Combined Effect of a Mixture of Silver Nanoparticles and Calcium Hydroxide against *Enterococcus faecalis* Biofilm

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ABSTRACT

Introduction: The aim of this study was to evaluate the antibiofilm effectiveness of calcium hydroxide ($Ca[OH]_2$) mixed with 0.02% silver nanoparticles (AgNPs) in comparison with 1 mg/mL triple antibiotic paste (TAP), $Ca(OH)_2$, and 0.02% AgNPs against *Enterococcus faecalis* using confocal laser scanning microscopy. **Methods:** Ninety dentin disks were prepared, sterilized, and inoculated with *E. faecalis* to establish a 3-week-old biofilm model. The samples received 1 mg/mL TAP, a mixture of $Ca(OH)_2$ + 0.02% AgNPs, $Ca(OH)_2$, or 0.02% AgNPs (n = 20/group). Specimens in each group were equally subdivided into 2 groups and incubated for 2 and 4 weeks. Untreated dentin disks (n = 10) were exposed to sterile saline solution and acted as a positive control. Sterile dentin disks (n = 10) were incubated anaerobically in brain-heart infusion broth and served as a negative control. At the end of each observation period, the specimens were stained with LIVE/DEAD *Bac*Light dye (Molecular Probes, Eugene, OR) and analyzed with confocal laser scanning microscopy to determine the proportion of dead cells in the biofilm. Statistical analysis was performed using the generalized linear model repeated measure and Tukey tests ($P < .05$). **Results:** A significantly greater proportion of dead cells was observed in the samples treated with 1 mg/mL TAP (90.39% and 99.41%) and a mixture of $Ca(OH)_2$ + AgNPs (90.85% and 98.49%) than those in the samples treated with $Ca(OH)_2$ (76.14% and 91.71%) and AgNPs (62.83% and 88.07%) at 2 and 4 weeks, respectively. A significant difference in the antibiofilm effectiveness was observed among the groups ($P < .05$), except for 1 mg/mL TAP and the mixture of $Ca(OH)_2$ + AgNPs ($P > .05$). All medicaments showed a significant difference in antibiofilm efficacy at the 2 time points. **Conclusions:** The mixture of $Ca(OH)_2$ + AgNPs showed a high antibiofilm effect and was not significantly different from 1 mg/mL TAP. Furthermore, long-term contact between intracanal medicaments and bacterial cells achieved significant antibiofilm efficacy. *(J Endod 2020;46:1689–1694.)*

KEY WORDS

Biofilm; calcium hydroxide; confocal laser scanning microscopy; intracanal medicament; silver nanoparticles; triple antibiotics

SIGNIFICANCE

A mixture of calcium hydroxide with silver nanoparticles exhibited a significantly potent antibiofilm effect against 3-week-old *E. faecalis* that is comparable with the effects from triple antibiotic paste. Moreover, no discoloration of dentin was noticed after exposure to the mixture compared with triple antibiotic paste. Thus, the use of this mixture could be considered in the future.

The main objective of root canal treatment is to achieve favorable outcomes through the removal of endodontic biofilms, which are considered the major cause of both primary and secondary root canal infection[1]. Studies have shown that bacterial biofilms remain inaccessible after chemomechanical debridement[2,3]; therefore, intracanal medicaments have been advocated in between visits to reduce or eliminate the remaining persistent bacterial biofilm in the root canal system[4]. Calcium hydroxide ($Ca[OH]_2$) is commonly used as an intracanal medicament because of its broad antimicrobial spectrum[5]. However, $Ca(OH)_2$ does not effectively remove a well-established *Enterococcus faecalis* biofilm in cases of persistent root canal infections[6,7]. Antibacterial drugs are also suggested for eradicating obligate anaerobic bacteria isolated from necrotic pulp and periradicular pathosis[8]. Triple antibiotic paste (TAP) containing a combination of metronidazole, ciprofloxacin, and minocycline has been proposed as an intracanal medicament in endodontic regenerative procedures[9]. Studies have shown the effectiveness of low concentrations of TAP (1, 0.1, and 0.01 mg/mL) to eradicate a well-established biofilm[9–12]. However, 1 of the drawbacks of TAP is discoloration[13].

