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Antibacterial Efficacy of Polymer-Coated Ceramic Microparticles Loaded with a Modified Combination of Antibiotics on the *Enterococcus faecalis* Biofilm

Nazanin Zargar [a], Ardavan Parhizkar [b], Mohammad Javad Nasiri [c], Sara Saedi [d*]

a Department of Endodontics, School of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran; b Iranian Center for Endodontic Research, Research Institute for Dental Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran; c Department of Microbiology, School of Medicine, Tehran, Iran; d Department of Endodontics, School of Dentistry, Guilan University of Medical Sciences

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***Corresponding author**:

Sara Saedi, Department of Endodontics, School of Dentistry, Guilan University of Medical Sciences, Rasht, Iran

E-mail: sara_saedi@yahoo.com

Introduction: Nano-technology applied for the local delivery of different agents and/or drugs has made its path to endodontics. In the current study, the antibacterial efficacy of biopolymer-coated ceramic microparticles loaded with a modified combination of triple antibiotics, i.e. Penicillin G, Metronidazole and Ciprofloxacin (PMC), was evaluated against two strains of *Enterococcus faecalis* (*E.faecalis*); a standard clinical strain obtained from previously root-filled teeth with persistent periapical lesions, and compared to the most common antimicrobials used in endodontics. **Methods and Materials:** After synthesis of the polymer-coated microparticles loaded with antibiotics, the 21-day release of antibiotics were evaluated and a stock solution was produced using the maximum released amount of drugs and distilled water. The antibacterial activity of PMC, triple antibiotic paste (TAP), calcium hydroxide (CH), chlorhexidine (CHX) and sodium hypochlorite (NaOCl) against two bacterial strains was determined using "Minimum Inhibitory Concentration" and "Agar Diffusion Test". Additionally, "Microtiter Plate Assay" was performed to assess anti-biofilm properties. **Results:** Minimum inhibitory concentration values reported for TAP and PMC were 1/256. PMC showed the maximum diameter of growth inhibition in both strains (33 mm and 35 mm), while CH had the minimum diameters (13 mm and13 mm). Based on microtiter plate assay, TAP showed higher biofilm formation than PMC. Biofilm formation was higher in the standard strain for PMC; however, NaOCl, CHX and CH completely inhibited biofilm formation. **Conclusions:** Based on the findings of the present study, it could be concluded that PMC and TAP were the most effective medicaments against *E.faecalis* in its planktonic form; however, none could inhibit its biofilm formation. Further studies using larger sample size and "Confocal Scanning Laser Microscopy" are recommended.

Keywords: Antibiotics; Biofilm; Enterococcus Faecalis; Microparticle; Microtiter Plate Assay

Introduction

Endodontic infection is considered a biofilm-induced pathosis, and its complete removal is essential for successful endodontic treatment [1, 2]. Consequently, different chemomechanical preparations of the root canal system in the practice of endodontics have been evaluated for their disinfection purposes. However, no specific methods have succeeded to provide a sterile root canal space in necrotic and infected cases as yet [3]. This is mainly due to the complex anatomy of root canal system and the multi-species complicated biofilm structures, which are resistant to antimicrobials [2].

Nanotechnology is deliberated as a novel approach in local drug delivery systems (LDDS). The ultimate aim of LDDS is the delivery of the agent and/or drug to a specific target tissue, and direct, sustained and slow release of drug to reduce the adverse effects and increase the efficacy and effectiveness of the medication. Therefore, nanoparticles have been recently used in endodontics [4] and elaborated as novel disinfection strategies [5, 6]. An innovative drug delivery system has been presented by Parhizkar et al.; in which ceramic microparticles with a poly (lactic-co glycolic acid) (PLGA) coating have been introduced to transfer a modified triple antibiotic combination of penicillin G,

metronidazole and ciprofloxacin (PMC) to radicular space [7]. Long-term sustained release of the loaded antibiotics suggests that the state-of-the-art LDDS can be effectively used as intracanal medication, in root canal(s) and pulp regeneration treatments.

