Effect of Endodontic Irrigants and Medicaments Mixed with Silver Nanoparticles against Biofilm Formation of *Enterococcus faecalis*

Yasamin Ghahramani [a] (iD), Fariba Yaghoobi [b] (iD), Ramin Motamedi [b] (iD), Akram Jamshidzadeh [c] (iD), Abbas Abbaszadegan [a*] (iD)

a Department of Endodontics, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran; *b* Dentist, Graduate school of Dentistry, Shiraz University of medical sciences, Shiraz, Iran; *c* Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

ARTICLE INFO	ABSTRACT
  Corresponding author: Abbas Abbaszadegan, Department of Endodontics, Dental School, Shiraz University of Medical Sciences, Shiraz, Iran. *Tel*: +98-713 6263192 *Email*: abbaszade@sums.ac.ir 	**Introduction:** The aim of this study was to evaluate the effectiveness of chlorhexidine (CHX), sodium hypochlorite (NaOCl), calcium hydroxide (CH) and double antibiotic paste (DAP) mixed with silver nanoparticles (AgNPs) against *Enterococcus faecalis*. **Methods and materials**: Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and biofilm formation inhibition (after 72 h) of the experimental substances alone or mixed with AgNPs were measured against *E. faecalis* using microtiter plate method. Bacterial cultures turbidity was measured using a spectrophotometer. All procedures were performed in triplicates. **Results**: The MIC values for CHX, NaOCl, CH and DAP were equal to 0.012, 1.25, 1.6 and 0.156 mg/mL, and their MBC's were 0.025, 2.5, 0 and 0.625 mg/mL. After mixing them with AgNPs, the MIC's for CHX, NaOCl, CH and DAP were reduced to 0.0032, 0.158, 0.2 and 0.0391 mg/mL, while their MBC's were reduced to 0.0064, 0.0632, 0.401 and 0.0156 mg/mL. Biofilm formation inhibition occurred in higher dilutions of all irrigants and medicaments as they were mixed with Ag NPs. **Conclusions**: Adding AgNPs resulted in an increased antimicrobial activity at the tested dilutions for all experimental substances .More investigations in *in vivo* conditions are required to confirm the results of this study. **Keywords:** Calcium Hydroxide; Chlorhexidine; Double Antibiotic Paste; *Enterococcus Faecalis*; Silver Nanoparticles; Sodium Hypochlorite

Introduction

The ultimate purpose of a root canal therapy is to eradicate microorganisms and to avoid recontamination of pulpal space [1]. Studies have shown that instrumentation debrides only 70% of the canal walls; hence, one of the final goals in root canal treatment is to facilitate entrance of irrigants to dentinal tubules to enhance the course of disinfection [2]. Common endodontic irrigants are used during treatment, which include sodium hypochlorite (NaOCl), chlorhexidine digluconate (CHX), mixture of tetracycline, acid and detergent (MTAD), and ethylenediaminetetraacetic acid (EDTA). However, there are some disadvantages associates to each.

NaOCl is regarded as a gold standard endodontic irrigant thanks to its antibacterial potency and organic dissolving characteristics [3]. Its drawbacks are the unpleasant odor and taste, toxicity and reduction in dentine flexural strength [4]. CHX is another common irrigant, which has antibacterial potency and good substantivity [5, 6], but the organic components in root canal system can negatively affect its antibacterial activity [7].

In addition to canal instrumentation and irrigation, intracanal inter appointment medicaments seem to be necessary for eradicating the infected tissues and microorganisms in some cases [8, 9]. Calcium hydroxide (CH) is the most common intracanal medicament with high pH that leads to its

antibacterial property. However, it is shown that its material effectiveness against some microorganisms is limited [10, 11].

