Original Article

Efficacy of Double Antibiotics in Hydroxypropyl Methylcellulose Gel against Enterococcus faecalis in Root Canals: An *in vitro* Study

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Abstract

Ciprofloxacin and metronidazole exert an antibacterial activity against Enterococcus faecalis *(E.f.).* However, applying paste containing these antibiotics into the root canals is not convenient for endodontists. This study aimed to study the possibility of hydroxypropyl methylcellulose (HPMC) as a vehicle for antibiotic delivery. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the two antibiotics were determined. A 1:1 ratio of each antibiotic was mixed with 2 % HPMC to form a gel, and the *in vitro* drug release was tested by a Franz diffusion cell. Fifty-four roots of the mandibular premolars were mechanically instrumented by Protaper Next rotary files until X3 and divided into three groups (18 each) (i) non-infected roots, treated with gel base, (ii) infected roots with *E.f.* for 21 days, treated with gel base, and (iii) infected roots with *E.f.* for 21 days, treated with gel containing the combined antibiotics for 14 or 28 days. DNA and RNA were isolated from the ground roots. The absolute quantity of *E.f.* DNA and relative mRNA expressions of *E.f.*-specific sequence and *pbp5* were determined by qPCR and RT-qPCR, respectively. MIC and MBC of the double antibiotic solution were 5 and 250 µg/ml, respectively. There was a significant decrease in the *E.f.* DNA content in group (iii) at 14 and 28 days ($p<0.001$). mRNA expression of *E.f.*-specific sequence was significantly reduced in group (iii) at both periods ($p<0.01$), whereas *pbp5* expression was significantly increased ($p<0.01$). This study demonstrated an *in vitro* efficacy of the combined antibiotics in the HPMC gel against *E.f.*, proposing an application for endodontic treatment.

Keywords: Biodegradable gel, Double antibiotics, Enterococcus faecalis, Intracanal medication, Root canal treatment

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Introduction

Infections with mixed bacteria play a major role in the development and progression of pulp and periapical diseases.[1,2] A major goal for root canal treatment is to eliminate bacterial populations. Controlled asepsis by means of mechanical preparation and chemical agents is important for successful healing of the periapical lesions.[3] Accordingly, intracanal medication with antibiotics is recommended to effectively remove residual bacteria after mechanical instrumentation.[4] The success rates of endodontic treatment for teeth without apical periodontitis range from 82.8 to 97.3 %, whereas those for teeth with apical periodontitis are lower varying from 75.6 to 87.77 %[5-7], as a result of persistent infections with gram-positive and facultative anaerobes.[8] Among these anaerobes, *Enterococcus faecalis* (*E. faecalis*) is the most prevalent bacteria found within previously treated canals.[9,10] *E. faecalis* is isolated alone or with a few other bacteria[11], and can penetrate and reside in the dentinal tubules, which helps resist killing by calcium hydroxide.[12] Moreover, a proton pump is critical for *E. faecalis* survival at high pH.[13] *E. faecalis* can enter the viable, but non-culturable (VBNC) state, in which it reduces metabolisms and synthesis of proteins, except penicillin binding protein 5 (*pbp5*), and remains dormant until necessary nutrients are available for its later growth.[14,15] Therefore, *pbp5* expression is used to determine *E. faecalis* in the VBNC state.[16]

Triple antibiotic paste (TAP), containing ciprofloxacin, minocycline and metronidazole in propylene glycol and macrogol, has been used as an intracanal medicament for selective root canal treatment and pulpal revascularization. Several studies have, however, shown that minocycline causes visible crown discoloration.[17] Therefore, minocycline is omitted from the paste, and called double antibiotic paste (DAP). *In vitro*, DAP was as effective as TAP against E. *faecalis* in the root canal[18,19], as evidenced by the resolution of periapical lesions by intracanal medication with DAP as reported by Iwaya *et al.,*[20] and Hargreaves *et al.,*[21] Nevertheless, mixing minocycline and ciprofloxacin with propylene glycol and macrogol as traditional vehicles makes the paste too viscous to be readily delivered or to fill up the root canal, limiting the penetration of antibiotics into the dentinal tubules. In addition, propylene glycol cannot control a sustained drug release.[22]

In this study, we wanted to determine the possibility of using hydroxypropyl methylcellulose (HPMC) as a vehicle for antibiotic delivery. HPMC, a water-soluble and biodegradable polymer derived from cellulose, is the most abundant polymer in nature and is used in food, drug and dietary supplements.[23] A previous study revealed a sustained release of drugs by using HPMC as a vehicle, which effectively prolongs their therapeutic effect.[24] To the best of our knowledge, HPMC has not yet been introduced for dental uses and no study has so far investigated the antibacterial activity of ciprofloxacin and metronidazole in HPMC gel. Therefore, this study aimed to examine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the two antibiotics against *E. faecalis in vitro*, to evaluate the release of these antibiotics from the gel, and to assess the antibacterial efficacy of the combined antibiotics in the gel against *E. faecalis* at 14 and 28 days.

