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Evaluation of antibacterial properties of nano silver Iranian MTA against *Fusobacterium nucleatum*

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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 Fusobacterium nucleatum, a Gram-negative anaerobic bacterium, which is one of the most prevalent taxa in the infected apical root canal system, chronic suppurative apical periodontitis and endodontic flare-ups. We combined NS (0.1 mg/mL) with IMTA at 3, 6 and 12% concentrations. To evaluate the antimicrobial effect, agar diffusion and broth dilution methods were used. In the agar diffusion test, IMTA and NS–IMTA showed zones of inhibition against F. nucleatum. The MIC of NS was 0.003 mg/mL. In broth dilution assay, IMTA reduced the growth of F. nucleatum by 71.3% and 88.9% after 48 h and 72 h, respectively. For 3% NS IMTA, the reduction in the number of viable bacteria was as the same as IMTA after 72 h. IMTA containing 6% and 12% NS completely inhibited the proliferation of F. nucleatum. A statistically significance difference ($P<0.05$) was found between IMTA and IMTA containing 6% and 12% of NS. In conclusion, NS–IMTA could completely inhibit the proliferation of F. nucleatum in dose-dependent manner that may have a significant effect in prognosis of root perforation.

Key words: Antimicrobial Agents, *Fusobacterium nucleatum*, MTA, Silver Nanoparticles

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INTRODUCTION

Perforation of the root creates non-natural connections between the root canal system and the periodontal ligament. Such perforations can be complications resulting from over instrumentation during endodontic procedures, internal or external resorption or caries invading through the floor of the pulp chamber. Of all root perforations, 47% were found to be related to endodontic procedures, and 53% were related to prosthodontic treatment [1].

In periodontal disease, microorganisms most commonly enter the root canal system via Periodontal disease may have an effect on the pulp through root perforation. Endodontic diseases are polymicrobial and predominantly caused by anaerobic bacteria [2]. *Fusobacterium nucleatum*, an anaerobic gram-negative bacterium is a dominant micro-organism within the periodonticum. Moreover, it is one of the few oral bacteria that are constantly associated with periodontitis, one of the most common infections in humans [3, 4]. *F. nucleatum* in combination with multiple other oral bacterial species plays a crucial role in biofilm formation. Indeed, the presence of *F. nucleatum* is critical in physical interactions between Gram-positive and Gram-negative bacteria during biofilm formation. This is in

addition to its role in preparing the proper redox potential required for the emergence of oxygen-labile anaerobes. Generation of biofilm is concerned with the occurrence of persistent infections. It is known that biofilm-forming microorganisms such as *P. acnes*, *S. epidermidis*, *P. aeruginosa* and *F. nucleatum* are involved in the development of persistent apical lesions. Studies have reported the enhanced attachment of *Porphyromonas gingivalis*, the most frequent etiological agent of chronic periodontitis, to human fibroblasts via *F. nucleatum* [5]. Numerous studies have reported the isolation of *F. nucleatum* from the apical canal of primary infections of the root canal system, root-filled teeth associated with periradicular lesions, chronic suppurative apical periodontitis and also abscess of dental origin [6, 7]. Moreover, *F. nucleatum* is one of several gram-negative bacteria that may be a causative and/or contributing agent in endodontic flare-ups [8]. *F. nucleatum*, a periodontal pathogen, could be accompanying endodontic infections due to the periodontic-endodontic interrelationships [2].

Control of infections in both periodontal and endodontic tissues is crucial to the management of combined endodontic–periodontal lesions [2]. After perforation, dentists should seal site of 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] and has been used successfully in the apical filling in teeth with post-core indications, and keep away from overextension of the filling material in teeth with external resorption as well as vital pulp-capping, an apical plug in one-visit apexification cases [12]. Many studies have documented the biocompatibility of MTA, remained when MTA was mixed with the different additives [13, 14]. The antimicrobial activities of MTA have been comprehensively evaluated, with conflicting reports [15].

