Original Article

Comparative evaluation of antimicrobial efficacy of chitosan nanoparticles and calcium hydroxide against endodontic biofilm of *Enterococcus faecalis*: An *in vitro* study

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Abstract

Aim: The aim of the study was to assess and evaluate the antimicrobial effectiveness of chitosan nanoparticles (CSNPs) with calcium hydroxide in the elimination of *Enterococcus faecalis*.

Materials and Methods: Using the broth microdilution method, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of calcium hydroxide and CSNPs were measured. The antibiofilm effect of calcium hydroxide and CSNPs against *E. faecalis* biofilm was qualitatively analyzed using a crystal violet assay. A 7-day-old biofilms of *E. faecalis* grown on dentine discs were assigned to the following three groups (n = 11 dentine discs), normal saline (group I), calcium hydroxide (group II), and CSNPs (group III). Quantification of live and dead cells using confocal microscopy was done to evaluate the antibiofilm efficacy of the medicaments included in the study.

Results: MIC of calcium hydroxide and CSNPs against *E. faecalis* was observed at 2.5 mg/mL and 0.31 mg/mL, respectively. MBC of calcium hydroxide and CSNPs was observed at 2.5 mg/mL and 0.31 mg/mL, respectively. Using Crystal Violet (CV) assay, calcium hydroxide and CSNPs showed biofilm inhibition at concentrations of 2.5 mg/mL and 0.625 mg/mL, respectively. Confocal laser scanning microscopy analysis found that both calcium hydroxide and CSNPs showed a significant decrease in viable cells at their MBC values compared to the control group's normal saline. CSNPs showed a significantly lower percentage of live cells than calcium hydroxide ($P < 0.05$).

Conclusion: The study results reveal that the antimicrobial efficacy of CSNPs is better than calcium hydroxide and normal saline against *E. faecalis* biofilm.

Keywords: Biofilm; calcium hydroxide; chitosan nanoparticles; *Enterococcus faecalis*

INTRODUCTION

Over the past few years, several studies have revealed that endodontic infections are complex and primarily biofilm based.[1] Although biomechanical preparation removes microorganisms mechanically, complete elimination is not possible. Therefore, chemomechanical preparation is utilized to eradicate as many microorganisms from the root canal as possible.[2]

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Various intracanal medicaments have been used during multiple visits to endodontic interventions, including calcium hydroxide, chlorhexidine, phenols, formaldehyde, halogens, steroids, and antibiotics. Among these, calcium hydroxide is commonly used for maintaining sterility in the canal space.[3] Its antimicrobial action is related to its high pH, which inactivates bacterial membrane enzymes. However, it is insufficient at eliminating microorganisms that have settled inside the dentinal tubules. Alkali-resistant pathogens, as well as the buffering activity of dentine, which neutralizes its alkali-killing impact on microbes, are other reasons that have questioned its antibacterial efficiency.[4]

Evidence suggests that nanoparticles of chitosan (chitosan nanoparticles [CSNPs]) have strong antimicrobial activity when compared to other antimicrobial powders. They cause cellular death by interacting with the surface of negatively charged bacterial cells.[5] They are also nontoxic to mammalian cells, biocompatible, cost-effective, readily available, and can be chemically modified.[6] Shrestha and Kishen discovered that dentine treated with CSNPs reduced *E. Enterococcus faecalis* adhesion significantly.[7]

E. faecalis is a major culprit for failed endodontic treatment and chronic periapical lesions. It is difficult to eliminate once it gains entry into bacterial biofilms due to its inherent capabilities and potential to acquire new defense mechanisms.[8] *E. faecalis* is a common bacterial species found in persistent endodontic lesions.[9] Therefore, *E. faecalis* has been used as a gold standard in the quantification of the antimicrobial efficacy of any chemical against endodontic biofilms.[10]

This study compared the antimicrobial effectiveness of CSNPs and calcium hydroxide against monospecies endodontic biofilms of *E. faecalis*.

