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Full Length Research Paper

Antimicrobial efficacy of nanosilver, sodium hypochlorite and chlorhexidine gluconate against *Enterococcus faecalis*

Mehrdad Lotfi[1,3],Sepideh Vosoughhosseini[1,2]*,Bahram Ranjkesh[3],Sajjad Khani[1],Mohammadali Saghiri[4] andVahid Zand[3]

[1]Research Center for Pharmaceutical Nanotechnology, Tabriz University (Medical Sciences), Tabriz, Iran.
[2]Department of Oral and Maxillofacial Pathology, Dental Faculty, Tabriz University (Medical Sciences), Tabriz, Iran.
[3]Department of Endodontics, Dental School, Tabriz University (Medical Sciences), Tabriz, Iran.
[4]Dental Material Department, Dental School, Azad University, Tehran, Iran.

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The purpose of this study was to compare the antibacterial efficacy of nanosilver (NS), chlorhexidine gluconate (CHX) and sodium hypochlorite (NaOCl) against *Enterococcus faecalis*. Two tests of minimum inhibitory concentration (MIC) and zone of inhibition were carried out using NS, NaOCl and CHX. 70-fold concentration of NaOCl is required for the same antibacterial effect of NS. CHX precipitated in contact with the culture medium and was excluded from MIC test. The means and standard deviations of the zones of inhibition for 5.25% NaOCl, 0.33% NaOCl, 25 µg/ml NS, 50 µg/ml NS, 4000 µg/ml NS and 2% CHX were 12.16 ± 1.46, 6.91 ± 0.66, 10.00 ± 0.42, 12.00 ± 0.60, 13.33 ± 1.23 and 24.80 ± 1.11, respectively. Statistical analysis using ANOVA showed significant differences among groups ($p < 0.001$). A post hoc Tukey test revealed no significant differences between 5.25% NaOCl and 4000 µg/ml NS ($p = 0.057$). However, the zones of inhibition for 2% CHX were significantly larger than those seen around the filter papers saturated with undiluted NaOCl and NS ($p < 0.001$ for both). This study revealed that NS in a remarkably lower concentration would possess the same bactericidal effect as 5.25% NaOCl.

Key words: Chlorhexidine gluconate, *Enterococcus faecalis*, nanosilver, sodium hypochlorite.

INTRODUCTION

Microorganisms are the main causes of pulp and periapical diseases (Kakehashi et al., 1965). Hence, root canal treatment mainly focuses on thorough elimination of bacteria from the root canals. Mechanical preparation is the main mechanism to reduce the bacterial load in canals, which is enhanced by intracanal irrigants. In spite of these procedures, some bacteria might persist within the canals. *Enterococcus faecalis* is the most common and dominant bacterial species and sometimes the only bacteria isolated from failed endodontic treatment cases and teeth with persistent periodontitis (Rocas et al., 2004). Consequently, current *in vitro* studies have placed great emphasis on the effectiveness of irrigants against *E. faecalis.*

Several antimicrobial solutions are used in endodontic treatment, including sodium hypochlorite (NaOCl), chlorhexidine gluconate (CHX) and recently, Biopure MTAD. Zender et al. (2002) reported that 0.0005% concentration of NaOCl could eliminate bacteria in 3 min. Portenier et al. (2005) concluded that 0.0001% NaOCl killed *E. faecalis* almost promptly after contact. However, some researchers (Vianna et al., 2004; Radcliffe et al., 2004; Retamozo et al., 2010) showed that more contact time and higher NaOCl concentration are necessary to eliminate *E. faecalis*. Instrumentation and irrigation with sodium hypochlorite could eliminate bacteria in 50 to 75%

*Corresponding author. E-mail svosough.rare@yahoo.com. Tel: +98 411 3355965. Fax: +98 411 3346977.

