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# Antibacterial effect of nano-chlorhexidine on *Enterococcus faecalis* biofilm in root canal system: An *in vitro* study

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## ABSTRACT

**Background:** A new method to improve the properties of the materials is nano-encapsulation, which improves the biological properties, antibacterial activity along with reduction of toxicity. Due to the spread of nano-knowledge, the present study was performed to evaluate the antibacterial effect of nano-chlorhexidine (CHX) on *Enterococcus faecalis* biofilm in the root canal system.

**Materials and Methods:** In this *in vitro* experimental study, 55 matured single-root mandibular premolars were decoronated and the canals were prepared by single length method up to #F3 ProTaper Universal system. Five teeth were selected as negative control. Then, the teeth were randomly divided into three experimental groups ($n$ = 15) and a positive control group ($n$ = 5). The experimental groups were irrigated with 2% nano- CHX gel, 2% CHX solution, and 5.25% sodium hypochlorite (NaOCl), respectively. Finally, the number of colonies was counted. Kruskal–Wallis test was used to compare the number of colonies among groups. The level of significance was set at $P < 0.05$.

**Results:** The mean number of colonies in the groups of nano-CHX, NaOCl, CHX, and positive control were obtained as 17.73 ± 18.69, 35.53 ± 36.42, 38.8 ± 31.8, and 96.8 ± 22.52, respectively. There was a significant decrease in the number of colonies in all the experimental groups compared to the control group ($P < 0.05$). However, difference in the number of colonies among these three groups was not significant ($P > 0.05$).

**Conclusion:** The use of nano-CHX in removing *E. faecalis* biofilm from root canal is as effective as the use of CHX and NaOCl.

**Key Words:** Chlorhexidine, *Enterococcus faecalis*, nanoparticles, sodium hypochlorite

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## INTRODUCTION

The presence of bacteria and their toxins in the root canal system and their penetration into dentin tubules are known among the main causes of root canal treatment failure. The ultimate goal of endodontic treatment is to prevent or to treat apical periodontitis. Biofilm formation at the advanced stages can cause

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the resistance of infection to the host immune system.[1] *Enterococcus faecalis (E. faecalis)* is resistant to intracanal drugs and can survive in the root canal without causing any synergistic effect on other bacteria. Accordingly, this micro-organism is commonly isolated from teeth with the failed endodontic treatment.[2]

The mechanical preparation of the canal is insufficient alone to reduce the bacterial load.[3] Therefore, numerous chemicals, including sodium hypochlorite (NaOCl), calcium hydroxide, and chlorhexidine (CHX), are employed for canal disinfection.[4] NaOCl with a wide range of activities against endodontic microorganisms has the tissue dissolving ability and desirable hemostatic properties. However, it has some significant disadvantages such as bad taste and odor, cytotoxicity, and causing negative effects on the bond strength of bonding systems and cements. In addition, the extrusion of this substance beyond the root apex can consequently cause a severe periapical reaction.[5,6]

Compared to NaOCl, CHX has no unpleasant odor or taste, does not irritate periapical tissues, and its antimicrobial effectiveness on endodontics has been well established. Among the unique properties of CHX, its long-term substantivity and low toxicity compared to other substances can be named.[7] However, CHX is not able to dissolve organic materials. In addition, it has a potential of hydrogen-dependent activity, and its effectiveness is greatly reduced in the presence of organic matters like dentin particles.[8] Contradictory results regarding the efficacies of both NaOCl and CHX on the removal of *E. faecalis* biofilm have been reported in previous studies.[9,10] In Elakanty's study, no significant difference was observed between CHX and NaOCl in terms of their effects on *E. faecalis*.[9] However, in the Gomes' study, NaOCl was reported as more effective on removing *E. faecalis*.[10]

A new method proposed recently to improve the properties of chemicals is nano-encapsulation. Using this method, the drug is delivered to the target site by a nanoscale carrier. In addition, the biological impact, drug uptake, antibacterial activity, penetration, and toxicity are consequently improved because of their higher surface to volume ratio and electrical charge density.[11,12] In a previous review study, promising results have been reported regarding the effectiveness of nanoparticles as endodontic irrigants.[12] Several studies have focused on investigating the effects of nanoparticles such as silver, zinc, chitosan, and hydroxyapatite, in endodontics.[13-15] Hydroxymethylcellulose (starch), which is a cheap and available hydrophilic organic polymer with the ability to become a gel, has been used in previous nano studies as a CHX-compatible carrier.[16,17]

Given the importance of the removal of maximal biofilm from the root canal as well as the importance of *E. faecalis* as a bacterium resistant to endodontic infections, along with the need for providing a new safer and less hazardous irrigant with the ability to overcome the biofilm structure, the present study aimed to evaluate the effect of nano-CHX on the biofilm of *E. faecalis* and also to compare the potency of this material with that of the conventional canal irrigants. In the current study, zero hypothesis was that there is no difference between the experimental irrigants in terms of their antimicrobial activity against *E. faecalis*.

