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ORIGINAL ARTICLE

Reduction of viable bacteria in dentinal tubules treated with a novel medicament (Z-Mix)

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Abstract *Background/purpose:* The 3Mix-MP formulation (a mixture of metronidazole, ciprofloxacin, and minocycline; macrogol and propylene glycol) has been used to kill residual bacteria in dentin caries. This study aimed to investigate the dentin disinfection and cytotoxicity of a novel zinc oxide (ZnO) based medicament, Z-Mix.
Materials and methods: Z-Mix was prepared as a prefilled syringe of materials containing mainly ZnO, incorporated with amoxicillin, ciprofloxacin, and metronidazole (1 g% of each antibiotic). Drug penetration was measured at 24 hours and 72 hours. *Streptococcus mutans*, *Lactobacillus acidophilus*, or *Enterococcus faecalis* were inoculated into dentinal tubules for 30 days and were then subjected to Z-Mix or 0.2% chlorhexidine (CHX) for 48 hours. Viable bacteria in the dentin were determined using fluorescence staining. Their cytotoxicity against human dental pulp cells was assessed using an MTT assay.
Results: Z-Mix obviously diffused into dentinal tubules and the root apex, compared to the 3Mix-MP ($P < 0.05$). Fluorescence staining demonstrated a reduction of viable bacteria at 100 μm and 500 μm below infected cavities after treatment with Z-mix or CHX for 48 hours. Live and dead bacteria ratios indicated that Z-Mix exhibited markedly antimicrobial effects on inoculated bacteria in dentin samples ($P < 0.05$). There was no significant difference in the antimicrobial property between Z-Mix and CHX ($P > 0.05$). An acceptable level of cytotoxicity was observed in Z-Mix and its ingredients.

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Conclusion: Z-Mix, a soft shapeable paste containing a mixture of three antibiotics, is successfully. It can penetrate to the root apex and exhibits antimicrobial properties.

Introduction

Streptococcus mutans and *Lactobacilli* have been considered to be significant in contributing to the initiation and progression of dental caries, respectively.[1–3] *Enterococcus faecalis* is also often found in carious lesions, however it is not directly involved in the formation of caries.[4,5] *E. faecalis* is also prevalent in endodontic infections, in which the pulp may become infected via carious lesions.[6,7] Recently, The Cariology Research Unit of The Niigata University, Niigata, Japan has developed the concept of "Lesion Sterilization and Tissue Repair" therapy.[8] This concept suggests that tissue repair will occur when the lesions are effectively disinfected. Using 3Mix (a mixture of metronidazole, ciprofloxacin, and minocycline) or 3Mix-MP (3Mix with macrogol and propylene glycol) resulted in the disinfection of the lesions and clinical success in treating deep carious lesions with and without pulp involvement.[9–11] At present 3Mix and 3Mix-MP are not available as commercial products in other countries except Japan. Furthermore, after mixing the three antibiotics with macrogol and propylene glycol, the mixture must be used on the day of treatment.[8,9] The mixture is not economically feasible for use in a dental practice because it is short-lived. In addition, it should be noted that there is a disadvantage of using 3Mix or 3Mix-MP because minocycline may effect tooth color.[12] Therefore, amoxicillin may be used instead of minocyclines.[13] Zinc oxide (ZnO) displayed antimicrobial properties against *S. mutans* and *Lactobacilli*.[14] For this reason, we incorporated ZnO powder into a polyethylene glycol ointment containing the antibiotics. Z-Mix, a soft shapeable paste containing a mixture of the three antibiotics (amoxicillin, ciprofloxacin, and metronidazole), is successfully prepared in a prefilled syringe that is practical to use.

In order to provide a new medicament for treating dental caries and endodontic infection, that is more convenient and economical to use in a dental practice compared with the fresh preparation of an antibiotic paste, this study evaluated the *in vitro* antibacterial effect of Z-Mix on caries-associated bacteria inoculated in dentin samples. The penetration of Z-Mix into dentinal tubules and the root apexes, and the biocompatibility of the medicament were also assessed in the study.