Materials and Methods

E. faecalis Strain and Medium

Blood agar plates (Merck KGaA, Darmstadt, Germany) were used to grow and maintain the colonies of *E. faecalis* ATCC 29212. A single colony was picked and inoculated in Brain-heart infusion (BHI) broth (HiMedia Laboratories Pvt. Ltd., Mumbai, India) supplemented with 5 g/L of yeast extract (HiMedia Laboratories Pvt. Ltd.) at 37°C for 24 h in an anaerobic chamber (Bactron, Shel lab, Lonay, Switzerland) for subsequent experiments.

Minimum Inhibitory and Bactericidal Concentrations

The double antibiotic solution was prepared by mixing 50 mg of ciprofloxacin and 50 mg of metronidazole (USP), generously obtained from the Siam Pharmaceutical Co., Ltd., Bangkok, Thailand, in 10 ml of deionized water, as recommended by Sabrah *et al.,*[19] The solution was serially diluted, ranging from 1:10 to 1:32000. *E. faecalis* cultures in BHI broth were treated with these dilutions in triplicate for 24 h in sterile 96-well microtiter plates

(Thermo Fisher Scientific, Waltham, MA, USA). The turbidity of *E. faecalis* cultures was determined by an optical density (OD) at 540 nm using a spectrophotometer (Tecan Austria GmbH, Grödig, Austria). MIC was determined as the fifty percent of growth inhibition[25] by the following equation: % of inhibition = (OD control - OD blank) - (OD sample - OD blank) x 100/(OD control - OD blank), where control = *E. faecalis* in BHI broth, blank = BHI broth alone, sample = *E. faecalis* in BHI broth treated with the solution. To determine MBC, bacterial cultures from the wells that contained different aforementioned dilutions were streaked onto blood agar plates, incubated at 37°C for 24 h in an anaerobic chamber. The lowest concentration of ciprofloxacin and metronidazole that resulted in no visible bacterial colony on the plates was considered as MBC.

In vitro Release of Double Antibiotics

An equal amount of ciprofloxacin and metronidazole (1:1) was dissolved in deionized water for the final concentrations of the combined antibiotics at 5 and 10 mg/ml. Two hundred mg of the HPMC powder (2 % w/v; S. Tong Chemicals Co., Nonthaburi, Thailand) was added to 10 ml of the double antibiotic solution and left at 4°C overnight to form the gel-like mixture. The *in vitro* release of both antibiotics from the mixture was performed using a Franz diffusion cell (Perme Gear, Inc., Hellertown, PA, USA) and artificial tubular cellulose membranes with a 0.4-µm pore size (Membrane Filtration Products, Inc., Sequin, TX, USA). Before being used, the membranes were hydrated with deionized water, as a medium, overnight. Two gram of the gel was loaded in the donor compartment, and 0.2 ml of the medium was withdrawn from the receptor compartment at various time points, including 0.25, 0.5, 1, 2, 4, 8, 12, 18 and 24 h. An equal volume of the fresh medium (deionized water) was immediately added into the receptor compartment at each sampling time to maintain sink condition. The samples were analyzed for the drug content using a spectrophotometer (UV-2450, Shimadzu Corporation, Kyoto, Japan).The standard curve of five known concentrations for each antibiotic was first established, and the concentrations of each antibiotic in the unknown samples were computed by comparing their OD values using the spectrophotometer with those of the five known concentrations from the standard curve. The OD readings, thus, indicated the accumulated concentrations of antibiotics at various times because some, but not all, medium was taken from the receptor compartment.

Preparation of Human Dentin Specimens

Fifty-four intact single-rooted premolars extracted for an orthodontic reason were collected, rinsed with normal saline and stored in 0.1 % (w/v) Thymol solution. The research protocol (#18/2018) of using discarded human premolars was approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University. The root of each tooth was resected horizontally below the cementoenamel junction until the length of each root was equal to 10 mm. The equivalent size of each root canal was determined by using a K-file #15 (Dentsply Maillefer, Ballaigues, Switzerland), being fitted in the root canal at the apical one-third with the working length at 9 mm. The root canal was prepared by ProTaper Next rotary files from X1 to X3 (Dentsply Maillefer) following the manufacturer's instruction. The smear layer was removed by treatment with 17 % EDTA and 5.25 % NaOCl for 4 min each in an ultrasonic bath (UC-1050, TPC Advanced Technology, City of Industry, CA, USA). To test the sterility, all root samples immersed in BHI broth were autoclaved for 15 min, and a few samples were randomly selected and incubated in fresh BHI broth at 37°C for 24 h. No microbial contamination was found.