Enterococcus faecalis (E. faecalis) is a gram-positive, facultative anaerobe cocci; frequently isolated from root-filled canals with persistent peri-radicular lesions [8]. The ability to survive prolonged starvation, resist in high alkaline environment (due to its proton pump), and invade dentinal tubules can define its prevalence in persistent infections [9]. Therefore, it is widely used as the target species in studies focusing on disinfection and antimicrobial evaluation [10]. Corresponding experimental studies have investigated the effect(s) of irrigants and medications mixed with nanoparticles, and shown promising results against *E. faecalis* biofilm [11, 12]. It has been reported that calcium hydroxide nanoparticles have a deep penetration in dentinal tubules and provide high antimicrobial efficacy against *E. faecalis* [13].

The current study was designed to evaluate the efficacy of the novel drug delivery system in the disinfection of *E.faecalis* bacteria and biofilm, and compare it to the most common antimicrobials used in endodontics; namely calcium hydroxide (CH), triple antibiotic paste (TAP), sodium hypochlorite (NaOCl) and chlorhexidine (CHX). The tests were performed on two strains of *E. faecalis*, a standard strain and a clinical one obtained from previously root-filled teeth with persistent radicular disease.

Materials and Methods

This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences with code No.: IR.SBMU.DRC.REC.1399.001.

Synthesis of biopolymer-coated microparticles loaded with antibiotics

The protocol followed hereby for the synthesis of the biopolymer-coated microparticles loaded with antibiotics has been described in an investigation by Parhizkar *et al.* [7]. Briefly, the ceramic microparticles were synthesized by mixing hydroxyapatite (HAP) (AH0050 Aprin Co. Tehran, Iran) with β-tricalcium phosphate (β-TCP) (Sigma-Aldrich Chemicals, USA) with a 65:35 weight percentage. The mixture was filtered using a sieve #60 with an aperture size of 250 μm, gently added to a 6% aqueous gelatine solution (Sigma-Aldrich Chemicals-G9391, USA) and stirred at 300 rpm for 2 h on a magnetic stirrer (Eyela, Japan) to perfectly form the particles. Next, the gelatine slurry containing β-TCP/HAP microparticles was dispersed in a Petri dish holding paraffin oil (Dr. Mojallali, Iran). The excess paraffin was removed and the remaining β-TCP/HAP microparticles were dried for in room temperature 24 h. Then, the obtained microparticles were heated in a ceramic furnace at 550°c for 1 h (to remove the remaining gelatine) and 950°c (to solidify and strengthen) for 1 hour. The morphology and size of microparticles were evaluated using scanning electron microscopy (VAT; 5402-GE02-0002/6891, Austria)[14].

Poly lactic co-glycolic acid (PLGA) (Resomer RG 504 H, Evonik, Germany) was dissolved in chloroform (Dr. Mojallali, Iran) to prepare a 5% PLGA/chloroform solution. Afterwards, the previously defined amounts of Penicillin G (Titrachem, Iran), Metronidazole (Titrachem, Iran) and Ciprofloxacin (Titrachem, Iran) were added to PLGA/chloroform solution 5% and mixed thoroughly on a vortex (Labnet International, USA). Five milligrams per mL of the previously prepared and dried microparticles were added to the mixture and vigorously mixed on the vortex. Then, the final mixture was centrifuged (Boeco U-320 R, Germany at 4500 rpm) at room temperature for 20 minutes to separate the loaded/coated microparticles. The loaded/coated microparticles were then dried at room temperature for 48 h.

Long-term Evaluation of Drug Release

Phosphate buffer saline (PBS) and 13 mg of the loaded/coated microparticles (per 3 mL of PBS) were added to a Petri dish and placed in a shaker incubator (Heidolph Promax 1020, Germany) with 70 rpm at 37°C. The PBS solution was collected and refreshed every 48 h for 21 days, and the collected extracts were read by UV-Spectrophotometry (UV- 2501 PC, Shimadzu, Japan) to analyse the amount of each antibiotic. The wavelengths of 200 nm, 271 nm and 319 nm were used for Penicillin G, Ciprofloxacin and Metronidazole, respectively [7].

