Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2013, 5(10):65-71

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

An *in vitro* assessment of the antibacterial properties of nanosilver Iranian MTA against *Porphyromonas gingivalis*

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ABSTRACT

The purpose of this study is to evaluate the antimicrobial effect of Iranian mineral trioxide aggregate (IMTA) and NanoSilver-IMTA (NS-IMTA) at clinical concentrations against Porphyromonas gingivalis, a Gram-negative anaerobic black-pigmented bacterium that is strongly associated with acute symptoms and signs in endodontic infections. We combined NS (0.1 mg/mL) with IMTA at 6, 12 and 25% concentrations. To evaluate the antimicrobial effect, agar diffusion and broth dilution methods were used. The data were analyzed using one-way ANOVA and post hoc analysis. In the agar diffusion test, 12% and 25% NS-IMTA showed zones of inhibition against P. *gingivalis, while IMTA and 6% NS-IMTA showed no zone. In the broth dilution assay, IMTA reduced the growth of* P. *gingivalis by 30.8% and 36.2% after 48h and 72 h, respectively. For 6% NS-IMTA, although the reduction in the number of viable bacteria was slightly higher than that of the IMTA after 72 h, there was no significant difference. After 48 h, IMTA containing 12% and 25% NS completely inhibited the proliferation of P. gingivalis. A statistically significance difference ($p < 0.05$) was found between IMTA and IMTA containing 12% and 25% NS. In Conclusions, NS-IMTA completely inhibits the proliferation of* P. *gingivalis in a dose-dependent manner that may have a significant effect in prognosis for root perforation.*

Key words: MTA; NanoSilver; Antimicrobial effects; *Porphyromonas gingivalis*

INTRODUCTION

Root perforation is defined as the non-natural connections between the root canal system and the periodontium. Such perforations can be complications resulting from medical treatment or progressive resorption and caries. Iatrogenic root perforations, occurring in approximately 2-12% of endodontic treatments, occur as a result of access opening, canal orifice search, excessive dentin removal from the “danger zones”, misdirected files during canal negotiation and misaligned instruments during post-space preparation. Of all root perforations, 47% were found to be related to endodontically treated teeth, and 53% were related to prosthodontic treatment. Various risk factors that may lead to root perforations include tooth anatomy, curved canals, tooth location, and the practitioner’s lack of experience [1].

In periodontal disease, microorganisms most commonly enter the root canal system via root perforations [2]. Endodontic diseases (pulpitis and periradicular infections) are polymicrobial and predominantly caused by strict

anaerobic bacteria [3]. The isolation rate of black-pigmented Gram-negative anaerobes in endodontic infections varies from 25% to >50%. *Prevotella* species are the most commonly found pigmented Gram-negative anaerobes, followed by *Porphyromonas* species. *Porphyromonas gingivalis* and *Porphyromonas endodontalis* are strongly associated with acute endodontic infections, whereas other black-pigmented Gram-negative anaerobes are not [4]. The most frequent etiological agent of chronic periodontitis is *P. gingivalis*, which can also colonize the tissues and cells of the gingival epithelium [5]. *Porphyromonas gingivalis* is one of the most frequently detected anaerobic bacteria in subgingival plaque samples from combined (periodontal-endodontic) lesions and necrotic pulps [6, 7]. In endodontic infections, PCR-based identification has shown higher detection rates of *P. gingivalis* (38%) than culture (1%) [8]. Infection control in both periodontal and endodontic tissues is essential to the management of combined endodontic–periodontal lesions [2]. After performing endodontic treatments, dentists should seal perforations immediately with a biocompatible material that is insoluble in tissue fluids, resistant to resorption, radiopaque, exhibits antimicrobial activity and allows regeneration of surrounding tissues [9].

At present, mineral trioxide aggregate (MTA) is considered to be the material of choice for the repair of root perforations [10]. MTA was introduced to endodontics by Torabinejad et al [11] in 1993 and has been used successfully in the repair of lateral root and furcal perforations, as well as vital pulp-capping, an apical plug in one-visit apexification cases. It has also been used to avoid overextension of the filling material in teeth with external resorption and as an apical filling material in teeth with post-core indications [12, 13]. Many studies have documented the biocompatibility of MTA, remained when MTA was mixed with the various additives [14, 15]. The antimicrobial activities of MTA have been extensively evaluated, with inconsistent reports [16, 17]. The antibacterial effect of MTA against some facultative and anaerobic bacteria has been documented, however its antimicrobial effect on *P. gingivalis* has not yet been reported.

