Original Article

Antibacterial activity of chitosan and its combination with other irrigants on Enterococcus faecalis: An in vitro study

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ABSTRACT
Objective: The aim of the in vitro study was to compare the antibacterial efficacy of sodium hypochlorite (NaOCl), chlorhexidine (CHX), chitosan and their combinations in vitro.

Materials and Methods: A total of 60 extracted single-rooted teeth were selected and used for the study. After the access cavity preparation and working length determination, the apical foramina of the samples were sealed with epoxy resin to prevent bacterial leakage, and the teeth were mounted in stone blocks. Biomechanical preparation was done using crown-down technique up to master apical file size of #50. Enterococcus faecalis (ATCC 29212) was used to contaminate the root canals. After incubation, samples were divided into six groups according to the solutions used for irrigation, that is, CHX, NaOCl, chitosan, alternating solution of chitosan and hypochlorite, alternating solution of chitosan and CHX, and saline. Antibacterial efficacy was assessed by obtaining the samples from root canal before and after the irrigation using paper points, culturing them on blood agar plates, and measuring the number of colony-forming units (CFUs) formed.

Results: All the statistical analysis was done using SPSS version 16. P <0.05 was considered statistically significant. Comparison of mean values before and after the irrigation was done using the paired t-test. Comparison of percentage reduction among the groups was done using ANOVA with post hoc Games–Howell test. A statistically significant difference was found in the number of CFU between experimental groups compared to the control group and also among five experimental groups (P > 0.001). Maximum antibacterial activity was seen when chitosan was used alternatively with CHX and NaOCl. Independently, hypochlorite showed maximum antibacterial activity followed by CHX and chitosan which showed almost similar antibacterial activity.

Conclusion: According to this study, there is synergistic antibacterial activity when chitosan is used alternatively with 2% CHX or 5% NaOCl.

Keywords: Antibacterial efficacy, chitosan, chlorhexidine, Enterococcus faecalis, root canal irrigants

INTRODUCTION

Microorganisms predominantly bacteria are the primary etiological factor in the development of pulp and periapical lesions. Successful root canal therapy depends on thorough chemomechanical debridement of pulpal tissue, dentin debris, and microorganisms. Almost half of the canal walls are left unprepared with usage of stainless steel files and nickel–titanium rotary instruments. Therefore, irrigation is an essential part of the root canal treatment. Chemical debridement is especially needed for teeth with complex internal anatomies such as fins, cul de sacs, intercanal communications, and other irregularities.

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Among the various root canal irrigants available, sodium hypochlorite (NaOCl) is by far the most commonly employed and suggested because of its tissue dissolving property and antibacterial activity. However, at any concentration, hypochlorite was found to be less effective against endotoxins during root canal disinfection.[3] Chlorhexidine (CHX) is another substance commonly recommended as root canal irrigant due to its wide-spectrum antimicrobial activity. It is effective against Gram-positive and Gram-negative bacteria and yeasts as well. However, it does not dissolve organic tissues. Antibacterial efficacy of CHX has been tested against that of hypochlorite and results from these studies are not conclusive due to differences in the methodologies employed.[4] Moreover, NaOCl and CHX cannot be combined because of the formation of para-chloroaniline.

Chitosan is a natural polysaccharide usually obtained by alkaline deacetylation from chitin, which is the basic component of crustacean exoskeletons.[5] It is a nontoxic cationic biopolymer with biocompatible, bioadhesion, and biodegradable properties. In vitro studies have already demonstrated the significant antibiofilm efficacy as well as the chelating property of chitosan nanoparticles (CNPs).[6,7] However, literature reveals that there are no studies where the effects of chitosan in combination with other irrigating solutions were assessed. Considering the fact that CNPs have both antibacterial as well as chelating properties, the objective of the study was to assess the antibacterial efficacy when CNPs are used alternatively with NaOCl and CHX.

MATERIALS AND METHODS

Sample preparation
A total of 60 freshly extracted single-rooted teeth were collected cleaned of debris, soft-tissue remnants, and stored in saline solution and used for the study. All teeth were sterilized and decoronated with a diamond disc using a low-speed straight handpiece. Endodontic access cavities were prepared with Endo Access Bur in a high-speed handpiece, and working length was determined. Apical foramina were sealed with epoxy resin to prevent bacterial leakage and teeth were mounted in dental stone blocks. Biomechanical preparations were done using K-files until 1 mm short of anatomical apex up to master apical file size #50.[8] NaOCl (Prime dental products, India) and normal saline were used as irrigants alternatively during biomechanical preparation. Teeth mounted in dental stone blocks were sterilized using autoclave at a temperature of 121°C and 15 lbs pressure for 15 min.

