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FULL PAPER

Nanosilver gel as an endodontic alternative against *Enterococcus faecalis* in an *in vitro* root canal system in Mexican dental specimens

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SUMMARY

Nanotechnology has become a research area with promising results for technological innovation. Endodontics can benefit from this field of research by increasing the success rate of the treatment, which currently ranges between 86% and 98% and has varied very little over the years. One of the causes of endodontic treatment failure is based on the presence of *Enterococcus faecalis*. The objective of this investigation is to evaluate the antibacterial effect of a gel preparation containing silver nanoparticles (Ag-NP) against *E. faecalis* present in the walls of the root canal. 60 extracted human uniradicular teeth that were instrumented with Wave One Gold (Denstplay/USA) and subsequently contaminated with *Enterococcus faecalis*. For antibacterial evaluation, intra-canal conducting was placed, and several groups were formed:
a) Ag-NP 300 ug/MI gel;
b) Ag-NP 500 ug/MI gel;
c) Ca (OH) 2 (Ultracal from Ultradent/USA) and the control group.
They were incubated at 37°C and a sample was taken every 24 h for 7 days. The Ag-NP gel showed antimicrobial activity against *E. faecalis* with a value of minimum inhibitory concentration and minimum bactericidal concentration of 300 g/ml and 900 g/ml, respectively. When the Ag-NP gel was used as an intra-canal conducting drug in an in-vitro model, its antimicrobial effect at 300 g/ml and 500 g/ml was equivalent to the action of $Ca(OH)_2$.

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INTRODUCTION

Bacteria and their by-products are an important factor in the initiation, spread and persistence of pulp and periapical disease. For this reason, the elimination of microorganisms remaining in the root-canal system is critical. *E. faecalis* is correlated with persistent infections because it can subsist in hostile conditions with a small amount of oxygen and nutrients, and can form biofilm and survive in alkaline environments such as calcium hydroxide, which is the most commonly used intra-root-canal drug (McHugh *et al.,* 2004). *E. faecalis* infection in the root canal can evade biomechanical instrumentation because the colonization can remain at a depth of 300 microns inside the dentinal tubules and can cause persistent lesions (Love and Jenkinson 2002), with a prevalence of up to 77% of failed endodontic treatments (Sedgley *et al.,* 2005; Zhang *et al.,* 2015). Some alternative antimicrobial treatments for root disinfection have been achieved, including antimicrobial photodynamic therapy (aPDT) (Diogo *et al.,* 2019), chlorophyll derivatives (Diogo *et al.,* 2018), bacteriophages, drug delivery systems (cellulose, chitosan, alginate foam/fyclodextrinas, dlectrolyzed water, hydrogel, lipid delivery system, oil based emulsions,) metal-organic frameworks (Diogo *et al.,* 2019), and recently nanoparticles (Franci *et al.,* 2015), nanoparticles being an excellent antimicrobial agent in medical treatments, e.g., to cure diabetic foot ulcers (Almonaci- Hernández *et al.,* 2017). Silver nanoparticles have bactericidal activity to combat a wide range of microorganisms because they act on multiple cellular functions in the bacterial cell, interacting with the sulfhydryl groups of various proteins, as well as with DNA. These nanoparticles modify the permeability and respiration in the bacterial cell, the transport of nutrients is interrupted, and the bacterium stops DNA replication (Chernousova and Epple 2013; Franci *et al.,* 2015; Le Ouay and Stellacci 2015). A noncytotoxic nanosilver gel as medication in endodontics can improve treatments for persistent infections with *E. faecalis*. The objective of this study is to evaluate

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Ag-NP, root-canal system, nanosilver gel, *Enterococcus faecalis*.

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the antibacterial effect of a non-cytotoxic nanosilver gel against *E. faecalis* as an intra-root canal drug in extracted teeth from patients in Michoacan, Mexico.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Enterococcus faecalis was donated by the Microbiology Laboratory associated with the Advanced Graduate Program in Endodontics of the Autonomous University of San Luis Potosí (UASLP)/México. Bacteria were grown in Brain Heart Infusion BHI (Fluka, Sigma/USA) liquid medium at 37°C. The purity of the culture was verified by Gram stain. Inoculum was prepared for 24 h and microbial density (optical density, or OD) was adjusted to 0.05 at 600 nm. Then 1-mL aliquots of the inoculum were added to experimental sets, which were prepared in 125-mL Erlenmeyer flasks containing 20 mL of BHI liquid medium. Bacterial growth was monitored over 72 h by measuring the OD at 600 nm to determine growth kinetics at 37°C.