Although the antimicrobial effects of silver are well known, the application of silver has been severely limited due to the toxicity of silver ions. Nevertheless, nanotechnology has provided production of smaller silver particles with increasingly large surface area-to-volume ratios, increasing the antimicrobial potency while reducing its toxicity. NanoSilver (NS), silver nanoparticles composed of bunches of silver atoms that range in diameter from 1 to 100 nm, have recently been identified as anti-inflammatory and accelerates wound healing. The biomedical application of NS is an emerging field of research, with a variety of commercially available products being used clinically, such as cardiovascular implants, neurosurgical and central venous catheters, bone cement and wound dressings [18]. This study was designed to evaluate antimicrobial effect of mixture MTA with NanoSilver against *P. gingivalis*.

EXPERIMENTAL SECTION

Bacterial Strain

The effects of the antimicrobial activity of freshly prepared Iranian MTA (IMTA; Tabriz, Iran) and NanoSilver-IMTA (NS-IMTA) were evaluated against *P. gingivalis.* Lyophilized *P. gingivalis* (ATCC 33270) was rehydrated with BHI broth and subcultured to Brucella-based (Merck, Darmstadt, Germany) sheep blood-agar plates supplemented with hemin (Sigma-Aldrich, Steinheim, Germany) and vitamin K (Sigma-Aldrich, Steinheim, Germany) (BHK) and incubated at 37 °C for 48 h. Colonies were harvested and suspended in BHI broth. Turbidity was adjusted by adding either *P. gingivalis* suspended in BHI broth or just pure BHI broth to equal a 0.5 McFarland turbidity standard, which corresponds to approximately 1.5×10^8 colony forming units per mL (CFU/mL). A sterile cotton-tipped swab was used to inoculate 0.1 mL of the suspension onto the surface of a BHK plate to achieve a lawn of bacterial growth [19].

Biosynthesis of Silver Nanoparticles (AgNPs)

The NanoAg was prepared using the Guangquan method [20]. Briefly, *Aspergillus terreus* was isolated from soil, and maintained on potato dextrose agar (PDA) medium (HiMedia, India) at 28 °C. The chemical silver nitrate ($AgNO_3$) and Nicotinamide Adenine Dinucleotide (NADH) were purchased from Sigma-Aldrich (Germany). Biomass of *A. terreus* was prepared in potato dextrose broth liquid medium at 28 °C on a rotary shaker (120 rpm) for 96 h. The biomass was harvested by filtration through Whatman filter paper No. 1, and then washed with distilled water to remove any components of the medium. 25 g biomass (wet weight) was placed in individual flasks containing 100 mL sterile distilled water. The flasks were incubated under the conditions described above for 24 h. Fifty ml of crude cell filtrate was dialyzed (molecular weight cut-off = 7 kDa) against distilled water for 48 h at 4 °C to remove small molecular weight compounds. 200 µL NADH (20 mmol/L) was then added to the dialyzed cell filtrate. Afterwards, 10 mL $AgNO_3$ solution (10 mmol/L) was added at 28 °C in dark for 24 h. AgNPs were

concentrated by centrifugation of the reaction mixture at 10,000 rpm for 10 min twice, and then were collected for further experiments.

Determination of the Minimum Inhibitory Concentration (MIC)

The antibacterial effects of NS were evaluated by determination of its MIC by the broth dilution method. AgNPs was freshly prepared before each experiment. One milliliter of the NS solution (0.1mg/mL) was mixed in 1 mL brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) medium and this mixture was diluted two-fold serially with the broth. *P. gingivalis* was precultured in BHI-broth anaerobically, 9-12 h prior to the test. Inocula were adjusted to 10^8 CFU (colony-forming units) /mL as measured by spectrophotometer (OD_{600} 0.7-0.8). Then, 0.1 mL aliquots of the inocula were added to 0.9 mL of each of the NS broth serial dilutions. The test tubes were incubated in an anaerobic atmosphere at 35-37 °C for 72 h. The MIC was defined as the lowest concentration of NS at which no visible bacterial growth was detected. The test was performed three times. Two or more similar results indicated the MIC of NS against *P. gingivalis* [21].

Chemical Exposure

After inoculation, four wells with 6-mm diameter and 4-mm depth were made by removal of agar with a puncher at equidistant points. IMTA (Tabriz, Iran) was mixed according to the manufacturer's instructions by using 1 g of powder for every 350 µL of sterile water. The NS-IMTA groups were mixed exactly the same way, substituting 25%, 12% and 6% colloidal solution of 0.1mg/mL NS for the sterile water. The selective concentration of NS was based on its MIC. Mixtures were prepared with a sterile spatula on a sterile glass slab. The resultant mixtures were transferred into the wells created on each plate using a sterile amalgam carrier and gently pressing it into each well. Two independent assays were performed. All plates were incubated at 37 °C in anaerobic conditions for 48 to 72 h as required for an even lawn of bacterial growth. A blinded, independent observer then measured the zones of inhibition around each well. The same procedure was conducted in a plate without the addition of bacterial seeding to detect contamination [21, 22].