Another intracanal medicament known as triple antibiotic paste (TAP) is a mixture of metronidazole, ciprofloxacin and minocycline that seems to be a promising medicament effective in root canal disinfection and revascularization [12, 13]. However, there is a potential risk of tooth discoloration related to the presence of minocycline in this mixture [14]. Hence, double antibiotic paste (DAP) was introduced later and regarded as an effective antibacterial medicament comparable to TAP [15]. Long period of contact time and bacterial resistance are some of the concerns with this antibiotic medicament [12]. Due to shortcomings of the aforementioned irrigants and medicaments in root canal treatment, their combination was employed to overcome the drawbacks, and also to improve their characteristics.

As most irrigants and medicaments are not clinically efficient to remove all endodontic pathogens at their minimum inhibitory concentration (MIC), it is appropriate to mixed them with silver nanoparticles (AgNPs) not only to use their lowest diluted form and decrease their side effects, but also to increase their anti-microbial potency.

Recently, nanotechnology has attracted the attention of researchers in the field of dentistry, especially in endodontic science. Amongst different nanoparticles, AgNPs have shown the highest antibacterial property and biocompatibility for root canal purposes [16, 17]. Moreover, studies have attested the enhancement in antibacterial capacity of different antibiotics when combined with AgNPs [18, 19]. Also, researches have shown higher antibacterial activity of CH in combination with AgNPs in comparison with CH alone [20, 21]. The aim of present investigation was to evaluate the synergistic antimicrobial effects of common irrigants and medicaments in combination with AgNPs against *Enterococcus faecalis* (*E. faecalis*) in planktonic and biofilm forms.

Materials and Methods

Bacterial strains and media
In this study, *E. faecalis* (PTCC1778) was used as the chief isolated bacteria from the root canal infections [22]. To culture this bacterium in anaerobic blood agar plates (CDC, BioMerieux, Durham, NC), brain-heart infusion (BHI) was used along with 5 g yeast extract/L and 5% v/v vitamin K+hemin (BHI-YE). To maintain 37°C anaerobic environment, gas-generating sachets (Gas-Pak EZ; Becton, Dickinson and Company, New Jersey USA) were used.

Preparation of experimental substances
Ag NPs were prepared according to a previous method explained by Abbaszadegn *et al.* [23]. In short, laboratory glassware were placed in 1:3 of HCl/HNO3 solution. Next, they were rinsed three times by distilled water. Then, a total of 1 mL of 0.01 M AgNO3 aqueous solution was added to 20 mL of 6.2 mM 1-dodecyl-3-methylimidazolium chloride ([C12mim][Cl]) aqueous solution, under constant stirring. Afterward, we added the prepared 0.4 M NaBH4 aqueous solution gradually until the observation of golden color in the solution. Subsequently, to remove the excess volume of ionic liquids, the solution was centrifuged for 20 min. Finally, the suspension was kept in room temperature and concentration was calculated as 5.7×10^{-8} Mol/L [24].

To prepare a saturated solution of CH paste 16 mg of CH powder (Golchai Co, Tehran, Iran), was mixed with 1 mL of distilled water or AgNPs.

A saturated solution of DAP was prepared by dissolving 300 mg USP grade antibiotic powder (Samen Medical, Mashhad, Iran) comprised of equal portions of ciprofloxacin and metronidazole in 3 mL distilled water (50 mg of each antibiotic/mL).

It is worth mentioning that CH and DAP mixture were stirred at room temperature for 4 h. Then to clarify the solutions, mixtures were centrifuged at 3000 rpm for 15 min.

NaOCl (2.25%), CHX (2%) (Cerkamed Dental-Medical company, Pawłowski, Poland) and CH were used alone or mixed with Ag NPs in the experimental groups.

In each group, 5 plates were prepared and the tests were performed in triplicates: Group 1; CH and 1 mL of distilled water, Group; CH and 1 mL of AgNPs, Group 3; DAP and 1 mL of distilled water, Group 4, DAP and 1 mL of AgNPs, Group 5; CHX, Group 6; CHX and 1 mL of AgNPs, Group 7; NaOCl, Group 8; NaOCl and 1 mL of AgNPs, Group 9; 1 mL of AgNPs (positive control) and Group 10; 1 mL of distilled water (negative control).