## MATERIALS AND METHODS

This *in vitro* experimental study was conducted after obtaining the ethical code (IR.QUMS.REC.1399.298) from the ethics committee of Qazvin University of Medical Sciences. In this experimental *in vitro* study, 55 mandibular premolars were included.

### Inclusion and exclusion criteria

The inclusion criteria were the followings: matured teeth with single root and canal, roots with round cross section, and no caries. The samples were excluded if the presence of any internal or external resorption, calcification of the canal or pulp chamber, and severe root curvature were observed.

### Preparation of samples

The collected teeth were firstly stored in Chloramine T for 48 h and then kept in the sterile saline solution at room temperature. Thereafter, the crown of the teeth was cut using a diamond disk (Teeskavan, Tehran, Iran). All the roots reached the standard length of 12 mm according to Mittal *et al*. study.[18] The working length was determined by placing K-file #15 (Dentsply Maillefer, Ballaigues, Switzerland) into the canal along with viewing the tip of the file from the apical foramen. The canals were prepared with length up to 0.5 mm shorter than working length using ProTaper Universal system (Dentsply Maillefer, Ballaigues, Switzerland) files from S1 to F3 via single length method. Afterward, 2 ml

of 5.25% NaOCl (Cerkamed Medical Company, Stalowa, Poland) was used between each 2 files. To remove the smear layer, the canals were irrigated with 1 ml of 17% ethylenediaminetetraacetic acid (Morvabon, Tehran, Iran) for 1 min, 5 ml of normal saline (SAMEN Co., Mashhad, Iran), and 1 ml of NaOCl 5.25% for 1 min, respectively. Next, the apical foramen was sealed using a self-cure Glass ionomer (Fuji IX GP, GC Corp., Tokyo, Japan). The root surface was sealed with 2 layers of nail polish, and each tooth was then transferred to a laboratory tube containing brain heart infusion (BHI) (Beijing Land Bridge Technology Co., Ltd., China) broth and sterilized in an autoclave for 15 min at 121°C. The tubes were firstly sealed and then incubated for 48 h at 37°C. Finally, 5 teeth were randomly selected as the negative control and then incubated in BHI broth for 24 h, indicating the sterilization accuracy and no bacterial growth.[18]

As well, *E. faecalis* (ATCC: 29212) was transferred to Broth Todd agar plate and then incubated for 24 h at 37°C. Thereafter, single colonies were inoculated in 10 ml of BHI broth and incubated for 24 h at 37°C. Moreover, a suspension was prepared as 0.5 McFarland ($1.5 \times 10^8$ colony-forming unit [CFU]/ml). Next, 0.01 ml of the suspension was injected into the canals through an insulin syringe. The obtained samples were then incubated for 2 weeks at 37°C under anerobic condition and the culture medium was replaced every day. After the incubation process, the teeth were randomly divided into 3 experimental groups ($n$ = 15) as well as a positive control group ($n$ = 5). In the experimental groups, the canals were firstly dried by sterile paper cone (#40) and root canals were then irrigated with 5 ml of 2% nano-CHX gel, 5 ml of 2% CHX solution, and 5 ml of 5.25% NaOCl for 1 min. Notably, no irrigation was performed in the positive control group and in this regard, only the CFU was counted. Sample's preparation was done by an experienced operator who was blind to the group categorization.

## Microbiological sampling

For microbiological sampling, sterile piezoreemer #2 (Mani Inc., Tochigi-ken, Japan) was used with low-speed handpiece for 20 s, and a new sterile piezoreemer was employed for each sample. Two sterile paper cones #40 were used to transfer dentin debris from the canal to the culture medium. The paper cones were then transferred into a laboratory tube containing 10 ml of normal saline. The saline was repeatedly diluted in several tubes for 10 times and then 100 µl of the samples was transferred to BHI medium through a sampler. The microbiological sampling was done by the same operator who was blind to the group categorization. The obtained samples were then incubated for 48 h at 37°C. Finally, the colonies were blindly counted in a biological hood under magnification by an expert microbiologist.