Silver nanoparticles

A 20% suspension of Ag-NP (1.2% of metallic silver content and 18.8% of polyvinylpyrrolidone as coating agent, Argovit™/Russia), with a particle size distribution between 3 and 50 nm (average of 35 nm). These Ag-NPs were donated by Nanoscience and Nanotechnology Center (CNyN), Universidad Autonoma de Mexico (UNAM)/Mexico.

Ag-NPs bactericidal assays

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Ag-NP in BHI (Fluka, Sigma/USA) liquid medium with *E. faecalis* were determined using serial dilutions of *E. faecalis* 1x108 CFU/mL and OD 0.1 at 600 nm from 4 h culture in BHI broth. All samples were made in triplicate including positive controls consisting of BHI broth without Ag-NP plus bacterial culture and negative controls that were media at different concentrations without inoculation. All samples were incubated for 24 hours at 37°C in the dark, without agitation. Bacterial growth was monitored by measuring the OD at 600 nm to determine the MIC. To evaluate the MBC, 50 µL aliquots of the dilutions where no growth was observed were inoculated on nutrient agar plates and incubated at 37°C for 48 hours.

Preparation of dentin specimens

60 human premolars of uniradicular extraction with complete apical development were collected for this study, under an ethics protocol approved by the Universidad Michoacana de San Nicolas de Hidalgo (UMSNH/Mexico) (add approval file number). The extracted teeth were kept in aqueous solution with liquid glycerine (1:1) until they were manipulated. The crowns were removed with a high-speed diamond disc and cooling (Komet, Brasseler/Alemania) to standardize the specimens at 15 mm. The remains of periodontal tissue of the specimens were removed by ultrasonic treatment. The permeability of the conduits was verified with files type K #10 (Dentsply/USA) and Wave One Gold files (Dentsply/USA). The specimens were placed in an ultrasonic bath (BRANSON 1510/China) with 5.25% NaClO for 10 minutes. Finally, the samples were rinsed in distilled water and placed in an ultrasonic bath for 10 minutes with EDTA 18% (Ultradent/USA). The outer surface of the root was sealed with 2 layers of nail polish (Sally Hansen/USA) and autoclaved. The teeth were then incubated in BHI broth for 24 hours at 37ºC to ensure that there had been no bacterial contamination.

Biofilm formation

The teeth were placed in sterile Eppendorf tubes with 1 mL of BHI broth containing 1x108 CFU/mL (OD 0.1 at 600 nm) of *E. faecalis*. The teeth were incubated at 37°C for 21 days. Every 24 h, 500 µL of fresh BHI medium was added. After 21 days, the teeth were rinsed with sterile distilled water and dried in sterile conditions.

To confirm biofilm formation, four roots were prepared and analyzed by scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS) (Genesys/USA).

Antibacterial activity of the intra-conductive Ag-NP gel against E. faecalis

Under sterile conditions, an Ag-NP gel was prepared with carbomer, with two final concentrations at 300 and 500 µg/mL corresponding to 18 µg/mL and 30 µg/mL of Ag0, respectively. The gel was packaged in dark hypodermic syringes. The infected teeth were medicated by applying the Ag-NP gel (300 and 500 µg/mL) with syringes and 0.35 mm capillary needles (Ultradent/USA) and sealing with a sterile teflon swab and provisit (Casa Idea/Mexico). As a reference, the $Ca(OH)_2$ used was the commercial product Ultracal XS® (Ultradent/USA), generally used to disinfect wounds. Experimental sets consisted of four groups of 15 teeth, including infected specimen, specimens plus Ultracal XS® (Ultradent/USA), specimens with 300 µg/mL Ag-NPs gel and specimens plus 500 µg/mL Ag-NPs gel. The four groups were incubated at 37°C for 7 days, taking samples (n=3) every 24 h. The sterile teflon swab was removed, and the Provisit (Casa Idea/Mexico) was irrigated with 10 mL of sterile distilled water using an Endo-Eze® needle in order to remove the intraconduct drug. To collect *E. faecalis* cells adhered to the root dentin, a file type #40 (Dentsply Maillefer) was introduced in the root canal. The cells and the file were introduced in an eppendorf tube with 900 µL of sterile distilled water and plated by the dilution method in nutrient agar, incubated at 37°C for 0, 24 and 48 hours to evaluate growth.

