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

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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 dis-infection 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, electrolyzed 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 excel- lent 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 endo-dontics can improve treatments for persistent infections with E. faecalis. The objective of this study is to evaluate
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 (CyN), Universidad Autónoma 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. 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/México) (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 37°C 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 carborner, 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 packed 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®, specimens plus 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 Provitis (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-
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 on the surface 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
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itself is 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 intracanicular medicament 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 scanning 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 (Ultrapac X5® 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 intracanicular 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 intracanicular 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).
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.

References