Potential apply of hydrogel-carried chlorhexidine and metronidazole in root canal disinfection

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Microorganisms may persist in the root canal system after root canal therapy (RCT). The purpose of this study was to explore the metronidazole (MTR)- and chlorhexidine (CHX)-loaded hydrogels as the potential application in intracanal medicaments for root canal disinfection. Ultraviolet cross-linked hydrogels (gGels) were synthesized by GelMA solution and photoinitiator, which were loaded with MTR (MTR@gGels) and CHX (CHX@gGels). gGels, MTR@gGels and CHX@gGels were characterized by scanning electron microscopy. The antimicrobial activity against E. faecalis, S. mutans and P. intermedia was assessed. Meanwhile, the biocompatibility of human dental pulp stem cells (hDPSCs) was evaluated. DCT, CCK-8, CFU and live/dead-stained biofilm results showed that the viability of E. faecalis, S. mutans and P. intermedia was significantly reduced in MTR@gGels and CHX@gGels in vitro. CCK-8 results showed considerable biocompatibility with hDPSCs. The filling and clearance of gGels in root canals were demonstrated in vivo. Therefore, MTR- and CHX-loaded hydrogels may be a potential application in intracanal medicaments for root canal disinfection.

Keywords: Hydrogels, Metronidazole, Chlorhexidine, Antibacterial effect, Root canal

INTRODUCTION

Pulpitis is a common and frequent oral disease with acute and unbearable pain that greatly affects individuals’ health and quality of life. Pulpitis is inflammation of dental pulp tissue. Usually, bacterial infection causes dental caries that then develops into pulpitis, or even periapical periodontitis. Conventional root canal therapy (RCT) is a standard and effective endodontic treatment for pulpitis and apical periodontitis and involves removing infected dental pulp and dentin tissue through biomechanical instrumentation and killing bacteria with antibiotics and antibacterial materials1. However, microorganisms and residual pulp tissue may persist in the anatomical complexities of the root canal system after RCT, which may induce the failure of the treatment2-4. E. faecalis is found in 4% to 40% of primary endodontic infections5, especially in the case of refractory apical periodontitis6. Due to its ability to invade the dentinal tubules, and to survive under nutritional deprivation and highly resistant to antimicrobial strategies, E. faecalis biofilms in the root canal system and apical periodontitis lesions show high resistance to irrigants and medicaments, including sodium hypochlorite and calcium hydroxide7. Calcium hydroxide is the most commonly used intracanal medicament in RCT8. The antimicrobial property of calcium hydroxide is attributed to direct contact with bacteria8, which will greatly reduce the effect of the root canal system complex. The inhibitory activity of calcium hydroxide against E. faecalis is limited. Moreover, calcium hydroxide also leads to some side effects after an operation, such as pain, calculus formation and dentin breakage9,10. In addition, many other bacteria contribute to the development of the pulpitis, such as S. mutans, P. intermedia and A. actinomyctecomitans11. Endodontic bacteria may invade blood and induce bacteremia and even systemic diseases12. Therefore, it is very important to develop new intracanal medical treatments or strategies to potentially inhibit bacterial growth and improve the success rate of pulpitis and apical periodontitis.

Many drugs can effectively inhibit bacteria and biofilms and have been used in oral disease therapy, such as metronidazole (MTR)13 and chlorhexidine (CHX)14. Compound gargle solutions of CHX and MTR are very common medicines. CHX could change the permeation barrier of the bacterial cytoplasmic membrane, which causes the cell to break and the contents of the cell to leak out. Because of its positive charge, CHX can be absorbed by dental plaque and the oral mucosa surface, both of which are negatively charged; then, CHX can gradually be released to prolong the antimicrobial effect. MTR is an antimicrobial agent that is highly active against anaerobic bacteria15. It has been used in acute endodontic infections due to its favorable
pharmacokinetic and pharmacodynamic properties, low resistance rate and minor adverse effects\textsuperscript{15,16}. However, the antibacterial efficacy is short term for both MTR and CHX due to dilution and degradation. Therefore, we attempted to use appropriate materials that have low toxicity and long-term retention in root canals to carry and deliver CHX or MTR.