Statistical analysis

Basic statistical parameters and analyses of variance (ANOVA) were performed using the commercial statistical package JMP version 8 (SAS Spain). P values ≤0.05 were considered statistically significant.

RESULTS

Growth assays of E. faecalis

The growth of *E. faecalis* in BHI liquid media was evaluated to determine the logarithmic phase for testing the antibacterial action of Ag-NP. The lag phase was between 0 h and 2 h, followed by the logarithmic phase between 4h and 6h, and the stationary phase from 6h onwards. (*Figure 1A*). The minimum inhibitory concentration was determined by testing concentrations of Ag-NPs ranging from 0 to 1000 µg/mL (*Figure 1B*). At concentrations of 300 µg/mL and above, the growth of *E. faecalis* decreased by more than 90%, which suggests that this is the MIC for the bacteria. From 900 µg/mL onward, no growth was observed, indicating that 900 µg/mL is the minimum bac-

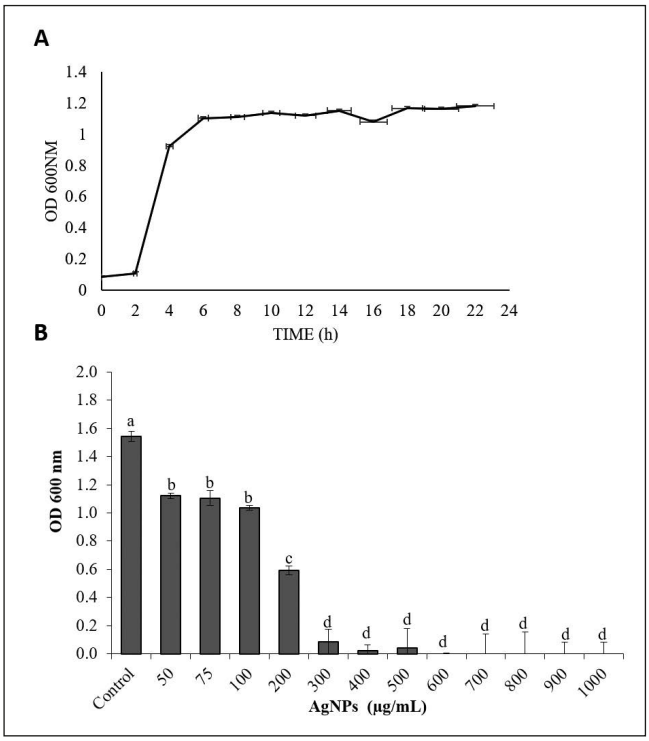

Figure 1 - *A) Growth curve of E. faecalis in liquid BHI at 37°C. Data are presented as average ± standard deviations (n=3). B) Growth of E. faecalis with different concentrations of Ag-NPs. Culture growth was performed in liquid BHI medium at 37°C plus 0-1000 µg/mLof Ag-NPs. Data are presented as average ± standard deviations (n=3), lowercase letters on top of the bars depict significant differences (p-value <0.05, ANOVA).*

