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Surface Modification of Endodontic Files with Silver Nanoparticles for Antimicrobial Purposes

Modificación de la Superficie de Limas Endodónticas con Nanopartículas de Plata para Fines Antimicrobianos

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ABSTRACT: The success of endodontic treatments is related to the neutralization of bacteria and their toxins, inside and outside the root canal system of a tooth. Usually this is achieved, under ideal conditions, by means of a chemical-mechanical action (endodontic files and irrigation with appropriate solutions). Silver nanoparticles (AgNPs) are well-known for their antibacterial properties, and, for this reason, the aim of this work was to modify the surface of Nickel-Titanium (NiTi) endodontic files with AgNPs, to confer them antimicrobial ability and avoid infections. For this purpose, the preparation and characterization of stable colloids of AgNPs were carried out. Ten NiTi files for endodontics (brand K3 XF, Sybron-Endo Keer) were divided into 2 groups; 5 for group 1 (control, files as received) and 5 for group 2 (files previously coated with AgNPs). Both groups were subjected to biofilm formation using a culture composed of *Candida albicans* and *Enterococcus faecalis*, in an anaerobic environment. Endodontic files were successfully modified with silver nanoparticles, and, after exposure under anaerobic conditions to the selected microorganisms. Scanning electron microscopy revealed the proliferation of microorganisms on the surface of the control files. In contrast, the modified and coated files showed no biofilm formation, and the bacteria exhibited changes in cell wall morphology, likely associated with wall rupture and subsequent cell death. The coating of silver nanoparticles on the surface of the files shows to change the morphology of the bacterial wall, causing death and thus inhibition in formation of microbiological biofilms. Since reactive oxygen species (ROS) are unlikely to be present under the anaerobic conditions used, the microbicidal effect of the nanoparticles indicates that ROS-promoted oxidative stress is not an important mechanism of action, and inactivation of thiol-containing proteins could be a more important mechanism.

KEY WORDS: antimicrobial activity, *Candida albicans*, endodontic files, *Enterococcus faecalis*, silver nanoparticles.

INTRODUCTION

Endodontics is the branch of dentistry that studies the diseases of the teeth pulp and the healing techniques. The success of the endodontic treatment depends of the appropriate cleaning and disinfection of the canal system. Treatments consist of removal of vital or necrotic pulp tissue, with or without bacterial load and generated toxins. This is usually accomplished using mechanical action (files) in the main duct, and through irrigation (chemical way) with different types of solutions. However, success is sometimes difficult achieve due to the anatomical complexity of the root canal system (Martins Justo *et al*., 2014). If the canal system is contaminated, endodontic fails because of the remaining bacteria, that, after a certain period of time and under the right conditions, reproduce. To achieve more effectiveness in the microorganism removal, there were attempts in modifying the instruments designs, changing and modifying the instrumentation techniques, and using different irrigants to remove organic tissue and dentinal mud, and achieve disinfection. Also, the use of irrigation techniques with manual, sonic and / or ultrasonic intra-conductive

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movement, or laser application, have been proposed (Prada *et al*., 2019).

A main problem is that disinfectants, in order to be effective, must be in direct contact with the surface of the bacteria, and this is particularly complicated in the apical portion of the root canal, and even more if this is narrow, since the irrigator usually does not reach these areas due to various factors such as surface tension, dentin mud and air bubbles. Besides, a vapor bubble could form by the mixture of ammonium and carbon dioxide generated by the interaction of sodium hypochlorite with organic material. The needles for irrigation usually do not reach the last 2 - 3 mm of the root apex, and it is even more difficult if there is a curvature, avoiding the eviction of the bubble and the dentinal mud. On many other occasions, the mud generated by the same instrumentation also remains in this area of the apical third, and, together with the multiple bacteria in an infected tooth and / or the multivariate biofilm, hinders the contact between the antimicrobial and the bacteria, avoiding a proper cleaning and disinfection of the root canal (Siqueira, 2001).

The antibacterial effect of silver ions has been known for a long time (Wong & Liu, 2010). Nevertheless, the action mechanisms of silver nanoparticles (AgNPs) as antimicrobial agents is still a subject of intensive scientific debate, particularly on the question of the specific role of the particles or the indirect effect by the slow release of silver ions from the oxidized particle surface. Some authors suggest that dissolved Ag+ accounts for most if not all of AgNPs' toxicity, and that the AgNPs could act as a source of ionic Ag+, mainly under aerobic conditions (Fewtrell *et al*., 2017; Burdu?el *et al*., 2018).