Although the antimicrobial effects of silver are well known, the application of silver salts such as silver nitrate (AgNO3) and silver chloride (AgCl) has been limited due to their toxicity. Toxic effects of silver salts are proportional to the rate of release of free silver ions. Nevertheless, nanotechnology has provided production of smaller silver particles with increasingly large surface area-to-volume ratios, which potentially results in high reactivity. The nano-size results in specific physicochemical features that may differ from those of the bulk silver of larger size. Because of these special features the use of substances in nano size may have advantages over the use of bulk materials. 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 [16]. This study was designed to evaluate antimicrobial effect of mixture MTA with NanoSilver against *F. nucleatum.*

MATERIALS AND METHODS

Bacterial strain

The effect of the antimicrobial activity of freshly prepared Iranian-MTA (IMTA) and NanoSilver-IMTA (NS-IMTA) were evaluated against a clinical isolate of *F. nucleatum.* The bacteria 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 *F. nucleatum* 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 0.1mL of the suspension onto the surface of a BHK plate to achieve a lawn of bacterial growth [17].

Determination of the Minimum Inhibitory Concentration (MIC)

The antibacterial effects of NS were evaluated by determination of its MIC by the broth dilution method. NS (Cat. # PL-Ag- S10-10mg, 10 nm diameter, PlasmaChem, GmbH, Germany) 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. *F. nucleatum* was precultured in BHI-broth anaerobically, 9-12 h prior to the test. Inocula were adjusted to contain 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 *F. nucleatum* [18].

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 12%, 6% and 3% 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 [18, 19].

Broth Dilution Test

For the broth dilution assay, 24-well cell culture plates were prepared according to the technique of Hernandez et al. [20] with some modifications. Suspensions with a turbidity equivalent to that of a 0.5 McFarland standard were prepared by suspending fresh growth of *F. nucleatum* 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 *F. nucleatum* served as 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 AND DISCUSSION

MIC

The MIC of NS was 0.003 mg/mL.

Agar Diffusion Test

The negative controls showed no bacterial growth throughout the experimental period, while the positive controls demonstrated entirely *F. nucleatum* growth. IMTA and NS–IMTA showed zones of inhibition. In our analyses, 12% NS–IMTA showed the greatest zone of inhibition averaging 23 mm in diameter, followed by 6% NS–IMTA (16 mm in diameter). However, 3% NS – IMTA exhibited the same inhibition as the IMTA (i.e. 11 mm). Statistical analyses showed no significant difference between the zone of inhibition of 6% and 12% NS – IMTA against *F. nucleatum* ($P>0.05$, ANOVA). Nonetheless, 12% NS–IMTA zones were significantly different from the 3% NS–IMTA zones; ($P<0.05$, ANOVA).

Broth Dilution Test

Overall results of the broth dilution tests are shown in Figure 1. Evaluation of the freshly mixed IMTA and 3% NS–IMTA in broth dilution test did not reveal any reduction in the number of viable *F. nucleatum* during the 0-1 h incubation. In contrast, reduction in viable bacteria was observed during the 1 h incubation of cultures with 6% and

12% NS–IMTA. However, in the IMTA samples, extending the incubation time led to detectable reduction in the number of viable bacteria. The mean reductions for the treatments after 48h and 72 h of IMTA exposure were 7.13% and 88.9%, respectively.

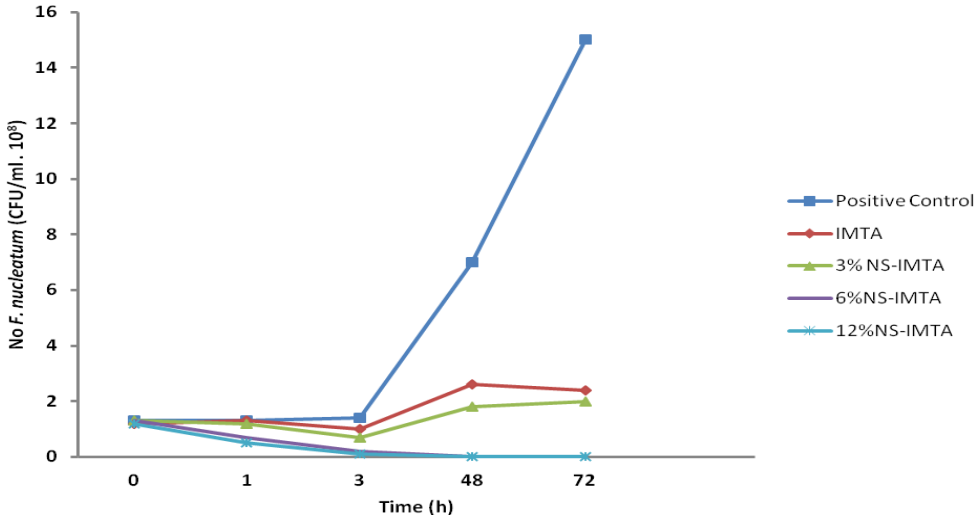

Figure 1: Antimicrobial effects of IMTA and NS-IMTA against *F. nucleatum* after 72 h exposure in broth dilution method

