

RESEARCH

Open Access



Synergistic mechanism of $\text{Ag}^+ - \text{Zn}^{2+}$ in anti-bacterial activity against *Enterococcus faecalis* and its application against dentin infection

Wei Fan^{1†}, Qing Sun^{1†}, Yanyun Li¹, Franklin R. Tay² and Bing Fan^{1*}

Abstract

Background: Ag^+ and Zn^{2+} have already been used in combinations to obtain both enhanced antibacterial effect and low cytotoxicity. Despite this, it is still unclear how the Zn^{2+} co-works with Ag^+ in the synergistic antibacterial activity. The main purposes of this study were to investigate the co-work pattern and optimum ratio between Ag^+ and Zn^{2+} in their synergistic antibacterial activity against *E. faecalis*, the possible mechanisms behind this synergy and the primary application of optimum $\text{Ag}^+ - \text{Zn}^{2+}$ co-work pattern against the *E. faecalis* biofilm on dentin. A serial of $\text{Ag}^+ - \text{Zn}^{2+}$ atomic combination ratios were tested on both planktonic and biofilm-resident *E. faecalis* on dentin, their antibacterial efficiency was calculated and optimum ratio determined. And the cytotoxicity of various $\text{Ag}^+ - \text{Zn}^{2+}$ atomic ratios was tested on MC3T3-E1 Cells. The role of Zn^{2+} in $\text{Ag}^+ - \text{Zn}^{2+}$ co-work was evaluated using a Zn^{2+} pre-treatment study and membrane potential—permeability measurement.

Results: The results showed that the synergistically promoted antibacterial effect of $\text{Ag}^+ - \text{Zn}^{2+}$ combinations was Zn^{2+} amount-dependent with the 1:9 and 1:12 $\text{Ag}^+ - \text{Zn}^{2+}$ atomic ratios showing the most powerful ability against both planktonic and biofilm-resident *E. faecalis*. This co-work could likely be attributed to the depolarization of *E. faecalis* cell membrane by the addition of Zn^{2+} . The cytotoxicity of the $\text{Ag}^+ - \text{Zn}^{2+}$ atomic ratios of 1:9 and 1:12 was much lower than 2% chlorhexidine.

Conclusions: The $\text{Ag}^+ - \text{Zn}^{2+}$ atomic ratios of 1:9 and 1:12 demonstrated similar strong ability against *E. faecalis* biofilm on dentin but much lower cytotoxicity than 2% chlorhexidine. New medications containing optimum $\text{Ag}^+ - \text{Zn}^{2+}$ atomic ratios higher than 1:6, such as 1:9 or 1:12, could be developed against *E. faecalis* infection in root canals of teeth or any other parts of human body.

Keywords: Silver, Zinc, Ion, Antibacterial, Biofilm, Dentin, *E. faecalis*

Background

Infectious diseases pose a major threat upon the health and well being of humans [1, 2]. The discovery and development of antibiotics in the last century has dramatically

changed the outcome of treatment for infectious diseases. However, the development of bacterial resistance against even the most powerful antibiotics is rapidly becoming a major challenge for the application of antibiotics in combating infectious diseases [3–5]. Because of this concern, stringent standards for the prescription of antibiotics have been established to minimize the development of bacteria resistance, particularly in the hospital environment [5, 6].

Antibacterial metal ions, such as silver ion (Ag^+) and zinc ion (Zn^{2+}), possess broad-spectrum antibacterial

*Correspondence: bingfan@whu.edu.cn

[†]Wei Fan and Qing Sun contributed equally to this work

¹The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) and Key Laboratory of Oral Biomedicine, Ministry of Education, School and Hospital of Stomatology, Wuhan University, Wuhan, People's Republic of China

Full list of author information is available at the end of the article

activities with no extensive development of bacterial resistance [7]. The silver ion has the most potent bactericidal activity among metal ions and has been used to treat bacterial infections for centuries [8–10]. However, the high cytotoxicity and discoloring potential of Ag^+ severely restricts its broad application in clinical medicine [11, 12]. To overcome this shortcoming, many researchers attempted to use Ag^+ with other metal ions, in most cases the Zn^{2+} , to achieve strong synergistic antibacterial effect and reduction in cytotoxicity [13, 14]. This is because Zn^{2+} is less toxic but also less bactericidal when compared with Ag^+ [10, 15]. This effort of synergistic use of Ag^+ and Zn^{2+} for infection control has been proven effective and efficient in biomedical, medical and dental studies [14, 16, 17]. Nevertheless, the mechanism by which Ag^+ and Zn^{2+} interacts synergistically with each other is not clearly understood. Moreover, the optimum ratio of Ag^+ and Zn^{2+} to achieve maximal synergistic antibacterial effect has not been established.

Enterococcus faecalis (*E. faecalis*) is a gram-positive facultative anaerobe that can cause refractory infections in the digestive system, urinary tract as well as root canals of human teeth [18–20]. Detection of *E. faecalis* in persistent infection of the root canals and around the root apex of teeth ranged from 24 to 77% [20], and is believed to be a major cause for the failure of root canal treatment [20, 21]. *Enterococcus faecalis* develops strong resistance to extremely harsh environments including highly-alkaline pH, nutritional deficiency and many current clinical intra-canal medications, such as calcium hydroxide [22]. This resistance has made the control of *E. faecalis* infection a great challenge for dentists worldwide.

Silver ion and chlorhexidine have been reported to be very effective in the control of *E. faecalis* infection in root canals [23, 24]. However, Ag^+ possesses high cytotoxicity and discoloring properties when it is in direct contact with the dentin of root canal walls and tissues surrounding the root apex [25]. Chlorhexidine is also cytotoxic to the apical tissues and produces toxic chemicals when it is used with sodium hypochlorite, a commonly used irrigant for canal debridement in root canal therapy [26, 27]. Chemicals containing Zn^{2+} have also been studied as an intracanal medication against *E. faecalis*, and were found to be more biocompatible but less effective in infection control when compared with medications containing Ag^+ [15, 26]. Studies on the combined use of Ag^+ and Zn^{2+} reported an improved antibacterial effect in both intracanal and dental implant surface infection control [28, 29]. Nevertheless, the mechanism and optimum ratio of Ag^+ and Zn^{2+} in their synergistic antibacterial activity are still elusive, especially in dealing with *E. faecalis* infection.

Membrane potential is a key feature of bacteria for maintaining membrane permeability, intracellular ionic balance, organelle function and quorum sensing, which are essential for the survival of bacterial communities [30–32]. Alteration of the bacteria membrane potential (i.e. hyper-polarization or de-polarization) is an important bactericidal mechanism for many antibacterial medications, including antibiotics [33]. Ion channels on the cell membrane regulate the trans-membrane potential, and the ionic strength in the extracellular matrix affects the activity of ion channels, resulting in the alteration of membrane potential [34]. It is not known whether alteration of membrane potential occurs in synergistic antibacterial activities involving Ag^+ – Zn^{2+} . Accordingly, the objective of the present study was to investigate the optimum ratio of Ag^+ and Zn^{2+} against *E. faecalis* biofilms on dentin and the potential mechanism behind this synergy. Silver ions, Zn^{2+} and chlorhexidine were used independently as controls in the study.

Methods

Colony-forming unit counting

The antimicrobial activity of Ag^+ , Zn^{2+} or Ag^+ – Zn^{2+} at different atomic ratios was first examined using a colony-forming unit (CFU) counting method. Briefly, for observation of the antibacterial effect of Ag^+ or Zn^{2+} , a 1 mL suspension [1×10^3 CFUs/mL] of *E. faecalis* (ATCC 29212, ATCC, Manassas, VA, USA) in a double-concentrated BHI broth was incubated with 1 mL either 1.6×10^{-2} , 3.2×10^{-2} , 6.4×10^{-2} , 12.8×10^{-2} , 25.6×10^{-2} , 51.2×10^{-2} or 102.4×10^{-2} mg/mL of silver nitrate solution (AgNO_3) (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) or 5.6×10^{-2} , 16.8×10^{-2} , 33.6×10^{-2} , 50.4×10^{-2} or 67.2×10^{-2} mg/mL zinc nitrate hexahydrate solution ($\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) (Sinopharm). For observation of the combined antibacterial activity of Ag^+ and Zn^{2+} , mixed solutions containing 3.2×10^{-2} mg/mL AgNO_3 and 5.6×10^{-2} , 16.8×10^{-2} , 33.6×10^{-2} , 50.4×10^{-2} or 67.2×10^{-2} mg/mL $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ were prepared, corresponding to the Ag^+ – Zn^{2+} atomic ratios of 1:1, 1:3, 1:6, 1:9 and 1:12. A 1 mL suspension [1×10^3 CFUs/mL] of *E. faecalis* in a double-concentrated BHI broth was incubated with 1 mL of the aforementioned mixed solutions, respectively. Solutions with only 3.2×10^{-2} mg/mL AgNO_3 or 67.2×10^{-2} mg/mL $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ were used as controls. All solutions containing Ag^+ were prepared, stored and operated in the absence of light.

After incubating at 4 °C for 24 h, 10 μL of the inoculum from each group was plated on brain heart infusion (BHI; Land Bridge Technology Co. Ltd., Beijing, China) broth agar (Biosharp, Hirono, Japan) and incubated

anaerobically at 37 °C for 24 h. Bacteria inoculum mixed with only sterilized deionized water was included as blank control group for each test. All tests were performed in sextuplicate for each group and CFUs of *E. faecalis* were counted for group comparisons.

Dynamic growth-curves

A dynamic growth-curve method was used to observe the dynamic antimicrobial effect of Ag⁺, Zn²⁺ or Ag⁺-Zn²⁺ solutions. Briefly, for Ag⁺, a 4 mL suspension [1×10^6 or 1×10^8 CFUs/mL] of *E. faecalis* was incubated with 4 mL of 1.6×10^{-2} , 3.2×10^{-2} , 6.4×10^{-2} , 12.8×10^{-2} , 25.6×10^{-2} , 51.2×10^{-2} or 102.4×10^{-2} mg/mL AgNO₃ solution at 37 °C. For Zn²⁺, a 4 mL suspension [1×10^3 CFUs/mL] of *E. faecalis* was incubated with 4 mL of 5.6×10^{-2} , 16.8×10^{-2} , 33.6×10^{-2} , 50.4×10^{-2} or 67.2×10^{-2} mg/mL Zn(NO₃)₂·6H₂O solutions at 37 °C. For Ag⁺-Zn²⁺, mixed solutions containing the same amount of AgNO₃ and Zn(NO₃)₂·6H₂O as described in CFU counting method were prepared. Then, 4 mL suspension [1×10^6 CFUs/mL] of *E. faecalis* was incubated with 4 mL of the mixed solutions and 67.2×10^{-2} mg/mL of Zn(NO₃)₂·6H₂O at 37 °C, respectively. In addition, a similar mixed solution with doubled concentration of Ag⁺ and Zn²⁺ but the same Ag⁺-Zn²⁺ atomic ratio was prepared. Another 4 mL suspension [1×10^8 CFUs/mL] of *E. faecalis* was incubated with 4 mL of this concentrated Ag⁺-Zn²⁺ mixed solution and 134.4×10^{-2} mg/mL of Zn(NO₃)₂·6H₂O at 37 °C, respectively. As in the CFU counting method, bacterial suspension was all prepared in a double-concentrated BHI broth before mixing with various AgNO₃ and/or Zn(NO₃)₂·6H₂O solutions.