Dentin Inoculation

A single colony of *E. faecalis* grown on a blood agar plate was picked and suspended in BHI broth for 3 h. The quantity of *E. faecalis* in BHI broth was determined from a 0.5-OD value of the McFarland standard that is equivalent to 0.5 x 10^8 cfu per ml.[26] Thirty six of 54 root specimens were inoculated with *E. faecalis* at 1x10^6 cfu per ml by injecting 20 µl of *E. faecalis* suspension into the root canal close to the root apex using a 3-ml syringe and a 27-gauge needle. After inoculation, the root samples were incubated in an anaerobic chamber, and 20 µl of fresh BHI broth was replenished every two days for 21 days.

Subsequently, all root samples were assigned into three groups (n=18 each) as follows: (i) non-infected roots with 2 % HPMC gel (negative control); (ii) *E. faecalis*-infected roots with 2 % HPMC gel (positive control); (iii) *E. faecalis*-infected roots with 5 mg/ml of ciprofloxacin and metronidazole in 2 % HPMC gel (experimental group). A syringe tip was used to fill up the root canal with the HPMC gel with or without the two antibiotics by inserting into the canal around 7 mm away from the root apex (~2-3 mm) in order to prevent leakage of the gel outside the canal. In addition, slow loading of the gel was conducted to prevent any air trapped between the gel and the canal. The root samples held in 1.5-ml Eppendorf tubes were incubated at 37°C in an anaerobic chamber for 14 or 28 days (27 for each period with nine for each of the three aforementioned groups). The reason for intracanal medication with double antibiotics for 14 or 28 days was because we wanted to simulate a long period of medication, especially for the case of pulp necrosis with chronic abscess, and some patients might not be convenient to come back for a 7-day follow-up visit. After incubation, the root samples were irrigated with 10 ml of sterile normal saline followed by being dried with sterile paper points. Ten of the 18 root samples in each group (total = 30) were used for determination of the antimicrobial efficacy of ciprofloxacin and metronidazole in the HPMC gel by absolute quantification of *E. faecalis* DNA and mRNA expression of *E. faecalis*-specific sequence, whereas the remaining eight samples in each group (total = 24) were used for scanning electron microscopy.

DNA and RNA Isolation

The root samples were ground into fine powder by autoclavable steel impactors, driven by dual electromagnets, and vials (6751 vial, SPEX CertiPrep Ltd., London, UK) using a bone mill (SPEX SamplePrep Freezer/Mills 6750, SPEX CertiPrep Ltd.). The powder was collected and weighed in a 15-ml tube for DNA and RNA extraction. DNA and total RNA were simultaneously isolated using 1 ml of TRIzol™ reagent (Thermo Fisher Scientific) per 100 mg of the powder. Zero point two ml of chloroform per 1 ml of TRIzol™ reagent was added, vortexed and incubated for 3 min. The mixture was centrifuged at 12000 g for 15 min at 4°C. The aqueous phase in the upper layer was aspirated and precipitated with 0.5 ml of isopropanol for RNA pellet, while the interphase was collected and precipitated with 0.3 ml of absolute ethanol for DNA pellet. Both RNA and DNA pellets, resulting from centrifugation at 12000 g for 10 min and at 2000 g for 5 min, respectively, were washed with 75 % ethanol twice, left to air dry, and resuspended in 25 µl of RNase- and DNase-free water (Thermo Fisher Scientific). DNA and total RNA amounts were determined by a NanoDrop™ spectrophotometer (Thermo Fisher Scientific) and then stored at -80°C until further analysis.