Broth Dilution Test

For the broth dilution assay, 24-well cell culture plates were prepared according to the technique of Hernandez et al [23] with some modifications. Suspensions with a turbidity equivalent to that of a 0.5 McFarland standard were prepared by suspending fresh growth of *P. gingivalis* from BHK plates in BHI broth. A 0.1-mL aliquot of each 1:10 serially diluted suspension was inoculated onto a BHK plate to determine the final CFU/mL of the inoculate. IMTA and NS-IMTA were prepared as described above.

In one set of MTA specimens, the fresh pellet (approximately 4 mm×4 mm) of each type of MTA was immediately enclosed in a sterile microbiologic filter (0.4-µm pore size). The filter was used to prevent direct physical interaction between the bacterial cells and the MTA while allowing for soluble compounds from the specimens to reach the cells. Then enclosed pellets were placed into wells containing 1 mL of bacterial suspension. In addition, 1 mL of bacterial suspension in a well free of IMTA and NS-IMTA served as positive control. A well without *P. gingivalis* served as a negative control. The tissue-culture plates were then incubated at 37°C under anaerobic atmosphere and then measured at 0, 1, 3, 48, and 72-hour time periods. Samples (0.1 mL) from each well were subcultured onto BHK plates to determine the bacterial concentration, reduction in live bacteria in treated wells relative to positive controls and to assess for contamination. All experiments were performed in duplicate.

Statistical Analysis

The results of the viable bacteria tests were analyzed using one-way ANOVA and Tukey's post hoc test using SPSS (version 13, Chicago, IL). Confidence level was set at $p < 0.05$.

RESULTS

Agar Diffusion Test

The negative controls showed no bacterial growth throughout the incubation period, while the positive controls demonstrated maximal *P. gingivalis* growth. The MIC of NS was 0.006 mg/mL.

IMTA with NS concentrations of 12% and 25% showed mean zones of inhibition of 9 and 13 mm, respectively, against *P. gingivalis* on inoculated BHK agar; these results were not significantly different ($p > 0.05$, ANOVA). IMTA and 6% NS-IMTA showed no zones of inhibition.

Broth Dilution Test

The overall results of the broth dilution test are shown in Figure 1. After 1 h of incubation, *P. gingivalis* growth was reduced by the 12% and 25% NS-IMTA, respectively. By 48 hours, these concentrations of NS-IMTA had completely eliminated all viable *P. gingivalis*. However, IMTA and 6% NS-IMTA broth dilution tests reveal that neither of these fillers reduced the number of viable *P. gingivalis* after 1 h incubation, but after 48h and 72 h of IMTA exposure, reductions were 30.8% and 36.2%, respectively. For 6% NS-IMTA, although the reduction in the number of viable bacteria was slightly higher than that of the IMTA after 72 h, no significant difference in the antibacterial effect was found between 6% NS-IMTA and IMTA. Most notably, there is a statistically significance difference ($p < 0.05$) between the antimicrobial action of IMTA and IMTA containing ≥12% NS.

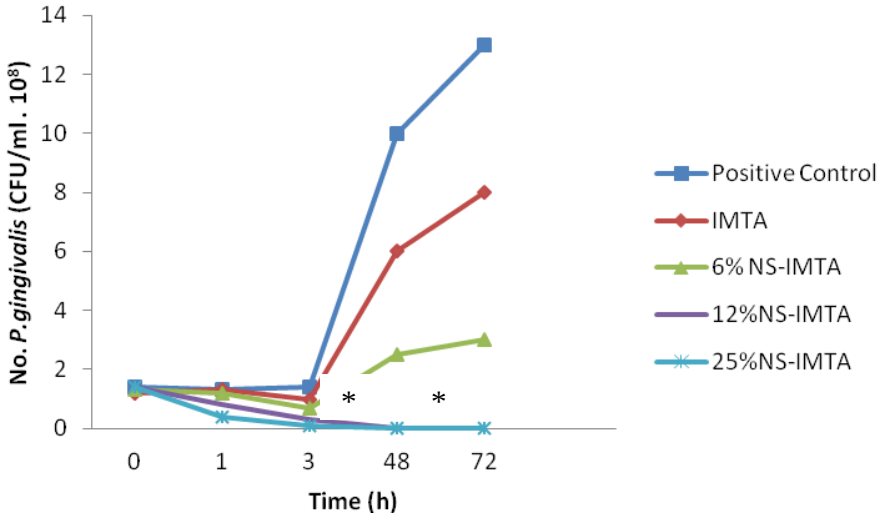

Figure 1. Antimicrobial effect of IMTA and NS-IMTA against *P. gingivalis* over a 72 h exposure by the broth dilution method