Hydrogel is a commonly used tissue engineering material with a tunable porous three-dimensional (3D) structure that can be loaded with small molecular compounds. It is easy to prepare and has good biocompatibility and affinity for biological fluids\textsuperscript{17}. Hydrogel causes a negligible inflammatory response, insignificant tissue damage and negligible thrombosis, it is easily molded into a precise shape and can be used for an extended period of time since it is inert\textsuperscript{18}. It also exhibits excellent performance in drug loading, long-term sustained release, and regeneration\textsuperscript{19}. Many reports have shown its various functions, including ectopic cartilage mimicking and therapeutic medicine delivery\textsuperscript{20,21}. Notably, hydrogels have good fluidity before curing, which is very suitable for filling the isthmus of root canal, type “C” root canal, apical ramification and lateral root canal, even can infiltrate into the dentinal tubules. Subsequently, hydrogels will solidify and efficiently deliver drugs to the complex root canal system to exert sustained antimicrobial activity till the next treatment. Therefore, an injectable UV light-sensitive GelMA hydrogel was chosen as the antimicrobial material carrier to slowly release CHX and MTR in this study.

In our study, injectable UV light-sensitive GelMA hydrogels loaded with CHX (CHX@gGel) and MTR (MTR@gGel) were synthesized. To explore its potential application in intracanal medicaments for root canal disinfection, the evaluation of the antibacterial effects on \textit{E. faecalis}, \textit{S. mutans} and \textit{P. intermedia} bacterial strains has been firstly shown in the present manuscript and the feasibility of root canal filling and material curing in root canal was tested \textit{in vitro}.

\textbf{MATERIALS AND METHODS}

The entire study was performed according to an informed protocol approved by the Ethics Committee of School & Hospital of Stomatology, Tongji University (No. 2017-037), and informed consent was obtained from the patients.

\textbf{Preparation of hydrogels}

GelMA was prepared according to a previous study\textsuperscript{22}. Briefly, 8 mL methacrylation was added into the dissolved gelatin solution in 100 mL phosphate-buffered saline (PBS) at 60°C and stirred for 3 h. Excess preheated PBS was added to the mix to terminate the reaction. The prepared solution was collected and dialyzed against ultrapure water at 40°C and then lyophilized to obtain GelMA. A mixture of 50 mg GelMA and 5 mg photoinitiator (PI, Sigma-Aldrich, St. Louis, MO, USA) in 1 mL H\textsubscript{2}O was exposed to UV light (365 nm, 30 mW/cm\textsuperscript{2}) for 1 min to obtain the gGel. CHX@gGel and MTR@gGel were prepared by mixing the abovementioned gGel with CHX (1 mg/mL) (CHX, Sigma-Aldrich) and MTR (10 μg/mL) (MTR, Sigma-Aldrich), respectively, before photoinitiation.

\textbf{CHX@gGel and MTR@gGel characteristics obtained by scanning electron microscopy (SEM)}

The gGel, CHX@gGel and MTR@gGel samples were serially dehydrated in ethanol and then prepared according to the special requirements of SEM\textsuperscript{23}. The images were obtained on a field emission scanning electron microscope (SU8220, HITACHI Co., Tokyo, Japan) operated at 10 kV.

\textbf{Bacteria preparation}

\textit{E. faecalis} (ATCC 29212), \textit{S. mutans} (ATCC 25175) and \textit{P. intermedia} (ATCC 25671) were obtained from American Type Culture Collection (Manassas, VA, USA). They were cultured in brain heart infusion medium (BHI) (Difco, Detroit, MI, USA) overnight under anaerobic conditions (80% N\textsubscript{2}, 10% H\textsubscript{2} and 10% CO\textsubscript{2}) at 37°C in anaerobic bags. Bacterial pellets were harvested by centrifugation at 2,500 rpm for 5 min. Then, they were diluted to $1\times10^8$ colony forming units (CFU)/mL based on a regression line derived from McFarland turbidity standards.

\textbf{Cell culture}

Primary hDPSCs were isolated from 3 healthy human premolars after extraction for orthodontic treatment (individuals were 12–14 years old); individuals provided informed consent before tooth extraction. Primary hDPSCs were cultured and identified according to our previous study\textsuperscript{24}. hDPSCs were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) (Thermo-Fisher, Waltham, MA, USA) with 10% fetal bovine serum (FBS, Thermo-Fisher) and 1% antibiotics (100 U/mL penicillin and 100 μ/mL streptomycin; Gibco-BRL, Grand Island, NY, USA) in a humidified atmosphere of 5% CO\textsubscript{2} at 37°C.

