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Anti Microbial Activity of Chitosan Nanoparticles with Chlorhexidine- An In vitro Study

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ABSTRACT

Introduction: Irrigants also play a crucial role in removing debris and smear layer from the root canal walls, in addition to antimicrobial activity which allows for better adhesion and penetration of root canal sealers and obturation materials (1). The most commonly used irrigants in endodontic treatment include sodium hypochlorite, chlorhexidine, and ethylenediaminetetraacetic acid (EDTA) [6]. However, each of these irrigants has its own limitations and potential side effects, such as cytotoxicity, allergic reactions, and dentin erosion (2). Therefore, the search for alternative irrigants that possess better antimicrobial activity and fewer side effects is ongoing in the field of endodontics.

Materials and Methods: The antimicrobial activity of the synthesised nano chitosan with chlorhexidine and Plain Chitosan with Chlorhexidine was evaluated using the agar well diffusion technique. Mueller Hinton agar plates were prepared and sterilised using an autoclave at 121°C for 15- 20 minutes. After sterilisation, the medium was poured on to the surface of sterile Petri plates and allowed to cool to room temperature. The bacterial suspension (E.faecalis) was spread evenly onto the agar plates using sterile cotton swabs. The wells were then filled with different concentrations of nanoparticles and plain chitosan solution. An antibiotic (e.g., Bacteria-Amoxyrite) was used as a standard. The plates were incubated at 37°C for 24 hours and 48 hours for bacterial cultures.

Results: Nanochitosan with chlorhexidine shows higher antimicrobial activity when compared to plain chitosan .Its activity increases with increase in dosage.10μl shows maximum antimicrobial efficacy. Increase in the time period showed increased antimicrobial efficacy. Antimicrobial efficacy at 10μl is comparable to positive control (sodium hypochlorite)

Conclusion: The irrigant nanochitosan with chlorhexidine showed better antibacterial efficacy than sodium hypochlorite and it can be used as an irrigant in endodontics. The several known advantages of this irrigant such as naturally available, non cytotoxic, biocompatible and low cost make it a good replacement of sodium hypochlorite as an irrigant.

Keywords: *Endodontic Irrigants,Chitosan, Chitosan nanoparticles , Natural Irrigant, Antibacterial Activity, Biocompatibility, E. feacalis,Root Canal Treatment*

INTRODUCTION

Irrigants also play a crucial role in removing debris and smear layer from the root canal walls, in addition to antimicrobial activity which allows for better adhesion and penetration of root canal sealers and obturation materials (1). The most commonly used irrigants in endodontic treatment include sodium hypochlorite, chlorhexidine, and ethylenediaminetetraacetic acid (EDTA). However, each of these irrigants has its own limitations and potential side effects, such as cytotoxicity, allergic reactions, and dentin erosion (2). Therefore, the search for alternative irrigants that possess better antimicrobial activity and fewer side effects is ongoing in the field of endodontics.

NaOCl is a commonly used irrigant solution in root canal therapy, known for its unpleasant taste. Despite its toxicity, it is still widely utilised for disinfecting root canals in many parts of the world (3–5). Typically, NaOCl is employed at concentrations ranging from 0.5% to 6.0%. It is valued for its ability to dissolve tissue and its antimicrobial properties (6,7). However, there are several issues associated with its use.

One challenge is the requirement for freshly prepared NaOCl to ensure optimal antimicrobial activity (8,9). Unfortunately, in many cases, NaOCl is purchased in large containers and stored at room temperature, leading to prolonged exposure to oxygen. This exposure can significantly diminish its effectiveness.Furthermore, the accidental extrusion of NaOCl into periapical tissues can result in severe injury to the patient (10,11).

An alternative irrigant solution to NaOCl is chlorhexidine (CHX), which has been utilised for caries prevention for the past 50 years (12). It is also commonly employed in periodontal. CHX possesses a broad-spectrum antibacterial action, sustained effectiveness, and lower toxicity compared to NaOCl (13). These favourable characteristics have led to its occasional use as a root canal irrigant (14). One notable advantage of chlorhexidine over NaOCl is its lesser cytotoxicity, making it safer for use in endodontic procedures. Furthermore, it does not have the foul smell and unpleasant taste associated with NaOCl (15). However, similar to NaOCl, chlorhexidine is not able to eliminate all bacteria and does not effectively remove the smear layer (16).