*Mean reductions for the treatments after 48h and 72 h of IMTA exposure were 30.8% and 36.2% respectively, compared with positive control. For 6% NS-IMTA, viable bacteria reduction was statistically the same as IMTA throughout the 72-hour experiment. IMTA containing 12% and 25% NS completely inhibited the proliferation of P. gingivalis. The difference in viable bacterial counts between cultures exposed to IMTA alone and IMTA containing ≥12% NS is statistically significant ($p < 0.05$). IMTA: Iranian mineral trioxide aggregate; NS: a colloidal solution of 0.1 mg/mL NanoSilver; 6% NS-IMTA: IMTA containing 6% of the NanoSilver solution; 12% NS-IMTA: IMTA containing 12% of the NanoSilver solution; 25% NS-IMTA: IMTA containing 25% of the NanoSilver solution; * indicates significant differences*

DISCUSSION

It has been reported that in root perforations where periodontal disease is present, several different microorganisms, including *P. gingivalis*, enter the root canal system [2, 4]. In endodontic disease, mixed infections associated with *P. gingivalis* influence the progress of endodontic lesions. *P. gingivalis* puts forth a synergistic interaction that might be involved in the pathogenesis of failed endodontic treatments [24]. MTA has been used successfully as a vital pulp-capping material in the repair of lateral and furcal root perforations [25]. A positive endodontic treatment prognosis depends on the successful elimination of the associated microorganisms and infected tissues as well as the effective sealing of the perforation.

The application of NanoSilver is already well-known in medicine, including its use in wound dressings and implantable medical devices, such as neurosurgical and venous catheters, but its use in endodontic treatment fillers has not yet been reported. NS exhibits remarkable biological properties, such as anti-inflammatory and antiviral activities, in addition to its more renowned antibacterial properties [18].

Several studies on the effect of MTA against microorganisms associated with endodontic disease have shown divergent results, which may be attributed to the methodology, various bacterial species tested, as well as the materials, concentration and method used to prepare the MTA [17, 26]. The antimicrobial activity of MTA was evaluated by Torabinejad et al [27] who reported that MTA, prepared according to their method at Loma Linda University, had an antibacterial effect on some of the facultative bacteria and no effect on any of the obligate

anaerobic bacteria (*Prevotella*, *Bactroides*, *Fusobacterium* and *Peptostreptococcus*) they tested. However, in a study by Stowe et al [22], MTA inhibited the growth of *F. nucleatum*. These varying results may be due to differences between the studies. First, in Torabinejad's study, MTA was placed on the surface of the agar. However, in Stowe's study, wells were formed in the agar into which the test materials were pressed, allowing them to use exact and reproducible volumes of MTA per sample and increasing the surface area for diffusion. The second explanation could be the different formulations of MTA. In Torabinejad's study, the MTA used might be different from the commercially marketed IMTA available later. Therefore, these differences make it difficult to compare the results of these two studies.

Most investigations of MTA antimicrobial activity have used the agar diffusion method, which only indicates the potential elimination of microorganism, but does not measure how long it takes to reduce growth. The broth dilution method is considered a more precise method to evaluate the antimicrobial properties of any filling material. This method allows direct and close contact between the test microorganism and materials. In addition, this method is considered appropriate when evaluating the antimicrobial activity of IMTA, which has low solubility and diffusibility [1].

While the mechanisms of cell damage of IMTA are still not well known, Ribeiro et al [25] concluded that MTA under aerobic conditions stimulated antimicrobial activity by the induction of reactive oxygen species (ROS). However, the most accepted explanation regarding the antimicrobial mechanism of IMTA is its high pH of 9.36 to >12.5 [28, 29], which is similar to that of calcium hydroxide [30, 31]. IMTA is a Portland-type cement that contains calcium oxide, which, in the presence of tissue fluid or water, is converted to calcium hydroxide. The release of hydroxide ions results in an increase in pH and calcium ions [28]. Although the antibacterial effect of IMTA against *P. gingivalis* is likely explained by its high pH, *P. gingivalis* might be relatively tolerant of high pH, and, if so, IMTA would not able to completely eliminate this microorganism [32].