Materials and methods

Preparation of Z-Mix and 3Mix-MP

Z-Mix was prepared as a triple combination of antibiotics (amoxicillin, ciprofloxacin, and metronidazole, 1 g% w/w each) with a ZnO ointment-based carrier. The ointment base consisted of ZnO powder, polyethylene glycol 4000, stearyl alcohol, glycerin, sodium lauryl sulfate, and distilled water. 3Mix-MP was prepared according to previous reports.[8,15] Metronidazole, ciprofloxacin, and minocycline powders were mixed in a ratio of 1:1:1. Macrogol and propylene glycol (1:1) were mixed to prepare MP ointment. The antibiotic mixture and MP ointment (1:7) were then thoroughly mixed to form 3Mix-MP.

Tooth preparation

The present study was approved by the Research Ethics Committee of Thammasat University, Pathum Thani, Thailand (Reference No. 028/2554). Experimental cavities (3 mm × 4 mm × 3 mm, width × length × depth) were slot cut from occlusal surfaces of caries-free human permanent premolars towards the dentino—enamel junction using a diamond fissure bur (No. 330; Dentsply Maillefer, Ballaigues, Switzerland) in a high-speed air turbine hand piece. The average thickness of the remaining dentin of the cavity floors was ~2 mm. Cavity floors and axial walls were placed in dentin.

Inorganic and organic components of the smear layer were removed by placing the specimens in 17% EDTA (pH 7.8) for 4 minutes, followed by 5.25% sodium hypochlorite for 4 minutes with ultrasonication.[16] The absence of a smear layer and the presence of open dentinal tubules were confirmed via a scanning electron microscope (JSM-35; JEOL, Tokyo, Japan). The specimens were autoclaved and incubated in tryptic soy broth enriched with 0.6% yeast extract (BD Difco, Franklin Lakes, NJ, USA) for 48 hours to monitor sterilization.

Penetration of dye in ZnO base, Z-Mix, and 3Mix-MP through the root apex

The method of this experiment was modified from a previous study.[17] The tooth samples were randomly divided into three groups ($n = 10$ in each group). Dentin cavities were prepared as mentioned previously. Test materials (ZnO, Z-Mix, and 3Mix-MP) were mixed with 0.1 mol/mL Safranin O solution (Sigma-Aldrich, St Louis, MO, USA) at the concentration of 1 g%. Then, 10 μL of the test materials were introduced into the prepared cavities. The tooth samples were sealed with occlusal indicator wax to avoid moisture contamination. They were then placed into a closed container with wet paper towels to maintain a humid atmosphere and incubated at 37°C. After incubation for 24 hours and 72 hours, the specimens were split vertically into two sections with a pair of straight crown scissors. Each section was then viewed under a stereomicroscope (Carl Zeiss, Vienna, Austria) with 100 × magnification. With an eye piece reticle calibrated in millimeters, the extent

(length) of penetration was measured from cavity floors to the most apical extent of dye penetration.

Dentinal tubule invasion by bacteria

S. mutans (GS-5) was grown in Müller Hinton broth (BD Difco) under 95% N_2 and 5% CO_2 at 37°C for 48 hours. *L. acidophilus* (HA122) was cultured in De Man Rogosa and Sharpe broth (BD Difco) at 37°C in an atmosphere of 10% CO_2 for 24 hours. *E. faecalis* (ATCC 47077/OG1RF) was cultured in tryptic soy broth (BD Difco) at 37°C in an atmosphere of 10% CO_2 for 24 hours. Twenty teeth were used in this experiment. Tooth cavities ($n = 5$ in each group) were prepared and sterilized as mentioned above. The specimens were kept in a flask with 30 mL of the appropriate culture medium containing each bacterial species (3×10^6 CFU/mL) for 30 days. The media were changed daily. Sterile broth was used in the control group ($n = 5$). Following treatment, tooth samples were split longitudinally into two halves. Microscopic analyses were done using a scanning electron microscope.

Bactericidal effect of Z-Mix on infected dentinal tubules

The potent antibacterial effect of Z-Mix against *S. mutans, L. acidophilus*, and *E. faecalis* was evaluated by placing 0.5 g of Z-Mix into the dentin cavities ($n = 10$ in each group), which infected them with each type of bacteria as mentioned above. After enveloping with aluminum foil, the tooth specimens were incubated in the appropriate media for 48 hours. Bacteria in dentinal tubules were obtained using a method modified from a previous study.[16] The dentin samples of cavity floors were collected using three different diameters of sterile steel round burs (International Standards Organization Nos. 016, 021, and 023, Dentsply Maillefer) run in a slow-speed hand piece. These burs excavated the dentin samples at < 100 μm, 100—350 μm, and 350—500 μm depth of dentin, respectively. The dentin samples were dislodged from the bur by shaking and dentin powder was removed from each sample by centrifugation at $100 \times g$ for 2 minutes. Supernatants were centrifuged at $10{,}000 \times g$ for 1 minute to pellet bacterial cells.