MATERIALS AND METHODS

Minimum inhibitory concentration and minimum bactericidal concentration test by microdilution method

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were assessed using the broth microdilution method in compliance with the Clinical Laboratory Standards Institute guidelines, 2019.[11] Fifty microliters of Mueller Hinton II Broth (Cation-Adjusted, BD-Difco) was added to a 96-well round bottom microtiter plate. Stock solutions of 20 mg/mL of calcium hydroxide and 20 mg/mL of CSNP were prepared. The solutions were serially diluted, and 25 µL of each dilution was added to the respective well. Twenty-five microliters of the bacterial suspension were added to each well, attaining an approximate quantity of 1.5×10^5 colony forming unit (CFU)/mL in each well. A 24-h incubation period at 37°C was used for the microtiter plate. The growth in each well was estimated using tetrazolium and resazurin dye.[12] The medicament with the lowest concentration in the well without any color change or visual growth turbidity was recorded as the MIC in mg/mL.

MBC was determined by placing a drop of 20 µL from each well of MIC plate on Mueller Hinton agar (MHA) plates. The smallest antimicrobial dose yielded no colonies on the MHA plate after 24 h. of incubation at 37°C by visual inspection and was documented as the MBC. Each experiment was performed in triplicate to reduce error.

Determination of antibiofilm activity by crystal violet staining assay

The microtiter dish assay for biofilm formation was done according to the protocol mentioned by O'Toole.[13] The *E. faecalis* bacteria adhered to the bottom and sides of the wells of a 96-well microtiter plate (flat bottom), formed the biofilm, and were subjected to different concentrations of calcium hydroxide and CSNPs. The treated wells were then analyzed using crystal violet staining each well to identify the concentration of the antimicrobial agent that can inhibit biofilm formation.

Determination of antibiofilm activity by confocal laser scanning microscopy

For confocal laser scanning microscopy (CLSM), imaging biofilm on dentine discs was established using the modified Chhibber *et al*. method.[14] Dentine discs were sterilized by autoclaving at 121°C for 20 min. The 96-well microtiter plate containing dentine discs and 100 µL of $10^{[8]}$ CFU/mL of *E. faecalis* in tryptic soy broth were under incubation at 37°C for 7 days, and the medium was replenished every 24 h. The effect of intracanal medicaments was studied on 7-day-old biofilm. The discs were then assigned to the following three groups of medicaments ($n = 11$ dentine discs): normal saline (group I), calcium hydroxide (group II), and CSNPs (group III).

The medicaments at a concentration of MBC values were added to different wells containing dentine discs. In a control well, 200 µL of normal saline was added. Incubation was carried out for 6 h at 37°C. The discs were subsequently stained with 5-(and 6-) carboxyfluorescein succinimidyl ester and propidium iodide and examined under confocal microscopy (CLSM, Olympus FV3000). The percentage of live and dead bacteria was determined using the Fiji Image J software.

RESULTS

Minimum inhibitory concentration and minimum bactericidal concentration by broth microdilution method

The study results show that CSNPs and calcium hydroxide

had MIC of 0.31 mg/mL and 2.5 mg/mL, respectively, against *E. faecalis*. MBC of CSNPs and calcium hydroxide against *E. faecalis* was 0.31 mg/mL and 2.5 mg/mL, respectively.

Antibiofilm activity by crystal violet staining assay

CSNPs and calcium hydroxide showed biofilm inhibition at a concentration of 0.625 mg/mL and 2.5 mg/mL, respectively. The untreated (UT) wells showed no biofilm inhibition.

Antibiofilm activity by confocal laser scanning microscopy

The result demonstrated that samples treated with normal saline had the highest number of live cells compared to both the calcium hydroxide and CSNPs group [Table 1 and Figure 1]. The CSNPs group had the least number of live cells [Table 1 and Figure 1].

The data were analyzed using ANOVA to find if there was any significant difference in the results obtained by treating the samples with normal saline, calcium hydroxide, and CSNPs. The results revealed a significant difference between the same ($P < 0.05$).

Post hoc analysis was done to find significant differences among the individual groups. The results showed that both calcium hydroxide and CSNPs exhibited significantly higher bactericidal effects than normal saline. CSNPs displayed a statistically significant increase in antimicrobial effect compared to calcium hydroxide ($P < 0.05$) [Table 2 and Figure 1].

