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Original Article

The ability of different diffusing enhancers to deliver chlorhexidine into dentinal tubules: An *in vitro* evaluation

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KEYWORDS
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Abstract *Background/purpose:* Root canal irrigants are difficult to diffuse deep into the dentinal tubules for root canal disinfection. The purpose of this study was to assess the ability of different diffusing enhancers to deliver chlorhexidine (CHX) into dentinal tubules.
Materials and methods: The diffusing property of five diffusing enhancers (acetone, DMSO, Triton X-100, JFC-E and azone) into dentinal tubules was firstly assessed using the Rhodamine B. The ability of the diffusing enhancers to deliver CHX into dentinal tubules was then evaluated both qualitatively and quantitatively. A deep dentinal tubule *Enterococcus faecalis* infection model was further established to test the bactericidal effect of CHX delivered by different diffusing enhancers. Finally, the *in vitro* cytotoxicity of these diffusing enhancers was tested using the CCK-8 method.
Results: Azone, Triton X-100 and JFC-E exhibited the largest maximum diffusing depths at all root canal parts ($P < 0.05$). Azone group also showed the highest percentage of diffusing depths for all root canal parts, followed by Triton X-100 ($P < 0.05$). The percentage of dead bacteria in dentinal tubules close to cementum layer was significantly higher in the CHX + azone group than in the other groups ($P < 0.05$), followed by CHX + Triton X-100 and CHX + JFC-E groups. The concentration of CHX diffusing onto the exterior surface of root was significantly higher in CHX + azone group than in the other groups ($P < 0.05$). All the diffusing enhancers showed relatively low cytotoxicity.
Conclusion: Azone showed the highest diffusing ability with low cytotoxicity and might be employed as a carrier agent for either intracanal irrigants or medications to achieve more thorough root canal disinfection.

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Introduction

The main purpose of root canal therapy is to thoroughly disinfect, hermetically seal the root canal system and prevent re-infection.[1,2] The anatomical abnormalities of root canal system such as the root canal isthmus, lateral root canals, apical bifurcation and apical delta create a number of physical obstacles for the thorough disinfection.[3–5] Present root canal instruments could not touch the whole root canal wall, always leaving about 10–30% untouched area.[6,7] Therefore, intra-canal irrigation and medication with antibacterial agents play a key role in the root canal disinfection. Sodium hypochlorite (NaOCl), ethylenediaminetetraacetate (EDTA) and chlorhexidine (CHX) are the most often-used irrigants in clinic, and their effects always rely on the direct contact with the tissue and bacteria.[8] However, the entrapment of air bubbles inside various anatomical abnormalities and dentinal tubules could hinder irrigants from diffusing into these areas.[9] Studies have confirmed that bacteria, such as *Enterococcus faecalis* (*E. faecalis*), could infiltrate and colonize into dentin tubules up to a depth of 500–1000 μm.[10] And mature *E. faecalis* biofilm consists of multiple layers of microorganisms in a self-produced extracellular polymeric substance (EPS) and utilizes various mechanisms to resist the action of antimicrobial agents.[11] These tenacious bacteria hiding in anatomical abnormalities and dentinal tubules, once conditions permit, will continue to proliferate and become a potential cause of canal re-infection and subsequent treatment failure. Even the CHX and NaOCl, often used as the "gold standard" for evaluating other antibacterial drugs in root canal disinfection, had been shown to be unable to eliminate the bacterial biofilm inside the deep dentin tubules.[12,13]

To solve the above problems, the assisted irrigation techniques such as sonic, ultrasonic or laser activated irrigating technology were applied to drive the irrigating solutions into complex root canal areas.[14] Despite this, these assisted irrigation methods have not yet achieved satisfactory intra-canal diffusion effects, especially in the apical root canal area.[15,16] Therefore, how to maximize the diffusing ability of irrigants inside the root canal system through an economic and easy way would be an important issue for promoting the disinfection effect of irrigants.

The addition of surfactants to irrigants to promote the diffusion ability of the irrigants has been one of endodontic research topics.[17] One research showed that under the same exposure time, the diffusion depth of NaOCl containing surfactants increased from 134 μm to 163 μm compared to the surfactant-free NaOCl at the same concentration.[18] As to the CHX, some studies ever evaluated the antibacterial effect of commercial CHX irrigant supplemented with an undisclosed surfactant ingredient, but they were limited to evaluating the diffusion of CHX into biofilm and did not assess its diffusing depth into dentinal tubules, let alone its antibacterial effect deep inside the dentinal tubules.[19] Therefore, more studies on the effective diffusing enhancers for CHX are still needed.