Reverse Transcription and qPCR

Complementary DNA (cDNA) was synthesized from total RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific). The reaction mixture contained 450 ng of total RNA, 1 µl of random hexamer primers and nuclease-free water up to 12 µl, 4 µl of 5x buffer reaction, 1 µl of Ribolock RNase inhibitor, 2 µl of 10 mM dNTP mix, and 1 µl of reverse transcriptase. The mixture was gently mixed and incubated at 25°C for 5 min followed by 42°C for 60 min. The reaction was terminated by heating at 70°C for 5 min. Two microliters of the resulting cDNA template were used for qPCR reaction in a total volume of 20 µl, containing 10 µl of 2x SensiFAST SYBR® No-ROX mix (Bioline, London, UK), 0.8 µl of 10 µM forward and reverse primers for *E. faecalis*-specific sequence, *pbp5* and *16s rRNA,* as a housekeeping gene (Table 1), and nuclease-free water. The reaction was performed in the LightCycler® 480 instrument (Roche, Basel, Switzerland) at 95°C for 10 min, followed by 45 cycles at 95°C for 20 s, 60°C for 1 min, and 72°C for 25 s. The cycle threshold (Ct) values of *E. faecalis*-specific sequence and *pbp5* were calculated using the LightCycler® 480 Software (Roche) and normalized by those of *16s rRNA* to obtain ΔC_t.

Table 1 *Oligonucleotide primers used in this study.*

		Oligonucleotide sequence
pbp5	Forward	5'-GATGCGCAATTAATCGG-3'
	Reverse	5'-CATAGCCTGTCGCAAAAC-3'
E. faecalis-specific	Forward	5'-CGCTTCTTTCCTCCCGAGT-3'
	Reverse	5'-GCCATGCGGCATAAACTG-3'
16s rRNA	Forward	5'-GATTAGATACCCTGGTAGTCCAC-3'
	Reverse	5'-TACCTTGTTACGACTT-3'

For absolute DNA quantification, 17 ng of DNA from each sample and of genomic DNA extracted from known concentrations (10^2 to 10^9 cfus/ml) of *E. faecalis,* for construction of a standard curve, were amplified in the qPCR reaction, containing 10 µl of 2x SensiFAST SYBR® No-ROX mix (Bioline), 0.8 µl of forward and reverse primers for *16s rRNA* (Table 1) and nuclease-free water. The qPCR conditions were 95°C for 10 min, followed by 45 cycles at 95°C for 20 s, 60°C for 1 min, and 72°C for 25 s. The quantity of *E. faecalis* DNA was derived by comparing the Ct value of each sample with the standard curve. Log concentrations of *E. faecalis* were plotted on a y-axis of the standard curve and Ct values of genomic DNA from *E. faecalis* were plotted on an x-axis.

Scanning Electron Microscope (SEM)

The remaining samples were split longitudinally with a chisel and a hammer into two pieces in order to view *E. faecalis* within the root canal and dentinal tubules. The root samples were fixed with 2.5 % glutaraldehyde in phosphate-buffered saline, dehydrated in serial dilutions of ethanol, dried with Polaron CPD7501 (Quorum Technologies Ltd., East Sussex, UK) and coated with gold using the gold-sputter-coated instrument (JFE-110E, JEOL, Tokyo, Japan). The coated samples were mounted on the stub and examined by an SEM (JSM-5410L, JEOL) at the magnification power of 7500x. The images were taken by a built-in digital camera attached to the SEM.

Statistical Analysis

Numerical data were checked for their distribution by the Kolmogorov-Smirnov test. Due to their normal distribution, the data were illustrated as mean percentages ± SD, representing log concentrations of *E. faecalis* DNA, and as means ± SD for mRNA expressions of *E. faecalis*-specific sequence and *pbp5* relative to that of *16s rRNA.* ANOVA and the Tukey's post-hoc tests were used to compare means with the significance level at *P*-values <0.05. Statistical analysis was performed using SPSS® software version 20 (IBM®, Armonk, NY, USA).

Results

MIC and MBC of the double antibiotic solution against *E. faecalis* were 5 µg/ml (2.5 µg/ml each; Table 2) and 250 µg/ml (125 µg/ml each; Fig. 1), respectively.

The maximum absorption of ciprofloxacin and metronidazole in deionized water was at 274 and 320 nm, respectively (data not shown), allowing simultaneous analysis of the antibiotics released from HPMC gel. Regarding the antibiotic release, 5 mg/ml and 10 mg/ml of the combined antibiotics in the HPMC gel were prepared according to Jenks *et al.,*[27] A sustained release of both antibiotics at these two doses was found up to 24 h (Table 3). Note that the concentrations of both antibiotics were close to the MIC and MBC at the same

time point, *i.e.* 15 min for the MIC and 2 h for the MBC, for both concentrations (Table 3). The concentrations of antibiotics released from 10 mg/ml of the combined antibiotics in the gel were higher than those from 5 mg/ml for every time point (Table 3).