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0099-2399/$ - see front matter

https://doi.org/10.1016/j.joen.2020.07.001

Accounting for TAP and $Ca(OH)_2$, the search for new, less harmful, and more potent medicaments has commenced. When considering more recent advances in the field of medicine, the affiliation with nanotechnology is surely 1 of them, and the ability to produce biomaterials with improved physical, chemical, and biological properties is truly 1 of its advantages. Nanoparticles have been an area of interest in dentistry with their improved physicochemical properties, including their ultrasmall size, large surface area/mass ratio, and enhanced chemical reactivity[14]. The application of nanoparticles in endodontics as an irrigant and a medication as well as an additive within root canal sealers has been explored[15]. Among the various existing nanomaterials, silver nanoparticles (AgNPs) have gained attention because of their distinctive physical and biochemical properties. Interestingly, the use of AgNPs as a vehicle with $Ca(OH)_2$ revealed better antimicrobial efficacy than that of $Ca(OH)_2$ against *E. faecalis*[16]. Similar findings reported that the combination of $Ca(OH)_2$ and AgNPs showed an obvious inhibitory effect on *E. faecalis*[17,18]. Using confocal laser scanning microscopy (CLSM), comparing 0.02% and 0.01% AgNP gels with $Ca(OH)_2$, 0.02% AgNPs gels were more effective than $Ca(OH)_2$ in removing *E. faecalis* biofilms with little to no residual cells[19].

To the best of our knowledge, there is a lack of comparative studies between TAP and the mixture of $Ca(OH)_2$ + AgNPs when used as an intracanal medicament. Therefore, the aim of this study was to compare the antibiofilm efficacy of the mixture of $Ca(OH)_2$ + 0.02% AgNPs with the effect of 1 mg/mL TAP, $Ca(OH)_2$, and 0.02% AgNPs against *E. faecalis* using CLSM.

MATERIALS AND METHODS

Specimen Preparation

This work was approved by the Institutional Review Board of King Saud University, Riyadh, Saudi Arabia (E-17-2691). For the study to have a power of 85% (based on $\alpha = 0.05$, estimated standard deviation = 0.6), it was calculated that 10 dentin disks would be required for the experimental group/observation period.

Single-rooted human teeth extracted for reasons not related to this study were collected. Roots with anatomic variations, curvatures, or previous endodontic treatment were excluded. Specimen preparation was adapted from a previous study[20]. The coronal and apical thirds of the root were cut off with a diamond disk to obtain the middle third, which was prepared with a size #35/0.06 ProFile Rotary File (Dentsply Maillefer, Ballaigues, Switzerland) to standardize the lumen size. The middle thirds were sectioned in a buccolingual direction under water cooling with a low-speed sectioning saw (IsoMet 2000 Precision Saw; IsoMet, Buehler, IL). One hundred 6 × 8 × 0.5 mm (width × length × thickness) dentin disks were prepared from the inner surface of each root half. The smear layer was removed by exposing the dentin disks to 17% EDTA in an ultrasonic bath for 1 minute. Then, gamma irradiation was used to sterilized the dentin disks at a dose of 25 kGy[21], and the efficacy of sterilization was checked by randomly selecting 2 dentin disks from each group and incubating them in brain-heart infusion (BHI) broth for 24 hours.

Biofilm Generation

A pure culture of *E. faecalis* (ATCC 29212; American Type Culture Collection, Manassas, VA) was grown in BHI broth and incubated in an anaerobic environment for 24 hours at 37°C. The colonies were diluted in fresh BHI broth, and the cell numbers were prepared to match the turbidity equivalent to a 0.5 McFarland standard, corresponding to an optical density of 0.08–0.1 absorbance at 600 nm in a spectrophotometer. Each sterilized dentin disk was transferred to a sterilized 12-well tissue culture plate (Nunc; Thermo Scientific, Darmstadt, Denmark) with the pulpal sides oriented outward, and then 3.0 mL *E. faecalis* suspension (1×10^8 colony-forming units/mL) was dispensed and incubated under anaerobic conditions for 3 weeks at 37°C. The growth medium was replenished every 72 hours to provide a constant rate of microbial growth during the incubation period. Gram staining and microscopic assessment of colony morphology were used to confirm the monoculture of *E. faecalis*. After incubation, the specimens were aseptically removed from the wells and gently rinsed with sterile phosphate-buffered saline for 1 minute.