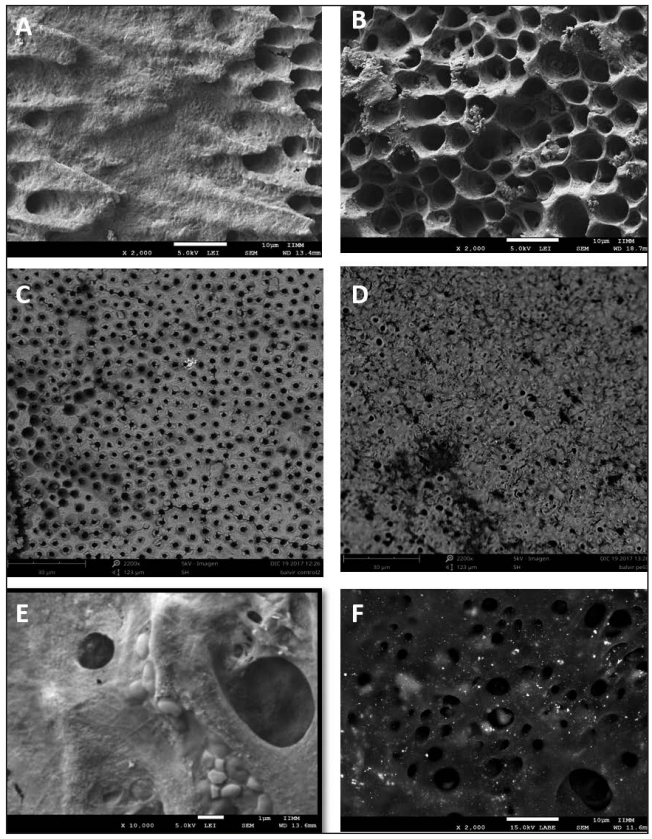

Figure 2 - *Representative SEM images of on dentin surface at 0h (A, C) and after 21 h incubation with Ag-NP gel (B, D). Panels A and C show permeable dentinal tubes without microorganisms, 2000X resolution by SEM. Panels B and D show biofilm of E. Faecalis formed on the surface of the root canal wall, (2200 X). E) E. faecalis in dentinal tissue. E. faecalis is identified on the basis of the morphology of the lobe in the dentinal tissue (10000x). F) Ag-NPs distribution in dentinal tissue after treatment. White dots indicate the distribution of Ag-NP at 500 ppm on root canal dentin (2000x) by EDS mapping.*

tericidal concentration, presenting a bacteriostatic effect from 300 µg/mL (data not shown).

Antibacterial activity of Ag-NP gel against E. faecalis

The biofilm formation of *E. faecalis* was verified by SEM. The micrographs of days 0 and 21 show the surface of the radicular wall of the dental organs (*Figure 2*). Panels A and C depict the surface of the wall of the root canal on day 0, showing that bacteria are absent on the open dentinal tubules. At day 21, a greater amount of biofilm can be observed the surface of the wall of the root canal compared to day 0 (*Figure 2B* and *2D*). Higher resolution micrographs (*Figure 2E* and *2F*) show records of rounded morphology (coccid) corresponding to *E. faecalis* on the surface of the radicular wall (*Figure 2E*) and the distribution of Ag-NPs in dentinal tissue (*Figure 2F*), respectively.

A clear antibacterial effect was observed with the various concentrations of Ag-NP at the different times. No statistically significant difference of the effect of Ag-NPs (500 µg/mL) was found in comparison with $Ca(OH)_2$ at 168 h (*Figure 3A*). The antibacterial effect of the Ag-NP gel (500 µg/mL) at 24 hours was greater compared to Ag-NP gel (300 µg/mL), and similar to $Ca(OH)_2$ at the same time point (*Figure 3*). In all the groups there was a difference in antimicrobial effect compared to the control group (*Figure 3A, B*). The antimicrobial effect of both Ag-NP gel and $Ca(OH)_2$ was maintained after 7 days of incubation (*Figure 3A, B).*

DISCUSSION

When analyzing the first clinical reports of the percentage of success in the treatment of endodontics (Seltzer *et al.,* 1963; Bender *et al.,* 1964; Oter *et al.,* 2018) and comparing them with recent reports (de Chevigny *et al.,* 2008; Ng *et al.,* 2011), we observed that the results are similar (low percentage of success in treatments). This situation is highly discouraging since in the last 50 years new technologies have been developed with the purpose of innovating and progressing in different areas; despite this, materials in which no progress has been made continue to be used in endodontics (Sjogren and Sundqvist 1987; Fouad 2017). *E. faecalis* is one of the microorganisms that inhabit the apical third of infected root canals, and there are even studies that indicate that it is the predominant bacterium in cases of failure in the treatment of ducts (Stuart *et al.,* 2006). For decades the first-choice intraconductive drug has been $Ca(OH)_2$, due to its capacity to the release of hydroxyl ions, which produce lethal effects on bacterial cells including protein denaturation and damage to bacterial and cytoplasmic membranes and DNA (Lana *et*