To determine bacterial viability, the bacterial pellet was resuspended in 300 μL of the staining kit (LIVE/DEAD BacLight bacterial viability stain; Molecular Probes, Eugene, OR, USA). Aliquots of stained bacterial suspensions were placed on glass slides under cover slips and observed under a fluorescence microscopy (FM Nikon Eclipse C1; Nikon, Mississauga, Ontario, Canada). Five random microscope fields were examined per slide. A volume of 100 μL from each sample was pipetted twice into a microtiter plate and fluorescence intensity was measured with a microtiter plate reader (DTX 880 Multimode Detector; Beckman Culture, Fullerton, CA, USA) with excitation at 485 nm and detection at 642 nm (red) and 535 nm (green), for propidium iodide and SYTO 9, respectively. Bacterial viability was expressed as the ratio of live and dead bacteria (green/red fluorescence). In this experiment, chlorhexidine [(CHX) Sigma-Aldrich] was used as the positive control and the culture medium served as the negative control.

Biocompatibility test

Dental pulp tissue explants were obtained from permanent premolars, extracted for orthodontic reasons. Tooth surfaces were cleaned and cut around the cementum—enamel junction using sterilized diamond stones to access the pulp chamber. The dental pulp tissue was diced into small tissue explants of ~ 1 mm^3 and kept in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (HyClone; Thermo Scientific, Cramlington, UK), 100 IU/mL/100 μg/mL penicillin/streptomycin and 10 μg/mL amphotericin-B at 37°C, 95% humidity, and 5% CO_2. Primary dental pulp cells between the third and sixth passages were used in this study.

One gram of ZnO base or Z-Mix was immersed in 5 mL of complete DMEM and incubated at 37°C for 24 hours to allow the soluble components to leach from the test materials into the cultured medium. The experimental media were then collected and filtered through 0.2-μm syringe filters to remove any particulate matter before use. One gram of each antibiotic (amoxicillin, ciprofloxacin, and metronidazole) was dissolved with 5mL of 0.02% dimethyl sulfoxide (DMSO) in complete DMEM. Primary dental pulp cells were seeded into 96-well plates at 5×10^4 cells/well in complete DMEM. Untreated primary human dental pulp cells served as the control. After 72 hours incubation with the test materials, the cell proliferation was measured using an MTT assay.[18] The mitochondrial function was then calculated as a percentage of the control.

Statistical analyses

All quantitative data were expressed as mean of ± standard error of the mean. Statistically significant differences ($P < 0.05$) were assessed using the one-way analysis of variance, followed by Dunnett or Bonferroni multiple comparison test when appropriate. Data analysis was performed using the GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Penetration of ZnO, Z-Mix, and 3MIX-MP

Figure 1 shows stereoscopic photographs of the test material penetration through the dentinal tubules and the root apex at indicated times. A pink color staining was considered as positive material penetration. All tested materials could not effectively diffuse from the floor of experimental cavities to the apical foramen at 24 hours (Figure 1A—C). However, ZnO base (Figure 1D) and Z-mix (Figure 1E), but not 3Mix-MP (Figure 1F), could penetrate to the root apex after 72 hours. For quantitative comparison, the penetration depths of the tested samples to the root apex at 24 hours and 72 hours were determined. Z-Mix and 3Mix-MP were significantly lower in penetration compared to ZnO at 24 hours (Figure 2A) and 72 hours (Figure 2B; $P < 0.05$). However, Z-Mix efficiently diffused to the root apex, compared to 3Mix-MP ($P < 0.05$).