Treatment of Infected Specimens

The dentin disks were randomly assigned into 4 groups ($n = 20$/group) according to the intracanal medicaments as follows: the TAP group, prepared by mixing equivalent portions of metronidazole, ciprofloxacin, and minocycline powders at a ratio of 1:1:1 with 1 mL sterile water to generate a concentration of 1 mg/mL (Xi'an Sgonek Biological Technology Co, Ltd, Shaanxi, China)[22]; the $Ca(OH)_2$ group, UltraCal (Ultradent Products, Inc, South Jordan, UT); the AgNP group, prepared by mixing AgNP powder (size = 10 nm) to form a suspension at a concentration of 0.02% (Zhengzhou Dongyao Nano Materials Co, Ltd, Zhengzhou, China)[19]; and a mixture of the $Ca(OH)_2$ + AgNP group, prepared by mixing $Ca(OH)_2$ paste with a 0.02% AgNP suspension at a proportion of 1:1[18]. Specimens in each group were equally subdivided into 2 groups and incubated anaerobically for 2 and 4 weeks at 37°C and 100% humidity. Untreated dentin specimens with 21-day-old biofilms ($n = 10$) were exposed to sterile saline solution and viewed by a confocal laser scanning microscope (Nikon $C2^+$ System; Nikon Instruments Inc, Melville, NY) to verify the viability of *E. faecalis* biofilms and served as a positive control. Sterile dentin disks ($n = 10$) were incubated anaerobically in 5.0 mL BHI broth at 37°C for 4 weeks to confirm the absence of any external bacterial contamination and served as a negative control.

At the end of each observation period, the specimens were irrigated with 5 mL sterile phosphate-buffered saline to remove the tested medication, stained with a fluorescent LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes, Eugene, OR), and viewed using CLSM. The 4 corners of each dentin disk were scanned with a 2-μm step size at a resolution of 1024 × 1024 pixels. Simultaneous dual-channel imaging was used to display the green (live cells) and red (dead cells) fluorescence. The confocal laser scanning microscopic images of the biofilms were analyzed and quantitated using Fiji software, which is an open-source platform for biological image analysis. The ratio of red to total fluorescence indicated the proportion of dead cells in each sample, from which the percentage of dead cells was obtained. All data were analyzed using SPSS Version 20 (IBM Corp, Armonk, NY). The obtained data were not normally distributed, so nonparametric statistical analysis was performed using the generalized linear model repeated measure to analyze the proportion of dead cells. The Tukey test was used for comparisons between medicaments at 2 time intervals ($P < .05$).

RESULTS

A homogenous, dense, and intact live *E. faecalis* biofilm was observed in the dentin surfaces of the positive control group (Fig. 1*A1–4*). The negative control group

confirmed the absence of any external bacterial contamination.

A significantly greater proportion of dead cells was observed in the samples treated with 1 mg/mL TAP (90.39% and 99.41%) and the mixture of $Ca(OH)_2$ + AgNPs (90.85% and 98.49%) than those in the samples treated with $Ca(OH)_2$ (76.14% and 91.71%) and AgNPs (62.83% and 88.07%) at 2 and 4 weeks, respectively. The Tukey test showed a statistically significant difference in the proportion of dead bacteria among the groups ($P < .05$) at both observation periods, except for TAP and the mixture of $Ca(OH)_2$ + AgNPs ($P > .05$). At 4 weeks, all medicaments showed a greater proportion of dead cells compared with those at 2 weeks (Table 1).

