bacterial membrane cells. (12) Nisin has demonstrated effectiveness against single-species biofilms of gram-positive organisms found in catheters, wounds, and food. (13) Moreover, it has shown promising results against oral pathogens, (12) including those involved in root canal infections. High-purity nisin (>95% purity) has been found to be more potent than low-purity nisin (2.5%) against multispecies oral biofilms. (11)

2. 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 grown on MacConkey agar plates (HiMedia, India). (Fig.1)

2.1 Preparation of the Medicaments:

2.1.a. Preparation of Nisin:
10% Nisin was prepared by diluting 0.1gm of pure form nisin in 1 ml of sterile water.

2.1.b. Preparation of Metronidazole:
Metronidazole (5%) was prepared by diluting 400 mg metronidazole in 8 ml of sterile water.

2.1.c. Preparation of Calcium Hydroxide:
Commercially available calcium hydroxide was selected for the study.

2.1.d. Preparation of Chlorhexidine:
Commercially available 2% Chlorhexidine was selected for the study.

The experimental groups used in this study were as follows:
1) Group I: 10% Nisin with 5% Metronidazole,
2) Group II: 10% Nisin with Calcium Hydroxide,
3) Group III: 10% Nisin with 2% Chlorhexidine,
4) Group IV: 2% Chlorhexidine.

2.2 Method:

2.2.a. AGAR DIFFUSION ASSAY
The agar diffusion assay was performed according to the guidelines of the Clinical Laboratories Standards Institute (CLSI) guidelines. Using the disc diffusion susceptibility test, the antibacterial efficacy was detected by challenging bacterial isolates with antibacterial agents impregnated on the disc placed on agar plates seeded with a lawn culture of E. faecalis at 24, 48, and 72 h. The disc impregnated with the antibacterial agents were placed on MHA agar plates and incubated at 37 °C. The zone of inhibition around each disc was measured in millimeters and tabulated at the end, 24, 48, and 72 h.

2.2.b. Statistical analysis:
The data was statistically analyzed using one-way ANOVA test and multiple comparisons among different groups were analyzed using the post hoc Tukey’s test. The level of significance was set at P<0.05.

3. Results:
The zone of inhibition was measured in millimeters using a Hi Media Hi - Antibiotic zone scale at three different time intervals: 24 hours, 48 hours, and 72 hours after the application of Antibiotic of Group I, Group II, Group III and groups IV (10% Nisin + 5% Metronidazole, 10% Nisin + Calcium Hydroxide, 10% Nisin + 2% Chlorhexidine, 2% Chlorhexidine respectively) on the agar plates inoculated with E. faecalis. (Fig. 2). The diameters of the zones of inhibition were recorded for each group and time point, and the results are presented in Table 1. (Fig. 3)
The results show that at the 24-hour time interval, the mean inhibition of E. Faecalis was maximum (15.30±1.34) in group 4, 2% Chlorhexidine followed by the group 3, 10% Nisin + 2% Chlorhexidine (14.90±2.69) while it was minimum in group 1, 10% Nisin + 5% Metronidazole (9.30±2.00). The significant difference was found in mean inhibition of E. Faecalis among the groups (p<0.001). Hence groups can be arranged according to inhibition of E. Faecalis as: Group 4 > Group 3 > Group 2 > Group 1

Similarly, at the 48-hour time interval, the mean inhibition of E. Faecalis was maximum in group 4, 10% Nisin + 2% Chlorhexidine (16.80±1.55) followed by the group 2, 10% Nisin + Calcium Hydroxide (15.50±1.43) while it was minimum in group 1, 10% Nisin + 5% Metronidazole (10.10±1.85). The significant difference was found in mean inhibition of E. Faecalis among the groups (p<0.001). Hence groups can be arranged according to inhibition of E. Faecalis as: Group 4 > Group 2 > Group 3 > Group 1

At 72 hrs, the mean inhibition of E. Faecalis was maximum in group 4, 2% Chlorhexidine (16.30±1.34) followed by the group 3, 10% Nisin + 2% Chlorhexidine (15.70±2.16) while it was minimum in group 1, 10% Nisin + 5% Metronidazole (10.50±1.65). The significant difference was found in mean inhibition of E. Faecalis among the groups (p<0.001). Hence groups can be arranged according to inhibition of E. Faecalis as: Group 4 > Group 3 > Group 2 > Group 1

<table>
<thead>
<tr>
<th>E. Faecalis</th>
<th>After 24hrs (mm) Mean</th>
<th>SD</th>
<th>After 48 Hrs Mean</th>
<th>SD</th>
<th>After 72 Hrs Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>9.30</td>
<td>2.00</td>
<td>10.10</td>
<td>1.85</td>
<td>10.50</td>
<td>1.65</td>
</tr>
<tr>
<td>Group 2</td>
<td>14.60</td>
<td>1.43</td>
<td>15.50</td>
<td>1.43</td>
<td>15.60</td>
<td>1.43</td>
</tr>
<tr>
<td>Group 3</td>
<td>14.90</td>
<td>2.69</td>
<td>15.30</td>
<td>2.41</td>
<td>15.70</td>
<td>2.16</td>
</tr>
<tr>
<td>Group 4</td>
<td>15.30</td>
<td>1.34</td>
<td>16.30</td>
<td>1.34</td>
<td>16.80</td>
<td>1.55</td>
</tr>
</tbody>
</table>