## Modification of rice particles and nanogel preparation

Raw rice powder was etherified with mixture of ethanol-water as a medium. The reaction was performed in a flask with a motor-driven stirrer. Fifty percent sodium hydroxide solution was mixed with ethanol at a 1:4 weight ratio. Following the addition of raw rice powder, the mixture was stirred and the temperature was elevated to 40°C for 30 min. Subsequently, monochloroacetic acid was added to the mixture. Afterward, the temperature of the mixture was raised to 50°C and stirred for 3 h. The solid mass was separated, neutralized with acetic acid, and then washed with 95% ethanol neutralized several times using glacial acetic acid until the time that the silver nitrate test for chloride of filtrate was negative.

0.5g of modified rice powder was weighed and then dispersed in 100 ml distilled water in two-neck round-bottom flask. Afterward, the flask lid was closed with a vacuum valve on the one side and a stopper on the other side, so that there was no air inside the container, and it was then put on the heater stirrer for 2 h at 90°C to be stirred, to obtain a homogenous and clear gel liquid. The appearance of the gel was observed visually after finishing the work, and the size and potential of Zeta were taken from the desired gel. After 2 h, the container was opened and after a little cooling, CHX powder was added to it.

## Size and surface charge determination

Dynamic light scattering was used to determine the size of nanocarriers. Scanning electron microscope was also applied to determine and confirm the diameter and morphology of the nanoparticles.

## Minimum inhibitory concentration and minimum bactericidal concentration determination

Minimum inhibitory concentration (MIC) of nanogel against *E. faecalis* was determined by the microbroth dilution method. One hundred µl of Müeller-Hinton broth medium was added to tubes 1–10. One hundred µl of nanogel (2000 µg/µl) was added to tube 1. After the vortex, 100 µl of nanogel

was added to the next tube, and the procedure was continued to tube number 10. In the next step, 100 µl of microbial suspension ($1 \times 10^6$) was added to each tube. The tubes were incubated at 37°C for 24 h. The lowest concentration that inhibited bacterial growth was MIC. To determine minimum bactericidal concentration (MBC), 100 µl of diluted solution with no sign of turbidity (prior to addition of microbial suspension) was cultured on Müeller-Hinton agar under aseptic conditions. The suspension was incubated at 37°C for 24 h. The colonies were counted, and the lowest concentration that destroyed 99.9% of isolates was determined as MBC.

### Statistical analysis

The obtained data were entered into SPSS software (IBM SPSS Statistics v. 22, IBM Corp., NY, USA). Kolmogorov–Smirnov test was then used to evaluate the normal distribution. Kruskal–Wallis nonparametric test was used to compare the colony counts among the three groups. The statistical significance level was considered as 0.05. According to Mittal *et al.*'s study,[18] the number of samples, considering the first type error = 0.05 and the second type error = 0.2, was calculated as 15 teeth for each experimental group and 5 teeth for both the positive and negative controls, each one using G*POWER software.

## RESULTS

### Comparison of experimental groups

The mean and standard deviation of the colony counts in each group are reported in Table 1. The maximum colony growth was observed in the samples obtained from the positive control group and no bacterial growth was found in the samples belonged to the negative control group.

The difference between the mean colony counts and the *P* values of the study groups is shown in Table 2 and Figure 1. The mean colony counts in all three experimental groups significantly reduced compared to that of the positive control group ($P < 0.05$).

The mean colony counts were obtained as 17.73 ± 18.69, 35.53 ± 36.42, and 38.8 ± 31.8 in the Nano-CHX, NaOCl, and CHX groups, respectively. Although the lowest mean colony counts were observed in the Nano-CHX group, the difference with the CHX group ($P = 0.216$) and NaOCl ($P = 0.356$) was not statistically significant. Furthermore, the difference between the mean colony counts between the CHX and hypochlorite groups was not statistically significant ($P = 0.990$).