Fabrega *et al*. (2009) showed that both AgNPs and Ag+ contribute to the antibacterial activity, but the specific mechanism of action of AgNPs is still not fully known as the contribution of each of them is difficult to elucidate, as both are present during exposure, mainly under aerobic conditions. The use of ligands to complex Ag+ and minimize their effect was attempted, in order to isolate the effect of AgNPs, but ignoring the possible interaction of the AgNPs surface with the ligands, where the toxicity is lowered after sorption of ligands onto the particles surface.

In the dental market there are no files made of metallic silver, nowadays, commercial brands commonly use alloys of nickel and titanium, but these chemical elements do not possess antibacterial properties. Therefore, Nickel-Titanium (NiTi) files coated with silver nanoparticles (AgNPs) could be more effective than conventional files in achieving complete disinfection of the root canal. On the other hand, one of the most common accidents during the endodontic treatment is the fracture and consequent permanence of the file inside the root canal, since it is often impossible to remove the broken file (Spili *et al*., 2005; Shen *et al*., 2009). If the endodontic files were previously modified with AgNPs, it is to assume, that the possibility of treatment failure due to the proliferation of microorganisms could be minimized due to the presence of the particles. Currently, there are no reports regarding the coating of NiTi files for endodontic treatment with of AgNPs, and therefore, the aims of this study were to perform the surface modification of endodontic NiTi files with AgNPs and demonstrate its antimicrobial ability in anaerobic media. We hope also to contribute with valuable information to the knowledge about the antibacterial properties and action mechanisms of silver nanoparticles in anaerobical conditions.

MATERIAL AND METHOD

Synthesis of silver nanoparticles (AgNPs)

All chemicals were Sigma-Aldrich reagent and laboratory grade. Silver nanoparticles were prepared by a modified synthesis protocol (Tashdjian *et al*., 2013), where 4 mL of a sodium borohydride alkaline solution (0.0076 g BH_4^-/mL in 0.01 M NaOH) were added dropwise and under sonication to 4 mL of a silver nitrate solution (0.12 g/mL), both prepared from aqueous solutions (0.01 g/mL) of polyvinylpyrrolidone (PVP, ~40,000 g/mol). For some experiments and the particles characterization, the nanoparticles were precipitated using acetone, redispersed in water and ethanol (50:50 v/v), precipitated again in acetone and redispersed in the alcoholic solution 5 times, and finally redispersed in water.

Characterization of AgNPs

The silver colloids were characterized optically and structurally. Optical characterization was carried out using an UV/VIS Spectrometer Lambda 35 (Perkin Elmer Inc.) in absorbance mode, in the range of 200-800 nm. The measurements were carried out using an aqueous dilution of 5 L of the as-prepared AgNPs colloids in 2 mL of deionized water. The pH was measured with a pH meter (Thermo Electron Corp. Orion 420ª +). The morphology and chemical

characterization of the AgNPs was performed using a Transmission Electron Microscope (TEM JEOL EM-21010). For this purpose, 20 L of the washed AgNPs were placed in 1 mL of isopropyl alcohol, dispersed in ultrasound bath for 1 minute, and mounted on a copper micro grid.

Modification of the file surface

In order to find the appropriate dilution of silver nanoparticles for the modification of the endodontic files, looking for best adherence to the file, and good coverage without particles agglomeration along the surface, various aqueous dilutions of the as-prepared AgNPs colloids were prepared (dilutions 1:5, 1:10, 1:15 and 1:20). Also, dilutions of the colloids obtained after washing of the AgNPs were used for files modification.

NiTi files from K3 XF brand (Sybron-Endo Keer) were chosen for the study. The files were extracted from the original package, washed in a water jet and with a brush, placed in an ultrasound bath for 10 min., dried and sterilized in an autoclave (121 °C for 30 min). After sterilization, under a laminar flow hood, they were immersed into an acetone bath, dried at room temperature, bathed in ethyl alcohol and subsequently dried. At random, the files were divided into 2 study groups; 6 for Group 1 (control, files not coated with AgNPs), and 6 for experimental Group 2 (files coated with AgNPs). Coating was achieved through immersion of the files into the AgNPs colloids at the selected dilutions, at 25 °C for 12 h in darkness. To observe the AgNPs on the surface of the file, and to confirm the presence of silver, a JEOL JSM-6510 Scanning Electron Microscope (SEM) with EDX analysis (Oxford X-act) was used.