Determination of minimum inhibitory and bactericidal concentrations
The bacterial tests were done according to Clinical Laboratory and Standard Institute (CLSI) instructions [25]. Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent, which inhibits the growth of a microorganism, and minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a particular bacterium. Two-fold dilution method was used to determine the MIC and MBC of each substance against *E. faecalis* [26]. BHI-YE-cultured *E. faecalis*

was treated with 0, 1:10 to 1:64000 dilutions of each experimental material in sterile 96-well plates under standard protocols for 24 h. A spectrophotometer was employed to measure the turbidity of the bacterial cultures based on optical density at 540 nm. In practice, MIC is defined as the lowest concentration of an agent that causes change equal to or less than 0.050 in turbidity [26, 27]. MBC was determined by bacterial cultures from the wells equal or more than MIC, and they were smeared on blood agar plates following 48 h of incubation.

MBC was defined as the lowest concentration of an antimicrobial agent that no visible bacterial colonies could survive on the agar plates after a 48-h incubation [27].

Determination of biofilm formation

E. faecalis cultures (10^6 colony-forming units/mL) in BHI-YE were treated in 96-well micro titer plates with 1:1 to 1:64 dilutions of each experimental solutions for 24 h [28]. Biofilm was washed twice with saline, fixed with 10% formaldehyde according to previous studies, then washed twice with saline again and stained with 0.5% crystal violet for 30 min. Biofilm was washed 3 times with saline, afterwards, the process of extracting crystal violet from the biofilm cells by 200 mL 2-propanol was done for 1 h. Then we diluted the extract (1:5) with 2-propanol and read at 490 nm with 2-propanol, which was used as a blank control. Biofilm formation was read at 72 h to assure the ability of the agent to inhibit biofilm formation over time. The actual bacterial biofilm mass was represented by optical absorbance of the diluted crystal violet stain. A higher absorbance shows higher biofilm mass.

Results

Table 1 shows the MIC and MBC values. With respect to biofilm formation, the inhibition was obtained at dilutions 8, 2 and 4, and 2 times higher than MIC's of NaOCl, CHX, CH and DAP, where they mixed with AgNPs.

Table1. MIC, MBC (mg/mL) of the experimental materials against *E. faecalis* (* not measureable)

Groups	MIC	MBC
CH	1.6	-
CH+AgNPs	0.2	0.401
DAP	0.156	0.625
DAP+AgNPs	0.0391	0.0156
CHX	0.012	0.025
CHX+AgNPs	0.0032	0.0064
NaOCl	1.25	2.5
NaOCl+AgNPs	0.0158	0.0632

Discussion

In this study, we found that adding AgNPs resulted in an increased antimicrobial activity for all the tested irrigants and medicaments. The present study was designed to investigate the synergistic effect of NaOCl, CHX, CH and DAP in combination with AgNPs against *E. faecalis* in planktonic and biofilm models.

E. faecalis is the dominant microorganism in persistent endodontic infections and it is found in 30 to 90% of treated root canals. Moreover, it is resistant to a variety of antibiotics as well as to high pH of intracanal medicaments. Furthermore, it invades dentin and binds to immobilized collagen [29, 30]. It is shown that NaOCl or CHX do not have sufficient ability to eliminate *E. faecalis* [31]. Also, Sabrah *et al.* [32] showed that DAP and TAP have a significant antibacterial effect against *E. faecalis*, although they were not effective against the established biofilm.