At 1, 2, 3, 4 and 5 h (2, 4, 6, 8 and 10 h for the Zn²⁺ only group using 1×10^3 CFUs/mL *E. faecalis*) after incubation, 1 mL of the suspension was retrieved for optical density (OD) measurement at 600 nm using a spectrometer (UV-2401PC, Shimadzu Corp., Japan). Bacteria inoculum mixed with sterilized deionized water was used as the blank control group. For each group, the test was repeated six times. All solutions containing Ag⁺ were prepared, stored and operated in the absence of light.

Determination of antibacterial efficiency of synergistic Ag⁺-Zn²⁺ against Ag⁺ only

The antibacterial efficiency of Ag⁺-Zn²⁺ synergy was determined by calculating the bactericidal percentage against same amount of Ag⁺ only, using the information derived from the aforementioned CFU counting and growth-curve tests. The bactericidal percentages against Ag⁺ were calculated for each test using the following equation:

Antibacterial efficiency (%)

$$= \left\{ 1 + \left(1 - \left[\frac{\text{CFUs or ODs (Ag + Zn)}}{\text{CFUs or ODs (Ag only)}} \right] \right) \right\} \times 100\%$$

Minimum inhibitory concentration (MIC) and bactericidal concentration (MBC)

Minimum inhibitory concentration and MBC of Ag⁺, Zn²⁺ or Ag⁺-Zn²⁺ at different atomic ratios were determined by a serial microdilution assay. Briefly, for Ag⁺, 25.6×10^{-2} mg/mL AgNO₃ solution was prepared. For Zn²⁺, 537.6×10^{-2} mg/mL Zn(NO₃)₂·6H₂O solution was prepared. For Ag⁺-Zn²⁺, Ag⁺-Zn²⁺ solutions (25.6×10^{-2} mg/mL AgNO₃) at different atomic ratios (1:1, 1:3, 1:6, 1:9 or 1:12) were prepared. From these starting solutions, a series of twofold dilutions were made by adding 50 μL suspension [1×10^5 CFUs/mL] of *E. faecalis* in double-concentrated BHI broth into each well of a 96-well plate with 50 μL Ag⁺, Zn²⁺ or Ag⁺-Zn²⁺ solutions at 37 °C for 24 h anaerobically. An optical density measurement was conducted by a micro-plate reader at 600 nm. Wells with the lowest Ag⁺, Zn²⁺ or Ag⁺-Zn²⁺ concentrations showing OD values most close to that of negative controls were determined to have MIC. MBC was determined by inoculating solutions from wells with Ag⁺, Zn²⁺ or Ag⁺-Zn²⁺ concentrations no less than MIC onto BHI agar plates and calculating the number of viable bacteria to be less than 4% of original bacteria. The test was repeated for three times.

Cytotoxicity of Ag⁺-Zn²⁺ combinations

To test the cytotoxicity of Ag⁺, Zn²⁺ or Ag⁺-Zn²⁺ combined antibacterial solutions, the cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) was used on MC3T3-E1 Cells (ATCC) according to manufacturer's instructions. Briefly, 5×10^3 cells suspension in 100 μL α-MEM (Thermo Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Thermo Scientific) were seeded in each well of a 96-well plate. After incubating at 37 °C with 5% CO₂ for 24 h, the medium was replaced with 100 μL of fresh α-MEM. For testing of Ag⁺ solutions, a 10 μL of 0.8×10^{-2} , 1.6×10^{-2} , 3.2×10^{-2} , 6.4×10^{-2} , 12.8×10^{-2} , 25.6×10^{-2} or 51.2×10^{-2} mg/mL AgNO₃ solutions was added respectively. Six wells were employed for each group. For Zn²⁺ solutions, 3×10^3 cells suspension in 100 μL of α-MEM supplemented with 10% FBS and 1% penicillin/streptomycin were seeded into each well of a 96-well plate. After the incubation at 37 °C with 5% CO₂ for 24 h, the medium was replaced with 100 μL of fresh α-MEM. Then, 10 μL

of 5.6×10^{-2} , 16.8×10^{-2} , 33.6×10^{-2} , 50.4×10^{-2} or 67.2×10^{-2} mg/mL $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ solutions was added to each well, respectively. The experiments were conducted in sextuplicate.

For $\text{Ag}^+ - \text{Zn}^{2+}$, 5×10^3 cells suspension in 100 μL α -MEM supplemented with 10% FBS and 1% penicillin/streptomycin were seeded into each well of a 96-well plate. The procedures described for Ag^+ or Zn^{2+} were subsequently followed. Then, a 10 μL aliquot of $\text{Ag}^+ - \text{Zn}^{2+}$ solution (3.2×10^{-2} mg/mL AgNO_3) of different atomic ratios (1:1, 1:3, 1:6, 1:9 or 1:12) was added. Cells exposed to only 3.2×10^{-2} mg/mL AgNO_3 or 67.2×10^{-2} mg/mL $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ were used as controls. Twelve wells were used for each group.

The medium in each well was removed after 24 h. The cells were washed three times with α -MEM and cultured with 100 μL of α -MEM and 10 μL of CCK-8 solution at 37 °C for 4 h. The absorbance at 450 nm was measured using a micro-plate reader (Power Wave XS2, BioTek Instruments, VT, USA). Wells containing only α -MEM were used for background recording. Cells cultured in medium mixed with 10 μL of sterilized deionized water or 2% chlorhexidine were used as control groups.

Inhibition of *E. faecalis* biofilm formation on dentin by $\text{Ag}^+ - \text{Zn}^{2+}$

Dentin slices (4 mm wide \times 4 mm long \times 1 mm thick) were prepared from human extracted wisdom teeth under a protocol approved by the Ethics Committee of School and Hospital of Stomatology, Wuhan University. All dentin slices were cleaned using an ultrasonic bath in deionized water, 5.25% sodium hypochlorite and 17% ethylenediaminetetraacetic acid successively and finally in deionized water. The cleanliness of the dentin surfaces was checked by a field emission scanning electron microscope (FE-SEM; Ultraplus; Zeiss, Oberkochen, Germany). The dentin slices were autoclaved for 20 min at 121 °C in deionized water and incubated anaerobically in BHI broth for 24 h at 37 °C to ensure no bacterial contamination. Sterilized dentin slices were soaked into 3 mL of *E. faecalis* suspension [1×10^8 CFUs/mL] and incubated under anaerobic condition at 37 °C for 4 weeks. Fresh BHI broth was replaced every second day to remove dead cells and to ensure bacterial viability. After incubation, the slices were rinsed with sterile phosphate buffered saline (PBS) to remove floating bacteria and culture medium. Two randomly-selected dentin slices were observed by FE-SEM to verify the presence of *E. faecalis* biofilm on the dentin surfaces.

After inoculation of the dentin slices, 0.15 g of methylcellulose (Aladdin Industrial Corp., Shanghai, China) was mixed with 2 mL of $\text{Ag}^+ - \text{Zn}^{2+}$ solution (3.2×10^{-2} mg/

mL AgNO_3) with atomic ratio of 1:1, 1:3, 1:9 or 1:12 (as described above), or 2% chlorhexidine. The latter was prepared by diluting 20% chlorhexidine (Adamas, Basel, Switzerland) with sterile deionized water. Addition of the methylcellulose enabled gels to be produced from those solutions. Dentin slices with *E. faecalis* biofilms were embedded in the gels and incubated anaerobically at 37 °C for 7 days in a 100% humidity environment. Gels prepared by mixing 2 mL of sterile PBS with 0.15 g methylcellulose were used as the negative control. Gels prepared by mixing 2 mL of 3.2×10^{-2} mg/mL AgNO_3 or 67.2×10^{-2} mg/mL $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ with 0.15 g methylcellulose were used as positive control groups. One hundred dentin slices were divided randomly into 10 groups ($N = 10$). After incubation, all slices were gently washed in sterile PBS for five times to remove remnant gels. Four dentin slices of each group were randomly selected to be observed by FE-SEM (sputter-coated with conductive carbon or gold) and the other six slices from each group were immersed in 8 mL of fresh BHI broth at 37 °C. At 2, 4, 6, 8 and 10 h after incubation, 1 mL suspension was retrieved for OD measurement at 600 nm using a spectrometer (UV-2401PC).

Zn^{2+} pretreatment

A Zn^{2+} pretreatment study was designed to further understand the role of Zn^{2+} in the synergistic antibacterial effect of $\text{Ag}^+ - \text{Zn}^{2+}$. Briefly, a 10 mL suspension [1×10^8 CFUs/mL] of *E. faecalis* was centrifuged at 8000 rpm for 5 min. After centrifuging, the supernatant was discarded. To remove the remaining culture medium, the bacteria pellet was suspended in 10 mL of sterilized deionized water and re-centrifuged. The supernatant was then discarded. For Zn^{2+} pretreatment, a 10 mL solution of 33.6×10^{-2} mg/mL $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ was added to the bacteria and incubated for 30 min at 4 °C. Sterilized deionized water (10 mL) was used as negative control. After 30 min, the Zn^{2+} pretreated bacteria and negative control groups were centrifuged; the bacteria pellet obtained from each group was re-suspended in 10 mL of sterilized deionized water and centrifuged once again to remove the remaining Zn^{2+} . The OD values of the suspensions were adjusted to 0.4. Then, 5 mL of Zn^{2+} -pretreated bacteria suspension was mixed with 5 mL of 3.2×10^{-2} mg/mL AgNO_3 solutions and 10 mL double-concentrated of BHI broth. For control groups, a 5 mL bacteria suspension from the negative control group was mixed with 5 mL of 3.2×10^{-2} mg/mL AgNO_3 solution or sterilized deionized water, together with 10 mL of double-concentrated BHI broth. At 1, 2, 3 and 4 h after incubation at 37 °C, 1 mL of the suspension was retrieved for OD determination. Experiments were conducted in sextuplicate.

Membrane potential and permeability of *E. faecalis* treated by Zn^{2+}

A BacLight bacterial membrane potential kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA) was used with the cell nucleus stain TO-PRO-3 (Molecular Probes) to determine the membrane potential and permeability of *E. faecalis* treated by Zn^{2+} [35]. Briefly, 1 mL suspension [4×10^5 CFUs/mL] of *E. faecalis* was diluted from a concentrated 1×10^8 CFUs/mL long-phase cultured bacteria suspension in BHI broth and mixed with $Zn(NO_3)_2 \cdot 6H_2O$ solutions (final concentrations: 5.6×10^{-2} , 16.8×10^{-2} , 33.6×10^{-2} , 50.4×10^{-2} and 67.2×10^{-2} mg/mL). Untreated suspension of *E. faecalis* was used as the control group. Subsequently, 10 μ L of 3 mM 3,3'-diethyloxycarbocyanine iodide (DiOC₂(3)) and 10 μ L of 50 μ M TO-PRO-3 were added simultaneously and incubated at room temperature for 25 min. A 1 mL untreated suspension of *E. faecalis* incubated previously with 10 μ L of 500 μ M carbonyl cyanide 3-chlorophenylhydrazone (CCCP) for 30 min was included as the positive control for DiOC₂(3); CCCP destroys membrane potential by eliminating the proton gradient [36]. Cells boiled at 100 °C for 10 min were used as the positive control for TO-PRO-3.