\textbf{CCK-8 analysis for cell viability}

CCK-8 detection (Dojindo Molecular Technology, Kumamoto, Japan) was used to analyze the viability of bacteria and hDPSCs in hydrogel liquid extracts. The gGel, CHX@gGel and MTR@gGel samples were soaked in BHI culture medium or DMEM (containing 10% FBS and antibiotics) for 7 days to obtain liquid extracts. Normal cultured bacteria or hDPSCs were collected and suspended in the above mentioned liquid extracts and then seeded in 96-well plates (100 μL per well, 5 replicate wells). After treatment for the appropriate time (2 days for bacteria and 1, 3, 5, 7 days for hDPSCs), 5 μL CCK-8 working solution was added into each well and incubated at 37°C for another 4 h. CCK-8 solution
without bacteria or cells was used as a blank control and the normal culture medium with hPDGs was set as a control group. Absorbance at 450 nm was measured to reflect the number of vital cells in each well.

**Antimicrobial activity by direct contact test (DCT)**

Sidewalls of 96-well plates (Corning, Corning, NY, USA) were coated with equal amounts (50 μL) of gGel, CHX@gGel and MTR@gGel solutions and initiated by UV light. Subsequently, the coated plates were disinfected by soaking in 75% ethanol for 5 min twice and washed with BHI culture medium three times before exposure to the bacterial suspension. Ten microliters of *E. faecalis*, *S. mutans* or *P. intermedia* bacterial suspension (1 × 10⁶ CFU/mL) was added into each well. BHI medium without bacteria placed on gGels was used as a blank control. Each group contained 6 replica wells. After incubation in a humid atmosphere at 37°C for 1 h, the evaporation of liquid was evaluated to ensure direct contact between bacteria and the material surface, and then 100 μL BHI medium was added gently into each well. After 24 h of incubation, the 96-well plate was placed in a microplate spectrophotometer (BioRad, Hercules, CA, USA) to determine the absorption value at 630 nm.

**CFU analysis**

According to the ISO10993 standard, the gGel-, CHX@gGel- and MTR@gGel-coated plates were soaked in BHI culture medium (1 mL for 6 cm² surface area) for 7 days to obtain liquid extract, which was kept in a refrigerator at 4°C for further use. One hundred microliters of *E. faecalis*, *S. mutans* or *P. intermedia* bacterial suspension was mixed with 2 mL of the abovementioned liquid extract, spread on agar plates and further incubated for 48 h at 37°C under anaerobic conditions. Bacteria cultured in BHI culture medium served as a control group. Subsequently, the CFU on the agar plate was counted for each group.

**Biofilm viability**

The gGel, CHX@gGel and MTR@gGel samples were prepared in confocal microscopy dishes (Corning) and washed with PBS solution 3 times after being sterilized by soaking in 75% ethanol for 5 min twice, as previously described. Two milliliters of bacterial suspensions of *E. faecalis* and *S. mutans* were added. All samples were incubated in an anaerobic environment at 37°C for 7 days. Subsequently, the *E. faecalis* and *S. mutans* biofilms grown in hydrogel samples (gGel, CHX@gGel and MTR@gGel) were stained using a LIVE/DEAD BacLight Bacterial Viability Kit (Yeasen, Shanghai, China) according to the manufacturer's instructions and then 3D imaged using confocal laser scanning microscopy (CLSM; Carl Zeiss, Oberkochen, Germany) at excitation wavelengths of 488 nm (Calcein-AM) and 555 nm (Propidium Iodide), respectively. For each disk, a 16-μm-thick 3D biovolume of interest from each biofilm image analysis was performed. The middle of the plot was chosen to analyze the percentage distribution of live and dead bacteria according to green and red fluorescence intensity. Images were obtained with a 20× objective, and at least three images were collected randomly from each sample.

**Hydrogel filling and clearance in teeth after injection in vitro**

Twelve single root canal premolars were obtained from healthy humans with informed consent after premolar extraction for orthodontic treatment. These teeth underwent bilateral groove procedures and dental crown removal by oral processing equipment *in vitro*. Subsequently, pulp tissue was removed, and root canals were expanded. After washing the root canal with 19% EDTA solution and ultrasonic treatment in water, the teeth were dried and prepared *in vitro*.