At 4 weeks, confocal laser scanning microscopic images showed that *E. faecalis* biofilms were almost destroyed in the 1 mg/mL TAP and the mixture of $Ca(OH)_2$ + AgNPs groups (Fig. 1*B1–4 and C1–4*), whereas some residual biofilm structure remained on the dentin surface of the $Ca(OH)_2$ group (Fig. 1*D1–4*). Compared with 4 weeks, the integrity of the biofilm structure was not destroyed completely after treatment with AgNPs at 2 weeks (Fig. 1*E1–4*).

DISCUSSION

The elimination of bacterial biofilms is an essential aspect for the successful treatment of

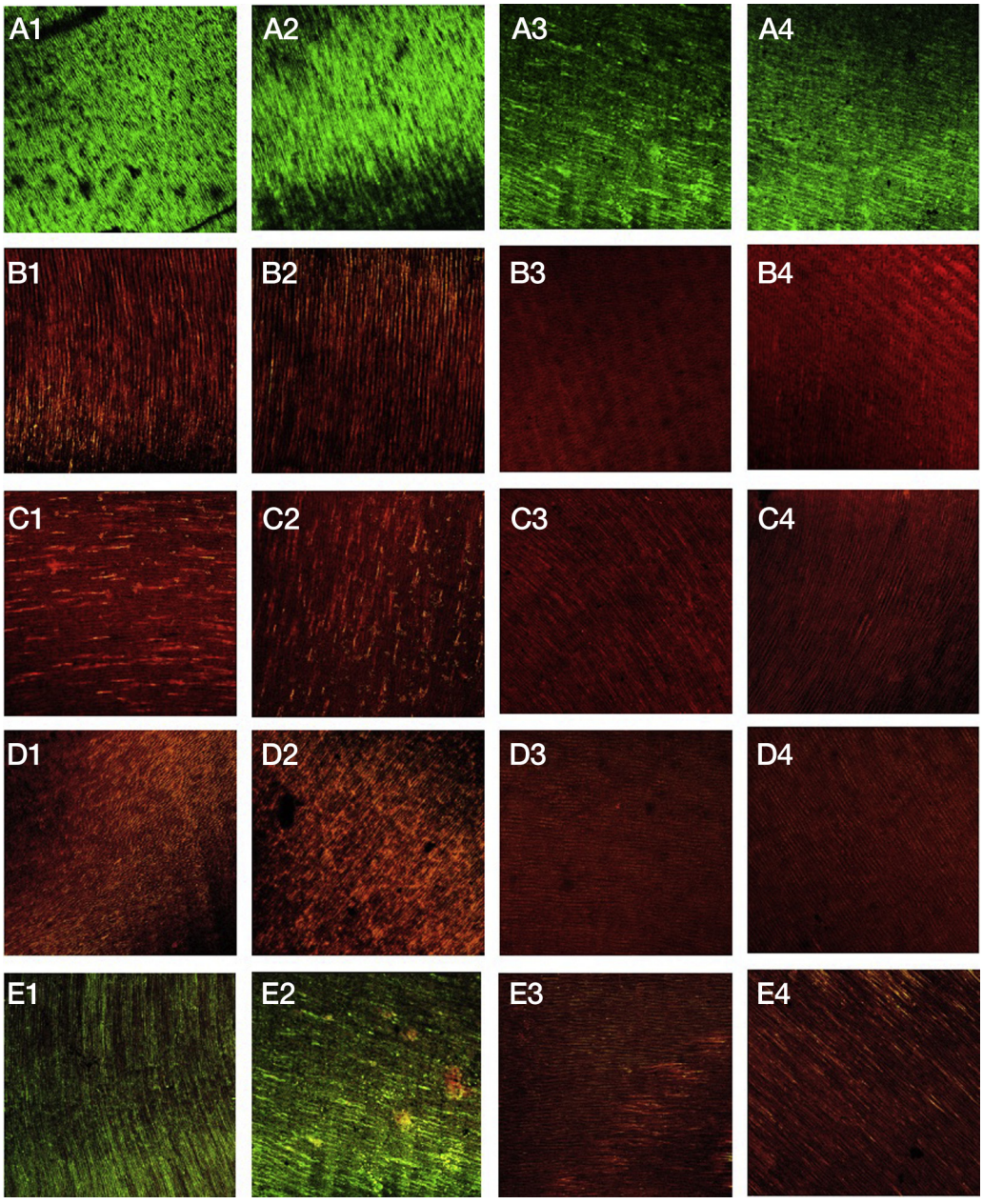

FIGURE 1 – (*A1–A4*) Confocal laser scanning microscopic images of 3-week-old *E. faecalis* infected dentin disks treated with saline *(A1–4)*. Dentin disks treated with TAP for (*B1* and *B2*) 2 weeks and (*B3* and *B4*) 4 weeks, $Ca(OH)_2$ + AgNPs for (*C1* and *C2*) 2 weeks and (*C3* and *C4*) 4 weeks, $Ca(OH)_2$ for (*D1* and *D2*) 2 weeks and (*D3* and *D4*) 4 weeks, and AgNPs for (*E1* and *E2*) 2 weeks and (*E3* and *E4*) 4 weeks. All images are shown at 25× magnification. The *green cells* represent cells with intact membranes, whereas the *red cells* are damaged or dead.

TABLE 1 - The Median and Range Values of Dead Cells (%) of the Positive Control Group and the Experimental Groups after Contact with the Experimental Medications for 2 and 4 Weeks

	Median (range, %)	
Positive control group	11.72 (8.09–14.94)	
Experimental groups	2 weeks	4 weeks
TAP	90.39 (87.23–92.08)[a]	99.41 (97.81–99.89)[a]
$Ca(OH)_2$ + AgNPs	90.85 (89.52–92.33)[a]	98.49 (97.13–99.72)[a]
$Ca(OH)_2$	76.14 (72.91–79.73)[b]	91.71 (89.01–93.82)[b]
AgNPs	62.83 (59.71–65.92)[c]	88.07 (84.26–90.63)[c]

AgNPs, silver nanoparticles; $Ca(OH)_2$, calcium hydroxide; TAP, triple antibiotic paste.
Different superscript letters in each column represent statistical significance ($P < .05$).