Abbreviations: NS, Nanosilver; **CHX,** chlorhexidine gluconate; **NaOCl,** sodium hypochlorite; **MIC,** minimum inhibitory concentration.

of infected root canals at the end of the first treatment session (Byström and Sundqvist, 1983; Peters et al., 2002). Moreover, Nair et al. (2005) concluded that even after instrumentation and irrigation with NaOCl in 88% of cases, infection in the root canals could be detected after obturation in one-visit treatment. On the other hand, negative findings regarding toxicity prompted recommendations to dilute 5.25% NaOCl to lower concentrations (Heggers et al., 1991). However, Harrison and Hand (1981) have demonstrated that diluting 5.25% NaOCl reduces antibacterial properties.

CHX has been shown to have a vast antimicrobial potential and has been recommended as an endodontic irrigant (Jeansonne and White, 1994; White et al., 1999), particularly because of the fact that its antibacterial effect would increase with time when it remains for several days within the canals (Lin et al., 2003; Delany et al., 1982). Aqueous solutions of 0.1 to 2.0% are used in periodontics, but 2% concentration has been considered as root canal irrigant in endodontic literature (Zamany et al., 2003). Moreover, it is safer and less caustic than NaOCl. However, data suggest that CHX is highly cytotoxic *in vitro* and caution should be exercised with the use of this antiseptic in the oral surgical procedures (Giannelli et al., 2008; Bonacorsi et al., 2004).

Various nano-particles have gained popularity as antimicrobial agents as a result of their broad spectrum of activity and biocompatibility (Neal, 2008). Recent studies have focused on using nano-particulate materials to disinfect root canals (Kishen et al., 2008; Cheng et al., 2004). Nanosilver (NS) shows antibacterial effect; it also exhibits novel physicochemical and biological activities (Kishen et al., 2008). In the medical field, there are wound dressings, contraceptive devices, surgical instruments and bone prostheses that are all coated or embedded with NS (Chen et al., 2003; Cohen et al., 2007; Lansdown, 2006). Previous studies have investigated the antibacterial effect of NS on both Gram positive and negative bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (Alt et al., 2004; Feng et al., 2000; Morones et al., 2005). However, the antibacterial effect of NS on *E. faecalis* has not been studied. The purpose of this study was to compare antibacterial efficacy of NS, CHX and NaOCl against *E. faecalis.*

MATERIALS AND METHODS

Two tests were completed to determine the antibacterial activity of NS. In the first one, the minimum inhibitory concentration (MIC) of materials against *E. faecalis* was measured.

Liquid (L)-form of NS (Nanocid Company, Tehran, Iran) was prepared. In compliance with manufacturer's instructions, NS was prepared using a two-step procedure. First, nano-particles were produced using a catalytic chemical vapors deposition procedure and then added to distilled water. No surfactant was used in the L-form NS fluid suspensions. The mixture was prepared using an ultrasonic homogenizer. The nano-particles used in this experiment were silver particles 35 nm (average) in size.

An overnight culture of *E. faecalis* (ATCC 2367) was harvested in brain-heart infusion (BHI) broth and the concentration was adjusted to optical density of 0.11 at 570 nm. The original concentration of stock NS fluid was 4000 µg/ml (0.4%). To investigate the MIC of materials, concentration of NS was adjusted to 400 µg/ml (0.04%). The concentrations of NaOCl (Pakshoma, Tehran, Iran) and CHX (FGM, Dentscare LTDA, Brasilia, Brazil) were 5.25 and 2%, respectively. To determine the MIC of the samples, the serial dilution method was used. Samples were diluted from 1:2 up to 1:2048 dilutions. Each of the twelve test tubes was filled with 5 ml of BHI broth. The first test tube (number 1) received 5 ml of the solution containing 400 µg/ml of silver and mixed thoroughly with the culture medium. The concentration of NS in the first test tube was 200 µg/ml (0.02%). Then, 5 ml of the content of the first test tube was added to the next test tube (number 2) and mixed completely. This process was performed serially to test tube number 12. At the end, 5 ml of the content of test tube number 12 was discarded. The same procedure was repeated for NaOCl and CHX samples. In the samples in tube number 1, 10 ml of 5.25% NaOCl and 2% CHX was filled purely without any medium to maintain the first concentration of solutions. The next tubes were filled with 5 ml of the medium and serial dilution method was carried out. Twelve tubes with the same concentration of samples were set as control groups to be compared for turbidity. Finally, 100 µl of standard microbial suspensions of *E. faecalis* were added to test tubes 1 to 12. The test tubes were incubated at 37°C for 48 h. Then, the microbial growth was determined by the presence or absence of turbidity after 6, 18, 24 and 48 h of incubation.