ANOVA F=21.30, p<0.001 F=24.57, p<0.001 F=26.85, p<0.001

Table 1: Diameters of the zones of inhibition were recorded for each group and time point.
4. DISCUSSION

Metronidazole exhibits greater efficacy against obligate anaerobic bacteria compared to aerobic and facultative anaerobic bacteria [14]. However, contrary to previous understanding, the present study demonstrates its antibacterial effects on E. faecalis, a facultative anaerobic bacterium. Anaerobic bacteria possess electron transport components, such as ferrodoxin, which possess a negative redox potential capable of donating electrons to metronidazole. This single electron transfer generates highly reactive nitro radical anions, which eliminate susceptible organisms through a radical-mediated mechanism targeting DNA and other biomolecules [14].

To completely eradicate E. faecalis, an increase in metronidazole concentration may be necessary [15]. However, in these studies, 1% metronidazole gel proved ineffective against facultative anaerobes despite its activity against strict anaerobes [16,17,18]. While metronidazole gel demonstrated effectiveness against all tested obligate anaerobes, it did not surpass the efficacy of calcium hydroxide overall.

Previous studies have indicated the superior resistance of E. faecalis to metronidazole. For instance, an in vitro study by Siqueira et al. (1996) observed the effectiveness of 10% metronidazole gel against all obligate anaerobes but found no effectiveness against facultative anaerobes, including E. faecalis.

In another in vitro study conducted by Krithika Datta et al. (2007) [19], it was reported that a 2% metronidazole gel achieved an overall reduction of approximately 86.5% in E. faecalis growth at depths of 200 and 400 micrometers. The white odorless powder known as calcium hydroxide has a pH range of 12.5-12.8 and exhibits bacteriostatic properties in its pure powder form. Originally developed by B.W. Herman as a pulp capping agent in 1920, it has since found applications in various endodontic procedures such as intracanal medicaments, endodontic sealers, apexification, and pulpotomies [20].

The effectiveness of a medicament in creating zones of inhibition depends on its toxicity against the tested bacteria and its ability to diffuse through the medium. Medicaments that rely on extreme pH values, such as acids or bases, may have their activity affected by the buffering capacity of artificial media. Due to its low solubility, calcium hydroxide does not diffuse easily and therefore takes a long time to raise the pH of the culture medium. However, in a clinical setting, the buffering capacity of blood, tissue fluids, and dentin may have similar effects.

Our study revealed that there was no increase in the antimicrobial effect of calcium hydroxide with longer exposure periods. Although calcium hydroxide has a pH of 12.5, the buffering effect of dentin makes it unlikely that such a high pH is maintained within the dentinal tubules, allowing E. faecalis to survive and multiply [21,22]. Our findings align with these observations.

Calcium hydroxide proves ineffective against E. faecalis primarily due to the organism's ability to act as a proton pump inhibitor [23]. Evans et al. demonstrated that the proton pump activity of E. faecalis confers resistance to the high pH of calcium hydroxide. The penetration of E. faecalis deep into the tubules is attributed to the bacterial adhesin Ace [24].

Furthermore, in the clinical context, calcium hydroxide fails to maintain its high pH within the dentinal tubules, allowing E. faecalis to persist and multiply in the intricate anatomy of the root canal system [23]. The poor performance of calcium hydroxide may also be attributed to its tendency to precipitate on the agar, reducing its diffusion. E. faecalis exhibited increased resistance to calcium hydroxide compared to nisin.

Several reasons have been proposed to explain E. faecalis' ability to survive intracanal treatment with calcium hydroxide [25]:

- E. faecalis passively maintains pH homeostasis through ion penetration across the cell membrane and cytoplasmic buffering capacity.
• E. faecalis possesses a proton pump that actively regulates pH homeostasis by pumping protons into the cell to lower the internal pH.

• To achieve a pH of 11.5 or higher, E. faecalis cannot survive. However, considering the buffering capacity of dentin, it is highly unlikely to maintain a pH of 11.5 using current calcium hydroxide techniques.

Chlorhexidine (CHX) is a positively charged bisguanide compound with broad antimicrobial activity, low toxicity to mammals, and a strong affinity for binding to dentin and mucous membrane. It interacts with negatively charged groups on the cell surface, leading to irreversible loss of cytoplasmic constituents, membrane damage, and enzyme inhibition. Ionic interactions likely occur between the positively charged CHX molecules and the negatively charged extracellular matrix.

In this study, CHX gel demonstrated the highest average zone of inhibition, consistent with other reports on its efficacy against E. faecalis.