However, the lower dose at 5 mg/ml of ciprofloxacin and metronidazole (2.5 mg/ml each) in the HPMC gel was selected for subsequent experiments because both 5 and 10 mg/ml yielded the MIC and MBC at the same time point and it is better to use the lowest dose of antibiotics that is still efficient in killing *E. faecalis* to avoid antibiotic resistance.[28] *E. faecalis* DNA amounts in the experimental group (iii) at 14 and 28 days were significantly decreased compared to those in the positive control group (ii) (p<0.001; Fig. 2A), but were not different from those in the negative control group (i) (Fig. 2A). mRNA expression of *E. faecalis*-specific sequence was significantly reduced in group (iii) at both periods compared to that in group (ii) (p<0.01; Fig. 2B). By contrast, mRNA expression of *pbp5* was significantly increased in group (iii) at both periods (p<0.01; Fig. 2C).

After dentin inoculation for 21 days, *E. faecalis* co-aggregated within the root canal and inside the dentinal tubules (positive control; Fig. 3). However, *E. faecalis* was dramatically eliminated by treatment with 5 mg/ml of ciprofloxacin and metronidazole in the HPMC gel with a few *E. faecalis* remaining in the dentinal tubules at 14 days (arrowheads; Fig. 3) and *E. faecalis* cell debris seen in the dentinal tubules at 28 days (arrows; Fig. 3). As anticipated, no *E. faecalis* was found inside the dentinal tubules in the negative control group (Fig. 3).

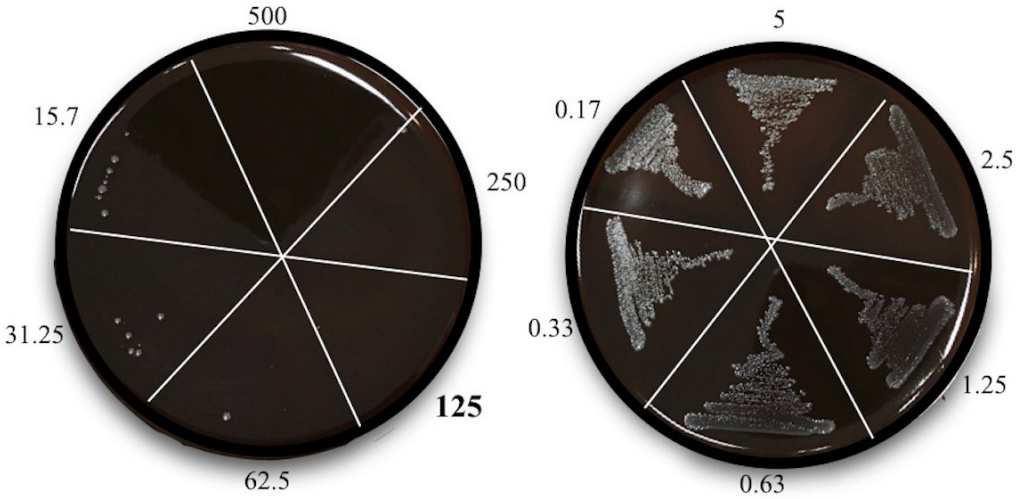

Figure 1 *Representative blood agar plates from three separate MIC experiments in Table 2 with similar results showing the minimum bactericidal concentration, in which no visible colony of E. faecalis was found at 125 µg/ml of each antibiotic (ciprofloxacin or metronidazole). Note E. faecalis cultures with twelve different concentrations of antibiotics in µg/ml from Table 2 were streaked onto different areas of two blood agar plates (six each).*

Table 2 *The percentage of E. faecalis inhibition by the double antibiotic solution, containing ciprofloxacin and metronidazole, in each dilution. Note that the fifty percent of inhibition regarded as the minimum inhibitory concentration was demonstrated at the dilution factor of 1:2000 or 5 μg/ml of the double antibiotic solution (bold). This experiment was done in triplicate on 3 separate times.*

Dilution	Double antibiotic (μg/ml)	Each antibiotic (μg/ml)	% of inhibition (mean ± SD)
1:10	1000	500	98.61 ± 0.91
1:20	500	250	96.35 ± 3.07
1:40	250	125	95.00 ± 3.93
1:80	125	62.5	89.84 ± 2.41
1:160	62.5	31.25	88.87 ± 1.80
1:320	31.25	15.7	86.64 ± 0.87
1:1000	10	5	76.00 ± 6.85
1:2000	**5**	**2.5**	**58.33 ± 4.40**
1:4000	2.5	1.25	44.94 ± 4.13
1:8000	1.25	0.63	34.26 ± 2.85
1:16000	0.63	0.33	23.34 ± 5.89
1:32000	0.33	0.17	13.86 ± 5.62