In the next experiment, zone of inhibition on plates inoculated with *E. faecalis* was investigated to determine the extent of antibacterial activity of the materials. An overnight culture of *E. faecalis* (ATCC 2367) was standardized to 0.11 OD measured at 570 nm. One hundred milliliter of the microorganism was spread onto a BHI agar (BHIA) plate using a sterile L-shaped glass rod. One-quarter-inch sterile S & S filter paper (Schleicher and Schuell) was placed in each of the four quadrants of the BHI plate. Twenty microliters of each of the following solutions were placed on the filter papers: 5.25% NaOCl, 0.33% NaOCl, 25 µg/ml (0.0025%) of NS, 50 µg/ml (0.005%) of NS, 4000 µg/ml (0.4%) of NS and 2% CHX. Vancomycin bacterial susceptibility papers were used as positive and sterile saline (Samen, Mashad, Iran) as negative control groups. Twelve replicas were prepared for each sample. The plates were incubated overnight at 37°C for 24 h and the zones of inhibition were measured in millimeters.

RESULTS

The results of MIC are listed in Table 1. Instant precipitation and turbidity of CHX groups occurred immediately after CHX and BHI mediums were mixed. Therefore, the CHX group was excluded. In the NS group, in the first 6 h after incubation, 12.5 µg/ml (0.00125%) NS could inhibit the growth of *E. faecalis*, whereas in NaOCl group, inhibition took place at 0.082% in 6 h. It means that 70-fold concentration of NaOCl was required to posses the same antibacterial effect of NS. After 18 h of incubation, the effective concentrations for NS and NaOCl were 0.005% (50 µg/ml) and 0.328%, respectively. In other words, approximately 70-fold concentration of NaOCl is required to exhibit the same effect as NS. These concentrations remained unchanged up to 48 h after incubation.

Table 1. Minimum inhibitory concentration dilutions of NS and NaOCl against *E. f*aecalis.

Antibacterial	6 h	18 h	24 h	28 h
NS	12.5 µg/ml (0.00125%)	50 µg/ml (0.005%)	50 µg/ml (0.005%)	50 µg/ml (0.005%)
NaOCl	0.082%	0.328%	0.328%	0.328%