Vianna et al [33] showed that $Ca(OH)_2$ paste, prepared by mixing calcium hydroxide powder with sterile water, eliminated the strict anaerobes *P. endodontalis* and *P. intermedia* within 30 s and *P. gingivalis* within 0.5 to 5 min. On the other hand, aerobic and facultative-anaerobic microorganisms, such as *Enterococcus faecalis* and *Staphylococcus aureus*, were much more resistant to $Ca(OH)_2$, with elimination times ranging from 4 to 24 h.

Our study showed that, although IMTA and 6% NS-IMTA in the broth dilution test did not show total inhibition of the test bacteria, some inhibition was observed after 2-3 days. The activity may be explained by the increase in pH caused by the IMTA and direct or close contact between the test microorganism and effective components of materials. In the agar diffusion test, the 12% and 25% NS-IMTA specimens had mean zones of inhibition of 9 and 13 mm, respectively, while 6% NS-IMTA and IMTA appeared to have no antibacterial action. The significant difference between the 6% and $\geq$12% concentrations indicate a dose-dependent effect of NS-IMTA. The broth dilution method also showed that the dose-dependent effect of NanoSilver, with 12% and 25% NS completely inhibiting the proliferation of *P. gingivalis* after 48 h. The silver ions in NS-IMTA might be released by the progressive hydrolysis of the cement surface, increasing the rate of release over time.

Alt et al [34] showed that the NanoSilver bone cement inhibited the proliferation of *S. epidermidis* and methicillin-resistant *Staphylococcus aureus* (MRSA), in a dose-dependent manner, without inducing *in-vitro* cytotoxicity. The bactericidal effect of NS is due to its interaction with four main components of the bacterial cell: the peptidoglycan cell wall, the plasma membrane, bacterial (cytoplasmic) DNA and bacterial enzymes such as the electron transport chain. NS also has native antibacterial effects that do not depend on the release of Ag+. There is evidence that NS produces reactive oxygen species (ROS), which might be the cause of the antibacterial activity of NS. Furthermore, bacterial resistance to NS is extremely rare, emphasizing the presence of multiple targets for NS. *In vitro* and animal studies have shown that the NS is toxic, but the successful widespread use of NS wound dressings in burn cases has not shown the toxicity found in *in vitro* and animal studies [18].

The prognosis of perforation repair depends on the location of the perforation, delay in perforation repair, and the sealing of the perforation. Delay in perforation repair can lead to microbial contamination of the perforation site and the affected periodontium, resulting in endoperiodontal lesions that are difficult to manage. So, perforation repair should precede definitive endodontic treatment. Following the repair of perforations, endodontic treatment should be performed with various irrigation solutions such as sodium hypochlorite to clean the root canal system. This procedure causes unavoidable contact of endodontic irrigant with the sealing material. In contrast to the well-

documented effect of routine irrigants on root dentin and smear layer, there is no data regarding the influence of these solutions on the integrity, sealing properties, surface corrosion and dissolution of NS-MTA [35].

Most cases of endodontic infections are polymicrobial, allowing several microbial species to attach to the dentin surface and form bacterial biofilms. Such biofilm formations are associated with persistent infections, antimicrobial resistance and microbial viability in the presence of root canal irrigants [36].

Although significant quantities of *P. gingivalis* have been found in the infected root canal system, no study has been conducted on the role of *P. gingivalis* in interacanal biofilm formation, persistant infection and the prognosis of a root perforation. On the other hand, further research is needed to investigate the effect of NS-MTA against other species of bacteria commonly found in primary endodontic diseases and microbial populations present after failed root canal therapy.

Biocompatibility and the ability of sealing material to enhance healing are the important factors when considering the prognosis of a perforation. Several studies have shown that MTA is biocompatible and enhances normal tissue regeneration in perforation sites [37-39]. The results of the present study demonstrate the increased antimicrobial activity of NS-MTA. Further research is needed to investigate the effect of fresh as well as set materials against other species of bacteria commonly found in endodontic infections. Additional studies are also required to investigate whether NS-MTA is biocompatible, enhances healing and is not subject to microleakage before this treatment can be recommended for clinical application. The prognosis of endodontic treatment using NS-MTA in an apically debrided canal system also remains to be determined.

CONCLUSION

The data collected from agar diffusion and broth dilution tests show that mixing IMTA with a $\geq$12% of a 0.1 mg/mL colloidal solution of NanoSilver instead of water enhances the antimicrobial activity of this material against *P. gingivalis*. If these results can be confirmed *in vivo*, NS-IMTA may have a significant effect in the prognosis of root perforation.

Acknowledgements
This study was supported in part by a grant from Tehran University of Medical Sciences. We thank Dr. J. Kharazi for performing the statistical analysis.

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