Diffusing enhancers are a kind of chemical materials applied to facilitate active ingredients to diffuse into tissues of human body. Water-soluble azone is a widely-used dermal diffusing enhancer since 1960s due to its colorless, tasteless, low toxicity as well as high-efficiency transdermal diffusing activity.[20] Acetone is a colorless and transparent liquid that has strong solvent capabilities. Research has shown that an effective solvent like acetone could enhance the diffusion of drugs.[21] Some studies have also suggested that dimethyl sulfoxide (DMSO) could help drugs diffuse into the skin rapidly with no cytotoxicity and genotoxicity.[22] Triton X-100, as a non-ionic detergent, is able to incorporate to liposomes of cell membranes, increasing the penetrability of membrane.[23] Aliphatic alcohol polyoxymethylene ether (JFC-E) is a new type of non-ionic surfactant that easily disperses or dissolves in water and possesses excellent wetting, emulsifying, and diffusing properties.[24] However, by now, the above diffusing enhancers have not yet been tested together on their diffusing ability inside the root canals, especially for delivering intra-canal irrigants.

Based on the above knowledge, acetone, DMSO, azone, Triton X-100 and JFC-E were used as diffusing enhancers of CHX in this study. Their surface tensions, the ability to diffuse and deliver CHX into dentinal tubules, antibacterial effect deep inside dentinal tubules as well as the *in vitro* cytotoxicity were then systematically investigated.

Materials and methods

The surface tension measurement

The surface tension of different diffusing enhancers was measured by the contact angle meter (DSA25, Krüss GmbH, Germany). Briefly, a drop of diffusing enhancer solution was suspended on a fine-diameter capillary tube and the rate of descent was measured to determine the surface tension of the solution. The groups were set as follows:

Group A: 1% acetone (Sinopharm, Shanghai, China) solution.

Group B: 1% DMSO (Sinopharm, Shanghai, China) solution.

Group C: 1% azone (Usolf, Qingdao, China) solution.

Group D: 1% Triton X-100 (Biofroxx, Einhausen, Germany) solution.

Group E: 1% JFC-E (Usolf, Qingdao, China) solution.

Group F: distill water (ddH_2O).

Dentinal tubule diffusing ability

The general procedure of this experiment was illustrated in Fig. 1.

Straight single-canaled human mandibular premolars with fully formed apices extracted for orthodontic reasons were collected under the approval of the Ethics Committee of School of Stomatology, Wuhan University. Teeth with more than one root canal, caries, root canal calcification, root resorption or cracks were excluded. Root canals of the teeth (n = 60) were instrumented using the Reciproc 25 (VDW GmbH, Munchen, Germany), and then the teeth were de-coronated and maintained 12 mm length. To ensure the smear layer removal, the canals were alternately irrigated by 2% NaOCl (Longly Biotech., Wuhan, China) and 17% EDTA (Longly Biotech., Wuhan, China) solutions during and after the canal instrumentation. Then the apical foramen was sealed with flowable resin (Shofu, Kyoto, Japan). All the samples were assigned into 6 groups randomly with 10 teeth in each group. The 20 μL mixture containing 1% diffusing enhancers (grouping as above) and 0.1% Rhodamine B (Aladdin, Shanghai, China) as the fluorescent marker was added into canals. After 5 min, the residual solutions were removed by normal saline irrigation. The root was cross-sectioned into three equal parts using low-speed precision cutter (Buehler, Chicago, IL, USA). The cross-sectional surface on buccal and lingual sides of each root part was observed under a confocal laser scanning microscope (CLSM, Leica, Mannheim, Germany) at 10 × magnification.[25] The excitation/emission wavelengths were 555/588 nm for Rhodamine B. The image J software (NIH, Bethesda, MD, USA) was used to measure and calculate the percentage of diffusing area and depth using the following formulas:

$$\text{Percentage of diffusing area}\ (\%) = \frac{\text{diffusing area}}{\text{total slice area} - \text{root canal area}} \times 100\%$$

$$\text{Percentage of diffusing depth}\ (\%) = \frac{\text{the maximum diffusing depth}}{\text{corresponding root canal wall thickness}} \times 100\%$$

CLSM evaluation on the antibacterial effect of CHX delivered into dentinal tubules

The general procedure of the experiments in this section was illustrated in Fig. 1.

Establishment of dentinal tubule deep infection model

Dentinal tubule deep infection model was established based on the published literature.[26] The teeth (n = 21) were prepared as above. And a 4 mm (length) middle-root segment was retrieved through cross sectioning, and the root surface of the specimens was polished using a 600-grit silicon carbide paper (Yingpai, Suzhou, China) to remove the cementum. Then each sample, after being cleaned by