Preparation of PMC Stock Solution

A stock solution of PMC in distilled water at the concentration of maximum amount of each antibiotic was prepared for antimicrobial evaluation [7].

Preparation of Other Antimicrobial Samples

Triple antibiotic paste was prepared by solving equal amounts of ciprofloxacin, metronidazole and minocycline (Apotex, Canada) to reach 1mg/mL concentration, which is the recommended dosage used in pulp regeneration protocols [15]. Calcium hydroxide (Morvabon, Iran) saturated solution was prepared based on the solubility point for CH [16]. The 1.60 mg/mL solution was oversaturated; consecutively, the amount of dissolved CH was reduced in a step by step manner, and 1 mg/mL CH was considered the saturated solution. Additionally, sodium hypochlorite 5.25% (NaOCl) (Morvabon, Iran) and chlorhexidine 2% (Morvabon, Iran) were used as experimental groups.

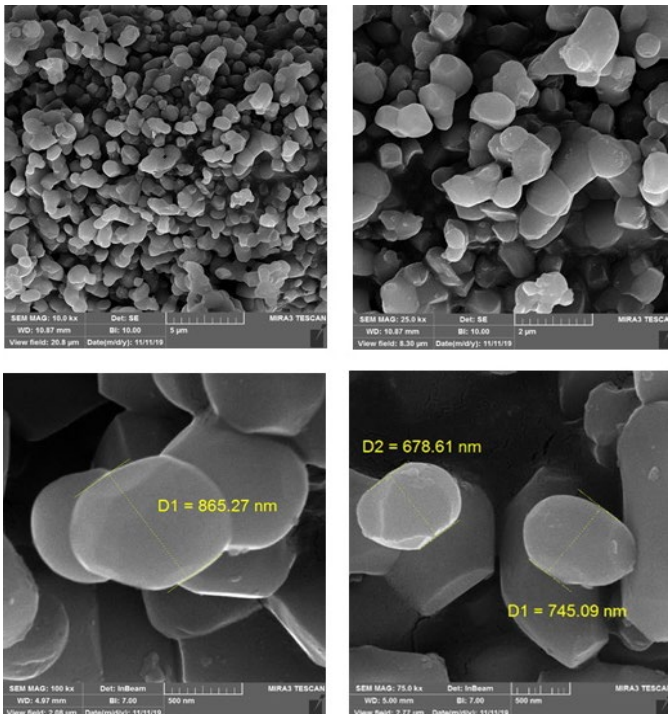

Figure 1. SEM micrographs of ceramic microparticles; exhibiting acceptable size and proper smooth surfaces; appropriate for drug delivery

Antimicrobial Properties

Two bacterial strains were used for the present study:

1. *E. faecalis* ATCC 2912 (*Standard*)
2. *E. faecalis* previously obtained from root-filled canals with persistent peri-radicular lesions. The strain was taken from patients who had fulfilled the inclusion criteria and were treated in "Department of Endodontics, School of dentistry, Shahid Beheshti University of Medical Sciences Tehran, Iran between 2019-2020" [17].

Experimental Groups:

1. PMC
2. TAP 1mg/mL
3. CH 1mg/mL
4. CHX 2%
5. NaOCl 5.25%

Agar Diffusion Test (ADT)

Bacterial colonies of *E. Faecalis* were separated after an overnight culture of each bacterial strain in Mueller Hinton Agar (MHA). The 0.5 McFarland suspensions of colonies were incubated in MHA at 37°C for 24 hours, and the prepared samples were placed in the designated wells in agar. The diameters of inhibition zones around each well were measured using a standard ruler [18].