Table 3 *A profile of the sustained drug release for ciprofloxacin and metronidazole from 0.25 to 24 h, expressed as means ± SD in μg per ml. Note approximately equivalent doses of both antibiotics released at each time point for either 5 or 10 mg/ml of the combined antibiotics in hydroxypropyl methylcellulose (HPMC) gel. The accumulated concentration of each antibi otic released from either 5 or 10 mg/ml of the combined antibiotics in the gel reached the minimum inhibitory concentration at 2.5 μg/ml (light gray highlight) shown in Table 2 at 0.25 h and the minimum bactericidal concentration at 125 μg/ml (dark gray highlight) shown in Figure 1 at 2 h. This experiment was independently repeated three times.*

Time (h)	5 mg/ml of combined antibiotics in HPMC gel		10 mg/ml of combined antibiotics in HPMC gel	
	Ciprofloxacin (μg/ml)	Metronidazole (μg/ml)	Ciprofloxacin (μg/ml)	Ciprofloxacin (μg/ml)
0.25	3.03 ± 0.48	3.03 ± 0.40	3.62 ± 0.30	3.90 ± 0.59
0.5	32.61 ± 2.62	33.22 ± 2.66	43.28 ± 2.34	41.28 ± 3.63
1	6.91 ± 0.57	76.22 ± 1.27	88.56 ± 15.32	87.55 ± 2.93
2	144.33 ± 1.73	154.89 ± 10.51	194.55 ± 8.77	208.01 ± 5.53
4	259.79 ± 5.77	229.63 ± 14.20	345.66 ± 29.43	338.49 ± 7.06
8	410.45 ± 8.02	331.64 ± 16.49	513.72 ± 43.28	590.64 ± 3.25
12	440.62 ± 13.72	392.02 ± 7.40	623.72 ± 12.49	609.83 ± 8.52
18	449.22 ± 9.08	405.14 ± 5.45	649.99 ± 8.18	611.83 ± 7.14
24	457.23 ± 5.74	410.04 ± 5.98	664.24 ± 7.42	620.00 ± 4.29

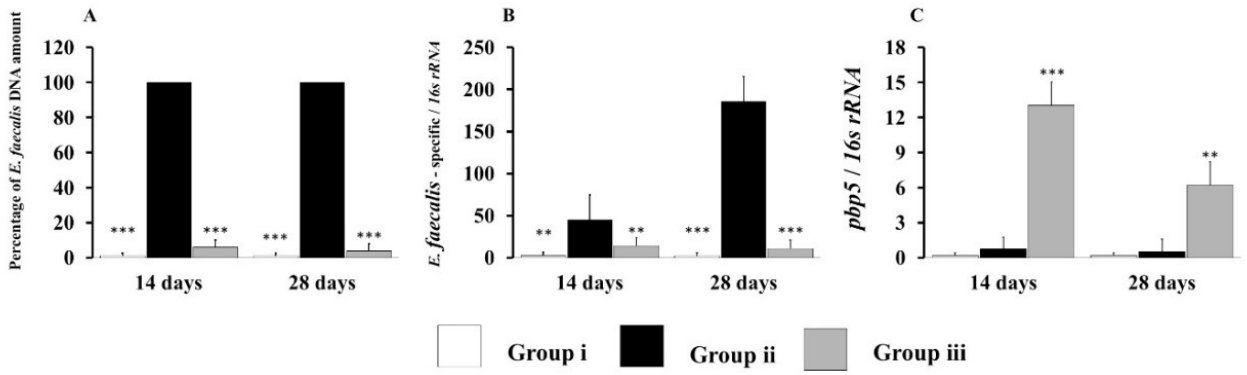

Figure 2 *Bar graphs demonstrating means ± SD (error bars) of the percentage of E. faecalis DNA (A), mRNA expression of E. faecalis-specific sequence normalized by that of 16s rRNA (E. faecalis-specific/16s rRNA; B), mRNA expression of pbp5 normalized by that of 16s rRNA (pbp5/16s rRNA; C) from five root samples in each group (n=5). ** = p<0.01; *** = p<0.001 for comparisons between group i or iii and group ii.*