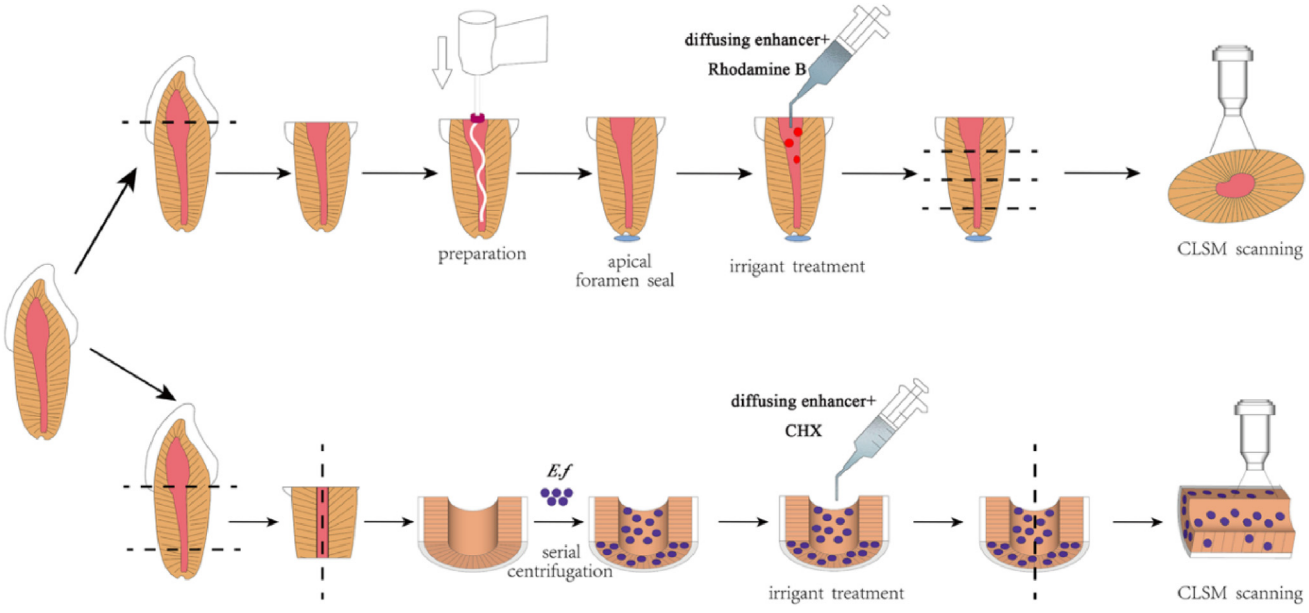

Figure 1 Illustration showing the experiment procedures on extracted teeth.

ultrasonic wash with 2% NaOCl and 6% citric acid (pH = 4.0, Aladdin, Shanghai, China), was sectioned into two halves (n = 42) in mesial-distal direction to obtain the thickness of 2 mm to fit a filter tube (Labselect, Hefei, China) with 0.45 μm pore size. Each semi-cylindrical dentin block was then placed in the filter tube with the canal wall facing up. The gaps between the specimen and the inner wall of the tube were filled with resin.

The tubes were centrifuged sequentially at 1400 ***g***, 2000 ***g***, 3600 ***g*** and 5600 ***g*** twice with each period being 5min. A fresh 500 μL E. *faecalis* (ATCC 29212, Manassas, VA, USA) suspension (3×10^6 CFU/mL) was added between the centrifugations. Subsequently, the tubes were incubated in fresh brain-heart infusion (BHI, Land bridge, Beijing, China) broth at 37 °C for 24 h. Then the dentin blocks were taken out, washed with phosphate buffer solution (PBS, Gibco, Waltham, MA, USA) for 1min and aired-dried. Finally, the cementum side of the dentin blocks was sealed with nail varnish.

CLSM evaluation

100 μL of mixture containing 1% diffusing enhancers (grouping as above) and 2% CHX (Yuanye, Shanghai, China) was added to the root canal surface. After 1min, the canals were rinsed with PBS for another 1 min. The dentin blocks were split longitudinally through the canal wall surface using a thin blade and hammer to expose fresh dentin surfaces for subsequent CLSM observation. Negative control group was defined as the dentin blocks being treated with PBS only.

The samples were stained with fluorescent LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The excitation/emission wavelengths were 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide. The biofilms were observed by CLSM at a 40× magnification. Image stacks containing 30 slices were acquired at 1 μm z-step from 5 randomly selected areas within 300 μm to the root surface. The Imaris software (Bitplane Inc, St Paul, MN, USA) was used for 3D reconstruction and volume calculation.

Detection of CHX delivered onto the root surface by diffusing enhancers

The teeth (n = 42) were prepared as above. 100 μL mixture containing 1% diffusing enhancers (grouping as above) and 20% CHX was added into the canals before the coronal opening was sealed tightly with resin. And one tooth in each group was randomly selected and kept in an upright position for 24 h. The dentin powder on the exterior surface of the root was scraped with a blade, then the CHX in the dentin powder was detected by Energy Dispersive Spectrometer (EDS, Oxford UltimMax 65, Oxford, UK). The left teeth(n = 36) were soaked in 1 mL ddH_2O at 37 °C on a shaker. After 24 h, the concentration of CHX diffused into the ddH_2O was analyzed through measuring the absorbance at 255 nm using a spectrometer (UV-2401PC, Shimadzu Corp., Kyoto, Japan).