Minimum Inhibitory Concentration (MIC)

The 2-fold broth dilution method was used to determine MIC amounts for each sample in a 96 microtiter plate. In the first well, 100 µL of Mueller Hinton Broth (MHB) was mixed with 100 µL of the experimental sample to produce the ½ dilution of sample; an approach which was considered to achieve other necessary concentrations for the needed wells. Then, 0.5 McFarland of the bacterial suspension was placed in each well. The turbidity of each well was assessed after incubation at 37°C at 24 h, and the first well with no detectable opacity was reported the MIC of the addressed sample [19].

Microtiter Plate Assay

To analyse the anti-biofilm properties of each sample, a "Microtiter Plate Assay" using crystal violet staining and submicron concentrations of each drug were used.

Three wells were assigned to each group. The first 3 wells were used as the negative control group and thus, only 200 µL of Tripton Soy Broth (TSB) was placed in them. The second 3 wells were assigned to the positive control group, and 197 µL of TSB mixed with 3 µL of the bacterial suspension were placed in them to observe the formation of bacterial biofilm. Each next 3 wells were dedicated to an experimental sample; 100 µL of TSB with 90 µL of submicron sample and 10 µL of bacteria were added to each well. After 24 hours, the wells were emptied and every well was washed 3-4 times with PBS. Then, 200 µL of methanol was added to each well to fix the biofilm structure. After 15 minutes, the wells were washed out and 200 µL crystal violet were added for 15 minutes to stain the attached biofilms. Afterwards, each well was washed with PBS and rinsed with 33% acetic acid to remove the stains. The optical density of each well was read by FLx 800 Multi-Detection Microplate Reader (BioTek Instruments Inc., US) at the wavelength of 492 nm. The obtained mean for each 3 wells, assigned to each group, was reported [20, 21].

Results

Morphology and Physical Structure of the Micro-particles

Figure 1 exhibits the SEM micrographs of HA/β-TCP ceramic microparticles. The structure and size of microparticles were evaluated using SEM; round to oval-shaped microparticles with soft edges were formed with magnitudes between 670-900 nm.

Determination of Antibiotics Release

Figure 2 depicts the release profile of the triple antibiotics. The drug release over the 21-day period was measured using the UV-spectrophotometry. All three antibiotics showed a burst release in the first 48 hours, and continued to gradually release to the end of the assigned period. The maximum amount of release was 992.07 µg/mL for penicillin G, 410.36 µg/mL for ciprofloxacin and 2564.28 µg/mL for metronidazole.

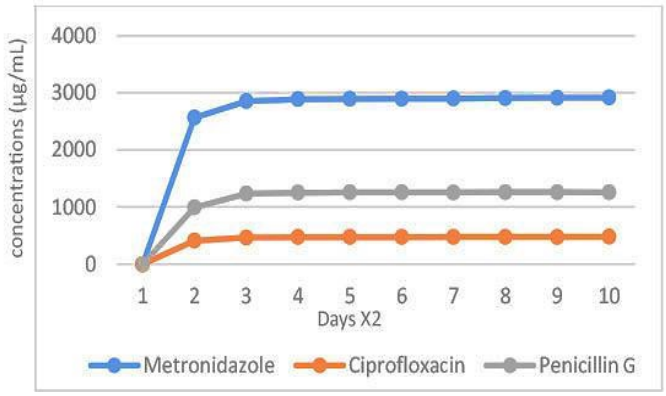

Figure 2. Drug release patterns of PMC components in 21 days. Note that the number of days on the horizontal access should be multiplied by 2. (PMC= Penicillin G, Metronidazole, Ciprofloxacin)