One notable advantage of chlorhexidine is its substantivity, which means it has a prolonged residual antimicrobial effect. Additionally, it does not promote the development of resistant microorganisms. Among various concentrations tested, 2% CHX has shown the highest efficiency. (26)

Basrani et al. [27] found that 2% chlorhexidine gel exhibited superior antimicrobial action compared to 0.2% chlorhexidine gel or calcium hydroxide mixed with 0.2% chlorhexidine. Another study showed that a 10-minute irrigation with 2% chlorhexidine before root canal obturation completely eliminated E. faecalis [28]. The sustained antibacterial activity of 2% chlorhexidine gel was observed from the first day of medication [29], which aligns with the findings of this study. Furthermore, Basrani et al. [30] reported lower contact angles in preparations containing chlorhexidine, facilitating better diffusion into the tubules.

Considering the limitations of the present study, it can be concluded that 2% chlorhexidine was the most effective against E. faecalis.

In the past, commercial synthetic medicaments were commonly used as interim intracanal medications. However, these synthetic options have been associated with toxic effects, the development of resistant strains, and a diminished immune response. Consequently, there has been a shift towards using naturally acquired medicaments, such as herbal extracts, which offer fewer side effects, easy availability, and renewability.

Nisin (NI), a therapeutic antimicrobial peptide, has demonstrated effectiveness against microorganisms resistant to common antibiotics. As a type-A antibiotic, NI exhibits potent antibacterial activity and proves effective against E. faecalis [31]. When compared to the control group, NI showed the lowest colony-forming units (CFU) of E. faecalis, with no statistically significant difference (p=0.930) in mean CFU when compared to 2% chlorhexidine (CHX). Studies have shown that NI effectively inhibits E. faecalis within one week, comparable to the action of CHX [32]. NI is a ribosome-synthesized peptide that undergoes posttranslational alterations, featuring one lanthionine, four-methylanthionine rings, and unusual residues like dehydroalanine and dehydrobutyryline [33].

NI exerts its bactericidal efficacy by forming pores that interact with a specific molecule called "Lipid II," thereby hindering cell wall synthesis. Lipid II is a major component of Gram-positive bacterial cell membranes. At the nanomolecular level, NI utilizes Lipid II as a "docking molecule" to create pores on the cell membrane, leading to the virtual elimination of bacteria [34]. In vitro studies have consistently demonstrated the efficacy of NI, a bacteriocin, in inhibiting E. faecalis and Streptococcus gordonii cells both in solution and within the root canal system. Comparatively, NI resulted in significantly lower levels of infected dentinal shavings of E. faecalis compared to calcium hydroxide [35]. Additionally, NI possesses anti-biofilm properties and can be synergistically used in combination with conventional therapeutic medicaments [36].

By utilizing Lipid II as a "docking molecule," nisin forms targeted pores on the surface of bacterial cell membranes, effectively killing bacteria at a nanomolar level [37]. This mechanism facilitates the penetration of other antibiotic molecules into microorganisms, enhancing their antibacterial effects when acting intracellularly. Previous studies have shown that the combination of nisin with a mixture of three D-amino acids increased its activity against Streptococcus mutans biofilms [38].

The combination of 2% CHX and high-purity nisin did not improve the activity of chlorhexidine against oral biofilms. This finding contrasts with previous studies where the combination of nisin and endodontic irrigants enhanced antibiofilm activity compared to using irrigants alone. The combination of low-purity nisin with the endodontic irrigant MTAD (mixture of doxycycline, citric acid, and Tween 80) exhibited better activity against E. faecalis biofilms compared to MTAD alone [39]. Similarly, combining high-purity nisin with low doses of sodium hypochlorite increased the activities of both antimicrobial agents against E. faecalis biofilms [40].

A previous study by Tong et al. (41) reported that nisin A (2.5%, Sigma-Aldrich) had a minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 1 and 2 mg/mL, respectively, against E. faecalis. Tong et al. (41,42) also evaluated the combined antimicrobial efficacy of a mixture of doxycycline, citric acid, and a detergent (Tween 80) with nisin as an intracanal medicament, showing that nisin (in combination with doxycycline) enhances the antimicrobial action of calcium hydroxide against pathogenic bacteria.

Nisin's mode of action differs from that of calcium hydroxide and does not rely on a highly alkaline environment for effective killing. While its mode of action is not fully understood, several proposed mechanisms include:

• Nisin inserting into the bacterial plasma membrane and triggering the activity of bacterial murein hydrolases, leading to damage or degradation of peptidoglycans and cell lysis [43].

• Interaction with the phospholipid membrane of target bacterial cells [44].

• Disruption of cellular mechanisms, resulting in the leakage of small intracellular contents from the cell [45].

CONCLUSION

• The results of the present study concluded that:

• Nisin can be used as a non-synthetic antibiotic medicament in conjunction with other intra canal medicaments.
Use of Nisin can help eliminating microorganism that have developed resistance to presently used medicaments.

Being a naturally derived product, it possesses less risk of toxicity and allergic reactions.

REFERENCES:


IMAGES:

Fig.1. Materials used.
Fig. 2. The zone of inhibition for each group was measured in millimeters using a Hi Media Hi-Antibiotic zone scale and tabulated at 24hrs, 48hrs and 72 hrs.

A. 

B. 

C. 

Fig. 3. The diameters of the zones of inhibition for each group 1, 2, 3, 4 at different time point, A: 24 hrs, B: 48hrs and C: 72 hrs.