Cytotoxicity

The primary human dental pulp cells (hDPCs) and periodontal ligament cells (hPDLCs) were obtained from freshly extracted wisdom teeth according to the approval of the Ethics Committee of School of Stomatology of Wuhan University, and the fourth passage was used for this test. Firstly, 200 μL of hDPCs and hPDLCs (5×10^4 cells/mL) were seeded into each well of a 96-well plate at 37 °C respectively, and each group included 6 repeated wells. After 24h, the culture medium was replaced with fresh α-minimum essential media (αMEM, Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific, USA), 1% penicillin-streptomycin (Thermo Fisher Scientific, USA) and different concentrations (0.001% and 0.002%, determined by the result of previous section) of diffusing enhancers. The cells cultured without diffusing enhancers were used as control. After 12h, the cells were washed by PBS. The agent of cell counting kit-8 (CCK-8, Biosharp, Hefei, China) was added into each well plate, and then the optical density at 450 nm (OD_{450}) was measured by a microplate reader (Power Wave XS2, BioTek Instruments, VT, USA). The relative proliferation rate of different diffusing groups was calculated as OD_{450} of diffusing enhancer groups/OD_{450} of control group × 100%.

Statistical analysis

The normal distribution and homoscedasticity of data were examined by the Shapiro-wilk and the modified Levene test. The data obeying the normal distribution and homoscedasticity were analyzed by one-way analysis of variance (ANOVA) and post-hoc Tukey multiple comparison tests. Otherwise, the Mann-Whitney U nonparametric and Dunn's multiple comparison tests were used. The analysis was performed using the SPSS version 27.0 software (SPSS Inc., Chicago, IL, USA). Significant difference was considered at $P < 0.05$.

Results

The surface tension of diffusing enhancers

The results showed that at the same concentration, the surface tensions of acetone (71.41 ± 0.07 mN/m), DMSO (71.86 ± 0.14), and ddH_2O (72.69 ± 0.04 mN/m) were similar to each other, but more than twice of the tension of azone (29.62 ± 0.02 mN/m), Triton X-100 (31.70 ± 0.01 mN/m) and JFC-E (26.64 ± 0.06 mN/m).

Dentinal tubule diffusing ability

As shown in Table 1, the diffusing ability of different enhancers was all found the highest at the coronal root canal part, followed by the middle part, and the lowest at the apical part. In terms of the percentage of diffusing area, azone, Triton X-100 and JFC-E groups showed the highest percentage ($P < 0.05$) at coronal part. At the middle part, azone group exhibited the highest percentage of diffusing area, followed by Triton X-100 and JFC-E ($P < 0.05$), and at

Table 1 Dentinal tubule diffusing ability of different diffusing enhancers.

Group	Percentage of diffusing area (%)			Maximum diffusing depth (μm)			Percentage of diffusing depth (%)		
	coronal	middle	apical	coronal	middle	apical	coronal	middle	apical
acetone	41.39 ± 3.13^{b}	28.84 ± 2.79^{b}	22.49 ± 1.87^{c}	1193.328 ± 246.709^{a}	1020.770 ± 107.451^{a}	747.428 ± 84.434^{b}	72.56 ± 3.82^{b}	48.39 ± 2.94^{b}	40.99 ± 2.56^{b}
DMSO	38.82 ± 3.82^{a}	25.18 ± 2.46^{a}	20.93 ± 1.76^{b}	1305.451 ± 233.681^{b}	998.115 ± 119.359^{a}	787.355 ± 87.398^{b}	58.97 ± 2.78^{a}	47.74 ± 3.99^{b}	40.40 ± 1.68^{b}
azone	71.75 ± 3.43^{c}	60.97 ± 2.69^{e}	40.69 ± 3.26^{e}	1744.961 ± 216.075^{c}	1410.721 ± 131.364^{b}	1187.192 ± 78.776^{d}	91.79 ± 3.55^{d}	82.39 ± 3.45^{e}	69.05 ± 2.78^{d}
Triton X-100	63.72 ± 2.84^{c}	58.21 ± 2.06^{d}	39.72 ± 2.28^{e}	1779.799 ± 196.913^{c}	1427.017 ± 116.372^{b}	1261.307 ± 106.242^{e}	85.72 ± 3.46^{c}	78.74 ± 2.12^{d}	59.03 ± 2.66^{c}
JFC-E	64.89 ± 3.75^{c}	48.15 ± 2.32^{c}	38.84 ± 2.32^{d}	1646.414 ± 181.400^{c}	1384.284 ± 91.521^{b}	1023.225 ± 76.961^{c}	84.42 ± 2.92^{c}	68.08 ± 2.97^{c}	58.27 ± 2.87^{c}
ddH_2O	40.84 ± 2.33^{b}	25.44 ± 2.73^{a}	15.97 ± 2.42^{a}	1171.743 ± 324.657^{a}	1028.656 ± 138.948^{a}	608.212 ± 61.810^{a}	60.83 ± 3.33^{a}	45.45 ± 2.63^{a}	37.10 ± 3.80^{a}

Values of diffusing area and depth variables were represented as the mean ± SD.
Different letters in the same column indicating significant differences between groups as determined, $P < 0.05$.

the apical part, azone and Triton X-100 groups showed the highest percentage ($P < 0.05$). Regarding the maximum diffusing depths, azone, Triton X-100 and JFC-E exhibited the largest diffusing depths at all root canal parts ($P < 0.05$). As for the percentage of diffusing depths, the highest percentage was also found in azone group for all root canal parts ($P < 0.05$). Fig. 2 showed the representative CLSM image of diffusing area for each diffusing enhancer group.