For each sample, fluorescence was acquired from 10,000 events by a flow cytometer (Beckman Coulter Inc., CA, USA). Signals were acquired with logarithmic amplification. DiOC₂(3) was excited at 488 nm, and its green fluorescence was detected through a 525 ± 40 nm band-pass filter and red fluorescence was detected through a 690 ± 50 nm band-pass filter [32]. TO-PRO-3 was excited at 638 nm, and its red fluorescence was detected through a 660 ± 20 nm band-pass filter. Mean fluorescence intensity values of each sample were used for analysis. Because the fluorescence of a bacterial cell or clump is size-dependent, membrane potential was determined by the red/green ratio of DiOC₂(3) and membrane permeability was evaluated by the values of TO-PRO-3 red/DiOC₂(3) green. For each group, the test was conducted in triplicate.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance analysis with a post hoc Student–Newman–Keuls test. When the normality or equal variance assumption of the data sets was violated, statistical analysis was performed using non-parametric Kruskal–Wallis analysis of variance with a post hoc Dunn test. For data in normal distribution, mean \pm standard deviation (SD) was used, while for data violating normality, median \pm quartile (i.e. P25–P75) was used. Statistical significance was preset at $\alpha = 0.05$.

Results

Synergistic pattern derived from CFUs counting

The CFUs formed on agar plates by *E. faecalis* incubated with Ag^+ were significantly less than the blank control except the 0.8×10^{-2} mg/mL group ($p < 0.05$; Fig. 1). Unlike the bactericidal effect of Ag^+ , no significant difference was observed in the CFUs among the blank control and the various Zn^{2+} group ($p > 0.05$; Fig. 2). There was no decline in CFUs with the increasing Zn^{2+} concentrations. All the $Ag^+ - Zn^{2+}$ groups except the 1:1 group had significantly less CFUs when compared with the blank control and the Zn^{2+} only group ($p < 0.05$; Fig. 3). The 1:9 and 1:12 $Ag^+ - Zn^{2+}$ groups with 1.6×10^{-2} mg/mL $AgNO_3$ showed significantly better antibacterial effect than the Ag^+ only group containing 1.6×10^{-2} mg/mL $AgNO_3$ ($p < 0.05$). The CFUs decreased extensively with increasing amounts of Zn^{2+} in the $Ag^+ - Zn^{2+}$ mixtures.

Synergistic pattern derived from dynamic growth-curve

Results from dynamic growth-curve method showed that in Ag^+ only groups tested with 1×10^6 CFUs/mL *E. faecalis*, groups with $AgNO_3$ concentrations of 3.2×10^{-2} , 6.4×10^{-2} , 12.8×10^{-2} , 25.6×10^{-2} and 51.2×10^{-2} mg/mL had significantly lower OD values than the blank control group ($p < 0.05$; Fig. 4a) at the 5th h. Among the Ag^+ only groups, those with 3.2×10^{-2} , 6.4×10^{-2} , 12.8×10^{-2} , 25.6×10^{-2} and 51.2×10^{-2} mg/mL $AgNO_3$ showed almost no bacteria growth after 5 h. In Ag^+ only groups tested with 1×10^8 CFUs/mL *E. faecalis*, groups with 6.4×10^{-2} , 25.6×10^{-2} , and 51.2×10^{-2} mg/mL $AgNO_3$ had significantly lower OD values than the blank control group ($p < 0.05$; Fig. 4c) at the 5th h. No statistical difference with the blank control group was detected in 12.8×10^{-2} mg/mL $AgNO_3$ group probably due to the relatively low test power of non-parametric statistics despite the similar no bacteria growth after 5 h as 6.4×10^{-2} , 25.6×10^{-2} , and 51.2×10^{-2} mg/mL $AgNO_3$ groups.

In Zn^{2+} only groups tested with 1×10^3 CFUs/mL *E. faecalis*, groups with 8.4×10^{-2} , 16.8×10^{-2} , 25.2×10^{-2} and 33.6×10^{-2} mg/mL $Zn(NO_3)_2 \cdot 6H_2O$ had significantly lower OD values than the blank control at the 10th h ($p < 0.05$; Fig. 4e).

At the 5th h, in the $Ag^+ - Zn^{2+}$ groups tested with 1×10^6 CFUs/mL *E. faecalis*, groups with 1:1, 1:3, 1:6, 1:9 and 1:12 $Ag^+ - Zn^{2+}$ ratios had significantly lower OD values than the blank control ($p < 0.05$; Fig. 4b). The OD values of 1:6 to 1:12 $Ag^+ - Zn^{2+}$ ratios were significantly lower than the values derived from the Zn^{2+} only group ($p < 0.05$; Fig. 4b). Likewise, OD values of groups with 1:6, 1:9 and 1:12 $Ag^+ - Zn^{2+}$ ratios were significantly lower than values derived from the Ag^+ only group

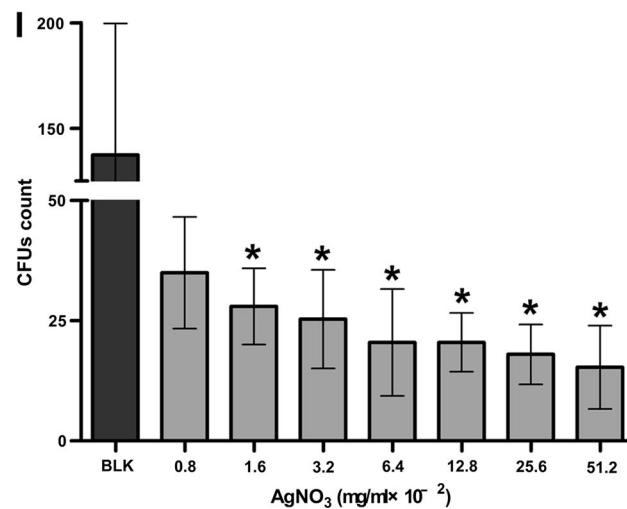
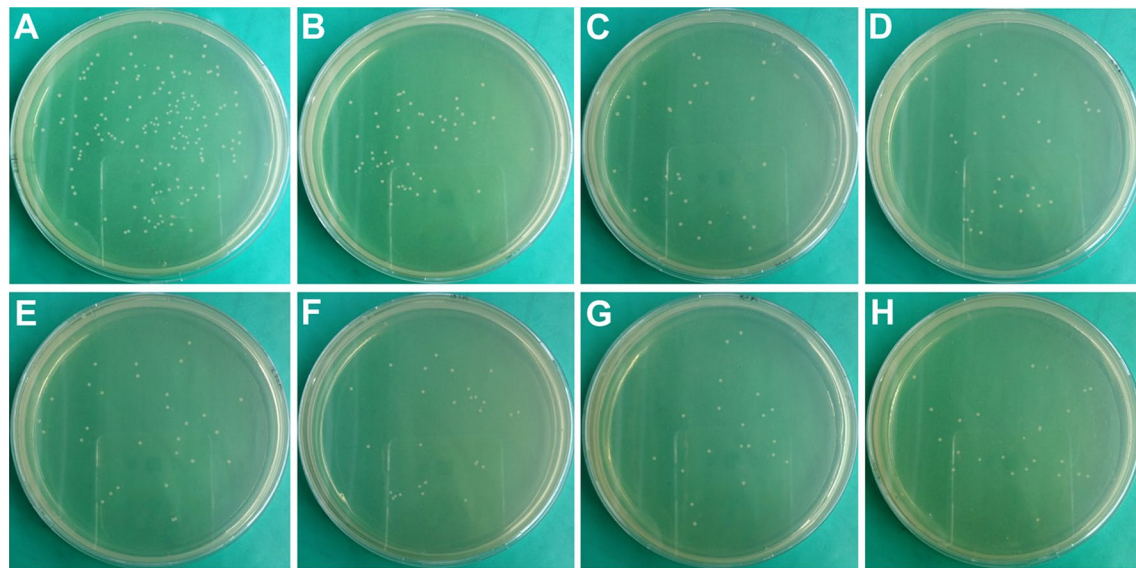


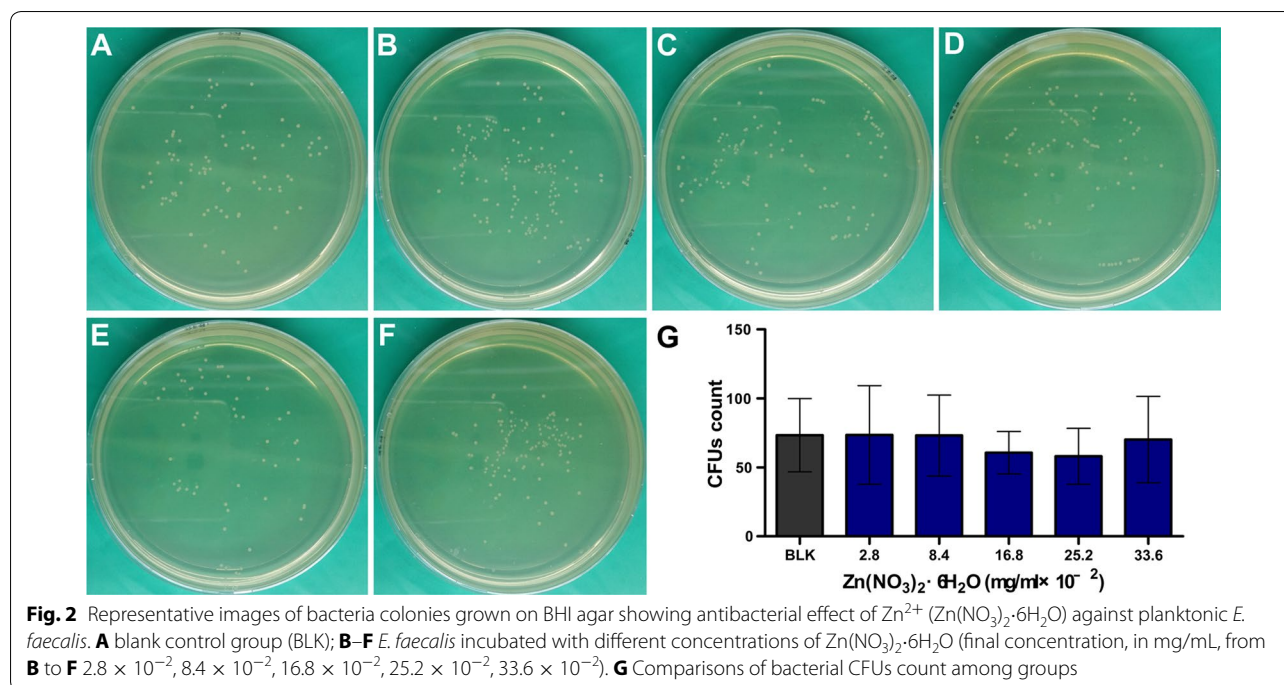
Fig. 1 Representative images of bacteria colonies grown on BHI agar showing the antibacterial effect of Ag⁺ (AgNO₃) against planktonic *E. faecalis*. **A** Blank control group (BLK); **B–H** *E. faecalis* incubated with different concentrations of AgNO₃ (final concentration, in mg/mL, from **B** to **H**: 0.8 × 10⁻², 1.6 × 10⁻², 3.2 × 10⁻², 6.4 × 10⁻², 12.8 × 10⁻², 25.6 × 10⁻², 51.2 × 10⁻²). **I** Comparisons of bacterial CFU count among groups. (Asterisk: significantly different when compared with the blank control group; *p* < 0.05)

(*p* < 0.05; Fig. 4b). In the Ag⁺–Zn²⁺ groups tested with 1 × 10⁸ CFUs/mL *E. faecalis*, groups with 1:1 and 1:3 Ag⁺–Zn²⁺ ratios were not significantly different from the blank control (*p* > 0.05; Fig. 4d). Optical density values of groups with 1:6, 1:9 and 1:12 Ag⁺–Zn²⁺ ratios were significantly lower than values derived from the Ag⁺ only group (*p* < 0.05).