Despite advancements in root canal treatment, the failure rate has remained relatively high, ranging from 18% to 26% over the past 50 years. One of the reasons for this is the current techniques' inability to effectively address the entire disease process and effectively eradicate bacterial biofilms within infected root canals. Consequently, researchers are exploring more advanced disinfection techniques and irrigants (17).Numerous studies have been conducted to evaluate new irrigants with the objective of finding solutions that are both more effective in their disinfection properties and less irritating to periapical tissues compared to NaOCl. These studies have explored various natural substances such as herbal solutions,Propolis, Chitosan (18,19), as well as antibacterial nanoparticles (20,21). These alternative substances are believed to possess comparable antibacterial efficacy to NaOCl while exhibiting lower toxicity and reduced irritation.

One of the naturally occurring polysaccharide is chitosan that is derived from the shells of crustaceans. It is characterised by its non-toxic, biodegradable and biocompatible properties. In the field of endodontics, chitosan has gained attention due to its broad-spectrum antimicrobial activity and significant chelating effects (22). Nanoparticles, on the other hand, exhibit enhanced antimicrobial activity due to their polycationic/polyanionic nature, along with their charge density and high surface area . These characteristics allow for increased interaction with bacterial cells, resulting in improved antimicrobial efficacy (23). As a result, chitosan nanoparticles (CNPs) have been utilised in various healthcare domains, including root canal therapy (24).

Numerous reports have demonstrated the favourable biocompatibility of chitosan and its derivative materials (25–28). Specifically, Seung-Yun Shin and colleagues have highlighted the excellent biocompatibility of chitosan at the nanometer scale (29,30). However, studies have shown variable antimicrobial activity of chitosan against Gram-positive and Gram-negative bacteria. Chitosan offers several advantages, including its antibacterial effects, biocompatibility, non-toxicity, biodegradability, and chelating potential. Nevertheless, its ability to penetrate is inferior to that of chlorhexidine (CHX), and at lower concentrations, it may not be equally effective compared to other irrigants.

Nano-sized chitosan materials are anticipated to exhibit enhanced penetration capabilities and improved disruption of bacterial cell membranes (31). Therefore, nano-chitosan is expected to be more effective against a wide range of organisms. The combination of nano chitosan with chlorhexidine is shown to have synergism and increases the bacterial cell penetration and the antibacterial activity. Hence, the purpose of this study was to assess the antibacterial effect of nanochitosan with chlorhexidine compared to those of NaOCl as an irrigant.

MATERIALS AND METHODS

Chitosan Synthesis

For this study the chitosan powder was obtained from dried exoskeleton of marine shrimps.

Preparation of Chitosan Nanoparticles

To prepare a solution of chitosan for use in a coating process,the dissolution mixture (500 mg of chitosan and 50 ml of 1% acetic acid solution) is stirred to get a clear solution at room temperature (1000 rpm for 25 minutes). To achieve a neutral pH of 5,the prepared solution was sonicated and titrated by adding either NaOH or HCl. The solution was then filtered using a 0.2 µ mesh. For the coating process, a solution of 5 ml of nano-magnetic solution was added to 75 mL of deionized water and sonicated for 10 minutes.The nanoparticle solution is further sonicated for 5 mins until a clear solution is obtained.

Preparation of Nanochitosan with Chlorhexidine solution

50 ml of 2% Chlorhexidiene was added to 50 ml of the prepared nano chitosan solution. The resulting solution was sonicated for 10 mins until the solution was clear.

Preparation of plain chitosan nanoparticles with chlorhexidine

To prepare a solution of chitosan and chlorhexidine, the dissolution mixture (500 mg of chitosan and 50 ml of 1% acetic acid solution) is stirred to get a clear solution at room temperature (1000 rpm for 25 minutes). The prepared solution was titrated and sonicated by adding either NaOH or HCl solution until a pH of 5 was achieved. The solution was then filtered using a 0.2 µ mesh. Next, 50 ml of 2% chlorhexidine was added to 50 ml of the prepared chitosan solution, and the resulting solution was sonicated for 10 minutes until it became clear.