CLSM evaluation of CHX against *E.faecalis* infection inside the dentinal tubules

Results of the CLSM evaluation showed the successful establishment of dentinal tubule deep infection model (Fig. 3G). Live *E.faecalis* (green) was found dominant inside the dentinal tubules in the acetone, DMSO and ddH_2O groups (Fig. 3A, B, F). However, the dead *E.faecalis* (red) was found dominant inside the dentinal tubules in the azone, Triton X-100 and JFC-E groups (Fig. 3C, D, E). In the analysis of the dead bacteria ratio in Fig. 3H, the percentage of dead bacteria in the azone group was significantly higher than other groups ($P < 0.05$), followed by Triton X-100 and JFC-E groups, and there was no statistically difference in the acetone, DMSO and ddH_2O groups ($P > 0.05$).

Concentration of CHX on root surface delivered by diffusing enhancers

The EDS results showed that the chlorine was detected in the dentin powders obtained from the azone, Triton X-100 and JFC-E groups, as illustrated in Fig. 4C—E. The CHX diffused into ddH_2O from root canal of azone group showed the highest concentration between 0.0015% and 0.002% (Fig. 4G), and the diffusing ratio (CHX concentration inside the root canal: CHX concentration outside the root canal) of the azone group was about 10000:1, which was also significantly higher than other groups ($P < 0.05$).

Cytotoxicity

Results of the CCK-8 tests (Fig. 4H) indicated that the relative proliferation rate in different diffusing enhancer groups remained 80%, and showed no significant difference ($P > 0.05$). However, as the concentration increased, a decrease trend in the relative proliferation rate could be observed ($P < 0.05$).

Discussion

Remaining infectious bacteria inside the dentinal tubules are thought to be a potential cause of treatment failure as these bacteria could incur the re-infection of root canal and periapical tissues when conditions become favorite.[27] Extending the bactericidal activity of intracanal irrigants and medications into the dentin tubules and irregular areas is essential for the long-term success of root canal treatment.[3—5,28] However, the diffusing ability of current intra-canal irrigants and medications is found quite limited.