al., 2009). However, antimicrobial activity of $Ca(OH)_2$ can be limited by dentin, the exudate of the periapical area and the bacterial biofilm (Haapasalo *et al.,* 2000). There is evidence that biofilm formation represents a mechanism of resistance to $Ca(OH)_2$ for *E. faecalis,* by which bacteria involved in a dense matrix are protected by the action of the compound (Distel *et al.,* 2002). It was also reported that *E. faecalis* can produce more extracellular matrix and protein in response to a high pH or an inhibitory dose of antimicrobials (Wilson *et al.,* 2015). Ag-NPs have the advantage of decreasing antimicrobial resistance since their mechanism of action has multiple targets in the bacterial cell (Franci *et al.,* 2015). To carry out the present study, Ag-NP of the trademark "Argovit™" (Russia) was used to prepare a nanosilver gel; its minimum inhibitory concentration against *E. faecalis* was determined as 300 µg/mL, and its minimum bactericidal concentration as 900 µg/mL (*Figure 1B*). Antimicrobial activity of Ag-NP gel was evident at 300-500 µg/mL concentration in an *in vitro* intraconductive medication model (*Figure 3A* and *3B*). Similar results were reported using different substrates such as dentin and bovine bone, hydroxyapatite and gutta-percha, with development of biofilm at 14 and 21 days, as observed by staining methods and confocal microscopy, and the greatest microbial development in hydroxyapatite (Guerreiro-Tanomaru *et al.,* 2013). Some SEM-based studies reported the formation of biofilm in human teeth after 14 days of incubation (Bulacio Mde *et al.,* 2015), as well as after 4 weeks (Wu *et al.,* 2014). In the present study, the antimicrobial action of Ag-NP gel was confirmed at both the concentrations studied; however, when used at 500 µg/mL the Ag-NP gel was more effective, showing an antimicrobial activity similar to $Ca(OH)_2$ (Ultracal XS® Ultradent/USA) at 24 and 168 h (*Figure 3A*). Further experiments were performed to determine Ag-NP bactericidal (98-100%) concentration against *E. faecalis* (*Figure 3B*). Differences in MIC values obtained in liquid cultures and the dentin model are likely due to the fact that, while in liquid cultures bacteria are in the planktonic state, in the extracted dental organs they form biofilms.

When used as an intraconductive medicine, $Ca(OH)_2$ leaves remnants that are attached to the walls of the root canal. It has been verified that these remnants of calcium hydroxide interfere with the penetration capacity in the dentin tubules of some sealants, affecting the quality of the filling and causing bacterial filtration later (Ma *et al.,* 2015). There is little probability that microorganisms develop resistance against Ag-NP because they interact with multiple targets in the microbial cell, such as the cell membrane, enzymes and plasmids, thus lowering the chances for the bacteria to acquire resistance (Chernousova and Epple 2013; Franci *et al.,* 2015). Microorganisms and their products, including endotoxins, cause severe inflammatory reactions in periapical tissues. The location of the biofilm within the root canal system of the tooth makes it inaccessible to the host's immune system, while the tubules provide physical protection to topically applied medications (Zhang *et al.,* 2016). In this regard, another of the advantages offered by the Ag-NP is their size, i.e., they can be moved into regions of the root canal that are not easily accessible and penetrate the dentinal tubules, using lasers or shaking by ultrasound, thus achieving greater efficacy against *E. faecalis* (Paiva *et al.,* 2012). Several investigations have explored the possibility of potentiating the antibacterial effect of Ag-NP against *E. faecalis* in the root canal by combining them with commercial calcium hydroxide; this interaction could raise the antimicrobial activity (synergistic effect) or alternatively lower it (antagonistic effect) (Javidi *et al.,* 2014; Alabdulmohsen and Saad 2017). Some reports indicate that alternative intraconductive antimicrobial treatments can be used, e. g., photodynamic antimicrobial chemotherapy PTD showed to be more efficient than NaOCl (0.5-6% concentration) (Diogo *et al.,* 2015). Therefore, when Ag-NP are combined with other antimicrobial agents, these interactions must be optimized, so that efficacy is improved rather than reduced (Ma *et al.,* 2015). In this work, the effect of non-cytotoxic Ag-NP gel against *E. faecalis* was detectable even at low concentrations on the root canal system (*Figure 3B*), possibly due to the good distribution of the Ag-NPs in the teeth (*Figure 2D*). Although there are still concerns about the possible toxicity of the particles at these concentrations, new techniques are being developed for their preparation with the aim of making them less harmful to humans and the environment. Ag-NP gel have low toxicity based on *in vitro* studies using cell lines. The Ag-NPs (Argovit™/Russia) have been approved by international organizations (Borrego *et al.,* 2016). The formulation of these nanoparticles has no genotoxic effects in cell lines at concentrations close to IC50 (3.5 µg/mL of metallic silver) (Juarez-Moreno *et al.,* 2017). However, for