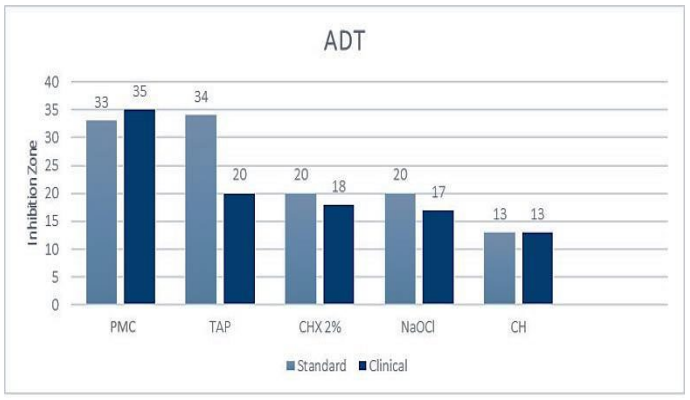

Figure 3. ADT results showing the diameters of zone of inhibitions around each sample (ADT: Agar Diffusion Test)

Antibacterial Activity

1. ADT

Figure 3 portrays the diameters measured for different zones of inhibition in each group. Both PMC and TAP were more effective on standard strains than other groups. TAP was less effective on the clinical strain than the standard strain. The diameter of inhibition zone of clinical strain was approximately equal in TAP, CHX and NaOCl. The least efficacy against both strains was observed in CH group.

2. MIC

Table 1 shows the MIC amounts for each antimicrobial sample. Both PMC and TAP were more effective against both strains of *E. faecalis* compared to other groups. CH required a higher concentration to inhibit the growth of the clinical strain of bacteria than the standard strain. Furthermore, 5.25% NaOCl inhibited the growth of both strains in a lower concentration than that of 2%CHX.

Table 1. The results of "Minimum Inhibitory Concentration" for clinical and standard bacterial strains; showing the efficacy of PMC and TAP against both strains of *Enterococcus faecalis* compared to other experimental groups

Minimum Inhibitory Concentration					
PMC	**TAP**	**CH 1 mg/mL**	**CHX 2%**	**NaOCl 5.25%**	***Enterococcus faecalis***
1/256	1/256	1/4	1/2	1/32	**Standard**
1/256	1/256	1/2	1/2	1/32	**Clinical**

PMC: Penicillin G, Metronidazole, Ciprofloxacin; TAP: Triple Antibiotic Paste; CH: Calcium Hydroxide; CHX: Chlorhexidine; NaOCl: Sodium Hypochlorite

3. Microtiter Plate Assay

The mean optical density of wells in each group is reported in Table 2. Based on the reported findings, CHX, NaOCl and CH inhibited the biofilm formation, while TAP and PMC showed biofilm growth. The amount of biofilm formation was higher in TAP than PMC. In PMC group, the standard strain showed lower biofilm formation than the clinical strain.

Discussion

The current study was designed to evaluate the antibacterial efficacy of biopolymer-coated ceramic microparticles loaded with modified combination of triple antibiotics against *E. faecalis* strains. The occurrence of genetic mutations and various phenotypes can result in altered resistance to antimicrobials. Therefore, besides the standard bacterial strain, a clinical strain obtained from root-filled canal with persistent peri-radicular infection was used for antimicrobial evaluations. The clinical strain showed different genotype profile than the available standard strain [17]. Moreover, the antimicrobial efficacy of the most common endodontic antimicrobials against the two bacterial strains and the present antibiotic-loaded drug delivery system was also compared.

Table 2. Results of Microtiter Plate Assay; showing the biofilm growth of *Enterococcus faecalis* in the presence of TAP and PMC. PMC, compared to TAP, showed higher efficacy on the prevention of biofilm formation.

Optical Density					
PMC	**TAP**	**CH 1mg/ml**	**CHX 2%**	**NaOCl 5.25%**	***Enterococcus faecalis***
1+	3+	N	N	N	**Standard**
2+	3+	N	N	N	**Clinical**

N represents negative biofilm formation. Higher OD shows higher amounts of biofilm; PMC: Penicillin G, Metronidazole, Ciprofloxacin; TAP: Triple Antibiotic Paste; CH: Calcium Hydroxide; CHX: Chlorhexidine; NaOCl: Sodium Hypochlorite

