Original Article

Comparative evaluation of antibacterial efficacy of nitrofurantoin, chitosan, and calcium hydroxide in combination with propylene glycol as an intracanal medicament against endodontic pathogen – An *in vitro* study

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Abstract

Objective: The objective is to evaluate and compare the antibacterial efficacy of nitrofurantoin, chitosan, and calcium hydroxide (Ca(OH)2) in combination with propylene glycol (PG) as an intracanal medicament against endodontic pathogens.

Materials and Methods: Fifty-two extracted single-rooted maxillary and mandibular anterior teeth and premolars were selected. The root canals were enlarged using Protaper universal rotary files. Clinical isolates of microorganisms collected from retreatment cases were used. Bacterial isolates obtained from infected root canals were introduced into brain–heart infusion (BHI) broth. Incubation of samples for 14 days was carried out to facilitate the development of mature biofilms. Intracanal medicaments were divided into four groups: Group 1 – Nirofurantoin+20% PG, Group 2 – Chitosan+20% PG, Group 3 – Ca(OH)2 + 20% PG, and Group 4 – 20% PG. The prepared root samples were incubated for 7 days. After collecting dentin samples, they were placed in a phosphate-buffered saline solution. Serial dilutions were then performed, and each dilution was plated on BHI agar. The plates were incubated for 24 h at 37°C. The antibacterial efficacy was assessed by calculating the percentage of remaining colony-forming units.

Results: Antibacterial efficacy of chitosan paste was significantly higher followed by nitrofurantoin as compared to other groups when used as an intracanal medicament.

Conclusions: In the root-canal biofilm model, the combination of chitosan and PG demonstrated a significant reduction in the viability of endodontic pathogens when employed as intracanal medication for 7 days. This suggests its potential as an effective intracanal medicament for endodontic retreatment.

Keywords: Antibacterial efficacy; chitosan; *Enterococcus faecalis*; intracanal medicament; nitrofurantoin; propylene glycol

INTRODUCTION

In endodontics, the major factor in endodontic treatment failure is persistent infection.[1,2] Therefore, ensuring

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effective disinfection of the root canal system is crucial. To eradicate microorganisms, intracanal medication is an important part of endodontic treatment that relies on the efficacy of antimicrobial agents.[2] Calcium hydroxide (Ca(OH)2) is often utilized as a standard intracanal medication. However, its effectiveness against *Enterococcus faecalis* is notably inadequate.[3] *E. faecalis*, a Gram-positive coccus, possesses a proton pump inhibitor mechanism that enables it to withstand the alkaline conditions induced by Ca(OH)2. It exhibits resistance across a broad pH spectrum, withstanding levels up to approximately pH 11.5, and may persist even after root canal obturation.[4,5] The other properties of *E. faecalis* are suppression of lymphocyte activity, formation of biofilm, attachment to collagen in serum and can invade dentinal tubules which protects them from destruction even after instrumentation and irrigation.[6-10] Chitosan is a natural polysaccharide obtained through the deacetylation process of chitin found in the shells of crustaceans. Chitosan has antimicrobial, and anti-fungal properties and enhances wound healing.[11] Chitosan can interact with cytoplasmic constituents, outer cellular components, and cell membranes.[12] Chitosan's attributes, including high biocompatibility and low toxicity, position it as a viable alternative medicament in endodontic treatment. Studies have demonstrated that chitosan exhibits inhibitory effects on both the planktonic form and biofilm of *E. faecalis*.[13,14] Certain derivatives of chitosan are effective against *E. faecalis*.[15-17] Hence, derivatives of chitosan hold the potential for use as intracanal medication. Propylene glycol (PG) can serve as a suitable vehicle for delivering chitosan-based intracanal medication, facilitating its passage through dentinal tubules and the apical foramen.[18,19] Thus, it may also contribute to the antimicrobial activity of the medication. Nitrofurantoin shows effectiveness against *E. faecalis*, aside from urinary tract infections. Moreover, nitrofurantoin is the preferred drug for treating infections arising from multidrug-resistant pathogens. To introduce nitrofurantoin into endodontics as an innovative intracanal medicament, it should be applied as a paste to effectively eliminate *E. faecalis*, a common culprit in failed endodontic therapy. This study aims to compare the antibacterial efficacy of nitrofurantoin, chitosan, and Ca(OH)2 in combination