The gGel solution was mixed with DAPI (15 μg/mL) and injected into the abovementioned teeth *in vitro* and then exposed to UV light to obtain the photocurable gel. Thereafter, six of these teeth were opened from the side grooves to determine whether the gel was fully filled. The root canals of the other six teeth were cleaned with a nickel-titanium file and irrigated with PBS buffer. Similarly, the teeth were opened from the side grooves to observe whether the hydrogel was easy to clean. Meanwhile, the opened teeth with fulfilled hydrogel were imaged using CLSM at excitation wavelengths of 405 nm. A 16-μm-thick 3D biovolume of interest from each biofilm image analysis was performed.

**Statistical analysis**

All tests were conducted three times independently. The results were analyzed with GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Statistical analyses were performed by SPSS 19.0 software (IBM, Armonk, NY, USA) at a significance level of p < 0.05. ANOVA was used to examine intergroup differences.

**RESULTS**

**The characteristics of the materials**

The SEM images of gGel, CHX@gGel and MTR@gGel samples (Fig. 1) showed the characteristics of the interconnected pore structures. These hydrogels had demonstrably good permeability and sustained-release properties due to their micrometer-scale pore size and were suitable for use as scaffolds. Compared with gGel samples, the structural characteristics of CHX@gGel and MTR@gGel samples showed little change, which meant that drug loading did not influence the characteristics of the interconnected pore structure of gGel samples.

**Biocompatibility of drug-loaded hydrogels**

HDPSCs were cultured in liquid extract from the hydrogels for CCK-8 detection. Cells grew normally for 7 days, with the optical density at 450 nm (OD450) increasing from less than 0.5 to over 1.5 daily. Moreover, there were no significant differences among the groups (Fig. 2). As previous work demonstrated that gGel samples had good biocompatibility, our findings suggested that the drug-loaded gGels were still
suitable for hDPSC growth. The result in Fig. 2 showed a representative experiment from three independent experiments from one donor.

**Antimicrobial activity of drug-loaded hydrogels**

To determine whether the material coating would affect the antimicrobial function of the compounds, the growth and viability of *E. faecalis*, *S. mutans* or *P. intermedia* on gGel, CHX@gGel and MTR@gGel samples were detected using several experimental methods. First, we detected the optical density at 630 nm (OD630) of *E. faecalis*, *S. mutans* or *P. intermedia* after treatment with these hydrogels for 24 h. Compared with the gGel samples, both CHX@gGel and MTR@gGel samples exhibited significant reductions in bacterial density (*p*<0.05). The actions of CHX@gGel samples were even better than those of MTR@gGel samples in both strains (Fig. 3A). Next, the viability of *E. faecalis*, *S. mutans* or *P. intermedia* cocultured with the liquid extracts from solid hydrogels was analyzed. Similar results were also found here. Compared with the gGel samples, the CHX@gGel and MTR@gGel sample liquid extracts showed significant antibacterial effects on *E. faecalis*, *S. mutans* or *P. intermedia*, and CHX@gGel samples provided the best results (Fig. 3B). The antibacterial effects of the liquid extracts were also identified by the CFU test. As
Fig. 4 The antibacterial effects of the drug-carried hydrogels by CFU.
(A) The colonies of the control group (gGel) were small and intensive, while those of the CHX@gGel and MTR@gGel groups were rare and scattered. (B) The statistical results exhibited the quantification of the colonies, implying that both CHX- and MTR-loaded hydrogels were potent antimicrobials (*, p<0.05).

In addition, *E. faecalis* and *S. mutans* biofilms were seeded and constructed on gGel, CHX@gGel and MTR@gGel to measure the live/dead bacterial ratio. The green and red fluorescence showed the live (green) and dead (red) bacterial distribution within the biofilms. After 24 h of treatment, the gGel group displayed mostly green fluorescence, with cell viabilities of 93.06% and 95.07% in the *E. faecalis* (A and C) and *S. mutans* biofilms (B and D), respectively. While about 75.13% and 45.03% in *E. faecalis* (A and C) and *S. mutans* biofilms (B and D) were killed in the MTR@gGel group. The live/dead bacteria ratio was strongly decreased in the CHX@gGel group, with almost 92.7% (*E. faecalis*) and 88.3% (*S. mutans*) of the bacteria being dead (Fig. 5). The above results revealed the potent antimicrobial activity of CHX@gGel and MTR@gGel in both contact and noncontact manners.