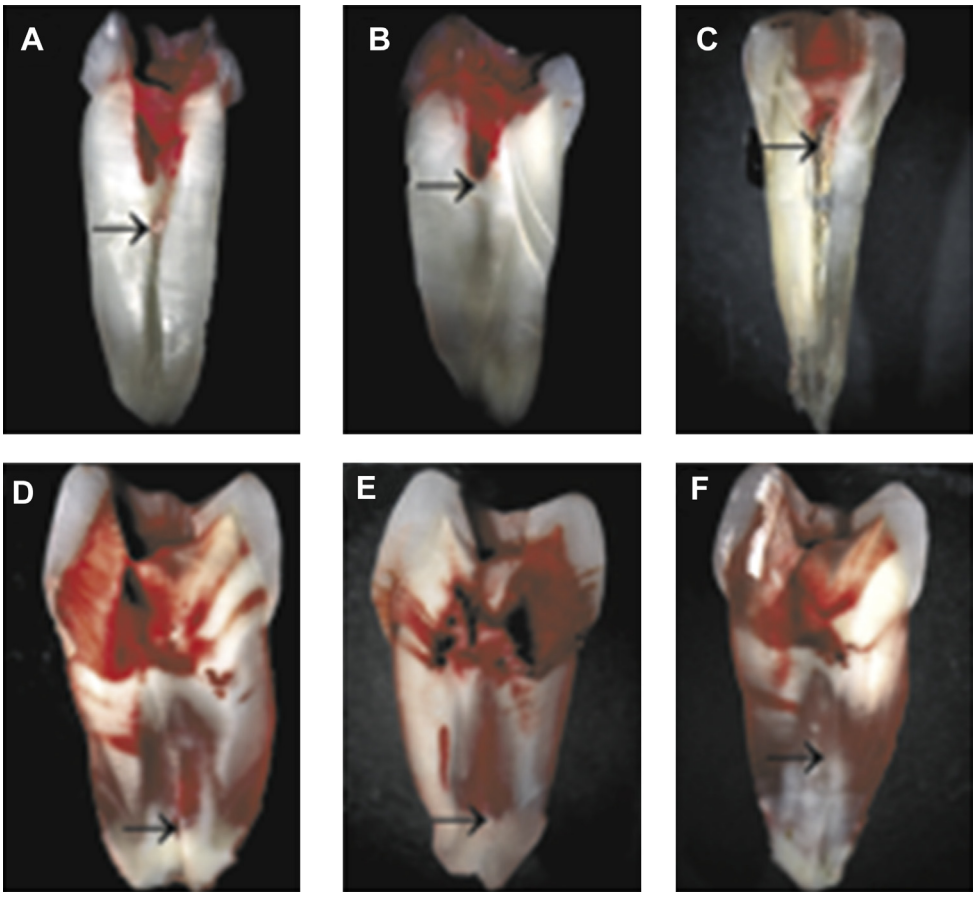

Figure 1 (A) Penetration patterns of the tested materials mixed with Safranin O dye (pink color) from the cavity floor to the root apex using ZnO ointment base at 24 hours; (B) Z-Mix at 24 hours; (C) 3Mix-MP at 24 hours; (D) ZnO ointment base at 72 hours; (E) Z-Mix at 72 hours; (F) 3Mix-MP at 72 hours. Arrows indicate the extent of the penetration of the diffused dye. (Stereo photograph, 100×).

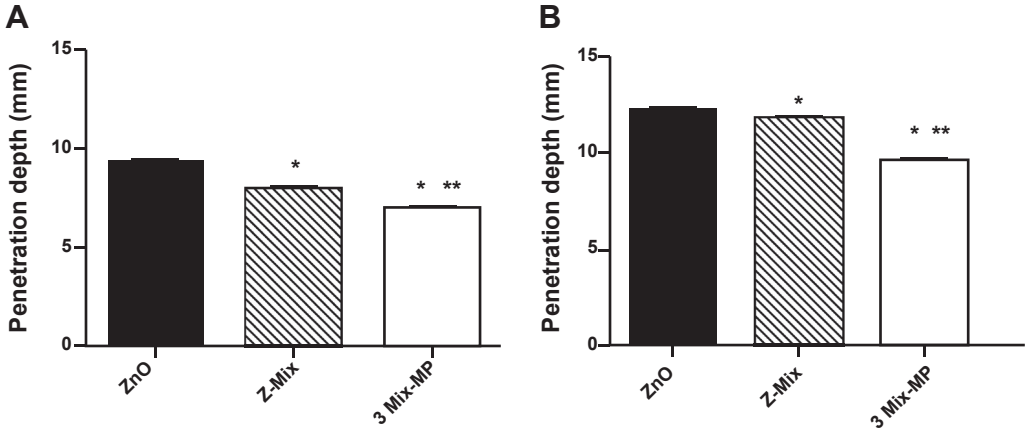

Figure 2 (A) Quantification of the penetration depth of the tested materials mixed with Safranin O dye from the cavity floor to the root apex at 24 hours; (B) and at 72 hours. Each value represents mean ± standard error of the mean ($n = 10$). * $P < 0.05$ versus the control, ** $P < 0.05$ versus Z-Mix.