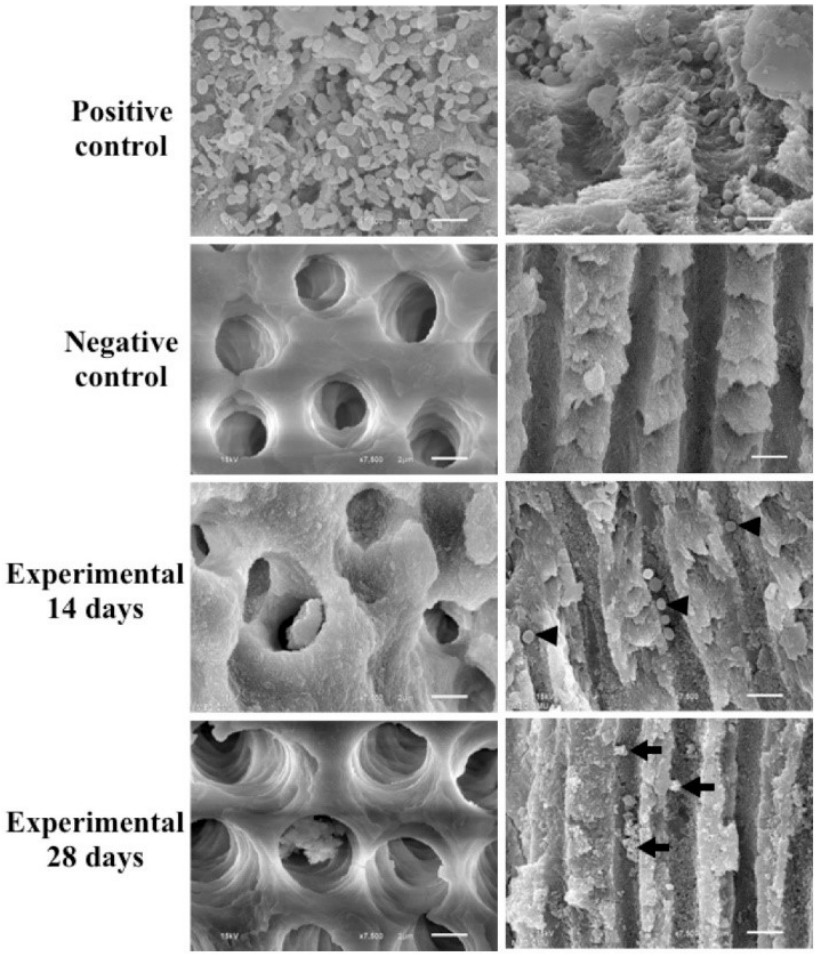

Figure 3 *Representative scanning electron micrographs from four different root samples in each group (n=4) with similar results showing almost eradication of E. faecalis by 5 mg/ml of ciprofloxacin and metronidazole in hydroxypropyl methylcellulose gel, at 14 and 28 days of treatment (experimental group). Arrowheads at 14 days showed some remaining E. faecalis in the dentinal tubules; arrows at 28 days indicated cell debris. The positive control was a root sample inoculated with E. faecalis for 21 days, while the negative control was a root sample without inoculation. Images on the left column were a cross-sectional view of dentinal tubules, while those on the right were a longitudinal view of the tubules. Bars = 2 µm.*

Discussion

In this study, MIC and MBC of the double antibiotic solution were 5 µg/ml (2.5 µg/ml each) and 250 µg/ml (125 µg/ml each), respectively. This MIC value is slightly higher than 1.4 µg per ml reported in previous study[18], due to different definition of MIC. The definition of MIC in this study was the fifty percent of *E. faecalis* inhibition, that was set as the lowest dose of ciprofloxacin and metronidazole that yielded a turbidity change ≤0.05.[18] However, the MBC value is approximately close to the MBC reported in previous study.[18] When ciprofloxacin and metronidazole were mixed with 2 % HPMC gel, a release of these antibiotics reached the MIC and MBC within 15 min and 2 h, indicating an immediate bacteriostatic action, followed by a bactericidal effect of the combined antibiotics in the HPMC gel. Moreover, a sustained release of both antibiotics up to 24 h was shown in Table 2. It is expected that these antibiotics can be released from the HPMC gel for a longer period of time than 24 h or until the antibiotics are completely released from the gel. Ciprofloxacin is a broad-spectrum antibiotic, whose structure is classified in the group of carboxy-fluoroquinoline. Its bactericidal action is due to the inhibition of two important enzymes, topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair, strand supercoiling repair, and recombination.[29] Metronidazole is cytotoxic to facultative anaerobic bacteria by disrupting their energy metabolism and hindering their DNA replication, transcription and repair process, resulting in bacterial cell death.[30]