Antibacterial efficiency of Ag⁺–Zn²⁺ against Ag⁺ only

The antibacterial efficiency of Ag⁺–Zn²⁺ combinations calculated using the results derived from CFU counting and growth curve determination are shown in Tables 1

and 2, respectively. In Table 1, antibacterial efficiencies of groups with 1:6, 1:9 and 1:12 Ag⁺–Zn²⁺ ratio were increased by 50.71, 62.28 and 60.05% respectively when compared with the use of only Ag⁺. In Table 2, despite the use of two bacteria concentrations (1 × 10⁶ CFUs/mL and 1 × 10⁸ CFUs/mL), the antibacterial efficiencies of Ag⁺–Zn²⁺ combinations exhibited the same trend as the results presented in Table 1. Antibacterial efficiency of the group with 1:12 Ag⁺–Zn²⁺ ratio was increased by 66.27% (1 × 10⁶ CFUs/mL) and 88.99% (1 × 10⁸ CFUs/mL) when compared with the use of only Ag⁺. Antibacterial efficiency of the group with 1:9



Ag⁺–Zn²⁺ ratio was found very close to that of 1:12 group.

MIC and MBC of Ag⁺–Zn²⁺ combinations

Minimum inhibitory concentration and MBC results were shown in Table 3. MIC and MBC of Zn²⁺ were undetectable for its limited antibacterial ability. MIC of Ag⁺ or Ag⁺–Zn²⁺ at different atomic ratios was 6.4 × 10⁻² mg/mL AgNO₃. MBC of each group was 12.8 × 10⁻² mg/mL AgNO₃ except for the 1:12 group with the MBC being 6.4 × 10⁻² mg/mL AgNO₃.

Cytotoxicity of Ag⁺–Zn²⁺ combinations

Results of the CCK-8 tests of Ag⁺ only on MC3T3-E1 cells indicated that 2% chlorhexidine and Ag⁺ only groups with 51.2 × 10⁻² mg/mL AgNO₃ had significantly lower cell growth rate when compared with the blank control group ($p < 0.05$; Fig. 5a). When the concentration of AgNO₃ exceeded 3.2 × 10⁻² mg/mL, cell growth decreased with the increasing Ag⁺ concentration. No significant difference was detected between the 25.6 × 10⁻², 51.2 × 10⁻² mg/mL AgNO₃ and 2% chlorhexidine ($p > 0.05$; Fig. 5a).

As the cytotoxicity of Zn²⁺ is low, 3 × 10³ cells were used for the test to facilitate the observation. It was found that the cytotoxicity of Zn²⁺ increased to the level of 2% chlorhexidine when the concentration of Zn(NO₃)₂·6H₂O reached 50.4 × 10⁻² mg/mL. No significant difference was detected between the 50.4 × 10⁻², 67.2 × 10⁻² mg/

mL Zn(NO₃)₂·6H₂O and 2% chlorhexidine ($p > 0.05$; Fig. 5b).

For CCK-8 tests of Ag⁺–Zn²⁺ combinations, all ratio groups except the 1:12 group were not significantly different from the blank control ($p > 0.05$; Fig. 5c). Although the 2% chlorhexidine group, Ag⁺–Zn²⁺ group with 1:12 ratio and Zn²⁺ only groups showed significantly lower cell growth rate when compared with the blank control group ($p < 0.05$), 2% chlorhexidine solutions exhibited the most suppressive effect on cell growth ($p < 0.05$) in all these three groups.

Although the Ag⁺–Zn²⁺ group with 1:12 ratio showed a slight suppressive effect on cell growth, its cytotoxicity was much lower than the 2% chlorhexidine group ($p < 0.05$; Fig. 5c).

Inhibition of *E. faecalis* biofilm grown on dentin using the optimum Ag⁺–Zn²⁺ ratio

Based on the aforementioned findings, the two most potent Ag⁺–Zn²⁺ ratios of 1:9 and 1:12 were used in this anti-biofilm test on dentin. The other two confirmed weaker ratios of 1:1 and 1:3 were also included for comparison. Optical density measurements after direct immersion of dentin slices into fresh BHI solution for 10 h indicated that there was significantly less biofilm accumulation on dentin slices treated with gels containing 2% chlorhexidine, Ag⁺ only or Ag⁺–Zn²⁺ combinations, when compared with the blank control group ($p < 0.05$; Fig. 6b). Dentin slices from the 2%

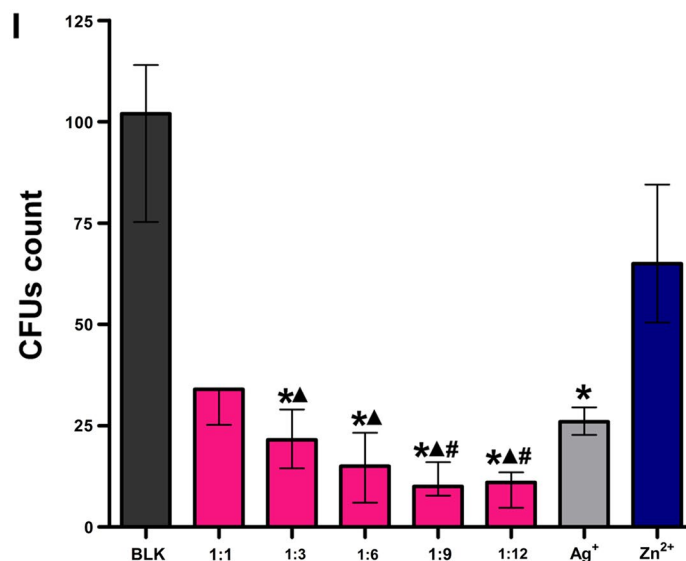
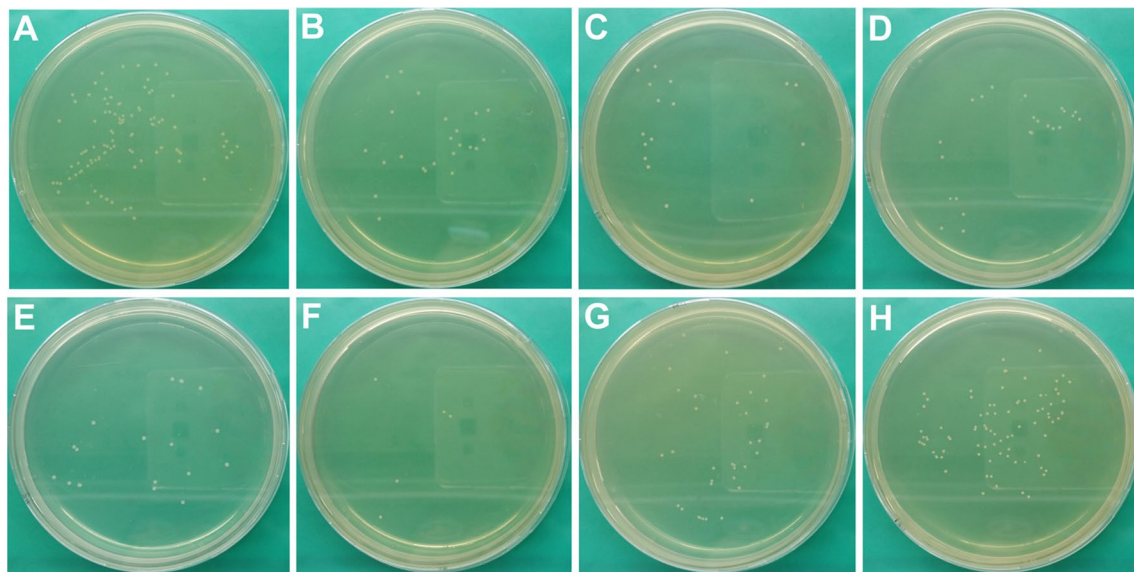


Fig. 3 Representative images of bacteria colonies grown on BHI agar showing the antibacterial effect of Ag^+ - Zn^{2+} combinations against planktonic *E. faecalis*. **A** Blank control group (BLK); **B-F** *E. faecalis* incubated with different atomic ratio of Ag^+ and Zn^{2+} (1:1; 1:3; 1:6; 1:9; 1:12); **G** *E. faecalis* incubated with AgNO_3 only (final concentration, 1.6×10^{-2} mg/mL); **H** *E. faecalis* incubated with $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ only (final concentration, 33.6×10^{-2} mg/mL). **I** Comparison of bacterial CFU counts among groups. (Asterisk: significant difference when compared with BLK; ▲significant difference when compared with the Zn^{2+} only group; #significant difference when compared with the Ag^+ only group; $p < 0.05$)

chlorhexidine group and the Ag^+ - Zn^{2+} group with 1:12 ratio had the least biofilm accumulation, especially when compared with the Ag^+ only group ($p < 0.05$; Fig. 6c). The Ag^+ - Zn^{2+} group with 1:1 ratio showed statistically higher OD value than the 2% chlorhexidine group and the Ag^+ - Zn^{2+} with 1:12 ratio ($p < 0.05$; Fig. 6c). Although no statistical difference was found among 1:3, 1:9, 1:12 and 2% chlorhexidine groups ($p > 0.05$; Fig. 6c),

the OD values decreased from 1:3 to 1:12 group with 2% chlorhexidine and 1:12 ratio groups showing the least biofilm accumulation on dentin. Scanning electron microscopy confirmed the differences in biofilm accumulation on dentin slices from different groups, except for those treated with 2% chlorhexidine as 2% chlorhexidine killed and fixed the bacteria biofilm on the dentin surface (Fig. 7) [37].