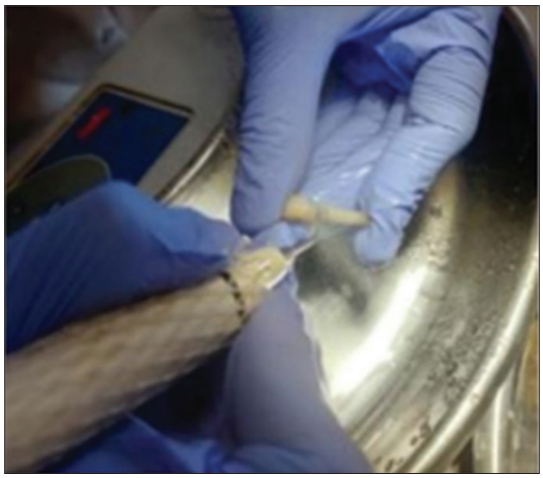


Figure 1: Cleaning of the samples

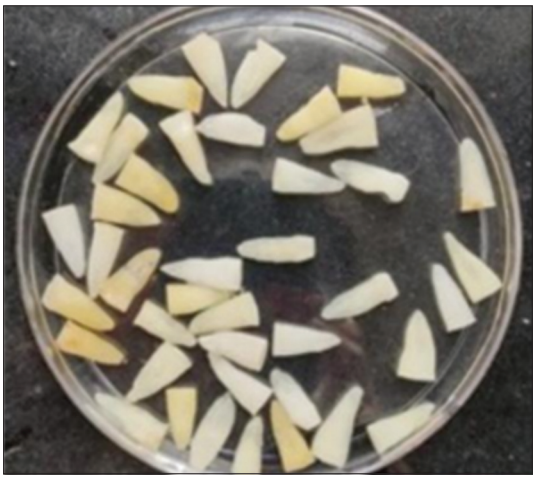


Figure 2: Tooth samples collected and decoronated

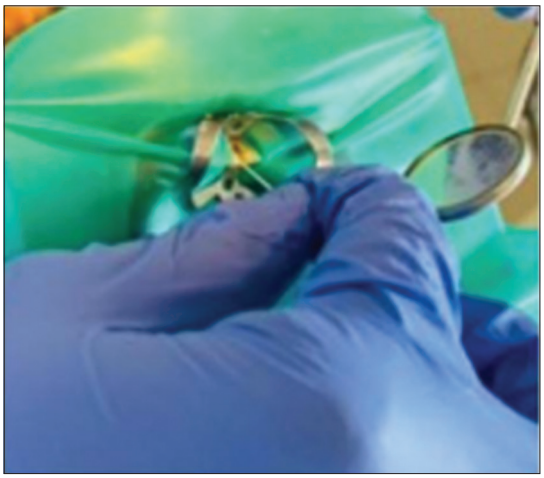


Figure 3: Collection of clinical isolates from the root canal

Figure 4: Plating of the collected sample

Figure 5: Infection of the root sample

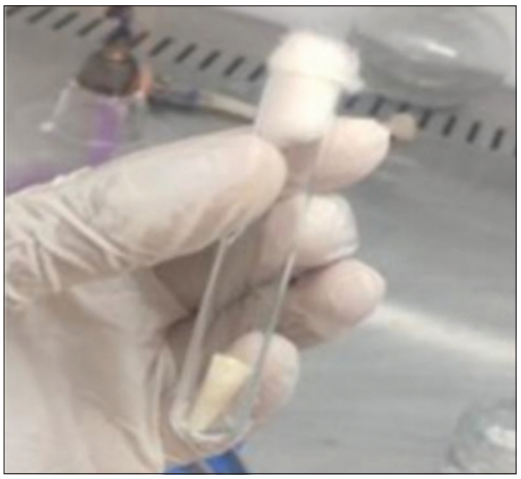
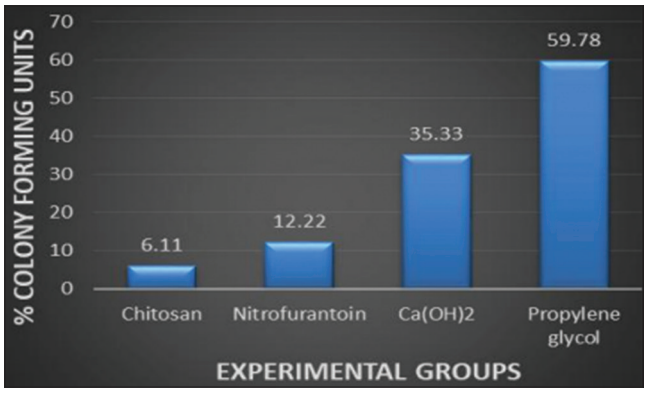


Figure 6: Sample kept into test tube for bacterial growth



Graph 1: Percentage of colony forming units

with PG as an intracanal medicament against endodontic pathogens.