In this study, the results of antimicrobial efficacy on the planktonic bacteria did not conform to the findings on biofilm. PMC and TAP, the two triple antibiotic combinations, were most effective on the planktonic bacteria based on MIC and ADT results; however, none were capable of inhibiting biofilm
formation. Despite the greater diameter of inhibition zone for TAP in the standard group, TAP induced a medium zone of inhibition in the clinical group. PMC, in which penicillin G replaced minocycline, was equally effective on both clinical and standard strains. This outcome can be justified by the higher concentration of antibiotics in the combination of PMC. In addition, Pinheiro *et al.* showed that the *E. faecalis* strains obtained from root-filled canals did not produce β-lactamase and were susceptible to penicillins, whereas there were strains resistant to the tetracycline family [22]. Thereby, the replacement ofminocycline with penicillin G has possibly resulted in the higher efficacy of PMC against *E. faecalis* biofilm formation and in ADT results.

Biofilms are complex structures, and are composed of bacteria and extracellular polysaccharide matrix [23]. Based on the results of the present study, CH had the lowest antimicrobial activity against the planktonic form of bacteria; however, NaOCl, CH and CHX all managed to inhibit biofilm formation. NaOCl and CH have the ability to dissolve organic tissue and structures; therefore, it can be hypothesized that, through the organic degradation of matrix, NaOCl and CH can both penetrate the biofilm structure and attack the residing bacteria. NaOCl is the standard and most commonly used antibacterial in endodontics for biofilm degradation; since it can kill the regional bacteria and disrupt the biofilm matrix.

In this study the MTP assay, an indirect quantitative method, was used to evaluate anti-biofilm properties. MTP assay is relatively easy to perform, reproducible and inexpensive. The crystal violet stain is claimed to be membrane permeable in gram positive and negative cells, and can colour the attached biofilm in MTP process. However, the main disadvantage of MTP is that it cannot differentiate live from the dead bacterial cells, which could be mentioned as a limitation of the present research [24].

On the other hand, CLSM (confocal laser scanning microscopy), a high cost technology with the use of dyes and fluorescence, allows obtaining information on spatial and temporal cellular viability/function without the destruction of biofilm [24]. In contrast to the results attained by MTP assay Zancan *et al.* reported that CH and CHX were not effective on *E. Faecalis* biofilm, while TAP and DAP (double antibiotic paste) were both effective based on CLSM [25]. Therefore, further investigations using CLSM technology is suggested to confirm the achieved results.

In the current study, the anti-biofilm activity of different drugs on the mono-species biofilm of *E.faecalis* was evaluated. However, the multi-species biofilms attached to the dentinal root wall are clinically the main source of endodontic
infections [26]. This may preclude applying the results of this study in a clinical setting. As previously reported, the effectiveness of antimicrobial agents against mono-species and multi-species biofilms could be different; even in in vitro environment [27]. These experiments were designed as an introduction to the analysis of the effectiveness of an innovative drug delivery system. Further studies as well as tests and experiments with greater sample size to statistically analyze the data are recommended to overcome limitations.

To conclude, it seems that the evaluation of anti-biofilm activity simulating clinical situation still stays a challenge and there is a paucity of a standard protocol to evaluate anti-biofilm activity of medicaments in endodontics. Moreover, the effectiveness of aforementioned micro/nanoparticles in drug delivery to a specific target can be assessed in further in vitro studies by evaluating the ability of the devised innovative drug delivery system on the *E .faecalis* biofilm in different dentinal depths in extracted human teeth.

Conclusions

Based on the findings of the current study, the antibiotic-loaded biopolymer-coated microparticles can have a strong antibacterial activity against *E. faecalis* bacterial strains. Nonetheless, the sole use of PMC at the concentration used in the present investigation without the dissolution of organic structures may not be sufficient to inhibit biofilm formation. Further studies using larger sample size and other state-of-the-art technologies, e.g. CLSM, are recommended to confirm the obtained results.

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Conflict of Interest: 'None declared'.

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