Antibacterial effects

Scanning electron micrographs (Figure 3A) revealed that there were no bacteria observed in dentinal tubules in the control group. Aggregation of *S. mutans* (Figure 3B), *L. acidophilus* (Figure 3C), and *E. faecalis* (Figure 3D) was observed in all areas of the dentin walls at 500 μm. Bacteria were also found in the dentinal tubules of all specimens.

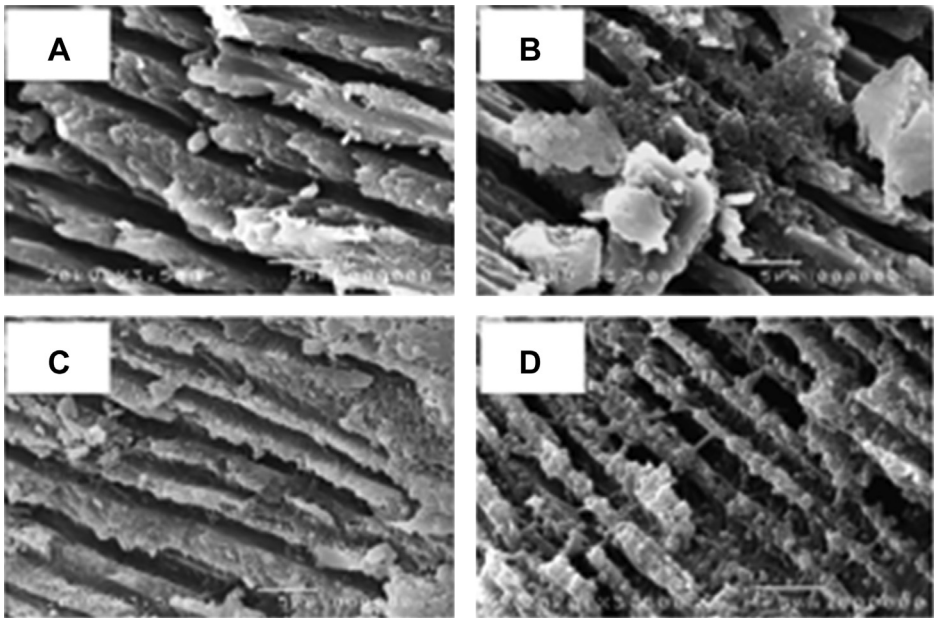

Figure 3 *In vitro* bacterial infection in dentin. (A) Scanning electron microscope photographs showing the bacteria in the dentinal tubules approximately 500 μm below the floor of prepared cavities after incubation for 30 days with appropriate control media; (B) incubation with *Streptococcus mutans*; (C) incubation with *Lactobacillus acidophilus*; (D) incubation with *Enterococcus faecalis*.

This indicated that the 30-day bacterial incubation in our model was enough for dentinal infection.

In our study, viable bacteria were observed from dentin samples infected with *S. mutans* (Figures 4A and 4D), *L. acidophilus* (Figures 4B and 4E), or *E. faecalis* (Figures 4C and 4F) at approximately 100 μm and 500 μm below the cavity floors. After treating infected dentin samples with Z-Mix for 48 hours, *S. mutans* (Figure 5D), *L. acidophilus* (Figure 5E), and *E. faecalis* (Figure 5F) in dentin samples from 500 μm below the cavity floors exhibited a more pronounced decrease in viable cell number compared with that of their control (Figures 5A—C). As expected, 0.2% CHX, the positive control, killed the bacteria in the infected dentin samples. After treatment with CHX, most of the *S. mutans* (Figure 5G), *L. acidophilus* (Figure 5H), and *E. faecalis* (Figure 5I) obtained from the dentin wall 500 μm