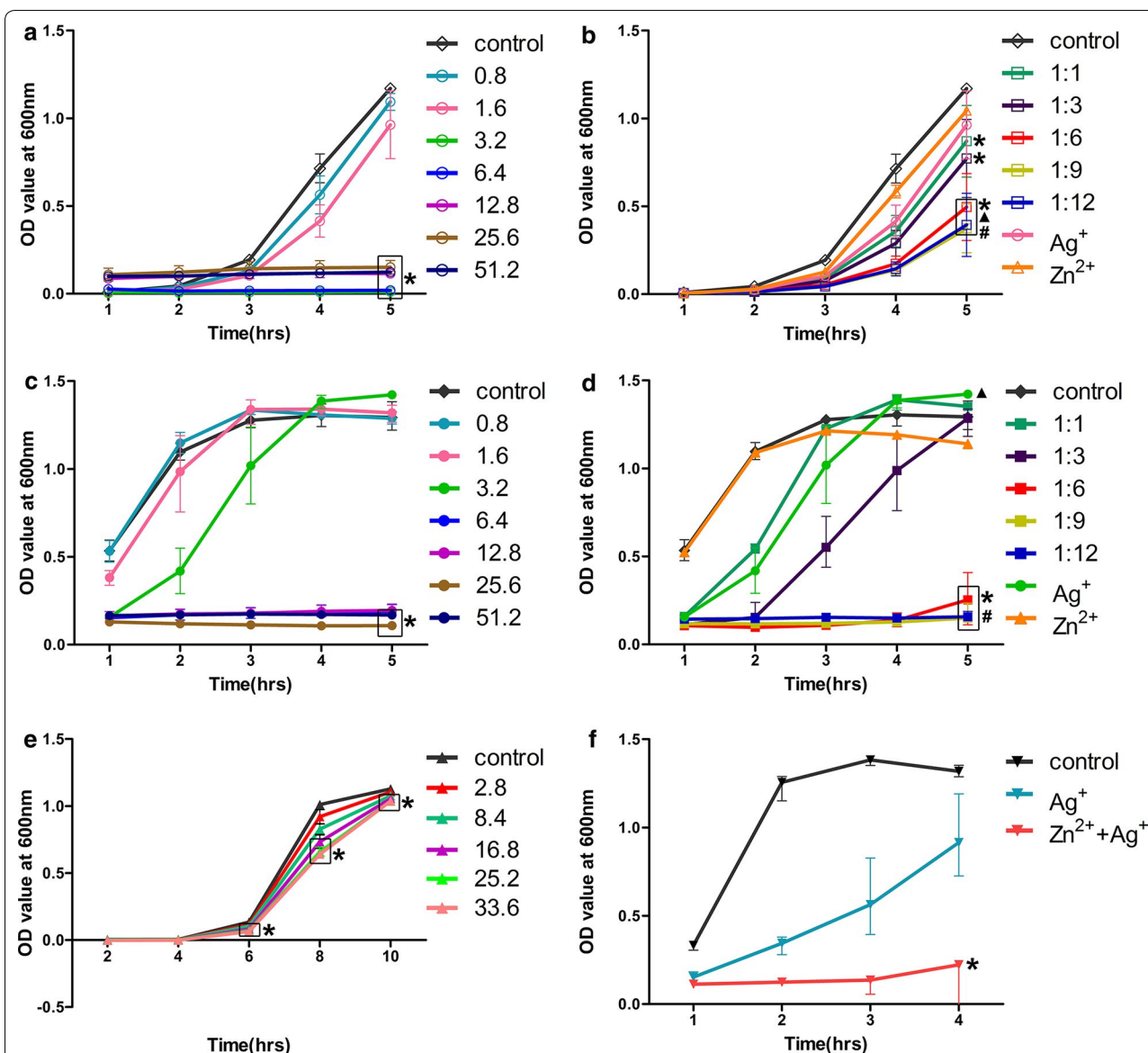


Fig. 4 Growth curve of *E. faecalis* under different conditions. OD optical density. **a** OD curve of *E. faecalis* (1×10^6 CFUs/mL) incubated with different concentrations of $AgNO_3$. **b** OD curve of *E. faecalis* (1×10^6 CFUs/mL) incubated with different Ag^+ - Zn^{2+} ratios. **c** OD curve of *E. faecalis* (1×10^8 CFUs/mL) incubated with different concentrations of $AgNO_3$. **d** OD curve of *E. faecalis* (1×10^8 CFUs/mL) incubated with different Ag^+ - Zn^{2+} ratios. **e** OD curve of *E. faecalis* (1×10^3 CFUs/mL) incubated with different concentrations of $Zn(NO_3)_2 \cdot 6H_2O$. **f** OD curve of *E. faecalis* (1×10^8 CFUs/mL) pretreated with Zn^{2+} only. (Asterisk: significant difference when compared with the blank control group ($p < 0.05$); \blacktriangle significant difference when compared with the Zn^{2+} only groups ($p < 0.05$); $\#$ significant difference when compared with the Ag^+ only groups ($p < 0.05$); Groups inside boxes in the corresponding chart had the same labels as the legend

Effect of Zn^{2+} pretreatment on the bactericidal activity of Ag^+

In Zn^{2+} pretreatment study, optical density measurement at the 4th h showed that only the Zn^{2+} pretreatment group had significantly lower OD values when compared with the control group ($p < 0.05$; Fig. 4f). The OD value of Zn^{2+} pretreatment group was also much lower than

the Ag^+ only group despite no statistical difference was detected by the non-parametric statistics in this test.

The membrane potential and permeability of *E. faecalis* under different Zn^{2+} conditions

In the membrane potential and permeability study, the membrane potential of *E. faecalis* exhibited a trend of

Table 1 Antibacterial efficiency of Ag⁺-Zn²⁺ combinations based on CFU counting

Atomic ratio (Ag ⁺ -Zn ²⁺)	AgNO ₃ + Zn(NO ₃) ₂ ·6H ₂ O (× 10 ⁻² mg/mL)	Antibacterial efficiency (Median (P25-P75) [%])
1:1	1.6 + 2.8	67.19 (63.37-76.52)
1:3	1.6 + 8.4	108.29 (83.11-146.85)
1:6	1.6 + 16.8	150.71 (118.46-174.25) ^a
1:9	1.6 + 25.2	162.28 (149.30-167.40) ^a
1:12	1.6 + 33.6	160.05 (156.20-174.95) ^a

Groups designated by same superscript are not significantly different (*p* > 0.05)

depolarization with increasing amounts of Zn²⁺ (Figs. 8 and 9a). However, membrane permeability was not altered significantly (*p* > 0.05; Fig. 9b). The largest degree of depolarization of the membrane potential occurred when the concentrations of Zn(NO₃)₂·6H₂O was more than 33.6 × 10⁻² mg/mL (*p* > 0.05 when compared with CCCP positive control group; Fig. 8).

Discussion

Silver ions has long since been used as a bactericidal metal element as its strong and broad-spectrum anti-bacterial ability. However, in this study, some *E. faecalis* were found still viable when the AgNO₃ concentration reached 51.2 × 10⁻² mg/mL. It might suggest that some strain of *E. faecalis* has developed the ability to resist the bactericidal effect of Ag⁺. On the other hand, the AgNO₃ concentration higher than 3.2 × 10⁻² mg/mL exhibited the cytotoxicity. Comparatively, the results from both CFUs counting and growth-curve methods indicated that the bactericidal ability of Zn²⁺ is much weaker than Ag⁺, generally being inhibitive rather than bactericidal to *E. faecalis*. It has been reported that when the Zn²⁺ was mixed with Ag⁺, the anti-bacterial effects would be significantly improved compared to either Ag⁺ or Zn²⁺ alone. However, the pattern and mechanism behind this synergistic activity is still unclear.

In this study, a series of atomic ratios between Ag⁺ and Zn²⁺ was produced by serial dilution, keeping the

amount of Ag⁺ constant while increasing the amount of Zn²⁺ in the mixture. Presumably, this helps to evaluate the role contributed by Zn²⁺ in promoting the antibacterial activity of the Ag⁺-Zn²⁺ synergy. Thus, based on the measured MIC of silver nitrate, 1/2 MIC and 1/4 MIC of silver nitrate were selected as the concentration of Ag⁺ in the Ag⁺-Zn²⁺ combinations in order to kept Ag⁺ at a relatively low level (1.6 × 10⁻² mg/mL AgNO₃ in which concentration of Ag⁺ is 1.0 × 10⁻² mg/mL) to facilitate evaluation of its synergistic antibacterial activity with Zn²⁺. Results from the CFU counting method indicated that the synergistic antibacterial effect of Ag⁺-Zn²⁺ combinations is significantly improved only when the Ag⁺-Zn²⁺ ratio is higher than 1:6, i.e. 1:9 and 1:12 in this study. Interestingly, there was no much difference in the antibacterial effect between the 1:9 and 1:12 ratios, which might suggest that these two atomic ratios reach the peak of the synergistic antibacterial ability of Ag⁺-Zn²⁺ combinations. Yang et al. reported a similar phenomenon when 1.7-7% Zn²⁺ was added to 0.3% Ag⁺-doped, heat-resistant honeycomb ceramic materials prepared from red mud industrial waste [13]. However, the combination ratios between the zinc and silver in that study were too

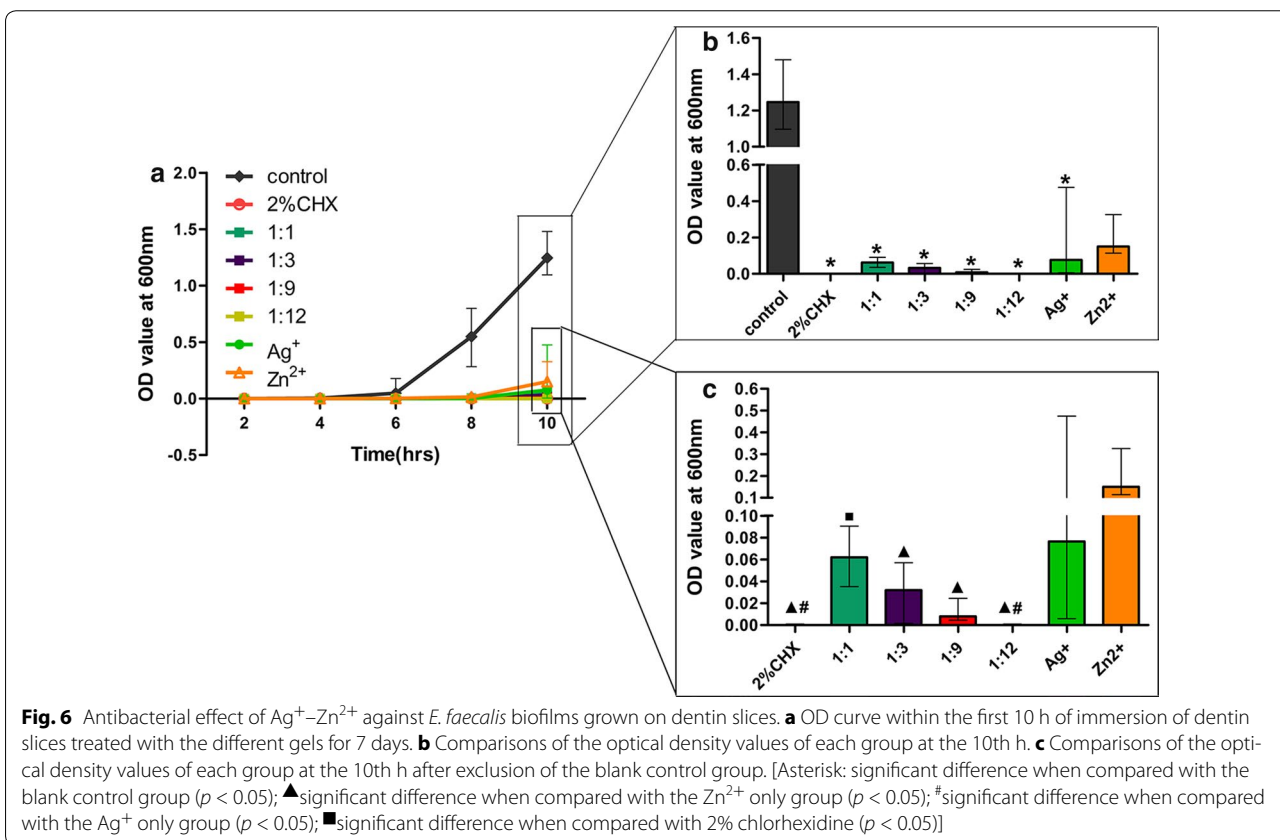
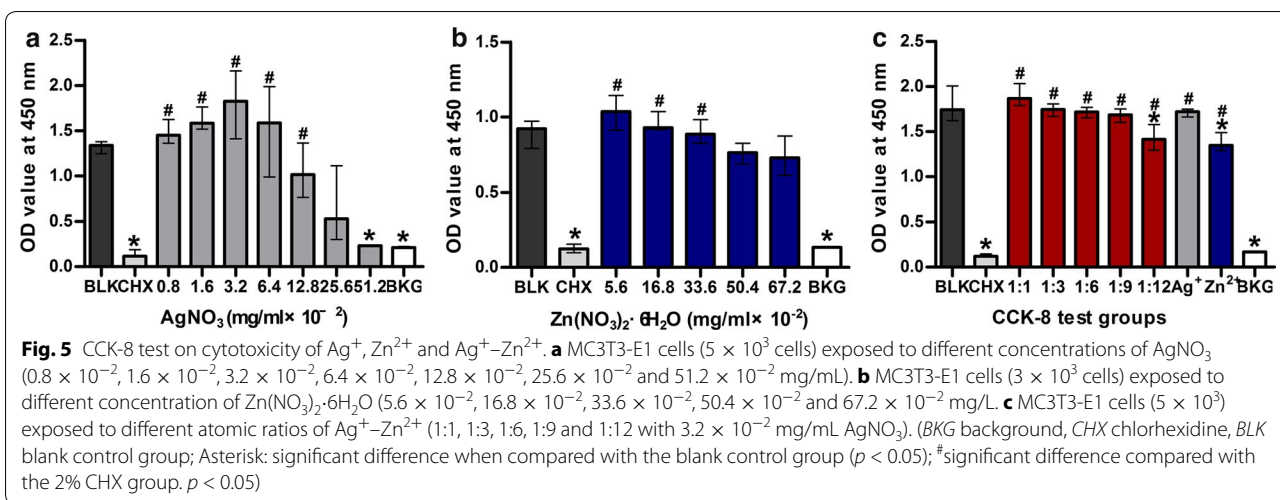
Table 3 MIC and MBC values of Ag⁺, Zn²⁺ and Ag⁺ + Zn²⁺ solutions against *E. faecalis*