DISCUSSION

Endodontic infection is a consequence of a biofilm-mediated infection.[15] Anatomic difficulties of the root canal system pose challenges in achieving complete endodontic disinfection, making instrumentation and irrigation techniques inefficient.[16] Therefore, intracanal medicaments are used to mitigate any bacteria that were not eliminated during canal preparation.[17] The

Table 1: Statistical analysis of percentage of live cells (mean values)

Groups	Mean±SD
Group 1: Normal saline	73.909±10.358
Group 2: Calcium hydroxide	40.545±11.021
Group 3: Chitosan nanoparticles	28.091±10.270

SD: Standard deviation

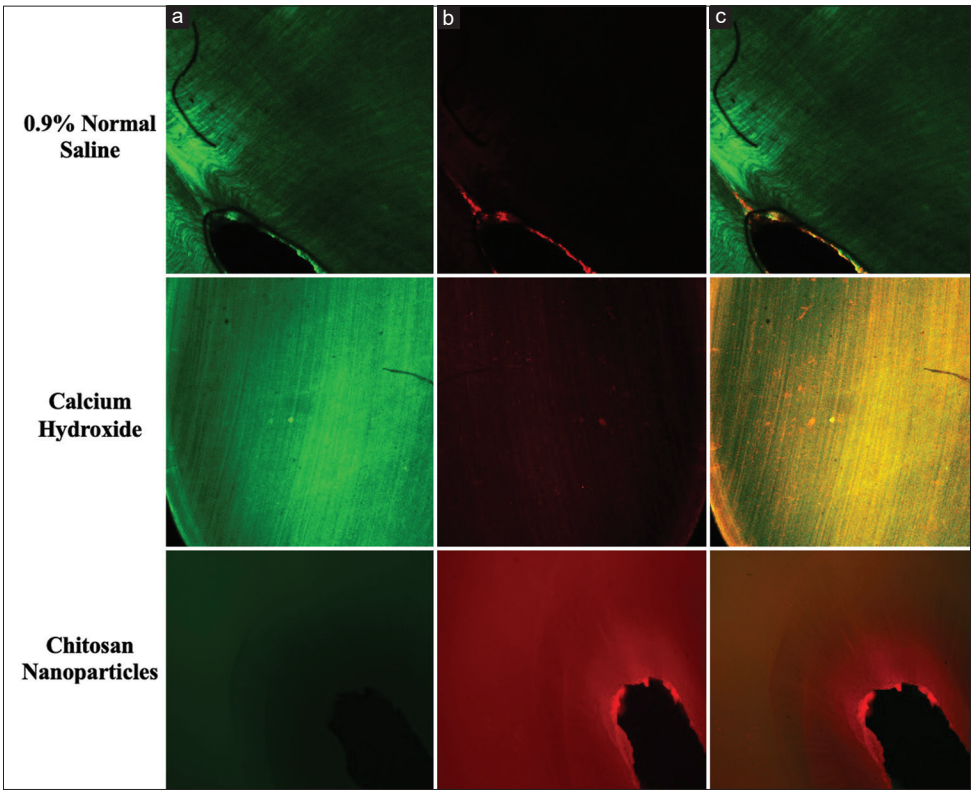

Figure 1: Confocal microscopy images of *Enterococcus faecalis* biofilm grown on human dentin discs after being treated with normal saline, calcium hydroxide, and chitosan nanoparticles. The same sections are displayed in columns (a and b), with 498-nm and 536-nm emission, respectively, to visualize live (green fluorescence) and dead (red fluorescence) bacteria. Column (c) displays merged images

Table 2: Intergroup comparison (*post hoc* analysis)

Groups (I)	Groups (J)	Mean difference (I−J)	95% CI for difference		*P*
			Lower	Upper	
Group 1	Group 2	33.36	22.26	44.46	<0.05
	Group 3	45.81	34.72	56.91	<0.05
Group 2	Group 3	12.45	1.35	23.55	0.02*

*The mean difference is significant at the 0.05 level. CI: Confidence interval

antibacterial effectiveness of calcium hydroxide and CSNPs was assessed in the present study against the planktonic and biofilm forms of *E. faecalis* ATCC 29212.