Antimicrobial activity

The antimicrobial activity of the synthesised Chitosan nanoparticles with Chlorhexidine and Plain Chitosan with Chlorhexidine was evaluated using the agar well diffusion technique. Mueller Hinton agar plates were prepared and sterilised using an autoclave at 121oC for 15- 20 minutes. After sterilisation, the medium was poured on to the surface of sterile Petri plates and allowed to cool to room temperature. The bacterial suspension (E feacalis) was spread evenly onto the agar plates using sterile cotton swabs. Wells of 9mm diameter were created in the agar plates using a sterile polystyrene tip. The wells were then filled with different concentrations (25 µg, 50 µg, 100 µg) of NPs and plain chitosan solution. An antibiotic (e.g Bacteria-Amoxyrite) was used as a standard. The plates were incubated at 37°C for 24 hours and 48 hours for bacterial cultures.The antimicrobial activity was evaluated by measuring the diameter of the inhibition zone surrounding the wells. The diameter of the zone of inhibition was measured using a ruler and recorded in millimetres (mm) and the zone of inhibition was calculated.

RESULTS

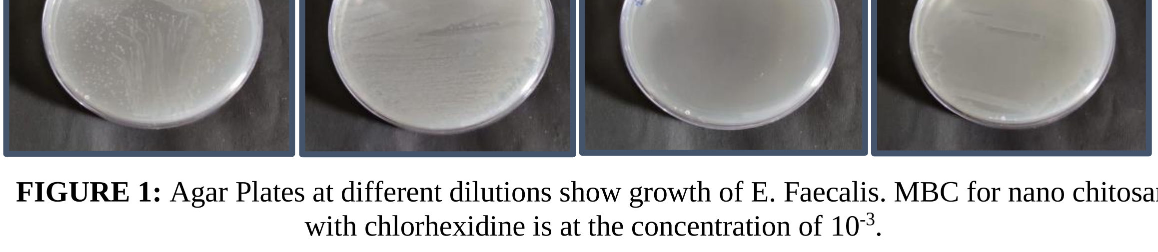


FIGURE 1: Agar Plates at different dilutions show growth of E. Faecalis. MBC for nano chitosan with chlorhexidine is at the concentration of 10^{-3}.