Inoculation of Enterococcus faecalis into prepared sample
About 1 ml of suspension of a pure culture of Enterococcus faecalis ATCC 29212 (in brain-heart infusion broth [BHIB] = 0.5 MFU unit) was used to contaminate the root canals in a laminar flow cabinet and then incubated at 37°C for 24 h.[9]

Sampling before irrigation
Root canals were sampled using paper points which were then transferred to tubes containing 1 ml BHIB and vortexed for 1 min. After ten-fold serial dilutions, aliquots of 0.001 ml were placed on to blood agar plates and incubated at 37°C for 48 h and then colony-forming units (CFUs) were counted using the colony counting meter.

Preparation of chitosan 0.2% solution
About 0.2% of chitosan solution was prepared by mixing 0.2 g of chitosan (Sigma-Aldrich, India) in 1% acetic acid solution, and the mixture was stirred in magnetic stirrer for 2 h.[10]

Irrigation of specimens
The specimens were randomly divided into six groups of ten each which were as follows:

- Group 1: CHX 2% (Safe Plus, Neelkanth Healthcare Pvt. Ltd.)
- Group 2: NaOCl 5%
- Group 3: Chitosan 0.2%
- Group 4: Alternating solution of chitosan 0.2% and NaOCl 5%
- Group 5: Alternating solution of chitosan 0.2% and CHX 2%
- Group 6: Saline (control).

- Group 1: 5 ml of CHX irrigating solution was placed in ten root samples and allowed to remain in canal for 5 min. Final irrigation was performed with 5 ml of normal saline in each root sample. Root canals were sampled before and after the irrigation using paper points
- Group 2: 5 ml of NaOCl irrigating solution was used and the procedure as in Group 1 was repeated
- Group 3: 5 ml of chitosan was used and the procedure as in Group 1 was repeated
- Group 4: 5 ml of chitosan solution was placed in ten root samples and allowed to remain in canal for 5 min. NaOCl was used as an irrigant alternatively and allowed to remain for 5 min. Final irrigation was performed with 5 ml of saline solution in each root sample
- Group 5: CHX solution was used as an irrigant alternatively after chitosan solution and the procedure as in Group 4 was repeated
- Group 6: 5 ml of saline was used and procedure as in Group 1 was repeated.

Sampling after irrigation
Root canals were sampled again using paper points. Paper
points were then transferred to tubes containing 1 ml BHIB and vortexed for 1 min. After ten-fold serial dilutions, aliquots of 0.001 ml were placed on to blood agar plates and incubated at 37°C for 48 h and then CFUs were counted using colony counting meter. All the CFU counts were log-transformed to ensure the normality before comparison.

All analysis was done using SPSS version 16 (SPSS-Inc, Chicago, IL, USA). P < 0.05 was considered statistically significant. Comparison of mean values of bacterial colonies before and after testing with test solutions was done using the paired t-test. Comparison of percentage reduction among the groups was done using ANOVA with post hoc Games–Howell test. ANOVA with post hoc Games-Howell test excluded from analysis as standard deviation is 0 due to 100% reduction in all the samples.

RESULTS

Mean counts, standard deviation, and post hoc Games–Howell test of number and percentage reduction of bacterial colonies between different experimental groups are shown in Tables 1 and 2. Mean CFU was compared in all the groups, and there was a statistically significant difference among different groups (P < 0.001). The mean colony count after irrigation ranged between 0 CFU/ml and 8.15 CFU/ml.

Post hoc test of number of colonies after irrigation suggested that Groups 4 and 5 had no CFU, followed by Group 2 whose mean CFU/ml was 0.40 after irrigation. Group 1 and 3 showed little difference with mean values 1.06 and 1.011, respectively. Group 6 showed the highest mean value 8.15.

DISCUSSION

There are over 700 species of bacteria present in the oral cavity, with any particular individual harboring 100–200 of these species, and only a selected group will become involved in an endodontic infection.[11] Classically, the black-pigmenting bacteria of the Bacteroides, Prevotella, Enterococcus, and Porphyromonas genera were thought of as the primary pathogens in endodontic infections. However, E. faecalis (ATCC29212) was selected as test microorganism, because it is the most commonly associated facultative anaerobe with root canal failure cases and persistent apical periodontitis, as it possesses certain virulence factors.