endodontic infections[1]. Therefore, every attempt should be made to eradicate microbes from the root canal system. In this study, the antibiofilm efficacy of 0.02% AgNPs in combination with $Ca(OH)_2$ against *E. faecalis* was evaluated using CLSM. The results of this study showed that the mixture of $Ca(OH)_2$ + 0.02% AgNPs revealed pronounced antibacterial efficacy against 3-week-old *E. faecalis* biofilms and surprisingly was not significantly different from 1 mg/mL TAP at the 2 time points. To the best of our knowledge, this is the first study that compares the antibiofilm efficacy of 1 mg/mL TAP with that of a mixture of $Ca(OH)_2$ + 0.02% AgNPs. It has been shown that silver has a high affinity for negatively charged molecules within bacterial cells, inactivates critical functions of bacterial cells, and subsequently prevents bacterial growth and biofilm formation[23]. The inhibitory effect of the mixture of $Ca(OH)_2$ + 0.02% AgNPs on *E. faecalis* biofilms is comparable with that found in several studies despite the difference in the evaluation method. Afkhami et al[16] showed that the mixture of $Ca(OH)_2$ + AgNPs resulted in a significant decrease in the number of colonies after 1 week of exposure. Furthermore, Zhang et al[17] found that the inhibitory effect of $Ca(OH)_2$ + AgNPs on the biofilms of *E. faecalis* was more significant than that of AgNPs alone and $Ca(OH)_2$ at 1 and 7 days using plate culture count and crystal violet biofilm assay methods. However, the exact mechanism of the antibacterial activity of the mixture of $Ca(OH)_2$ + AgNPs is yet to be fully understood.

The findings of this study showed that 1 mg/mL TAP attained marked antibiofilm efficacy that was enhanced over time and was significantly higher than that in the $Ca(OH)_2$ and 0.02% AgNPs groups. This finding is in agreement with results from previous studies[9,11,22]. The high antibiofilm effect of TAP has been attributed to the active antibiotic ingredients[24,25].