In this study, AgNPs were added to common irrigants and medicaments to investigate the possible antibacterial potency leading to microbial synergism against *E. faecalis*. Abbaszadegan *et al.* [24] revealed that AgNPs with a positive surface charge have effective antibacterial potency against *E. faecalis* as the most challenging microorganism. Furthermore, studies have proven that AgNPs are less harmful than the other metal nanoparticles and nontoxic to mammalian cells in dilutions less than 7.4×10^{-4} mol/L [33-35]. Besides, AgNPs has high bactericidal potency and good biocompatibility in low dilutions [36]. Therefore, in the present study AgNPs at 5.7×10^{-8} mol/L was selected as a test material. The synergistic effects of AgNPs with antibiotics were previously established through increasing bacterial bonds, resulting in antimicrobial potency [37, 38]. Also, it was shown that AgNPs have synergistic effect with CH against *E. faecalis* [21].

Although CH is the most common intracanal medicament and is still regarded as the gold standard inter appointment dressing, there are numerous studies that have shown its failure against *E. faecalis* and *C. albicans* [39-41]. We also reached the same findings; however, when it was combined with AgNPs, their combination resulted in an increased antibacterial activity confirmed by the reduction in its MIC and MBC and biofilm inhibition test. Our finding was in line with Javidi *et al.* [21].

Hoshino *et al.* [42] introduced triple antibiotic paste (TAP), and DAP as a potent intracanal medicaments in endodontics with siginificant antibacterial effects. Sabrah *et*

al. [15] demonstrated that DAP had similar antibacterial activity relative to triple antibiotic paste and was effective against *E. faecalis* and *P. gingivalis* without tooth discoloration. Therefore, we selected DAP to be included in this investigation. Some previous studies showed the synergistic effect of AgNPs and antibiotics against some microorganisms [37, 38]; however, no previous study was available to support the synergistic effects of TAP or DAP in combination with AgNPs in endodontics. In the present investigation, the efficacy of these antibiotic medicaments with or without AgNPs was confirmed.

To the best of our knowledge, in the literature there is no report on the evaluation of possible antimicrobial synergistic effect between endodontic irrigants and AgNPs. However, there are numerous studies demonstrating the bactericidal effects of NaOCl and CHX alone or in combination against *E. faecalis* in infected root canals and dentinal tubules [31, 43]. In the present study, NaOCl as the gold standard irrigant in endodontics was tested at concentration of 2.5% due to its matchless antibacterial activity, low toxicity and tissue dissolving capacity [44]. Estrela *et al.* [45] revealed that 2% CHX was the effective agent against most microorganisms. Consistently, we also found that 2.5% NaOCl and 2% CHX were efficient irrigants against planktonic and biofilm *E. faecalis.* Furthermore, we found that 2% CHX was more potent than 2.5% NaOCl. It is worth mentioning that there is a controversy regarding the higher antibacterial potency of CHX relative to NaOCl, which might be due to different methods, dilutions and contact time tested [46-49].

In our study, we showed that AgNPs was able to increase the antimicrobial activity of NaOCl and CHX. Han *et al.* [50] showed that NaOCl caused structural changes in bacterial membrane, and bacterial LPS have a protective mechanism against NaOCl. The synergistic effect of NaOCl and AgNPs were observed in the current study, this might have been due to the alteration of AgNPs in highly conserved lipopolysaccharide of *E. faecalis* leading to higher efficiency of NaOCl.

The possible mechanism for synergistic effect of AgNPs with CHX is the positive charge of these two irrigants that might have resulted in their higher antibacterial activity when they came in contact with each other. Amongst experimented combined materials, CH+AgNPs showed the least and CHX+AgNPs showed the most antibacterial potency.

Further studies should be performed on the cytotoxicity of these materials as they are merged as well as to investigate the possible interactions between such mixtures and root canal filling materials. Also, future researches should focus on animal or *in vitro* models in combination with microorganisms commonly found in infected root canal.

Conclusion

Within the limitations of this study, we found that adding AgNPs to endodontic irrigants and medicaments could lead to improvement in their antibacterial potency against *E. faecalis.* Further investigations are desired to evaluate other characteristics of these combined materials and their effects on dentin, periodontium and root canal filling material.

Acknowledgment

Conflict of Interest: 'None declared'.

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