Regarding the *in vitro* bactericidal effect of ciprofloxacin and metronidazole in the HPMC gel, the DNA content of *E. faecalis* inoculated in the root dentin was remarkably reduced by treatment with the combined antibiotics in the HPMC gel for 14 and 28 days, consistent with a significant decrease in mRNA expression of *E. faecalis*-specific sequence and a considerable reduction of *E. faecalis* within the dentinal tubules (Fig. 3). Nevertheless, mRNA expression of *pbp5* was significantly up-regulated by the treatment with these antibiotics in the HPMC gel for 14 and 28 days, probably owing to a response of *E. faecalis* to environmental stress from antibiotic treatment or a transition of *E. faecalis* to the VBNC state.[31] Although the *pbp5* mRNA expression was significantly increased in group iii (hydrogel + double antibiotics) as compared to that in group ii (only hydrogel; Fig. 2C), the total number of their viable cells was considerably decreased by the double antibiotic treatment as shown by a significant and dramatic decrease in the mRNA expression of *E. faecalis*-specific gene (Fig. 2B). A very small amount of viable *E. faecalis* may not survive in the root canal or be sufficient to cause subsequent infection after proper sealing by root canal obturation. The *pbp5* expression in the experimental samples that contained the two antibiotics in the HPMC gel at 28 days was lower than that at 14 days (Fig. 2C). This may be due to a lower number of viable *E. faecalis* left in the root samples after prolonged antibiotic treatment. Conversely, the expression of *E. faecalis*-specific sequence at 28 days in the positive control that contained no antibiotics in the HPMC gel was higher than that at 14 days (Fig. 2B), possibly due to the growth of *E. faecalis*.

The HPMC gel has so far attracted considerable attention from both scientists and academicians in the biomedical field because of its excellent biocompatibility to human tissues and cells and low toxicity.[32] The efficacy of ciprofloxacin and metronidazole in the HPMC gel at 5 mg/ml against *E. faecalis* in this study is comparable to that of these antibiotics in methyl cellulose gel at 5 mg/ml[28], which effectively eliminates the bacterial biofilm in necrotic pulps from mature and immature roots.[33] However, there are some differences in the physical properties between HPMC and methyl cellulose. The solubility of HPMC in cold water is higher than that of methyl cellulose, so the HPMC can prevent droplets and particles from agglomerating, thus inhibiting the

formation of sediments.[34] Moreover, the viscosity of HPMC is less affected by a temperature change than that of methyl cellulose, and the HPMC gel is more stable to salts and to a wide pH range than the methyl cellulose gel.[24] Therefore, it is of our interest to develop the HPMC gel as a new vehicle for antibiotic delivery in the root canal due to these advantages of HPMC over methyl cellulose. Moreover, the efficacy of the double antibiotics in the HPMC gel was not compared with that of calcium hydroxide in the present study because few previous studies have demonstrated that calcium hydroxide does not exert an antibacterial activity against *E. faecalis.*[13, 18]

PCR, especially qPCR, is recommended for microbial detection and quantification because it is more sensitive than the conventional bacterial culture.[16,35] Furthermore, the PCR technique is suitable for detecting *E. faecalis* that enters the VBNC state, in which *E. faecalis* does not form a colony as it is cultured. *E. faecalis* in this state is still alive and can produce some enzymes and acids necessary for maintaining its pathogenicity[36], resulting in persistent root canal infections that lead to endodontic treatment failure. Consequently, the culture method would underestimate the total quantity of *E. faecalis* in the root dentin. A bone mill and steel impactors were used to grind the whole root into fine powder for simultaneous DNA and RNA extractions. Due to the presence of *E. faecalis* in the dentinal tubules (Fig. 3), pulverizing the whole root using these tools would yield more reliable DNA and RNA contents of *E. faecalis* than scraping the root dentin surface.[37]

A period of dentin inoculation with *E. faecalis* for 21 days was chosen to mimic chronic root canal infection and to show the existence of *E. faecalis* in the dentinal tubules.[38] This was verified by an SEM in the positive control (Fig. 3). Although 5 mg/ml of ciprofloxacin and metronidazole in the HPMC gel was effective against *E. faecalis in vitro* as early as 14 days with a further reduction of *E. faecalis* quantities at 28 days, it is still necessary to further determine the real efficacy of this preparation for treatment of pulp and periapical diseases in clinical settings. A long-term intracanal medicament up to 28 days is suggested in this study to maximally eradicate *E. faecalis* in the root dentin; however, a clinician's decision for root canal obturation is not made by *E. faecalis* quantities, but by clinical signs and symptoms.

Conclusion

In summary, 5 mg/ml of ciprofloxacin and metronidazole in the HPMC gel can significantly decrease *E. faecalis* inoculated in the root dentin, and their bactericidal effect lasts for at least 28 days. The HPMC gel may be clinically beneficial as a new vehicle for delivery of the double antibiotics into the root canal as an injectable, easy-to-flow and ready-to-use preparation of antibiotics mixed in the gel.

Conflict of Interest

The authors deny any conflicts of interest related to this study.

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