MATERIALS AND METHODS

This study was initiated with the approval of the institutional ethical committee.

Root samples

Fifty-two intact single-rooted, maxillary, and mandibular anterior teeth and premolars were collected. Teeth with caries, fractures, cracks, or any other defects identified through magnifying loupes were excluded from the study's criteria. All soft-tissue remnants were meticulously eliminated from the tooth surfaces, crowns, and the coronal third of the roots, ensuring each root was reduced to a length of 15 mm [Figure 1]. Root canals were shaped using Protaper universal rotary files at a speed of 300 RPM and torque of 2 N [Figure 2].[20,21] The samples underwent irrigation with 3 mL of 2.5% sodium hypochlorite (NaOCl), followed by 1 mL of 17% ethylenediaminetetraacetic acid, to effectively remove organic and inorganic debris from the root canals. Following the irrigation process, all samples were flushed with 5 mL of distilled water to eliminate any remaining traces of prior irrigants. Substrate sterilization of the tooth specimens was done by autoclave at 121°C for 20 min. Nail polish was applied to seal the external surfaces of the samples. Subsequently, the samples were divided into four groups and inoculated with *E. faecalis*.

Preparation of microbial culture

Clinical isolates of microorganisms collected from patients with retreatment cases were used. Isolation of the selected tooth was done under a rubber dam. The operative field and the selected tooth were cleansed with 3% hydrogen peroxide solution and disinfected with 2.5% NaOCl solution for 30 s. Access opening was done using a sterile round bur. The pulp chamber was disinfected by applying a swab soaked in 2.5% NaOCl, and any residual NaOCl was neutralized using sterile 5% sodium thiosulfate. Bacterial isolates were sampled from the infected root canal by employing sterile paper points at the working length [Figure 3]. These paper points were left inside for 1 min to absorb the tissue fluid. These paper points were placed into 2 mL glucose broth, vortexed to separate the bacteria, and incubated anaerobically at 37°C for 48 h [Figure 4].

Infection of the root samples

A 30 µL log-phase culture of *E. faecalis* was introduced into the root canals to initiate inoculation [Figure 5]. Subsequently, the samples were incubated for 14 days to facilitate the formation of mature biofilms. All procedures were conducted within a biosafety cabinet (Bionics Scientific) [Figure 6].

Preparation of intracanal medicaments

The chitosan powder was mixed with 1% acetic acid to create a solution with a concentration of 20 mg/mL. To formulate the chitosan paste, 1 mL of the chitosan solution (20 mg/mL) was combined with 1 mL of PG (Purenso) and 3 mL of distilled water. The resulting chitosan concentration in the paste was 4 mg/mL, with a PG content of 20%. As a comparison, Ca(OH)2 (PRIME RC Cal) was used as the

intracanal medicament. The nitrofurantoin paste was prepared by mixing 100 mg nitrofurantoin powder with 1 mL of distilled water and 80 mg methylcellulose powder. The resultant concentration of nitrofurantoin paste was 100 mg/mL.

Antimicrobial assessment

After the designated incubation period, a sample from each culture was plated on solid media to evaluate the purity and viability of the microbes. Root samples contaminated with *E. faecalis* were then categorized into four groups as outlined below: Group 1 – Nirofurantoin+20% PG, Group 2 – Chitosan+20% PG, Group 3 – $Ca(OH)_2$ powder + 20% PG, and Group 4 – 20% PG. Each root canal received 30 µL of the designated medication. Following that, the root samples were placed in an incubator set at 37°C for 7 days. After the 7-day incubation period, 3 mL of sterile distilled water was used to rinse the canal. Protaper universal rotary files were employed at a speed of 300 RPM and 2N torque to eliminate the intracanal medicament. Dentin samples from the root canal surfaces were obtained using Protaper universal hand files and gathered in 1 mL of phosphate-buffered saline solution. After serial dilutions, 100 µL of each dilution was plated on brain–heart infusion agar and incubated for 24 h at 37°C. The colonies obtained were counted and expressed as a percentage of the remaining colony-forming units.