**Table 1: Mean and standard deviation of colony-forming unit values (colony-forming units/ml) in the groups**

| Group | Mean±SD (CFUs/ml) |
|---|---|
| Sodium hypochlorite | 35.53±36.42 |
| Nano-chlorhexidine | 17.73±18.69 |
| Chlorhexidine | 38.8±31.8 |
| Positive control | 96.8±22.52 |

CFUs: Colony-forming units; SD: Standard deviation

**Table 2: Comparisons of colony-forming unit values (colony-forming units/ml) between the groups**

| | Mean difference (CFUs) | *P* |
|---|---|---|
| Sodium hypochlorite | | |
| Nano-chlorhexidine | 17.80 | 0.356 |
| Chlorhexidine | −3.27 | 0.990 |
| Positive control | −61.27 | 0.001 |
| Nano-chlorhexidine | | |
| Chlorhexidine | −79.07 | 0.216 |
| Positive control | −21.07 | 0.000 |
| Chlorhexidine | | |
| Positive control | −58.00 | 0.002 |

CFUs: Colony-forming units

### Nanogel structure

The formation of nanogel structure was confirmed by scanning electron microscopic [Figure 2]. Nanogel showed homogeneous nanofibers morphology, and the particle size average was 100 nm (ranged from 70 to 300 nm), which is in agreement with dynamic light scattering values [Figure 3].

### Antimicrobial susceptibility testing

Antibacterial effect of nano-CHX against *E. faecalis* growth was determined by antimicrobial susceptibility testing. The results demonstrated that *E. faecalis* growth inhibition at concentration was 15.6 µg/ml. Furthermore, the MBC value was 31.2 µg/ml. On the other hand, the MIC and MBC values of CHX were reported as 62.5 µg/ml and 125 µg/ml, respectively.

## DISCUSSION

Canal irrigants used in endodontics should have both great antimicrobial properties and less destructive effect on surrounding tissues.[19] In the present study, the new material, namely nano-CHX, was used as a canal disinfectant with the aim of comparing it with other conventional canal disinfectants.

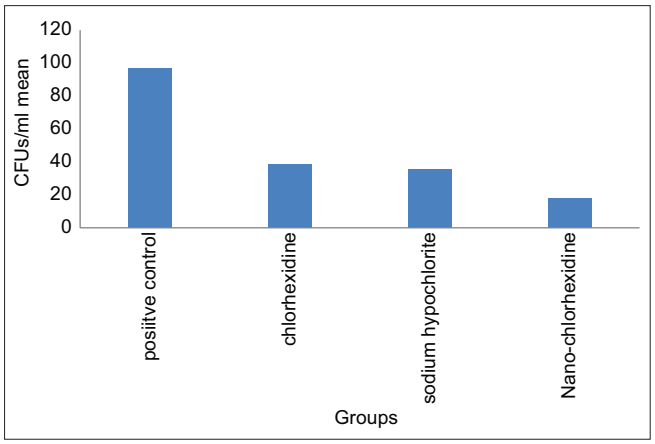


**Figure 1:** Comparisons of colony-forming units' values between the groups.

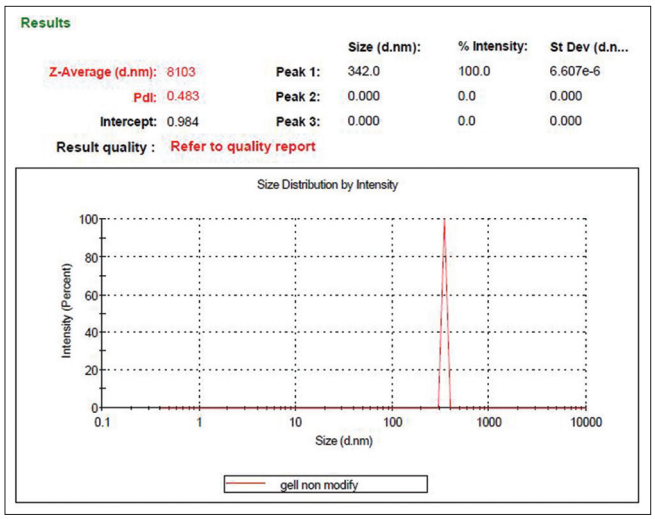


**Figure 2:** Size distribution profile of particles using dynamic light scattering.

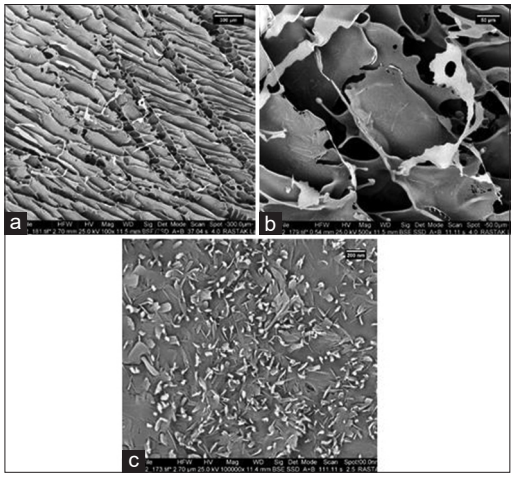


**Figure 3:** Scanning electron microscope images; (a) uniform distribution pattern of nano carrier at×100 magnification; (b) nanoparticles at×500 magnification; (c) morphology and dimensions of starch nanoparticles at×100000 magnification.