Groups	AgNO ₃ + Zn(NO ₃) ₂ ·6H ₂ O (× 10 ⁻² mg/mL)	
	MIC	MBC ₉₆
Ag ⁺	6.4 + 0	12.8 + 0
Zn ²⁺	-	-
Ag ⁺ -Zn ²⁺		
1:1	6.4 + 11.2	12.8 + 22.4
1:3	6.4 + 33.6	12.8 + 67.2
1:6	6.4 + 67.2	12.8 + 134.4
1:9	6.4 + 100.8	12.8 + 201.6
1:12	6.4 + 134.4	6.4 + 134.4

Table 2 Antibacterial efficiency of Ag⁺-Zn²⁺ combinations based on growth curve determination

Atomic ratio (Ag ⁺ -Zn ²⁺)	AgNO ₃ + Zn(NO ₃) ₂ ·6H ₂ O (× 10 ⁻² mg/mL)		Antibacterial efficiency (Median (P25-P75) [%])	
	1 × 10 ⁶ CFUs/mL	1 × 10 ⁸ CFUs/mL	1 × 10 ⁶ CFUs/mL	1 × 10 ⁸ CFUs/mL
1:1	1.6 + 2.8	3.2 + 5.6	111.68 (106.09-113.65)	104.92 (104.10-106.53)
1:3	1.6 + 8.4	3.2 + 16.8	123.58 (113.85-126.03)	109.43 (106.16-115.32)
1:6	1.6 + 16.8	3.2 + 33.6	155.21 (142.46-155.90) ^a	181.98 (172.57-191.55) ^a
1:9	1.6 + 25.2	3.2 + 50.4	165.85 (155.98-167.69) ^a	189.38 (186.85-190.60) ^a
1:12	1.6 + 33.6	3.2 + 67.2	166.27 (150.97-167.80) ^a	188.99 (188.67-189.05) ^a

Groups designated by same superscript are not significantly different (*p* > 0.05)



limited to identify the mechanism and optimum ratio for $\text{Ag}^+-\text{Zn}^{2+}$ synergy against infection. Furthermore, the antibacterial effect of $\text{Ag}^+-\text{Zn}^{2+}$ combinations in our study did not show a lower MIC than silver ions only (Table 3), and even the antibacterial effect of 1:12 group was found increased by only 88.99% (Table 2). Considering these findings, it might be more appropriate to use

the word “antiseptic” than “antibacterial” in this manuscript. Despite these, these findings could be meaningful in designing new antiseptic or antibacterial materials containing both $\text{Ag}^+-\text{Zn}^{2+}$.

The cytotoxicity test revealed that the 1:9 $\text{Ag}^+-\text{Zn}^{2+}$ ratios exhibited no suppressive effect on cell growth, while the 1:12 ratio showed a slight suppressive effect.

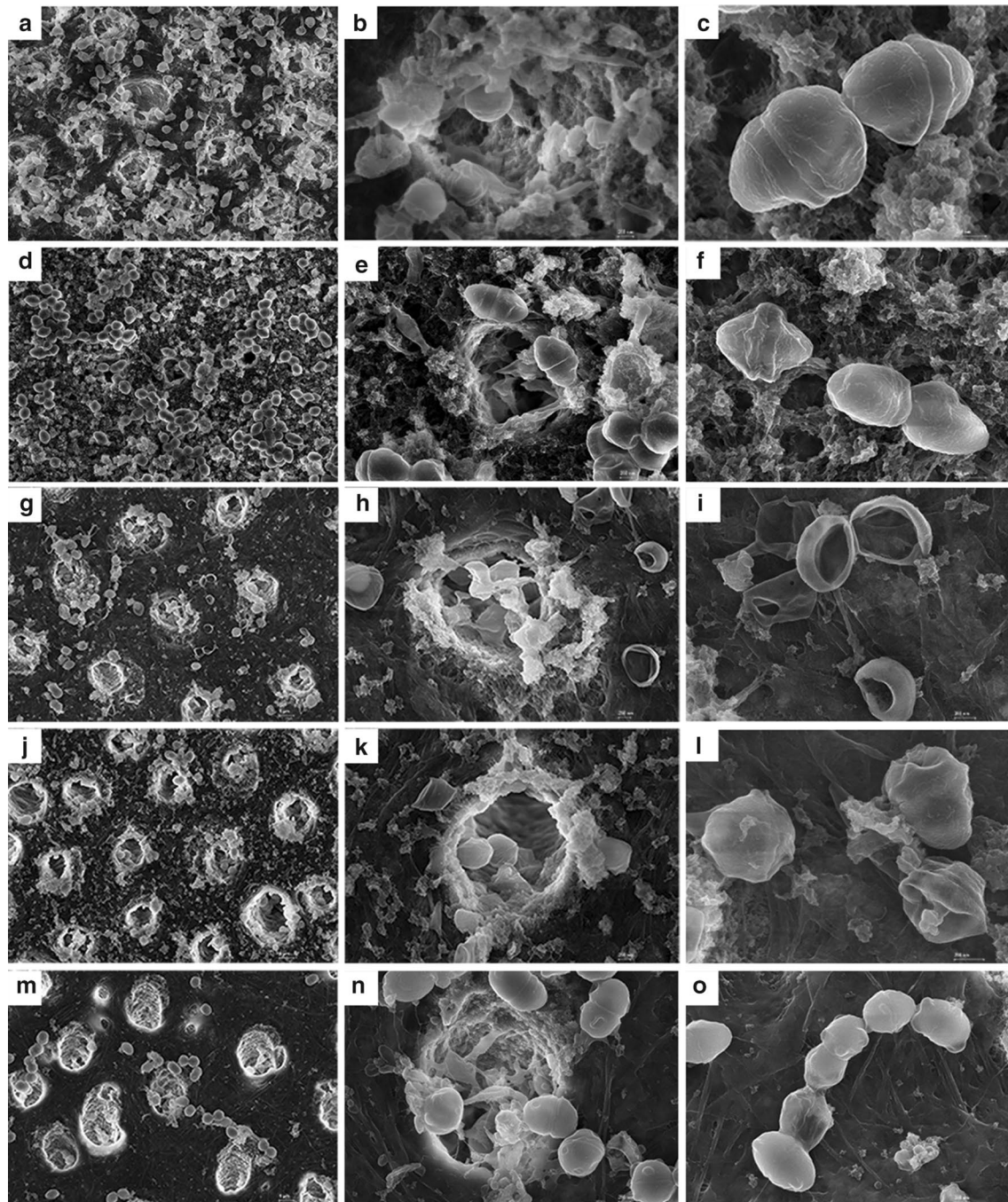
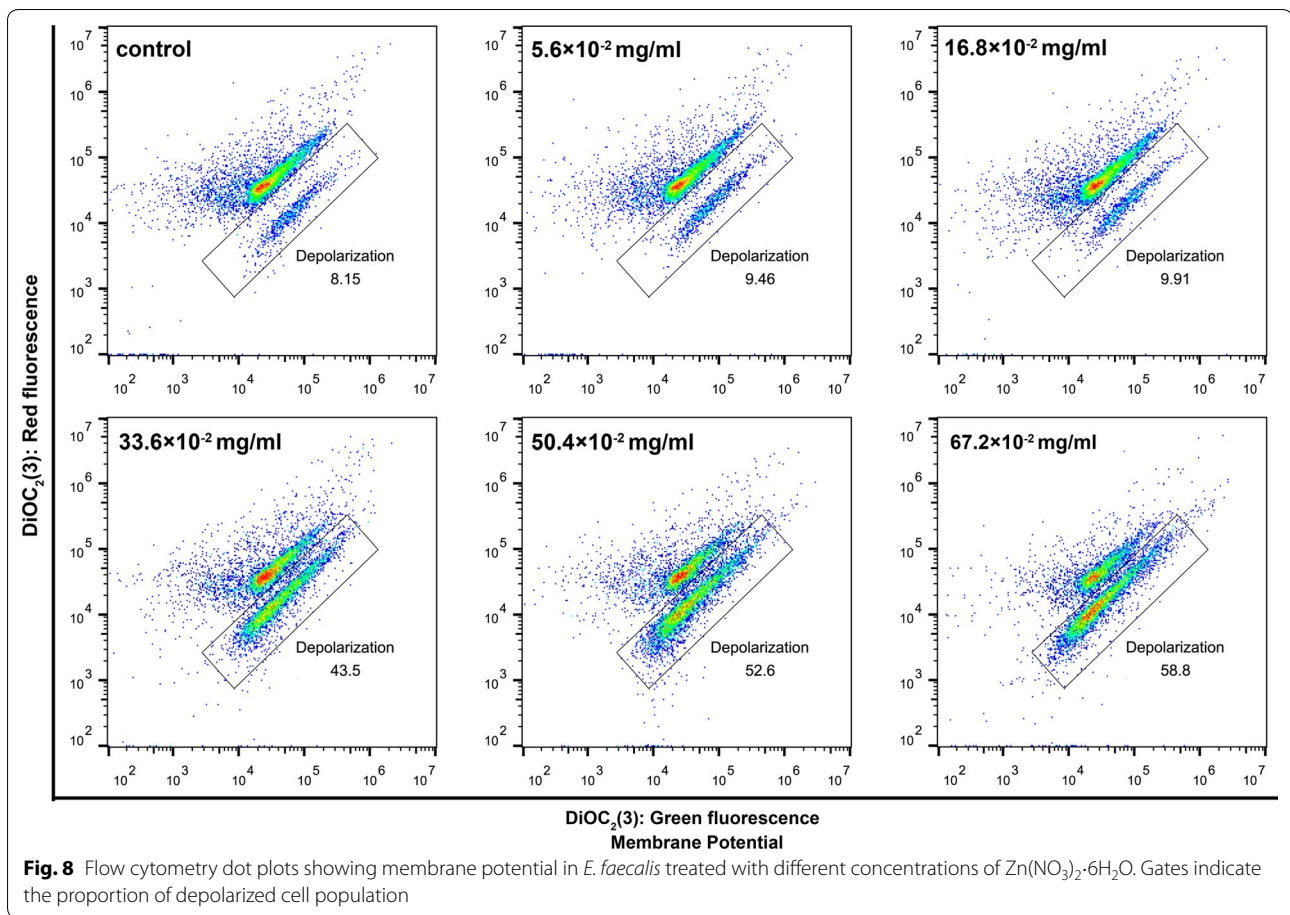