Properties of root canals filled with material in vitro
To solve the difficulty of drug filling and sterilization in the root canal isthmus, we attempted to investigate whether the hydrogels could fill the root canal and solidify. As mentioned above, twelve prepared *in vitro* premolars were injected with a liquid mixture of hydrogel and DAPI and solidified with UV light. The images of the ripped teeth were recorded with a camera. As shown in Fig. 6A, the root canals were filled with colorless transparent gelatinous hydrogel. After removal, the root canals were very clean, and little residual hydrogel was observed (Fig. 6B). Moreover, we measured the fluorescence images of the ripped teeth using laser confocal equipment. Blue spots were detected as hydrogel-DAPI signaling. The typical picture showed the interface of the dentin and hydrogel, which showed that the hydrogel was filling in the root canal and even...
According to other published work and our previous work, hydrogels showed good biocompatibility with S. mutans and had very intensive antibacterial effects on P. intermedia.

In addition, both CHX@gGel and MTR@gGel samples, suggesting the notable antibacterial effect of these hydrogels. Similar results were obtained for S. mutans, although the action was not as strong as that in E. faecalis. Importantly, the current concept in endodontic microbiology considers endodontic disease as a biofilm-induced infection, emphasizing the importance of biofilm formation in persistent endodontic infection. In this work, we detected permeating into dentinal tubules (Fig. 6C). The 3D image synthesis revealed the noteworthy fluidity of the hydrogel in the root canal system after curing (Fig. 6D).

**DISCUSSION**

The criterion for successful endodontic therapy is complete resolution of pulpal or periapical pathosis by delivering efficacious treatments to the patients. In the process of providing effective treatment, eliminating the bacteria and preventing persistent infection or reinfection are the key steps. There are still some deficiencies in the current use of root canal drugs in clinical practice. Thus, it is essential to develop advanced endodontic disinfectant strategies. In this study, an injectable UV light-sensitive gGel was generated as the antimicrobial material carrier to release CHX (CHX@gGel) and MTR (MTR@gGel) (Fig. 1). Our results showed that the gGel could fully fill and be solidified in the root canal, and the gGel was easy to clean using root canal file (Fig. 5). This characteristic suggests its potential use in RCT. In addition, both CHX@gGel and MTR@gGel samples had very intensive antibacterial effects on E. faecalis, S. mutans or P. intermedia in vitro (Figs. 3–5). Meanwhile, according to other published work and our previous work, hydrogels showed the good biocompatibility with stem cells, similar results were found in our drug-loaded hydrogels on hDPSCs (Fig. 2). Considering the advanced fluidity and filling properties, remarkable bacteriostasis, and good biocompatibility of the materials, our findings provide some support for their potential application in root canal dressings.

Pulpal pathosis is usually caused by multiple bacterial infections. Studies have demonstrated that E. faecalis was observed in root-filled teeth with periapical lesions with a prevalence ranging from 24% to 77%6. E. faecalis is a nonspore form of facultative anaerobic coccus belonging to Group D streptococci. The existence of E. faecalis in the tooth canal would greatly affect the success rate of RCTs, causing it to fall to 79%25. It is closely related to a high percentage of root canal failures and is able to survive in the root canal as a single organism or as a major component of the flora. E. faecalis can tolerate poor environments, including high-pH alkaline conditions and nutritional deficiency. Many current clinical intracanal medicines, such as CH, cannot adequately treat E. faecalis. Thus, eliminating E. faecalis is a challenge for dentists728. Another microorganism found in infected root canals associated with apical periodontitis is S. mutans, which is a major pathogen of dental caries. The microbiome of deep dentinal caries lesions is considered to be the primary pathogen that invades the pulp to induce endodontic infection because most species detected in deep dentinal caries are the same as those found in infected root canals27. These endodontic bacteria may enter the bloodstream, cause bacteremia and initiate systemic diseases29. S. mutans has been shown to be one of the most opportunistic microorganisms in the infected dentine model, P. intermedia, as an obligate anaerobe, has been reported as one of the major bacteria in pulpitis and apical periodontitis29. In particular, the capability of E. faecalis, S. mutans or P. intermedia in pulpitis and apical periodontitis makes them very suitable research objects.