Different approaches have been used to test the effectiveness of antimicrobial agents in the laboratory. These include incubation of broth cultures of selected bacteria with the antimicrobial agent, growth of selected bacteria as “lawn” on agar surfaces and the use of the disk diffusion method, the artificial infection of extracted teeth with selected bacteria, and using irrigation with test antimicrobial agent.[12] A combination of such approaches has sometimes been adopted but with discrepant findings. In the present study, prepared root canals were artificially infected with E. faecalis, and a standardized irrigation protocol was followed where a direct contact method of different experimental approach for evaluating the antibacterial activity of a selected chemical agent, in which a fixed volume of this agent is mixed with a bacterial suspension for 5 min.[8] The antibacterial activity is verified through culture of the resulting mixture in a nutrient medium (blood agar), and thus, the presence or absence of bacterial growth is evaluated for E. faecalis.

In the present study, apical preparations were done up to 50 K-file in all the specimens. Preparing the canals with #50 K-file helped the irrigating solutions better penetrate into the canals to ensure that irrigants reach till the apex.[8] Different methods have been suggested for the collection of microbial samples from the root canals which include the use of paper points, collection of dentinal shavings from the internal root surface using files, or external root surface using burs, pulverization of root tips in liquid nitrogen. Paper point cultures of the root canals detected bacteria more frequently than dentin filing cultures on files or reamers.[8]
The root canals were irrigated with normal saline prior to obtaining samples to physically remove the irrigants from the canal, thereby preventing the carryover of the antimicrobial solutions. In the present study, normal physiological saline was used as a positive control irrigant, as it is devoid of antibacterial action as compared to the other test solutions, which had some amount of known antibacterial activity.

NaOCl is the most commonly used endodontic irrigant and fulfills the bulk of the properties of an ideal irrigant. The antimicrobial effect and tissue dissolution process are a result of saponification, amino acid neutralization, and chloramination reactions that occur in the presence of microorganisms and organic tissue.[13] In the present study, NaOCl showed better antibacterial efficacy when compared to CHX. The results were in accordance with study by Karale et al., where the 3% NaOCl was used.[14] Whereas in the present study, 5% NaOCl was used since a few in vitro studies suggested better effectiveness of hypochlorite in higher concentrations.[15]

CHX interacts with the negatively charged phosphate groups on the microbial cell walls, thereby altering the osmotic equilibrium and cell permeability of microbial cell. This allows the CHX to penetrate into the bacteria. In the present study, mean value of CFUs after irrigation with CHX was 1.06 and percentage reduction of colonies was 87%. These results suggest that NaOCl showed better antimicrobial activity than CHX. Gomes et al. tested the antimicrobial activity of CHX gluconate (gel and solution) and NaOCl at different concentrations and found that CHX gel was more efficient than the liquid at equivalent concentrations. Furthermore, the growth inhibition halos produced by both forms of 2% CHX were significantly larger than those created by all concentrations of NaOCl, including 5.25% which are contradictory to the present study.[16] Results of many studies were inconclusive when the antibacterial efficacy of NaOCl and CHX was compared due to differences in the methodologies used.[14]

Chitosan is a natural polysaccharide, prepared by the deacetylation of chitin, which is obtained from the shells of crabs and shrimps. Chitosan is mainly used in areas of medicine and pharmaceuticals as an antibacterial and antitumor agent, drug carrier, wound-healing accelerator, biotechnology as enzyme and cell carrier, chromatography resin, agriculture as seed production, cosmetics and foods as iron and calcium absorption accelerator, and fiber source. It has a high chelating ability for various metal ions in acidic conditions.[5,6] Studies have already proven that chitosan has dual benefit of chelating as well as antibacterial properties and can be used as an effective alternative to routinely used irrigating solutions.[6,7] Moreover, the smear layer removal capability was equivalent to ethylenediaminetetraacetic acid and citric acid. However, there are no studies done assessing the antibacterial efficacy of chitosan in combination with other irrigants, so the present study was planned.

In the present study, the mean value of CFUs after irrigation with chitosan was 1.11 and percentage reduction of colonies was 86.4%, almost similar to that of 2% CHX. The results are similar to the study by Suzuki et al., where antibacterial effect of chitosan-citrate solution was achieved at 5 min.[17] In the present study, Group 4 and 5 when chitosan used alternatively with CHX and NaOCl, respectively, showed zero colonies and 100% reduction in CFU. It means that there is a synergistic antibacterial activity when chitosan is used alternatively with CHX or NaOCl. Malik et al. have found that when CHX is used in combination with chitosan it enhanced the sustained release property.[18]

Limitations
Further studies have to be done to know the possible chemical interactions between chitosan and NaCl, CHX, and their smear layer removal capability.

CONCLUSION
Within the limitations of this study, it can be concluded that 0.2% chitosan when used alternatively with NaOCl and CHX showed the synergistic effect with enhanced antibacterial activity than either 5% NaOCl or 2% CHX when used alone.

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Conflicts of interest
There are no conflicts of interest.

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