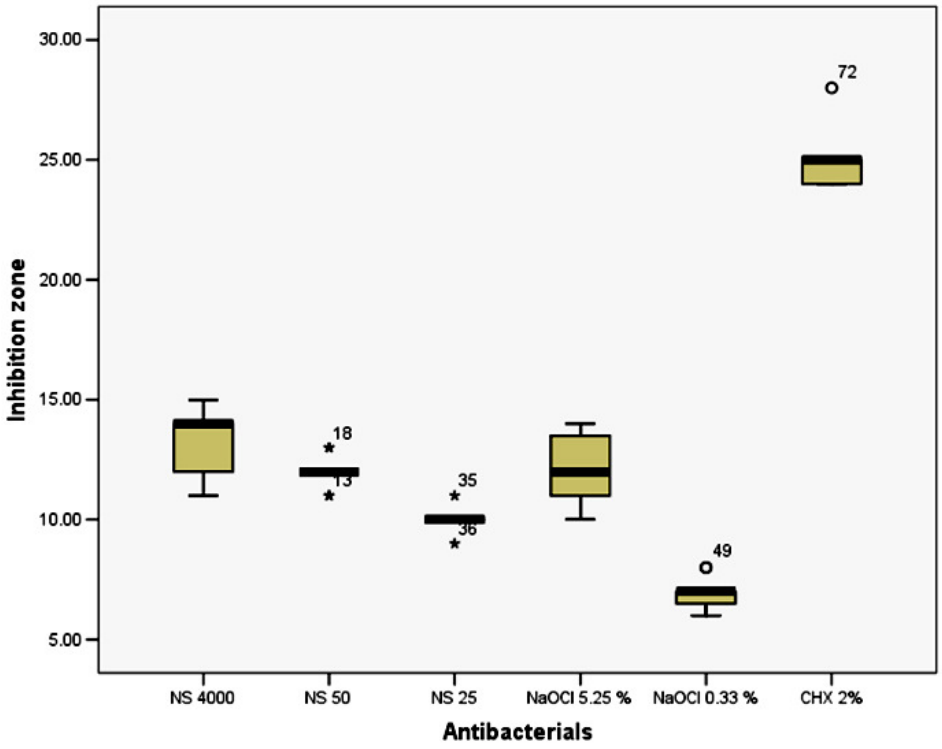

Figure 1. Box plots of the inhibition zone of different concentrations of antibacterial solutions in culture media contact, which illustrate the mean ± standard deviation of minimum and maximum zone of inhibitions, as well as the variance in each experimental group.

No zone of inhibition was observed adjacent to the filter papers saturated with sterile saline. The means ± standard deviations of the zones of inhibition for vancomycin susceptibility paper were 21.12 ± 0.14 mm. The means and standard deviations of the zones of inhibition for 5.25% NaOCl, 0.33% NaOCl, 25 µg/ml NS, 50 µg/ml NS, 4000 µg/ml and 2% CHX were 12.16 ± 1.46, 6.91 ± 0.66, 10.00 ± .42, 12.00 ± 0.60, 13.33 ± 1.23 and 24.80 ± 1.11, respectively. Statistical analysis using ANOVA showed significant differences among groups ($p < 0.001$). A post hoc Tukey test revealed no significant differences between 5.25% NaOCl and 4000 µg/ml NS ($p = 0.057$). However, the zones of inhibition for 2% CHX were significantly larger than those seen around the filter papers saturated with undiluted NaOCl and NS ($p < 0.001$ for both). Dilution of the solutions reduced the zones of inhibition for NaOCl and NS solutions, but at different rates. There were no significant differences among 5.25% NaOCl, 25 µg/ml NS, 50 µg/ml NS and 4000 µg/ml NS ($p < 0.05$). In other words, at 70-fold dilution, NS created a zone of inhibition that was not significantly smaller in diameter than undiluted NaOCl ($p = 0.99$) (Figure 1).

DISCUSSION

In this study, antibacterial effect of NS on *E. faecalis* was evaluated and compared with that of NaOCl and CHX. Two standard and routine microbiological tests, agar diffusion and MIC, were used in this study. In Agar diffusion test, the effectiveness of an antibacterial material against bacteria is measured in a grown culture. In this method, the diameter of the microbial inhibition zone depends on the solubility and infusibility of the test material; therefore, it may not exhibit its full potential. Direct and close contact between the microorganism and the samples are examined by direct contact tests, independent of the diffusion properties of the tested material and media, which is an advantage over other

tests similar to agar diffusion method (Estrela et al., 2001). MIC method uses serial dilutions of a solution to determine the lowest concentration of material that would still show antibacterial properties. Instant precipitation and turbidity of CHX groups immediately after CHX and BHI medium showed that this kind of medium is not suitable for MIC experiments. *E. faecalis* was selected because this bacterium is the most commonly isolated organism in endodontic retreatment cases with apical periodontitis (Rocas et al., 2004; Haapasalo et al., 2005).