In the present study, on comparing with calcium hydroxide (MIC 2.5 mg/mL), CSNPs showed a much lower MIC value (0.31 mg/mL) against planktonic *E. faecalis* bacteria. These findings show that CSNPs were more potent than calcium hydroxide against *E. faecalis*, showing a greater reduction in bacterial growth. The possible reasons may be due to the availability of more surface area and the polycationic nature of CSNPs that enables them to be tightly absorbed to the negatively charged bacterial surface, rupturing the membrane and causing the release of internal substances, which ultimately causes cell death.[18]

The MBC test, in contrast to the MIC test, reveals the smallest concentration of antimicrobial agent that significantly suppresses bacterial growth. The evaluations of the present study revealed that CSNPs were more effective than calcium hydroxide and acted in smaller amounts. A concentration of 0.31 mg/mL showed no microbial growth in the CSNPs group, while the calcium hydroxide group concentration of 2.5 mg/mL did not allow microbial growth. It can be concluded that CSNPs were more potent than calcium hydroxide in eradicating planktonic *E. faecalis* bacteria.

According to the CV assay, it was observed that the UT wells showed no biofilm inhibition. Calcium hydroxide showed biofilm inhibition at MBC value, i.e., 2.5 mg/mL. Similar results were also observed by Sabrah *et al*., in which biofilm inhibition with calcium hydroxide was seen at a 1.6 mg/mL concentration using crystal violet assay.[19] However when compared with CSNPs (0.625 mg/mL), calcium hydroxide showed an inhibitory effect at a much higher concentration (2.5 mg/mL). The high tolerance of endodontic microbial biofilms to an alkaline challenge may explain the comparatively poor susceptibility of *E. faecalis* biofilms to calcium hydroxide, as reported in previous studies.[20]

According to CLSM analysis, calcium hydroxide displayed a significant reduction in live bacterial cells at their MBC value compared to the control group (treated with normal saline) [Tables 1 and 2]. The mean percentage of live cells in the calcium hydroxide group was 41%. These findings correspond to the research conducted by Zancan *et al*. which found the mean of 45% live cells in the group treated by calcium hydroxide.[21]

In comparison to the control group of normal saline, CSNPs revealed a significant reduction in live bacterial cells at their MBC value [Tables 1 and 2]. The mean percentage of live cells in the CSNPs group was 28.09%. A similar study conducted by Kishen *et al*. revealed that the use of CSNPs significantly decreased the quantity of *E. faecalis* cells adhered to dentine.[22] Compared to samples treated with calcium hydroxide, the CSNPs group showed a significant decrease in the percentage of live cells at their MBC values [Table 2 and Figure 1]. Similarly, Del Carpio-Perochena *et al*. observed that the antibacterial efficacy of calcium hydroxide incorporated with CSNPs was significantly better than calcium hydroxide alone.[23] Louwakul *et al*. studied the antibiofilm efficacy under CLSM and observed that calcium hydroxide was less successful in eliminating E. faecalis inside dentinal tubules than the nanoparticles group. They observed that calcium hydroxide was less successful in eliminating *E. faecalis* inside dentinal tubules than the nanoparticles group.[24] This lower antibacterial efficacy of calcium hydroxide against *E. faecalis* was also observed by Wu *et al*. under confocal laser scanning microscopic study.[16] The lower efficacy could be due to the neutralization or buffering by the dentine and biofilm matrix of *E. faecalis*.[25]

The results of the present *in vitro* study are promising but some limitations of this study must be recognized. The study design was an *in vitro* model; therefore, the results cannot be directly extrapolated to clinical situations. A monospecies of the bacteria were used to establish the biofilm, whereas *in vivo* endodontic biofilms consist of multispecies of bacteria. Although this study may conclude that CSNPs were the most effective agent against *E. faecalis*, they cannot eradicate all bacteria from the biofilm. Further studies may be designed to improve the antibacterial effect of CSNPs, for example, using higher concentrations of CSNPs or CSNPs with other solvents or formulations.

CONCLUSION

The present study shows that both calcium hydroxide and CSNPs significantly decrease the percentage of live *E. faecalis* bacteria cells at their MBC values compared to the control group normal saline. Compared to the calcium hydroxide group, CSNPs displayed a significantly higher percentage of dead cells. Hence, these results confirm the earlier observations reported regarding the antimicrobial efficacy of CSNPs, and on its basis, an inference can be drawn that the antimicrobial efficacy of CSNPs is stronger than calcium hydroxide and Normal saline against *E. faecalis* biofilm. However, *in vivo* and clinical studies are needed to confirm the efficiency of these nanoparticles and to assess their biocompatibility and safety in human tissues.

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Conflicts of interest
There are no conflicts of interest.

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