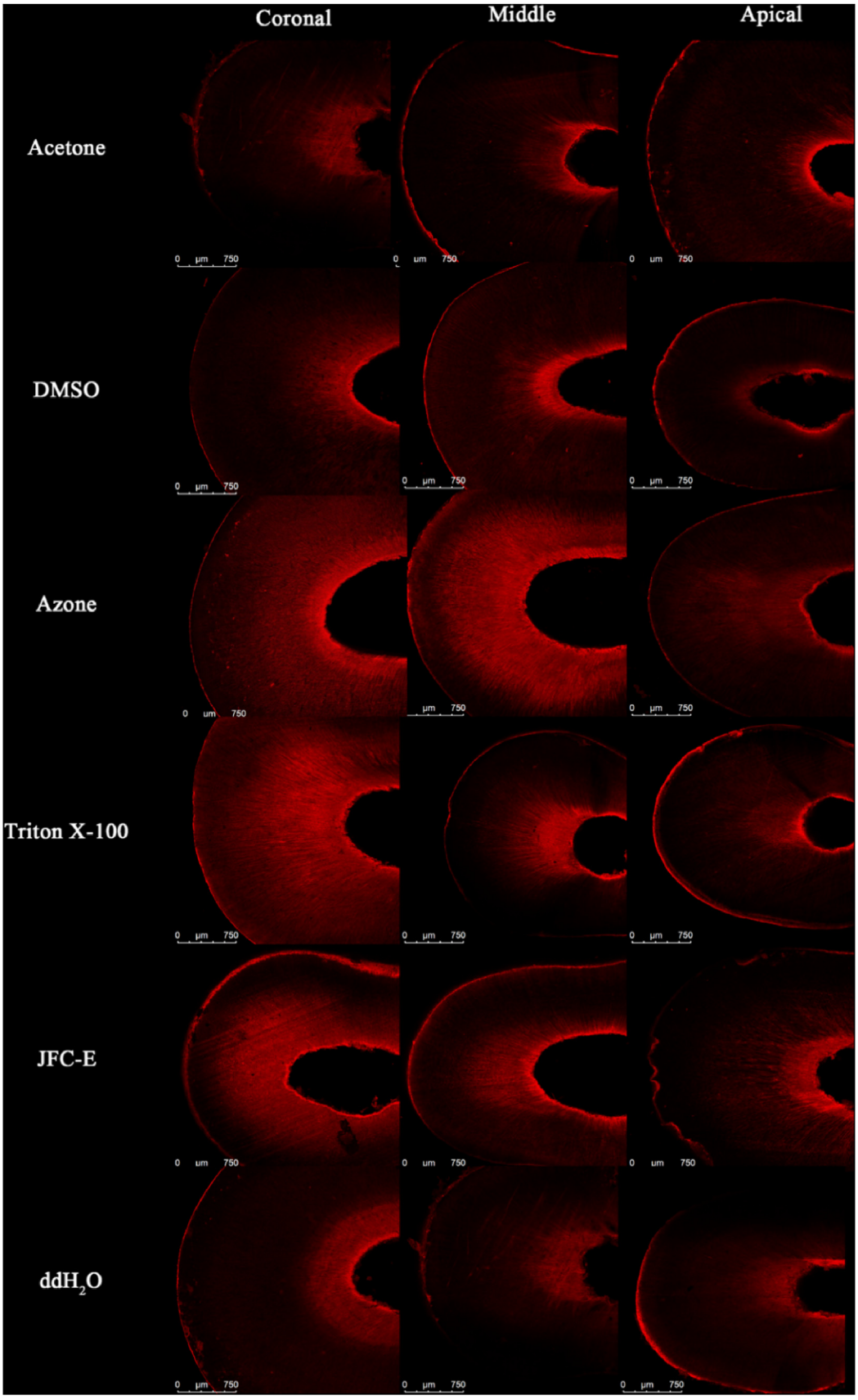

Figure 2 Representative CLSM images of different diffusing enhancer groups showing the Rhodamine B diffusing into the dentinal tubules in coronal, middle, and apical root canal parts.