In this study, 0.00125% NS showed inhibition effect against *E. faecalis* in MIC test in the first 6 h, whereas NaOCl could exert the same effect at a concentration of 0.082%. It means that the concentration of NaOCl should increase approximately 70 folds to demonstrate the same antibacterial effect as NS against *E. f*aecalis. These values reached 0.005 and 0.328% for NS and NaOCl, respectively, after 18 h and remained unchanged for 48 h. In other words, the proportion of 70 folds remained unchanged until the end of the study. Heling et al. (2001) and Bulacio et al. (2006) reported MIC values of 0.157 and 0.2% after 24 h for NaOCl. These values are lesser than the ones reported here. Various strains of bacteria can explain these differences. Another important finding was almost the same amount of inhibition diameter between undiluted NaOCl and 0.005% NS ($p > 0.05$). This result indicates that NS could exhibit same antibacterial potential as undiluted NaOCl even after 1:70 dilutions of the original solution; therefore, NS would possess the same bactericidal effect as 5.25% NaOCl in a remarkably lower concentration (0.005%), which is almost 1:1000 of NaOCl concentration. Data also confirmed that antibacterial properties of NaOCl and NS depend on the concentration of the solution. In direct contact test (MIC) of materials, effective NS concentration was also lower than those for NaOCl: 0.005% for NS and 0.33% for NaOCl; NaOCl is in a higher concentration as compared with NS (about 1:65). On the other hand, 0.33% NaOCl proved to be the effective concentration in MIC test, but in agar model, it showed the smallest zone of inhibition among the samples. In contrast, MIC of NS results (0.005%) showed almost the same performance of 0.4% NS. Recently, Hiraishi et al. (2010) compared the efficacy of 3.8% silver diamine fluoride with that of 5.25% NaOCl in eliminating *E. faecalis* and reported that both solutions showed the same potential. Therefore, they recommended the use of silver diamine fluoride as an intracanal irrigant or intersession medicament. In our study, 0.005% NS showed equal bactericidal action as 5.25% NaOCl. This considerable lower concentration of NS shows its ultrafine particle size, which causes its action.

Regarding the comparison of NaOCl and CHX, our findings do not coincide with those of Giardino et al. (2009). They showed that 5.25% NaOCl is more effective than CHX against *E. faecalis*. In contrast, Vianna et al. (2004) concluded that 2% CHX yields larger zones of inhibition as compared to NaOCl in different concentrations. These discrepancies among the results might be attributed to the experimental methods, various bacterial strains, biological indicators and exposure time.

Our findings confirmed the relationship between NaOCl concentration and its antibacterial potential, which can be extended to NS, as decreases in NS concentration led to decreases in antibacterial properties but at different rates when compared with NaOCl. By considering the results of both antibacterial tests, the concentration of 50 µg/ml (0.005%) NS showed an acceptable antibacterial action against *E. faecalis*.

The biologic effects of silver are believed to be closely related to silver ion (Chen and Schluesener, 2008; Matsumura et al., 2003). In an aqueous microenvironment, silver nano-particles continuously release silver ion (Lok et al., 2007). It is well known that smaller silver nano-particles show stronger and better bactericidal effect than larger particles because they have a larger surface area for interaction (Doty et al., 2005). Binding to essential cellular structural elements like enzymes and other proteins (Ghandour et al., 1988), particularly to their SH-groups (Grier, 1977; Petering, 1976) and interfering with the integrity of the bacterial cell (Schreurs and Rosenberg, 1982) are the main reasons for bactericidal properties of silver ions.

With regards to cytotoxicity of NS, Miura and Shinohara (2009) determined the biologic effects of NS exposure to mammalian cells. In their paper, Hela cells were evaluated by being exposed to different concentrations of NS. They concluded that 80 µg/ml concentration could be harmful for Hela cells. Alt et al. (2004) showed that 1% NS polymethylmetacrylate bone cement was free of *in vivo* cytotoxicity.

According to the results of this study, due to antibacterial effect of NS even in 50 µg/ml concentration, which is lower than 80 µg/ml, it seems that the use of NS as an antibacterial agent against *E. faecalis* is possible. Further studies are recommended to elucidate the interaction of NS in the presence of dentinal structures. By considering this study, a therapeutic window can be provided to use NS in 0.005% concentration as a novel intracanal irrigant. On the other hand, because of broad spectrum antibacterial action of NS and its influence on Gram positive and negative bacteria, more investigations are needed to consider NS as an irrigant in primary polymicrobial endodontic infections.

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