Statistical analysis

All collected data were inputted into a computer using a coding system and then meticulously reviewed for any potential entry errors. The compiled data were organized within an MS Office Excel Sheet (version 2019, Microsoft Redmond Campus, Redmond, Washington, United States). Subsequently, statistical analysis was performed using the IBM Corp. IBM SPSS Statistics for Windows, Version 29.0.2.0 (Armonk, NY: IBM Corp.). Descriptive statistics, including frequencies and percentages for categorical data, and mean values along with standard deviation for numerical data, were calculated and presented. The normality of the numerical data was assessed using the Shapiro–Wilk test, revealing that the data adhered to a normal distribution. Consequently, parametric tests were employed for comparisons. Intergroup comparisons were performed using a one-way ANOVA, followed by pair-wise comparisons utilizing a *post hoc* test. A significance level of $P < 0.05$ was deemed statistically significant, maintaining an α error of 5% and a β error of 20%, thus ensuring a study power of 80%. The symbols used to denote significance levels in the results are as follows [Tables 1-3]:

- * = Statistically significant difference ($P < 0.05$)
- ** = Statistically highly significant difference ($P < 0.01$)
- # = Nonsignificant difference ($P < 0.05$).

RESULTS

After a 7-day treatment with different medications, the present count of microorganisms in the root canals of the treated teeth was evaluated using plate count analysis. The groups were numbered as Group 1 – Chitosan, Group 2 – Nitrofurantoin, Group 3 – Ca(OH)2, and Group 4 – PG [Graph 1]. The chitosan group showed 6.11% of remaining viable microorganisms in the root canal infection model after 7 days. The percentages of remaining viable microorganisms in the root canal models were 12.22%, 35.33%, and 59.78% in nitrofurantoin, Ca(OH)2, and PG groups, respectively. Out of the four groups of intracanal medicaments used in the study against the coccus *E. faecalis*, the chitosan group showed significant results after 7 days.

DISCUSSION

Microbiological investigations of cases with a secondary infection found a diverse community of bacteria and fungi present in the endodontically teeth.[1,3] Utilizing larger-sized hand or rotary instruments for mechanical instrumentation may aid in the removal of bacteria within the root canals. However, it is insufficient for completely eradicating microbes within intricate root canal structures. Moreover, excessive instrumentation can compromise the integrity of the root dentin.[22] Therefore, the use of effective antimicrobial intracanal medicament can address the limitations of instrumentation and successfully diminish pathogens present in the intricate anatomy of root canals. Several studies using culture-dependent and molecular techniques have identified *E. faecalis* as the most prevalent microorganism in root canal-treated teeth with a prevalence of 36.6% and 77% using polymerase chain reaction.[3]

Table 1: Inter group Pair wise comparison using Tukey HSD *Post Hoc* Tests

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	*P*	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-6.111*	1.954	0.019*	-11.41	-0.82
	3	-29.222*	1.954	0.000**	-34.52	-23.93
	4	-53.667*	1.954	0.000**	-58.96	-48.37
2	3	-23.111*	1.954	0.000**	-28.41	-17.82
	4	-47.556*	1.954	0.000**	-52.85	-42.26
3	4	-24.444*	1.954	0.000**	-29.74	-19.15

There was a statistically highly significant/significant difference seen for the values between all the pairs of groups ($P<0.01$, 0.05)

Several studies have shown that multispecies biofilms demonstrate increased resistance to antimicrobial treatment (antibiotics as well as disinfectants) compared to monoculture biofilms. Thus, multispecies biofilms provide more complexity, more resemblance to the clinical reality, enhanced metabolic capacity and stress tolerance, more resilience, and a greater challenge toward biofilm eradication.[23] Hence, this multi-species biofilm model was selected as it was feasible and reproduce more of a clinical condition.