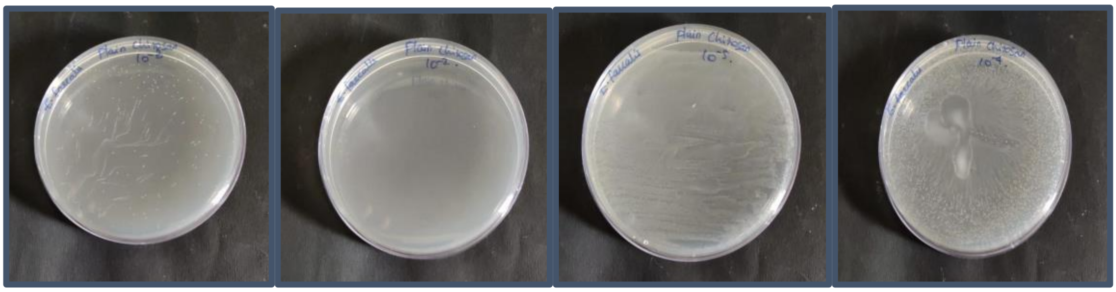
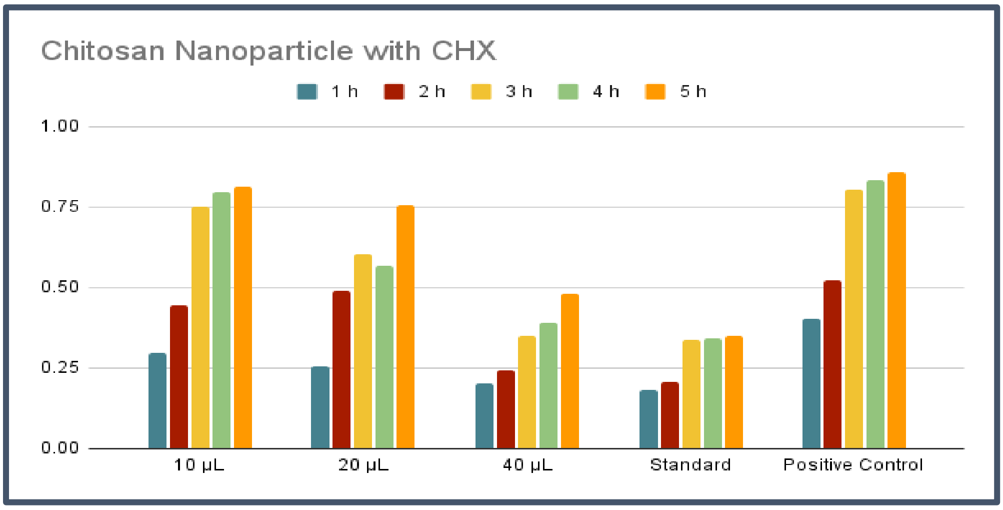
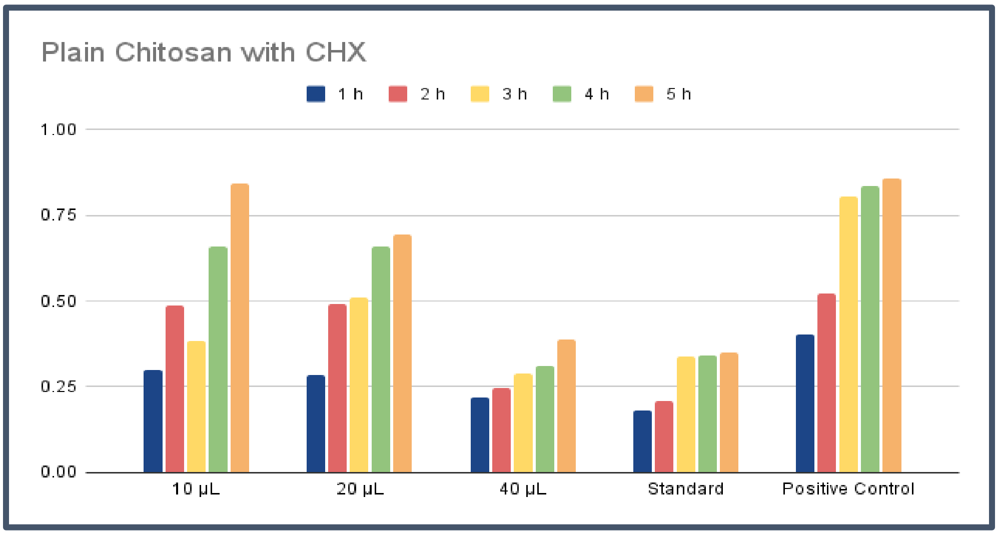


FIGURE 2: Agar Plates at different dilutions show growth of E. Faecalis. MBC for plain chitosan with chlorhexidine is at the concentration of 10^{-2}.



GRAPH 1: Graph represents anti microbial efficacy of nano chitosan with chlorhexidine at different dilutions. 10µl shows maximum antimicrobial efficacy. Increase in the time period showed increased antimicrobial efficacy. Antimicrobial efficacy at 10µl is comparable to positive control (sodium hypochlorite)



GRAPH 2: Graph represents anti microbial efficacy of chitosan nanoparticles with chlorhexidine at different dilutions. 10µl shows maximum antimicrobial efficacy. Increase in the time period showed increased antimicrobial efficacy. Antimicrobial efficacy at 10µl is comparable to positive control (sodium hypochlorite)

DISCUSSION

The process of mechanically preparing a root canal involves getting rid of any tissues, whether they are living or dead, in order to eliminate any infection in the pulp space. However, due to the complex anatomy of the root canal system, there may be hidden areas that are difficult to reach with traditional endodontic instruments. Thus, chemical disinfection is necessary to supplement mechanical preparation, resulting in what is known as chemo-mechanical preparation (32).

NaOCl remains the preferred and widely used irrigating solution in the field of Endodontics, considered as the gold standard. Therefore, in the present study, it was chosen as a reference for comparison. Specifically, a concentration of 5.25% NaOCl was utilised because previous research demonstrated that only this particular concentration was effective in completely eliminating Enterococcus faecalis, a highly resilient microorganism commonly found in endodontic infections. In contrast, lower concentrations of 1.3% and 2.5% were found to be inadequate in achieving the same level of eradication (33).