For 3% 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. 6% NS-IMTA and 12% NS-IMTA completely inhibited growth of *F. nucleatum.* No significant difference in the antibacterial effect was found between 6% and 12% of NS–IMTA. Most notably, there is a statistically significance difference ($P<0.05$) between the antibacterial action of IMTA and IMTA containing ≥6% NS. Statistical analysis showed a highly significant difference in the mean bacterial counts between the negative and the positive control groups ($P<0.05$).

In perforations of root where periodontal disease is present, several different microorganisms, including *F. nucleatum*, enter the root canal system [2]. Mixed infections associated with *F. nucleatum* influence the prognosis of endodontic disease. *F. nucleatum* puts forth a synergistic communication that might be involved in the pathogenesis of failed endodontic treatments [21]. Endodontic treatment prognosis will depend on elimination of the associated microorganisms and infected tissues as well as the effective sealing of the perforation. MTA has been used successfully as a vital pulp-capping material in the repair of root perforations [22].

The application of Nano-Silver is already well-known in medicine, including its use in wound dressings and implantable medical devices, such as catheters, but its use in endodontic treatment fillers has not yet been reported. NS exhibits amazing biological properties, such as anti-inflammatory and antiviral activities, in addition to its more renowned antibacterial properties [16, 23-26].

Several investigations on the effect of MTA against microorganisms associated with endodontic disease have shown conflicting results, which may be attributed to the methodology, various microbial species tested, as well as the materials, concentration and method used to prepare the MTA [27]. The antimicrobial effect of MTA was evaluated by Torabinejad et al. [28] 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. [19] 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.

Our study showed that NS – IMTA and IMTA might exert antibacterial effects in fresh specimens when assessed by in the agar diffusion test. We observed a dose-dependent effect for NS in NS–IMTA preparations, with the higher concentrations of NS being associated with the enhanced NS–IMTA antibacterial effects. In particular, mixture with a concentration of 12% of 0.1mg/mL Nano-Silver showed greater zones of inhibition when compared with lower (i.e. %3) NS levels. Although IMTA and 3% of NS–IMTA in broth dilution test did not show total inhibition of the test bacteria, some activity was observed. This activity might be due to increased pH of the environment and direct or close contact between the test microorganism and effective components of the materials.

Most studies 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 effects 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].

In our study, the data collected of broth dilution test showed that the bacterial reductions for the treatments after 72 h of IMTA and 3% of NS–IMTA exposure compared to the control proliferation. While the mechanisms of antimicrobial effect of IMTA are still not well known, Ribeiro et al. [22] 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 effect of IMTA is its high pH of 9.36 to >12.5, which is similar to that of calcium hydroxide [29]. IMTA 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 [29]. Although the antimicrobial effect of IMTA against *F. nucleatum* is likely explained by its high pH, Vianna et al. [30] 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, the effect of NanoSilver was also dose-dependent. Mixtures with concentration of 12% and 6% NanoSilver were the only NS–IMTA mixtures that completely inhibited the proliferation of *F. nucleatum* after 72h of exposure. 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. [31] 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 [16]. A recent study showed that incorporation of silver-zeolite may enhance the antimicrobial activity of MTA against *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Porphyromonas gingivalis* [32]. For the preparation of silver zeolite, silver nitrate has been used. As mentioned above, application of silver has been limited in biomedical procedures due to the toxicity. However, NanoSilver (NS) particles have large surface areato-volume ratios, with higher antimicrobial potency and exhibit very low levels toxicity for humans.

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 [33].

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 [34]. Although significant quantities of *F. nucleatum* have been found in the infected root canal system, no study has been conducted on the role of *F. nucleatum* in interacanal biofilm formation, persistant infection and the prognosis of a root perforation. The results of the present study demonstrate the increased antimicrobial activity of NS–MTA against *F. nucleatum*. 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 [35, 36]. 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$6% of a 0.1 mg/mL colloidal solution of NanoSilver instead of water enhances the antimicrobial activity of this material against *F. nucleatum*. If these results can be confirmed *in vivo*, NS-IMTA may have a significant effect in the prognosis of root perforation.

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