To evaluate the antibacterial effect of CHX@gGel and MTR@gGel samples against E. faecalis, S. mutans or P. intermedia, gGels and their liquid extracts were evaluated. Bacteria were treated with liquid extracts from the hydrogels and then used for CCK-8 and CFU analyses. The antibacterial activities of the CHX@gGel and MTR@gGel liquid extracts were significantly stronger than that of the control gGel. Since microorganisms that adhered to the biofilm were more insensitive to antibacterial agents than their suspended status, we observed the antibacterial effects of CHX@gGel and MTR@gGel against the bacterial biofilms. In this work, 3D images of the bacteria-hydrogel coculture complex clearly characterized live and dead bacterial distributions within biofilms. As Fig. 6 shows, E. faecalis hardly lived in CHX@gGel and MTR@gGel samples, suggesting the notable antibacterial effect of these hydrogels. Similar results were obtained for S. mutans, although the action was not as strong as that in E. faecalis. Importantly, the current concept in endodontic microbiology considers endodontic disease as a biofilm-induced infection, emphasizing the importance of biofilm formation in persistent endodontic infection. In this work, we detected
the antibacterial effect of CHX@gGel and MTR@gGel on two kinds of key oral pathogenic bacteria, and their potent antibacterial functions were observed in various statuses, including direct antibiosis and liquid extract antibiosis, suspended bacteria and adhered biofilms. Both CHX and MTR still played a strong antibacterial role in the material coating conditions.

It is difficult to effectively eliminate endodontic polymicrobial infection because of the complex root canal system and innate resistance capacity of biofilms. The gGel solution had good fluidity and easily filled the entire root canal system, especially for collateral pulp canals or accessory root canals (Fig. 6). Most interesting, the gGel maybe infiltrate into the dentin tubules over 100 μm in Fig. 6. gGels have been used in tissue engineering and medicine release carriers due to their great biocompatibility and porosity. We investigated the injection and solidification of gGel in detached teeth in vitro. gGel was cured when exposed to a certain intensity of UV light, suggesting that the material would be convenient for dentist use. The preparation and removal of the hydrogel was easy to perform, and there was no obvious residue in the root canal after removal and flushing. Blue fluorescence with clear boundaries and uniform color by the dye-mixed gGel revealed a suitable level of root canal occupation (Fig. 6).

MTR is the standard criterion for anaerobic infection therapy. CHX is a common medicine for disinfection with bacteriostatic effects at low concentrations and bactericidal functions at high concentrations. CHX combined with MTR is usually used as an oral gargle. Meantime, gGel showed very notable flowability and biocompatibility (Fig. 2). Therefore, combining gGels and the antibacterial medicine via CHX@gGels and MTR@gGels, which might be useful as intracanal medication during RCT, root canal filling material, or dental pulp regeneration material in young permanent teeth.

Our work provided some evidence that could be used to develop new applications of drug-loaded hydrogels in endodontics. For further study, to develop CHX@gGel and MTR@gGel as commercial products, the penetrating ability and localization of drug-loaded gGels into dentinal tubules should be considered. Also, the filling and antibacterial activity in the root canal infection model, for the atypical and obstructed root canal system in particular, and the release of medicine from gGels and its long-term antibacterial effect should be considered in future.

In conclusion, this study showed the feasibility of synthesizing an antibacterial UV light-sensitive hydrogel. CHX@gGels and MTR@gGels had obvious antimicrobial effects against E. faecalis, S. mutans or P. intermedia observed by DCT, CCK-8, CFU, and biofilm CLSM analysis in vitro. This drug-loaded material might be a promising material for root canal disinfection due to its good biocompatibility. This pilot study laid the foundation for further study using CHX- and MTR-loaded hydrogels in root canal infection models in vivo.

ACKNOWLEDGMENTS

The authors thank Dr. Xin Wei from the Department of Operative Dentistry and Endodontics, Affiliated Hospital of Stomatologic, Nanjing Medical University, for providing E. faecalis and S. mutans. The authors thank professor Xiujun Zhang from Oral Medicine Research Center Affiliated Shandong University (Jinan, China) for providing P. intermedia. The authors do not have any conflicts of interest related to this study. This study was supported by grants from Chongming Municipal Commission of Health and Family Planning Program (No. 20184Y0110) and Shanghai Municipal Commission of Health and Family Planning Program (No. 202040095). We obtained informed consent from the patients for publication. The authors declare that they have no competing interests.

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