Biofilm formation starts with the surface attachment of bacteria, followed by microcolony development, secretion of extracellular polymeric substance, different stages of biofilm maturation, and dissociation. When comparing "young" (several hours to days) versus "old" biofilms, differences can be observed in the biofilm biomass/thickness, the cell count, and its antimicrobial resistance.[23]

In secondary apical periodontitis, *E. faecalis* was found in a higher proportion compared to primary infection, although it was not the most prevalent bacteria identified through microbiological studies. This highlights the persistence of these microorganisms in root canals, which presents challenges for endodontic treatment.

E. faecalis, a Gram-positive coccus, possesses a proton pump inhibitor mechanism that enables it to withstand the alkaline conditions induced by Ca(OH)2. It exhibits resistance across a broad pH spectrum, withstanding levels up to approximately pH 11.5, and may persist even after root canal obturation.[4,5] The other properties of *E. faecalis* are suppression of lymphocyte activity, formation of biofilm, attachment to collagen in serum and can invade dentinal tubules which protects them from destruction even after instrumentation and irrigation.

Thus, there's a pressing need for an effective antimicrobial medicine targeting *E. faecalis* present in canals of root canal-treated teeth.[5] Although Ca(OH)2 is routinely and widely utilized as intracanal medication, it has proven ineffective against *E. faecalis*, a bacterium often associated with persistent infections in the root canal.[24-26] In this study, it was observed that chitosan paste showed an antimicrobial effect against *E. faecalis* pathogen present in the root canals. The findings suggest that chitosan paste holds promise as a potential alternative antimicrobial intracanal medication for cases characterized by persistent infection. In this study, we developed a paste containing 4 mg/mL of chitosan in 0.2% acetic acid for use as an antimicrobial intracanal medication. The addition of 20% PG was aimed at enhancing the flowability of the mixture, facilitating easier handling, and promoting improved penetration into dentinal tubules. The combination of chitosan and PG (Chitosan + PG) exhibited a more significant antibacterial effect against *E. faecalis* biofilm compared to PG alone (PG), and it also demonstrated greater efficacy than $Ca(OH)_2$. Chitosan, with its positive charge, adheres to the negatively charged cell membrane of microbes, facilitating interaction, and potential disruption of the microorganism. Meanwhile, PG serves a dual purpose by aiding the intracanal medication in penetrating deeper into dentinal tubules and exhibiting germicidal activity.[19] Chitosan shows promise as a viable alternative for intracanal medication, whether applied alone or in conjunction with other active ingredients. This is particularly significant in cases of secondary infection, where persistent *E. faecalis* infection may play a crucial role. Nitrofurantoin is an antimicrobial agent and showed effectiveness against *E. faecalis*, aside from urinary tract infections. In addition, it is recognized as a preferred drug for treating infections caused by multidrug-resistant pathogens. It helps to eradicate *E. faecalis*, causing failed endodontic therapy. Biofilm comprises microorganisms that exhibit increased tolerance to antimicrobial agents, and this is the predominant form found within root canals.[8] Therefore, in this study, we replicated a biofilm-like condition by inoculating extracted human root specimens with *E. faecalis* and providing ample time for mature biofilm development and penetration into dentinal tubules, which spanned 14 days.[10] Our study results revealed that the use of chitosan paste led to a significant decrease in the presence of *E. faecalis* within this root canal biofilm model.

Table 2: Groups are numbered as

1	Chitosan
2	Nitrofurantoin
3	Ca(OH)2
4	PG(%)

CONCLUSIONS

Developing a multi-species biofilm intraorally or from in

Table 3: Inter group comparison of outcome variables

Group	*n*	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	*F*	*P* of one way ANOVA
					Lower Bound	Upper Bound				
1	9	6.11	1.833	0.611	4.70	7.52	4	9		
2	9	12.22	2.991	0.997	9.92	14.52	8	18	312.636	0.000**
3	9	35.33	4.243	1.414	32.07	38.59	30	42		
4	9	59.78	6.200	2.067	55.01	64.54	48	68		
Total	36	28.36	21.824	3.637	20.98	35.75	4	68		

There was a statistically highly significant difference seen for the values between the groups ($P<0.01$) with higher values in group 4

vivo inocula is highly clinically relevant and it is the closest method that can get to mimicking natural conditions in laboratory biofilm models. In the root canal biofilm model employed, the application of chitosan paste for 7 days significantly reduced the viability of E. faecalis. This suggests that chitosan may serve as an effective intracanal medication for endodontic retreatment. Further studies can be done for its in-vivo results and its scope in clinical scenario.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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