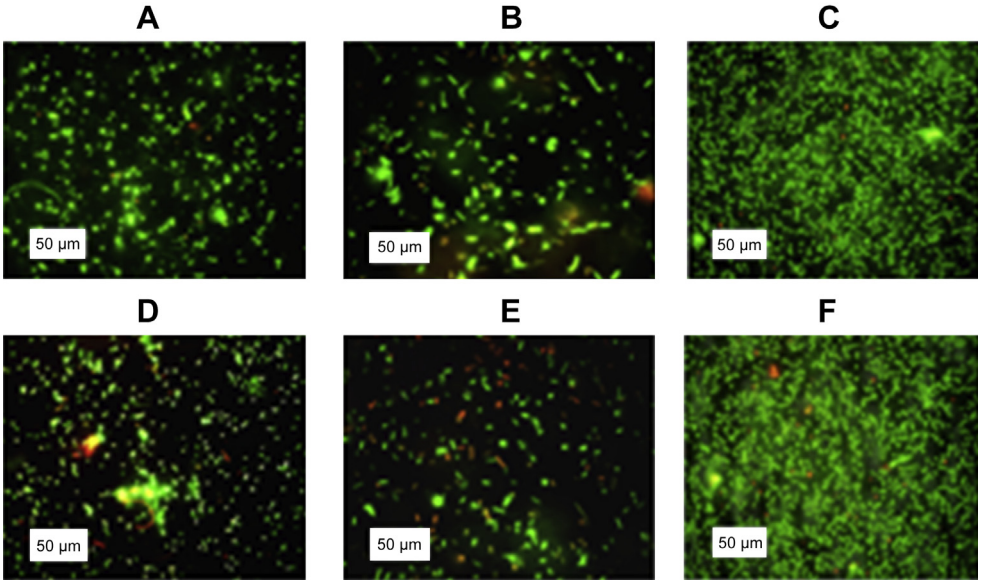

Figure 4 Live and dead bacteria in infected dentin samples. Fluorescence microscopic images showing viable (green) and dead (red) bacteria obtained from dentin samples (A—C) approximately 100 μm below the cavity floors; (D—F) 500 μm below the cavity floors. Prepared cavities were incubated with *S. mutans* (A and D), *Lactobacillus acidophilus* (B and E) or *Enterococcus faecalis* (C and F) for 30 days. Viable and dead bacteria were stained with DNA-binding dyes (Syto9 and propidium iodide).