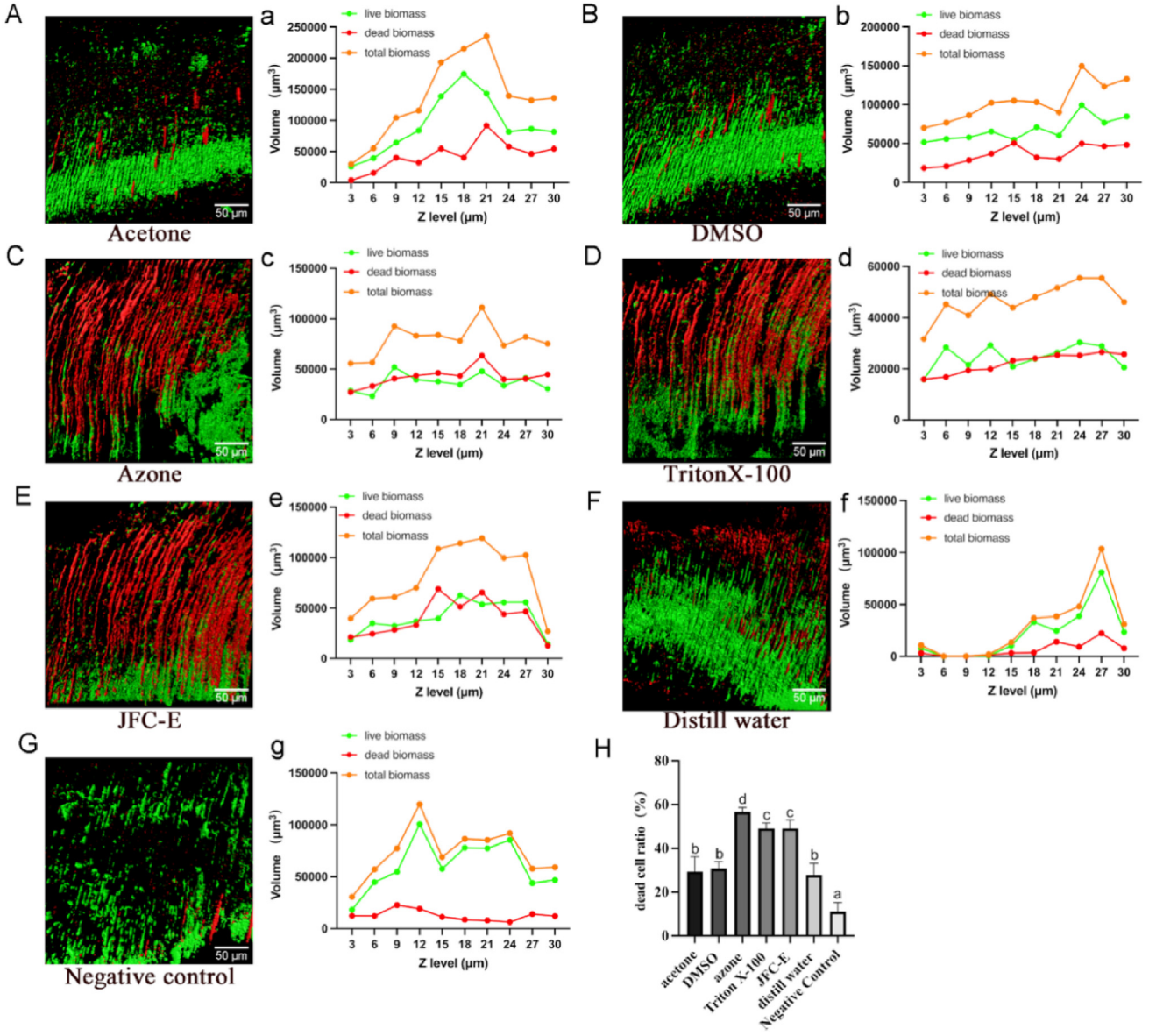

Figure 3 CLSM evaluation of CHX against *E.faecalis* deep infection inside the dentinal tubules among different diffusing enhancer groups. (A-G, a-g) Representative 3D CLSM images and corresponding biomass distribution of total, live and dead bacteria of different groups (green-live bacteria; red-dead bacteria); (H) Dead cell ratio of different groups. (Different letters indicating significant differences between groups as determined, $P < 0.05$.)

NaOCl could reach from 38.8 to 411 μm depth in dentinal tubules,[29] and the mean diffusing depth of CHX was reported to be 138 μm, 80 μm, and 44 μm in the coronal, middle, and apical canal third,[30] which severely compromises the antibacterial effects inside the root canal system.[31,32] Some studies ever added surfactants such as Triton X-100, cetrimide and polypropylene glycol into the NaOCl solution, but the diffusing depth was still found limited within 200 μm into the dentinal tubules.[18,33] In this study, however, the azone, JFC-E and Triton X-100 increased the diffusing depth into the dentinal tubules up to more than 1100 μm. The disparity in results may be due to the differences in research methodologies. This study employed Rhodamine B as a fluorescent marker to indicate diffusion depth, while previous studies utilized the oxidizing properties of NaOCl to bleach the dye inside dentinal tubules, thereby determining the diffusion depth of NaOCl. However, the oxidizing ability of NaOCl is correlated with its concentration. NaOCl diffusing into deep area of dentinal tubules may exhibit reduced concentration and bleaching effect, probably resulting in an underestimation of the diffusing depth.

In this study, two new diffusing enhancers azone and JFC-E were tested along with aceton, DMSO and Triton X-100, which are also often used as diffusing enhancers.[20] The result showed that azone, Triton X-100 and JFC-E significantly facilitated the diffusion of fluorescence in all root canal parts. Even in the apical part, azone showed significantly enhanced diffusing area and depth. Although the pro-diffusing mechanisms of diffusing enhancers are still