Fig. 7 Representative scanning electron microscopy images showing the inhibition of *E. faecalis* biofilms grown on dentin. **a–c** A biofilm treated with PBS gel for 7 days (**a** $\times 5000$; **b** $\times 25,000$; **c** $\times 50,000$) (negative control). **d–f** A biofilm treated with 2% chlorhexidine gel for 7 days (**d** $\times 5000$; **e** $\times 20,000$; **f** $\times 35,000$). **g–i** A biofilm treated with $\text{Ag}^+ - \text{Zn}^{2+}$ gel with 1:12 $\text{Ag}^+ - \text{Zn}^{2+}$ ratio (3.2×10^{-2} mg/mL AgNO_3) for 7 days (**g** $\times 5000$; **h** $\times 20,000$; **i** $\times 35,000$). **j–l** A biofilm treated with gel containing only Ag^+ (3.2×10^{-2} mg/mL AgNO_3) for 7 days (**j** $\times 5000$; **k** $\times 20,000$; **l** $\times 50,000$). **m–o** Biofilm treated with gel containing only Zn^{2+} [67.2×10^{-2} mg/mL $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$] for 7 days (**m** $\times 5,000$; **n** $\times 20,000$; **o** $\times 25,000$)

Considering the 1:9 ratio having the similar strong antibacterial activity as the 1:12 $\text{Ag}^+ - \text{Zn}^{2+}$ ratio, the 1:9 $\text{Ag}^+ - \text{Zn}^{2+}$ ratio might be more appropriate for in vivo applications against *E. faecalis* infection.

In the study against *E. faecalis* biofilm on dentin, 2% chlorhexidine gel was used as the positive control because it is an effective clinical antibacterial agent against *E. faecalis* in root canals [24]. SEM images showed that there

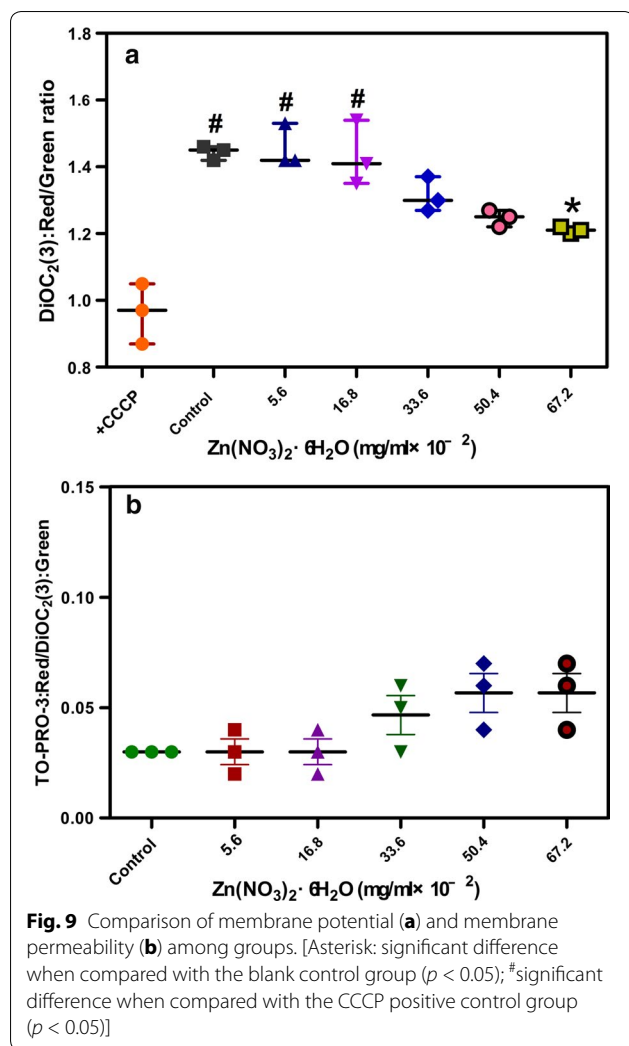


seemed still many bacteria left on the dentin surface treated by 2% chlorhexidine, which is different from that of metal ions-treated dentins (Fig. 7). The reason for this difference is because 2% chlorhexidine could kill as well as fix the bacteria biofilm on the dentin surface [37]. The OD values of the $\text{Ag}^+ - \text{Zn}^{2+}$ group with 1:9 and 1:12 ratios were similar to that of 2% chlorhexidine, which might suggest that $\text{Ag}^+ - \text{Zn}^{2+}$ combination with a 1:9 or 1:12 atomic ratio was as efficacious as 2% chlorhexidine in eradicating *E. faecalis* biofilms on dentin. Considering that 2% chlorhexidine is significantly more cytotoxic than the $\text{Ag}^+ - \text{Zn}^{2+}$ combination with 1:9 or 1:12 atomic ratio, the latter may be used to produce new medication against *E. faecalis* biofilm infection on dentin. The bactericidal mechanism of chlorhexidine is different from that of metal ions. Chlorhexidine could kill the bacteria through binding to acidic groups on the surface of the bacteria [38, 39], while metal ions normally enter the cell and combine with the enzyme or other proteins inside the cells, resulting in the cellular malfunctioning and death [40].

To better show and understand how the $\text{Ag}^+ - \text{Zn}^{2+}$ co-worked in antibacterial activities, a single *E. faecalis* bacteria strain was selected in this study to avoid

interferences from different bacteria strains. The synergistic antibacterial effect of $\text{Ag}^+ - \text{Zn}^{2+}$ combinations could be different on other bacteria or on other materials surfaces with different surface topography [41]. Rathnayake et al. ever reported that multi-drug resistance in clinical isolates (71.2% of *E. faecalis* and 70.3% of *E. faecium*) was much higher than water isolates (only 5.7% *E. faecium*) [42]. Hence, further studies need to be conducted on clinical isolates and multi-species biofilms on different material surfaces, which may express resistance trait against $\text{Ag}^+ - \text{Zn}^{2+}$ effect.

The Zn^{2+} pretreatment experiment was designed to clarify whether the Zn^{2+} really improves the antibacterial activity of Ag^+ in the $\text{Ag}^+ - \text{Zn}^{2+}$ combination. In this experiment, a low Ag^+ concentration (0.8×10^{-2} mg/mL AgNO_3) was used to facilitate observation of the enhanced antibacterial effect produced by Zn^{2+} pretreatment. The results suggested that Zn^{2+} pretreatment significantly enhanced the sensitivity of *E. faecalis* to the bactericidal activity of Ag^+ . This phenomenon may partly explain why combining Ag^+ and Zn^{2+} together produces a much stronger antibacterial effect than the use of Ag^+ alone.



To further investigate how Zn²⁺ makes *E. faecalis* more sensitive to Ag⁺, the membrane potential and permeability of *E. faecalis* was monitored by flow cytometry after the cells were exposed to different amounts of Zn²⁺. This is because membrane potential and permeability are closely related to the sensitivity of bacteria to ionic environment; ion homeostasis affects the proliferation, communication, metabolism and survival of bacteria [30, 34]. Zinc ions have been reported to alter the mitochondria membrane potential of rat brain cells [43, 44]. Nano-zinc oxide, for example, changes the permeability of the cell membrane [45, 46]. The results of this study suggested that the Zn²⁺ depolarizes the membrane potential but does not cause the bacterial cell membrane to rupture and leak. It is consistent with cytotoxicity testing, in which Zn²⁺ showed an inhibitive instead of destructive effect against bacteria. The largest degree of depolarization happened when the concentrations of Zn(NO₃)₂·6H₂O was more than 33.6×10^{-2} mg/mL,

which might probably explain why the Ag⁺–Zn²⁺ combination was more potent when its atomic ratio was between 1:6 and 1:12.

Maintenance of membrane potential is dependent on normal functioning of the proton pumps and ion channels in the cell membrane [47, 48]. Energy derived from adenosine triphosphate (ATP) is required for homeostasis of membrane potential, including ion transportation through the cell membrane and excretion of toxic heavy metal ions such as Ag⁺ [49]. When the concentration of extracellular Zn²⁺ increases as in the present study, the bacteria will transport some cations, including Na²⁺ and Zn²⁺, into the cell to maintain their membrane potential. However, Zn²⁺ is an inhibitor of ATP synthesis and oxidative phosphorylation in cells [50–52]. P-type ATPases are the essential enzymes involved in bacterial resistance to heavy metal ions [49, 53, 54]. Ion transportation through the cell membrane is hampered when ATP synthesis is inhibited by Zn²⁺. This may result in the accumulation of cations within the cell, including toxic Ag⁺. Intracellular cation accumulation, especially the heavy metal Ag⁺ ions, could result in depolarization of membrane potential and ultimately destruction of cells. The potential pathways through which Zn²⁺ influences Ag⁺ transportation and accumulation requires investigation in future studies.

Conclusions

Based on the findings of the present study, it may be concluded that the synergistic antibacterial effect of Ag⁺ and Zn²⁺ is dependent upon the amount of Zn²⁺ present in the medium. Atomic Ag⁺–Zn²⁺ ratios higher than 1:6 (i.e. 1:9 and 1:12 in this study) appear to be optimum ratios against both planktonic *E. faecalis* as well as single-species *E. faecalis* biofilms. This synergistic antibacterial effect may be attributed to the ability of Zn²⁺ to depolarize the bacterial cell membrane. The 1:9 and 1:12 Ag⁺–Zn²⁺ combinations are as efficacious as 2% chlorhexidine against *E. faecalis* biofilms that accumulate on dentin. New medications containing an optimal Ag⁺–Zn²⁺ ratio may be developed against *E. faecalis* infection of tooth root canals as well as infections in other parts of the human body.