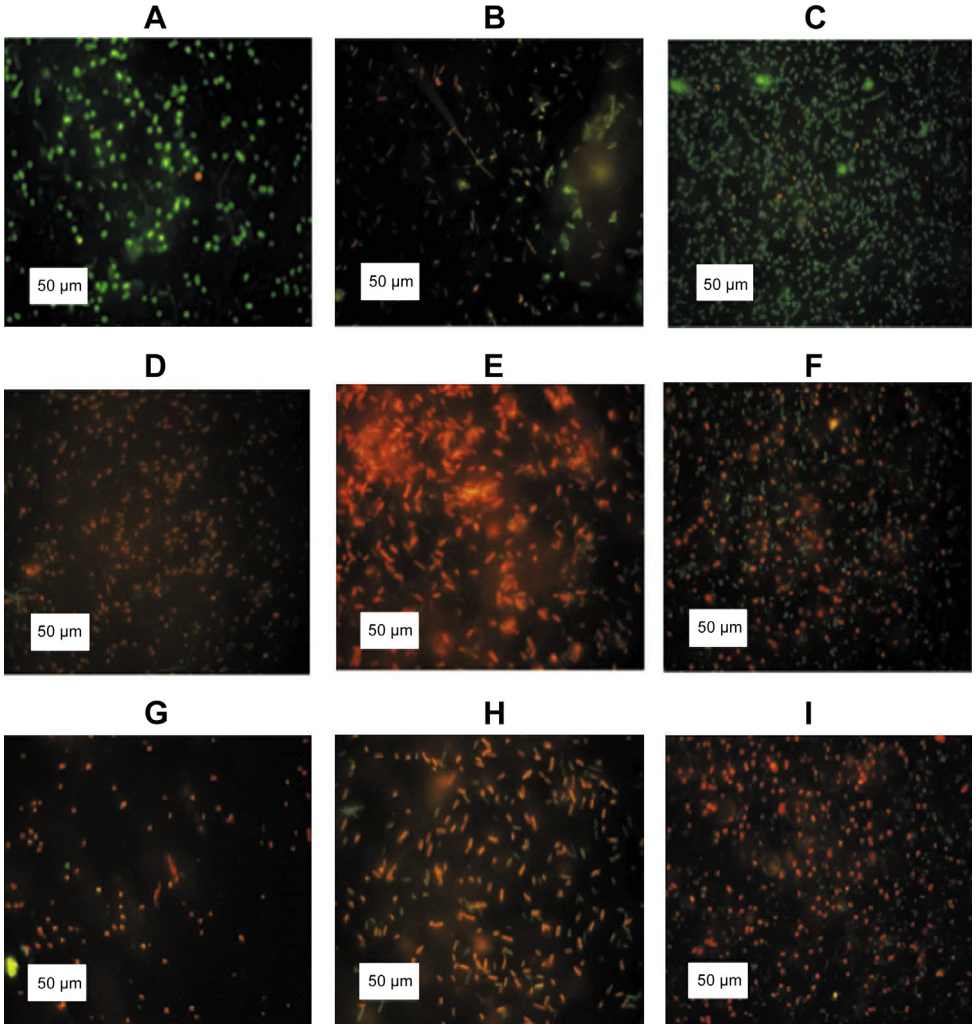

Figure 5 Bactericidal effects of Z-Mix or 0.2% CHX on bacteria inoculated in dentin. Fluorescence microscopic images showing viable (green) and dead (red) bacteria obtained from dentin approximately 500 μm below the prepared cavities. (A—C) Control groups; (D) cavities infected with *Streptococcus mutans*; (E) *Lactobacillus acidophilus*; (F) *Enterococcus faecalis* for 30 days, followed by treatment with Z-Mix (0.5 g) for 48 hours; (G) cavities infected with *S. mutans*; (H) *L. acidophilus*; (I) *E. faecalis* for 30 days, followed by treatment with 0.2% CHX for 48 hours. Viable bacteria were decreased approximately to the levels in the corresponding control groups. Viable and dead bacteria were stained with DNA-binding dyes (Syto9 and propidium iodide).

below the infected cavities were dead, compared with those in the corresponding control groups (Figures 5 and 6). Figure 6 demonstrated that the percentage of live/dead *S. mutans*, *L. acidophilus*, and *E. faecalis* significantly decreased after treatment of infected dentin samples with 0.5 g Z-mix or 0.2% CHX, compared to that of the control ($P < 0.05$). No significant differences in the bactericidal activity against all tested bacterial species were found between the Z-Mix and CHX groups ($P > 0.05$).

Biocompatibility

MTT assays show that the viabilities of dental pulp cells were 83.5%, 73.5%, 72.7%, 70.6%, and 74.6% for ZnO base (12.5 mg/mL), Z-Mix (12.5 mg/mL), amoxicillin (100 μg/mL), ciprofloxacin (100 μg/mL), and metronidazole (100 μg/mL), respectively (Figure 7). Although a significant difference was observed between test substances and the corresponding control, the cell viability was in the range of 60—90%, indicating slight cytotoxicity of the test substances.[19] It should be noted that the final concentration of DMSO in each antibiotic extract was < 0.01%. Therefore, there was no cytotoxicity due to DMSO.

Discussion

This experiment aimed to evaluate the penetration ability, antibacterial effects, and biocompatibility of a novel medicament, Z-Mix. We found that Z-Mix could penetrate into dentinal tubules and disinfected residual bacteria in dentin walls. The antimicrobial medicament should effectively deliver to dentinal tubules, therefore the remaining bacteria may not be capable of causing recurrent infection.[20] Our findings that propylene glycol effectively penetrated to the root apex were in agreement with a previous report.[17] It should be noted that in our

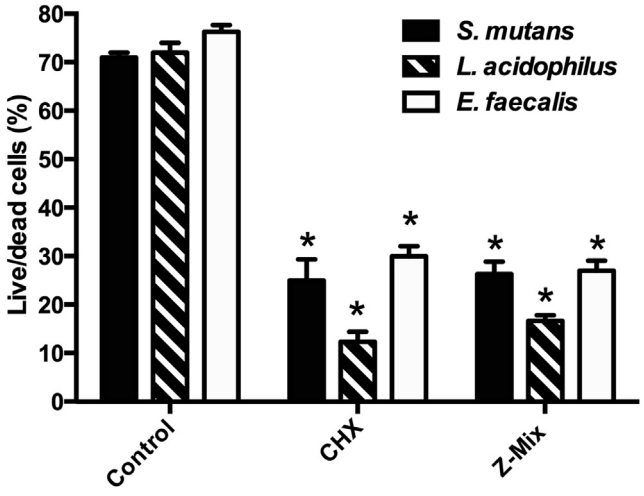

Figure 6 Effect of Z-Mix or CHX on the viability of inoculated bacteria in the dentin samples. The live/dead ratio (%) of *Streptococcus mutans*, *Lactobacillus acidophilus*, and *Enterococcus faecalis* recovered from dentin samples approximately 500 μm below the infected cavities after no treatment (control group) or treatment with Z-Mix or 0.2% CHX for 72 hours. Each value represents the mean ± standard error of the mean ($n = 3$). * $P < 0.05$ compared to their corresponding controls. The live/dead bacteria ratios of all tested bacteria were comparable in the Z-Mix and CHX groups.