The study incorporated a 2% concentration of CHX (chlorhexidine) as a root canal irrigating solution due to its extensive antimicrobial properties and ability to remain active for an extended period of time. Additionally, CHX addresses the limitations associated with the use of NaOCl (34,35). Furthermore, the study utilised CNPs (calcium nanoparticles) with a size range of 50 ± 5 nm at a concentration of 3%. It has been proposed that a 3% solution of CNPs exhibits a positive bactericidal effect as a root canal irrigant against Enterococcus faecalis, and this effect is comparable to that of a 2.5% NaOCl solution (25).

Furthermore, previous research has indicated that CNPs with an average size of 97 nm possess potent bactericidal properties against both Gram-negative and Gram-positive bacteria (26). Based on this information, the study aimed to investigate the potential of CNPs as a carrier for delivering CHX into dentinal tubules. Previous studies have demonstrated that the antimicrobial efficacy of CNPs can be enhanced by loading them with other antimicrobial agents (19,22). Therefore, in this study, a combination of 2% CHX and 3% CNPs was employed to explore the synergistic effects and the ability of CNPs to facilitate the delivery of CHX into the dentinal tubules.

There are several theories explaining the antibacterial action of chitosan. One such theory is the contact-mediated killing theory, which suggests that chitosan, with its positive charge, interacts with the phosphoryl group present in the bacterial cell membrane. This interaction leads to an increase in membrane permeability, causing proteins and cellular components to leak out and ultimately resulting in bacterial cell death (36). Additionally, chitosan, being a chelating agent, has been proposed to inhibit bacterial growth through metal chelation. By binding to metal ions, chitosan reduces the activity of certain enzymes necessary for bacterial survival, thereby impeding bacterial growth. At the nanoscale, CNPs have the ability to enter bacterial cells and bind to their DNA. This interaction hinders the transport of RNA, which is essential for protein synthesis, thereby disrupting bacterial processes. Additionally, CNPs can impede enzymatic degradation, which is the mechanism by which bacteria penetrate into dentinal tubules. By blocking this process, CNPs contribute to inhibiting bacterial invasion into the dentinal tubules (37).

The study's results indicated that 3% NaOCl demonstrated a significantly stronger antibacterial effect against bacteria compared to 2% CHX. This finding is consistent with the research conducted by Agrawal et al. (30) and Arias et al. (38). However, it contradicts the findings of Rocas et al, who reported no significant difference in effectiveness between the two solutions. It is important to note that this discrepancy could be attributed to the variation in NaOCl concentration used in the different studies. The NaOCl concentration utilised in the current study (3%) was higher compared to the concentration (2.5%) employed in the studies by Rocas et al. (39,40). NaOCl exerts its antibacterial effects through multiple mechanisms. Firstly, the hydroxyl ions released by NaOCl act to destroy bacterial cell membranes and nucleic acids. Additionally, the high pH of NaOCl leads to the denaturation of bacterial proteins (39). Furthermore, the chloride ions released by NaOCl play a significant role in its antimicrobial activity as they dissolve organic materials, including bacterial biofilms (40,41).

On the contrary, the mode of action of CHX involves interfering with the bacterial cell walls by attaching to proteins containing phosphate. This attachment weakens the cell wall, making it more susceptible to damage. Once inside the bacterial cells, CHX forms irreversible compounds with bacterial ATP and DNA, disrupting their normal functions. Consequently, these actions lead to the demise of the bacterial cells. (40). Our team has extensive knowledge and research experience that has translated into high quality publications (42–51)). The combination chitosan with chlorhexidine shows substantivity and it synergistically increases the efficacy of the irrigant. The results of the present study show that nano chitosan with chlorhexidine showed better antimicrobial activity when compared to sodium hypochlorite.

CONCLUSION

The irrigant nanochitosan with chlorhexidine showed better antibacterial efficacy than sodium hypochlorite and it can be used as an irrigant in endodontics. The several known advantages of this irrigant such as naturally available, non cytotoxic, biocompatible and low cost make it a good replacement of sodium hypochlorite as an irrigant.

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