Authors' contributions

WF and QS performed the study design, acquisition of data and manuscript writing. WF also provided funding support for this study. YL helped collect and analyze data and FRT participated in the study design and manuscript editing. BF is the corresponding author who designed the study, reviewed and revised the manuscript, provided funding support. All authors read and approved the final manuscript.

Author details

¹The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) and Key Laboratory of Oral Biomedicine, Ministry of Education, School and Hospital of Stomatology, Wuhan University, Wuhan, People's

Republic of China. ² Department of Endodontics, The Dental College of Georgia, Augusta University, Augusta, GA, USA.

Acknowledgements

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and supporting materials

The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

This manuscript is approved by all authors for the submission.

Ethics approval and consent to participate

The teeth samples used for making dentin slices were collected under the approval of ethics committee of School and Hospital of Stomatology, Wuhan University.

Funding

This study was financially supported by the National Natural Science Foundation of China (Grant Nos. 81570969 and 81470732).

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 19 July 2017 Accepted: 19 January 2018

Published online: 31 January 2018

References

- Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science*. 1999;284:1318–22.
- Weiss RA, McMichael AJ. Social and environmental risk factors in the emergence of infectious diseases. *Nat Med*. 2004;10:570–6.
- Neu HC. The crisis in antibiotic resistance. *Science*. 1992;257:1064–73.
- Smith RA, M'ikanatha NM, Read AF. Antibiotic resistance: a primer and call to action. *Health Commun*. 2015;30:309–14.
- Williams RJ, Heymann DL. Containment of antibiotic resistance. *Science*. 1998;279:1153–4.
- Goossens H, Ferech M, Vander Stichele R, Elseviers M, Group EP. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet*. 2005;365:579–87.
- Lemire JA, Harrison JJ, Turner RJ. Antimicrobial activity of metals: mechanisms, molecular targets and applications. *Nat Rev Microbiol*. 2013;11:371–84.
- Chernousova S, Epple M. Silver as antibacterial agent: ion, nanoparticle, and metal. *Angew Chem Int Ed Engl*. 2013;52:1636–53.
- Franci G, Falanga A, Galdiero S, Palomba L, Rai M, Morelli G, Galdiero M. Silver nanoparticles as potential antibacterial agents. *Molecules*. 2015;20:8856–74.
- Nieboer E, Richardson DH. The replacement of the nondescript term 'heavy metals' by a biologically and chemically significant classification of metal ions. *Environ Pollut Ser B Chem Phys*. 1980;1:3–26.
- Lansdown AB. A pharmacological and toxicological profile of silver as an antimicrobial agent in medical devices. *Adv Pharmacol Sci*. 2010;2010:910686.
- White JM, Powell AM, Brady K, Russell-Jones R. Severe generalized argyria secondary to ingestion of colloidal silver protein. *Clin Exp Dermatol*. 2003;28:254–6.
- Yang S, Zhang Y, Yu J, Zhen Z, Huang T, Tang Q, Chu PK, Qi L, Lv H. Antibacterial and mechanical properties of honeycomb ceramic materials incorporated with silver and zinc. *Mater Des*. 2014;59:461–5.
- Jia H, Hou W, Wei L, Xu B, Liu X. The structures and antibacterial properties of nano-SiO₂ supported silver/zinc-silver materials. *Dent Mater*. 2008;24:244–9.
- Hernandez-Sierra JF, Ruiz F, Pena DC, Martinez-Gutierrez F, Martinez AE, Guillen Ade J, Tapia-Perez H, Castanon GM. The antimicrobial sensitivity of *Streptococcus mutans* to nanoparticles of silver, zinc oxide, and gold. *Nanomedicine*. 2008;4:237–40.
- Galeano B, Korff E, Nicholson WL. Inactivation of vegetative cells, but not spores, of *Bacillus anthracis*, *B. cereus*, and *B. subtilis* on stainless steel surfaces coated with an antimicrobial silver- and zinc-containing zeolite formulation. *Appl Environ Microbiol*. 2003;69:4329–31.
- Casemiro LA, Gomes Martins CH, Pires-de-Souza Fde C, Panzeri H. Antimicrobial and mechanical properties of acrylic resins with incorporated silver–zinc zeolite—part I. *Gerodontology*. 2008;25:187–94.
- Klein G. Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *Int J Food Microbiol*. 2003;88:123–31.
- Swaminathan S, Alangaden GJ. Treatment of resistant enterococcal urinary tract infections. *Curr Infect Dis Rep*. 2010;12:455–64.
- Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. *Enterococcus faecalis*: its role in root canal treatment failure and current concepts in retreatment. *J Endod*. 2006;32:93–8.
- Love RM. *Enterococcus faecalis*—a mechanism for its role in endodontic failure. *Int Endod J*. 2001;34:399–405.
- Evans M, Davies JK, Sundqvist G, Figdor D. Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. *Int Endod J*. 2002;35:221–8.
- Fan W, Wu D, Tay FR, Ma T, Wu Y, Fan B. Effects of adsorbed and templated nanosilver in mesoporous calcium-silicate nanoparticles on inhibition of bacteria colonization of dentin. *Int J Nanomed*. 2014;9:5217–30.
- Gomes BPFA, Ferraz CCR, Vianna ME, Berber VB, Teixeira FB, Souza-Filho FJ. In vitro antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of *Enterococcus faecalis*. *Int Endod J*. 2001;34:424–8.
- Ahmed HMA, Abbott PV. Discolouration potential of endodontic procedures and materials: a review. *Int Endod J*. 2012;45:883–97.
- Samiei M, Farjami A, Dizaj SM, Lotfipour F. Nanoparticles for antimicrobial purposes in endodontics: a systematic review of in vitro studies. *Mater Sci Eng C*. 2016;58:1269–78.
- Mohammadi Z, Abbott PV. The properties and applications of chlorhexidine in endodontics. *Int Endod J*. 2009;42:288–302.
- Shayani Rad M, Kompany A, Khorsand Zak A, Javidi M, Mortazavi SM. Microleakage and antibacterial properties of ZnO and ZnO: Ag nanoparticles prepared via a sol–gel method for endodontic sealer application. *J Nanoparticle Res*. 2013;15:1–8.
- Chang YY, Lai CH, Hsu JT, Tang CH, Liao WC, Huang HL. Antibacterial properties and human gingival fibroblast cell compatibility of TiO₂/Ag compound coatings and ZnO films on titanium-based material. *Clin Oral Investig*. 2012;16:95–100.
- Strahl H, Hamoen LW. Membrane potential is important for bacterial cell division. *Proc Natl Acad Sci USA*. 2010;107:12281–6.
- Penyige A, Matko J, Deak E, Bodnar A, Barabas G. Depolarization of the membrane potential by beta-lactams as a signal to induce autolysis. *Biochem Biophys Res Commun*. 2002;290:1169–75.
- Novo D, Perlmutter NG, Hunt RH, Shapiro HM. Accurate flow cytometric membrane potential measurement in bacteria using diethyloxycarbocyanine and a ratiometric technique. *Cytometry*. 1999;35:55–63.
- Silverman JA, Perlmutter NG, Shapiro HM. Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*. *Antimicrob Agents Chemother*. 2003;47:2538–44.
- Prindle A, Liu J, Asally M, Ly S, Garcia-Ojalvo J, Suel GM. Ion channels enable electrical communication in bacterial communities. *Nature*. 2015;527:59–63.
- Novo DJ, Perlmutter NG, Hunt RH, Shapiro HM. Multiparameter flow cytometric analysis of antibiotic effects on membrane potential, membrane permeability, and bacterial counts of *Staphylococcus aureus* and *Micrococcus luteus*. *Antimicrob Agents Chemother*. 2000;44:827–34.
- Chen J, Peng H, Wang X, Shao F, Yuan Z, Han H. Graphene oxide exhibits broad-spectrum antimicrobial activity against bacterial phytopathogens and fungal conidia by intertwining and membrane perturbation. *Nanoscale*. 2014;6:1879–89.
- Clegg MS, Vertucci FJ, Walker C, Belanger M, Britto LR. The effect of exposure to irrigant solutions on apical dentin biofilms in vitro. *J Endod*. 2006;32:434–7.

38. Rolla G, Melsen B. On the mechanism of the plaque inhibition by chlorhexidine. *J Dent Res*. 1975;54(Spec No B):B57–62.
39. Jones CG. Chlorhexidine: is it still the gold standard? *Periodontology*. 2000;1997(15):55–62.
40. Hall JL. Cellular mechanisms for heavy metal detoxification and tolerance. *J Exp Bot*. 2002;53:1–11.
41. Rivera_Gil P, Yang F, Thomas H, Li L, Terfort A, Parak WJ. Development of an assay based on cell counting with quantum dot labels for comparing cell adhesion within cocultures. *Nano Today*. 2011;6:20–7.
42. Rathnayake IU, Hargreaves M, Huygens F. Antibiotic resistance and virulence traits in clinical and environmental *Enterococcus faecalis* and *Enterococcus faecium* isolates. *Syst Appl Microbiol*. 2012;35:326–33.
43. Dineley KE, Richards LL, Votyakova TV, Reynolds IJ. Zinc causes loss of membrane potential and alters production of reactive oxygen species in isolated brain mitochondria. *J Neurochem*. 2002;81:104.
44. Dineley KE, Richards LL, Votyakova TV, Reynolds IJ. Zinc causes loss of membrane potential and elevates reactive oxygen species in rat brain mitochondria. *Mitochondrion*. 2005;5:55–65.
45. Salem W, Leitner DR, Zingl FG, Schratte G, Prassl R, Goessler W, Reidl J, Schild S. Antibacterial activity of silver and zinc nanoparticles against *Vibrio cholerae* and enterotoxigenic *Escherichia coli*. *Int J Med Microbiol*. 2015;305:85–95.
46. Zhang LL, Jiang YH, Ding YL, Povey M, York D. Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (ZnO nanofluids). *J Nanoparticle Res*. 2007;9:479–89.
47. Griniuvienė B, Chmieliauskaitė V, Grinius L. Energy-linked transport of permeant ions in *Escherichia coli* cells: evidence for membrane potential generation by proton-pump. *Biochem Biophys Res Commun*. 1974;56:206–13.
48. Ren D, Navarro B, Xu H, Yue L, Shi Q, Clapham DE. A prokaryotic voltage-gated sodium channel. *Science*. 2001;294:2372–5.
49. Silver S. Bacterial resistances to toxic metal ions—a review. *Gene*. 1996;179:9–19.
50. Dineley KE, Votyakova TV, Reynolds IJ. Zinc inhibition of cellular energy production: implications for mitochondria and neurodegeneration. *J Neurochem*. 2003;85:563–70.
51. Klein C, Sunahara RK, Hudson TY, Heyduk T, Howlett AC. Zinc inhibition of cAMP signaling. *J Biol Chem*. 2002;277:11859–65.
52. Kasahara M, Anraku Y. Inhibition of the respiratory chain of *Escherichia coli* by zinc ions. *J Biochem*. 1972;72:777–81.
53. Nies DH, Silver S. Ion efflux systems involved in bacterial metal resistances. *J Ind Microbiol*. 1995;14:186–99.
54. Rensing C, Ghosh M, Rosen BP. Families of soft-metal-ion-transporting ATPases. *J Bacteriol*. 1999;181:5891–7.

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at
www.biomedcentral.com/submit