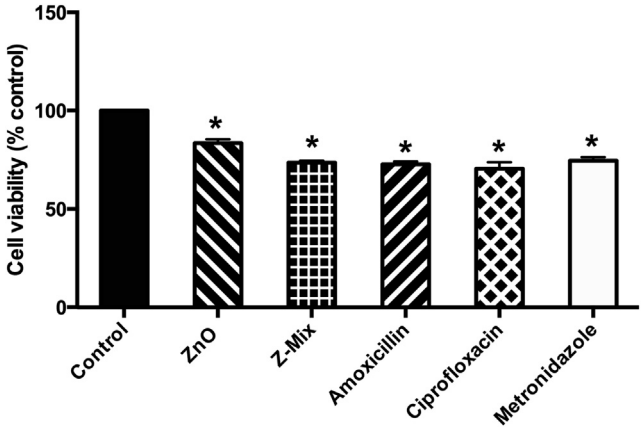

Figure 7 Viability (% of the control) of primary human dental pulp cells after treatment with the tested materials. Cells were treated with the ZnO base extract (12.5 mg/mL), the Z-Mix extract (12.5 mg/mL), amoxicillin (100 μg/mL), ciprofloxacin (100 μg/mL), or metronidazole (100 μg/mL) for 72 hours. Cytotoxicity was assessed by the MTT assay. Each value represents the mean ± standard error of the mean ($n = 3$). * $P < 0.05$ versus the control.

experiment, we used not only polyethylene glycol 4000 but also sodium lauryl sulfate as vehicles. Therefore, the effective penetration ability of Z-Mix is probably due to the presence of both compounds.

Previous reports demonstrated that at least 2–3 weeks were required for bacteria to invade the dentinal tubules.[21,22] Therefore, in this study, tooth samples were inoculated with tested bacteria for 30 days. We found that each bacterial species could penetrate into dentinal tubules at least 500 μm from the cavity floors. Our results indicated that amoxicillin, ciprofloxacin, and metronidazole still possessed their antimicrobial properties when incorporated into Z-Mix. Z-Mix exhibited remarkably bactericidal effects against *S. mutans*, *L. acidophilus*, and *E. faecalis*, which were involved in dentin and pulp infection.[1,2,6,7]

Our findings regarding the antimicrobial property of Z-Mix are in agreement with previous reports. Ciprofloxacin, metronidazole, and polyethylene glycol vehicles exhibited antimicrobial properties against 23 microbial strains including *S. mutans* and *E. faecalis*.[23] Metronidazole itself was not capable of eliminating any tested microorganisms. However, antimicrobial effects were found in a metronidazole and ciprofloxacin combination. A combination of these antibiotics was sufficient to disinfect infected root dentine *in situ*.[11] Our findings demonstrating that CHX could kill residual bacteria in dentinal tubules are in accordance with previous reports.[24–26] The antimicrobial properties of Z-Mix are comparable to that of 0.2% CHX. This means Z-Mix can be considered as a suitable antibiotic paste to eliminate bacteria involved in the deeper layers of carious lesions, pulpal infections, and periapical lesions.[27] Over 75% cell viability was observed for the Z-Mix medications, indicating slight cytotoxicity.[28] However, the level of cytotoxicity reported here indicates that Z-Mix can be regarded as a safe dental material and *in vitro* biocompatible.[29–31]

In conclusion, this study showed that Z-Mix could effectively penetrate from the cavity floor to the root apex *in vitro*. It exhibits an antimicrobial property against bacteria involved in dental caries and endodontic infection. Z-Mix has no cytotoxicity. Our findings suggest that Z-Mix might be used for the disinfection of dentinal tubules. In order to confirm this, additional studies should be carried out to further evaluate the efficacy of Z-Mix.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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