Bacteria can penetrate into the tubules to a depth of 1000 μm, so they cannot be removed from the tubules using some conventional irrigating methods.[20] In the present study, *E. faecalis* biofilm was used due to its ability to create a "live but uncultivable" condition following performing the clinical treatment processes that can reduce the effect of antibacterial agent.[21] A recent systematic review has shown that despite the effectiveness of both hypochlorite and CHX, as irrigants, they are not able to remove the infection from the canal completely.[7]

According to the results of the present study, all 3 irrigation solutions, including nano-CHX, CHX, and NaOCl, significantly reduced the colony counts compared to the control group, indicating their effectiveness on the biofilm of *E. faecalis*, which is in agreement with the results of previous studies in this field.[22-24] In the present study, the activity of nano-CHX in the removal of *E. faecalis* was found to be similar to the effect of NaOCl, which is in line with the results of previous studies.[13,25] According to the results of the Moghadas' study, there is no significant difference in the mechanism of nanoparticle irrigants with that of NaOCl, as canal disinfectants, in removing *E. faecalis*.[26] On the other hand, some studies have previously found that the use of nano-carrier irrigant in destroying *E. faecalis* biofilm and other bacteria in the biofilm is not as effective as NaOCl.[27,28] Differences in the obtained results may be attributed to the differences in studies' design, duration of nanoparticle irrigation, and the type of carrier used.

In the present study, 2% nano-CHX gel was used with the aim of eliminating the limitations of the common irrigants used in the removal of microbial biofilm from the root canal system. In some recent studies, CHX has been used with several carriers such as polylactic-co-glycolic acid[29,30] and silica.[31] Due to the reported cytotoxicity in some carriers and their high cost,[32,33] in the present study, starch was used as the carrier of choice for CHX nanoencapsulation. Accordingly, starch is an inert, highly water-soluble, nonionic substance used in the form of gel.[16,17] Vivacqua Gomes *et al*.[34] showed that the gel form of CHX, unlike that of NaOCl, cannot impair the sealing ability of cements. Furthermore, it was shown that a final flush of 5 ml with distilled water can lead the dentin walls to be almost free of the smear layer, which consequently compensates the inability of CHX in dissolving organic tissues. This nanoencapsulation process makes particles smaller than dentinal tubules.

Thereafter, it increases the penetration ability of antibacterial drugs into the tubules. Subsequently, the distribution of antibacterial particles in the matrix occurs with very weak polymeric bonds. Correspondingly, this fragile connection would be broken by causing a minimal shear stress such as the injection of the substance using a syringe, and thereafter, the antibacterial drug will be released. Moreover, this property makes the material an ideal candidate to be used as an irrigant.[22] The study by Priyadarshini *et al*.[23] has previously confirmed the efficacy of nano-CHX in the removal of bacteria existing inside the dentin microtubules due to its proper penetration and substantivity of the drug in the tubules. In addition, in another study, it was shown that nano-CHX could significantly reduce the activities of planktonic bacteria and mono-species biofilms such as *Streptococcus mutans*, *Streptococcus sobrinus*, *Fusobacterium nucleatum*, and. *E. faecalis*.[31]

In this study, although no significant difference was found among the study groups in terms of the effect of irrigants on *E. faecalis* biofilm, the mean colony counts for the nano-CHX group was lower than those of the CHX and NaOCl groups. Accordingly, this was expected and it can be justified due to the properties of nano-CHX as a substance with the hydrogel nanoparticle structure. Some properties such as hydrophilicity, high biocompatibility, permeability, and high substantivity of the material can be considered as potential factors causing further reduction in the mean number of colonies in the nano-CHX group.[22,30,31]

### Limitations

One of the limitations of the present study is small sample size leading to insignificant difference between the groups. Other limitation is short-time evaluation of the outcomes. It is recommended to conduct similar studies with greater sample size to evaluate the antimicrobial results in longer time intervals.

## CONCLUSION

Nano-CHX in disinfecting the canal was as effective as the conventional canal irrigating agents such as CHX and NaOCl. Due to the limitation of the present study, nano-CHX gel can be suggested as an alternative final irrigant with a high efficacy and less side effects.

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### Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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