In the current study, AgNPs demonstrated the minimum antibiofilm efficacy compared with other medicaments. However, their efficacy was improved with time. This observation is in agreement with the findings of previous studies that indicated that the concentration of nanoparticles and their interaction duration with bacterial cells play a major role in bacterial eradication[26,27]. On the other hand, this observation contradicts the study of Wu et al[19], who demonstrated that the application of a 0.02% AgNP gel as a medicament resulted in a significant disruption of *E. faecalis* biofilm compared with $Ca(OH)_2$. A possible explanation for such an inconsistency could be related to the vehicle of AgNPs (gel vs suspension). The size of the AgNPs used in this study was 10 nm, and this size showed an effective bactericidal potential against both gram-positive and -negative bacteria[28,29] and a successful inhibition effect on multidrug-resistant organisms[30].

Despite the effectiveness of AgNPs in root canal disinfection, their possible adverse effects, including tooth discoloration and cytotoxicity, make them a controversial agent for *in vivo* usage, especially for long-term application as a root canal medicament. However, Afkhami et al[31] demonstrated that a mixture of AgNPs and $Ca(OH)_2$ caused no significant change in tooth color compared with the application of $Ca(OH)_2$ alone when used as intracanal medicaments. Although the effect of the tested medicaments on dentin discoloration was beyond the scope of this study, it is worth mentioning that no discoloration of dentin discs was noticed after exposure to the mixture of $Ca(OH)_2$ and AgNPs compared with TAP at the 2-week observation period (Fig. 2*A–C*). Previous studies have also confirmed that the cytotoxicity of AgNPs is concentration and size dependent[32,33]. Nevertheless, these potential adverse effects should be investigated in further studies before the clinical application of the mixture can be considered.

This study revealed a statistically significant difference in antibiofilm effectiveness between the 2- and 4-week periods among all medicaments ($P < .05$). Therefore, long-term contact between

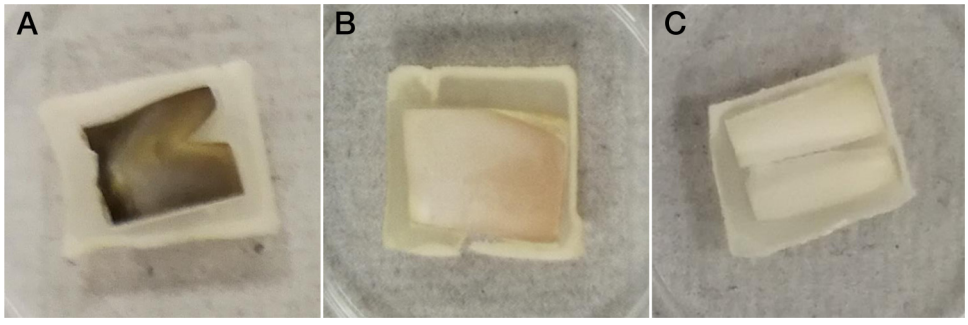

FIGURE 2 – Photographs of representative dentin disks treated with (*A*) 1 mg/mL TAP and (*B*) a mixture of $Ca(OH)_2$ + AgNPs for 2 weeks. Severe dentin staining was apparent in the dentin disk treated with 1 mg/mL TAP. There was no visible staining in the disk treated with a mixture of $Ca(OH)_2$ + AgNPs, and it was comparable with the positive control (*C*).

intracanal medicaments and bacterial cells was critical to achieve a significant antibiofilm effect.

In conclusion, within the limitations of this study, the mixture of $Ca(OH)_2$ + 0.02% AgNPs showed potent antibiofilm effects against *E. faecalis* and was comparable with the effects from treatment with 1 mg/mL TAP. Furthermore, long-term contact between intracanal medicaments and bacterial cells resulted in a marked destruction of the biofilm structure.

ACKNOWLEDGMENTS

The authors thank Dr Sayed Auda for his help during the preparation of the medicaments.

The authors thank Prince Naif Bin AbdulAziz Health Research Center for the financial support.

This study was performed in the Molecular and Cell Biology Laboratory (a core research facility of the King Saud University College of Dentistry in collaboration with the Prince Naif Bin AbdulAziz Health Research Center).

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

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