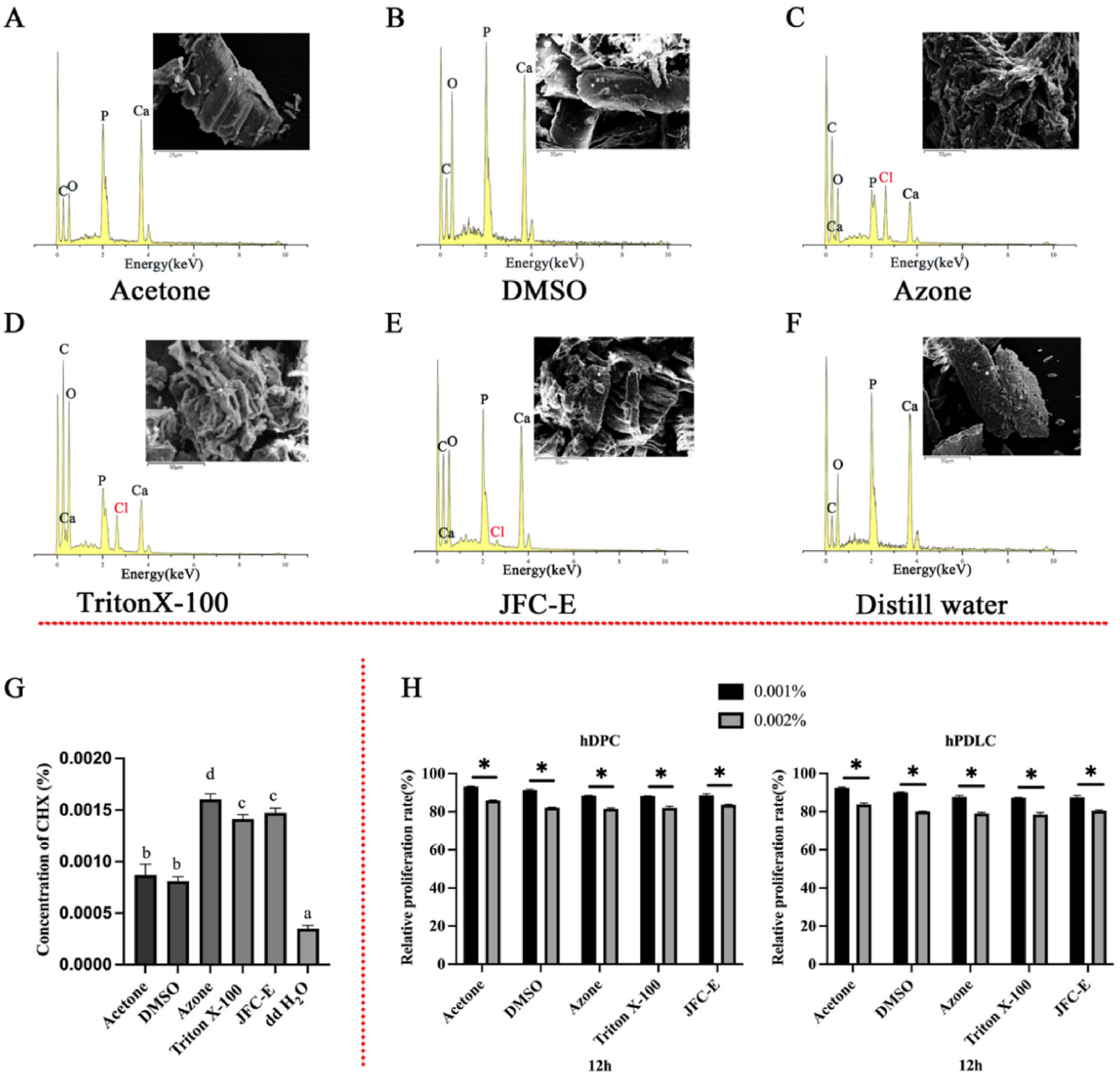

Figure 4 Ability of the diffusing enhancer to deliver CHX and cytotoxicity of the diffusing enhancers. (A-F) EDS detection of CHX in dentin powders from different groups; (G) The concentration of CHX diffused into ddH_2O of different groups (Different letters indicating significant differences between groups as determined, $P < 0.05$); (H) CCK-8 test on hDPCs and hPDLCs for 12 h (*indicating the significant difference, $P < 0.05$).

not fully clarified yet, the low surface tension of the enhancers could be a key factor to increase the diffusing ability of the added chemicals.[34]

Furthermore, the percentage of dead bacteria in dentinal tubules close to cementum layer was also significantly higher in the CHX + azone group, followed by CHX + Triton X-100 and CHX + JFC-E. Previous studies only evaluated the number of dead bacteria in the field close to the root canal surface,[35] which cannot show the antibacterial effect throughout the whole layer of dentin. To further clarify the antibacterial effect of the CHX delivered by diffusing enhancers throughout the whole layer of dentin, the dentin field within 300 μm to the cementum was selected for observation. The results confirmed that the CHX delivered by diffusing enhancers, especially by azone, was able to diffuse into the deep area of dentinal tubules and exert bactericidal effect.

Azone is able to solubilize skin lipids or denature skin proteins by acting with collagen proteins, thereby increasing the solubility and diffusion of drugs for transdermal applications.[36,37] Collagen I fibers are the major organic component of dentin. Therefore, it is speculated that azone plays similar roles on the dentin collagen fibers as in skin to promote the diffusing. On the other hand, azone could decrease the

resistance of the stratum corneum against diffusion through extracting skin lipids. Similarly, as dentin also contains 0.36% lipids, azone could possibly also increase the diffusing effect through this mechanism.[37,38]

The extra-radicular CHX detection experiments showed that the CHX could be delivered to the root surface by the azone, JFC-E and Triton X-100. Extra-radicular biofilm on the apical root surface was normally an extension of intra-canal biofilm and could inhibit the healing of apical and periapical tissues. It was difficult to be eliminated through the root canal treatment unless the apical surgery was performed.[39,40] The enhanced diffusing depth of CHX could probably facilitate the infection control either inside the canal or on the root surface.

To quantitatively analyze the amount of disinfectant diffusing onto the surface of the root, a high concentration of CHX (20%) was injected into the root canal. The results showed that with the assistance of the diffusing enhancer, the ratio of CHX concentration inside and outside the root canal was close to 10000:1, and the amount of CHX diffused onto the root surface showed very low cytotoxicity. This result might mean that the CHX diffusing to the surface of root may help inhibit the biofilm on the root surface while with the low cytotoxicity. Despite this, the in vivo diffusing ability of azone and other diffusing enhancers need to be further investigated.

Compared to ddH_2O and other diffusing enhancers, azone showed the highest diffusing ability and could significantly promote the diffusion and antibacterial effect of CHX solution inside the dentinal tubules at all root canal parts. Azone might be used as an effective diffusing enhancer for intra-canal irrigants or medications in the root canal disinfection.

Ethical approval

The study was approved by the Ethics Committee of School and Hospital of Stomatology, Wuhan University (2024LUNSHENZIB07).

Declaration of competing interest

The authors declare that they have no conflict of interest relevant to this article.

Acknowledgements

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