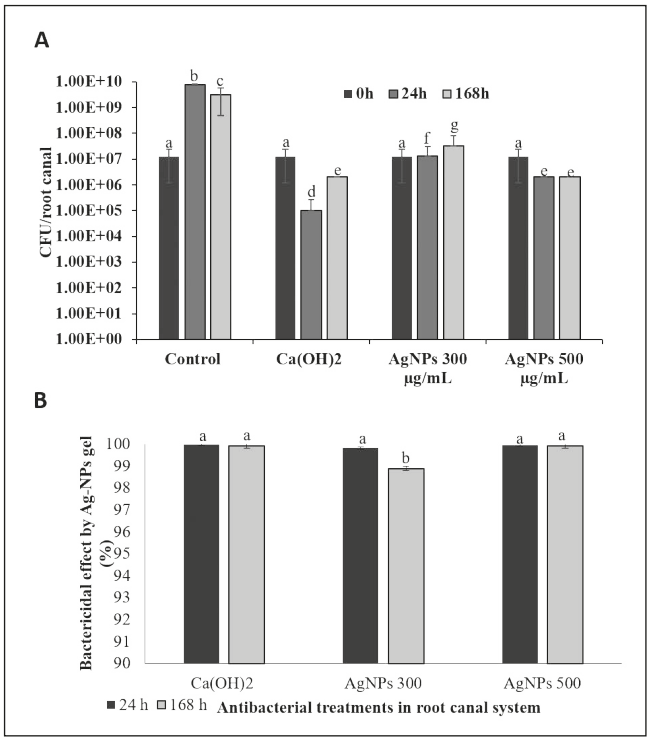

Figure 3 - A) Antibacterial effect of Ag-NP gel and $Ca(OH)_2$ against *E. faecalis*. Bars represent the number of CFU of *E. faecalis* per root canal at 0h, 24 h and 168h in each treatment condition. Data are presented as average ± standard deviations (n=3), lowercase letters on top of the bars depict significant differences (p-value <0.05, ANOVA). B) Percentage of reduction of *E. faecalis* compared to control after 24h and 168h treatment with Ag-NP gel and $Ca(OH)_2$. Data are presented as average ± standard deviations (n=3), lowercase letters on top of the bars depict significant differences (p-value <0.05, ANOVA).

a clinical use, preclinical studies in animals and clinical studies in humans are needed to evaluate the toxicological profile in greater detail.

Also, antimicrobial distribution during application is important to obtain an adequate root canal disinfection. On dentin discs, the use of chlorophyll derivative (chlorophyll-based photosensitizer (PS) Zn(II)chlorin e6 methyl ester (Zn(II)e6Me) performed better in biofilm removal (59.1%) than FotoSan® agent (commercial Toluidine Blue O formulation) (57.5%), but with lower efficacy than NaOCl (68.1%). Conversely, at the root block, the chlorophyll derivate (79.7%) presents better antimicrobial efficacy than NaOCl (75.5%) (Diogo *et al.,* 2018).

CONCLUSION

Antimicrobial activity of Ag-NP gel against *Enterococcus faecalis* is equivalent to Ultracal XS® in an intra-conductive model. Due to their small size, nanoparticles can penetrate to regions which may harbour bacteria and that are inaccessible to other medicines.

Silver nanoparticles have an advantage because the development of microbial resistance against them is unlikely, due to their mechanism of action. These nanoparticles could be used to give antimicrobial properties to endodontic materials, allowing them to be used in the different stages of therapy.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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