Antibacterial tests

To test the antibacterial action of the surface-modified files, the instruments of both study groups were immersed in culture broth (Heart Brain Infusion and Sabouraud Dextrose Broth), after inoculation with *Enterococcus faecalis* and *Candida albicans*. The microorganisms were isolated from patients in the clinic of the Master of Endodontics (UASLP), where the bacterial suspensions were adjusted to a turbidity of 1.5 X 10-8 Colony Forming Units/mL (CFU/mL), standardized to the McFarland scale. Subsequently, the files in each study group were taken to an anaerobic chamber (MAIC/2000 COY Laboratory). Both groups were kept under these conditions for 21 days to allow for the formation of a mature biofilm, replacing 80% of the broth (fresh sterile culture medium) every 2 days, and confirming with a Gram stain smear the bacterial structures by observation under a stereoscopic microscope. After the 21 days, the files were removed, rinsed with sterile deionized water and subjected to a fixation with 2 % glutaraldehyde and 1% Alcian blue, followed by dehydration with alcohol at different concentrations. The control and experimental files were observed by SEM at magnification of 5,000x. Also, to observe the wall characteristics of the bacteria on the files modified with AgNPs, an Electronic Scanning Ambient Microscope (ESAM, Quanta FEG 250, IPICyT) was used. The observations were performed always at the level of the second groove of the tip of the file towards the handle of the same, in its most concave part, this to standardize the visualization of the zone in all the instruments.

RESULTS

Characterization of AgNPs

Stable concentrated silver colloids were obtained, and optically characterized, presenting a peak corresponding to the silver surface plasmon resonance band (SPRB) at 398 nm (Fig. 1a), a typical value for nanometric silver (Tashdjian *et al*., 2013). The SPRB remains without variation even after 5 days of storage in the fridge. The peak maximum exhibits a blue shift compared to the typical value for large particles (~440 nm, Zhou *et al*., 2011). The absorption spectrum showed a narrow peak, giving a hint about the narrow size distribution and low particles agglomeration. The AgNPs observed by TEM showed (Fig. 1c) a spheroidal morphology, and bimodal size distribution, typical for silver nanoparticles synthesized by chemical methods (Green *et al*., 2002), and diameters ranging from 2 to 20 nm. The chemical analysis of the material through EDX indicated the presence of silver, and copper from the grid (Fig. 1b).

Files modification

Figure 2a shows SEM images of a control file (without AgNPs coverage, at x40, x500, x2000, x5000, and x10,000), where the typical irregular surface of the stretch marks of the file can be observed. Figure 3 shows SEM images of modified files, where the dispersion and distribution of the silver nanoparticles along the surface of the instrument can be observed. For modification, the NiTi endodontic files were immersed in several dilutions (most of them not shown) of the as-prepared and the washed AgNPs, but

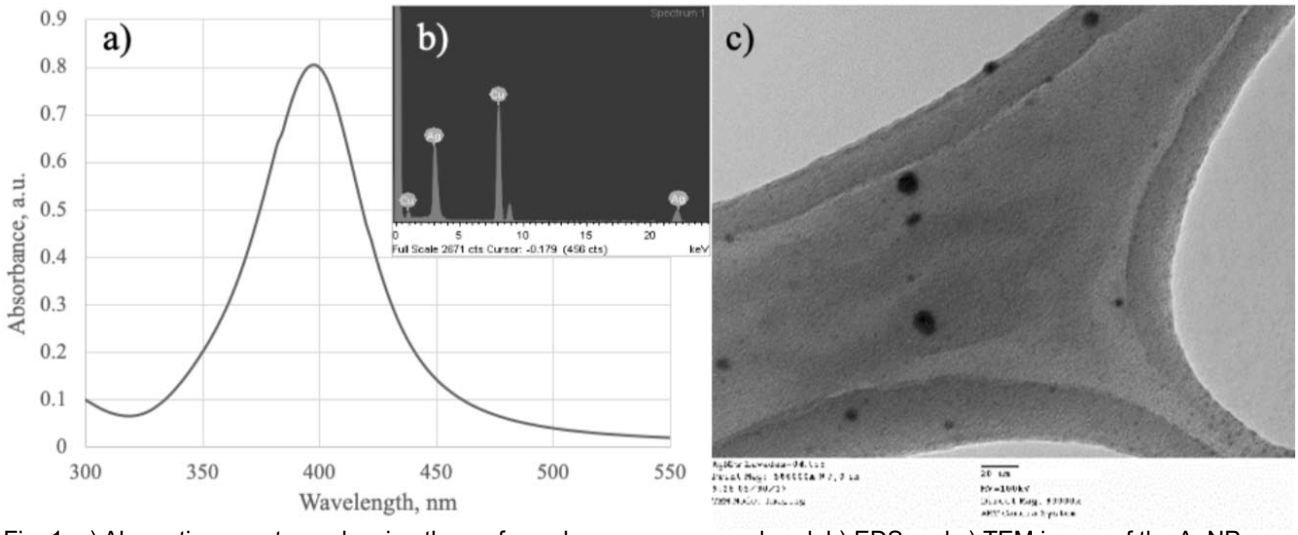

Fig. 1. a) Absorption spectrum showing the surface plasmonresonance band, b) EDS and c) TEM image of the AgNPs.

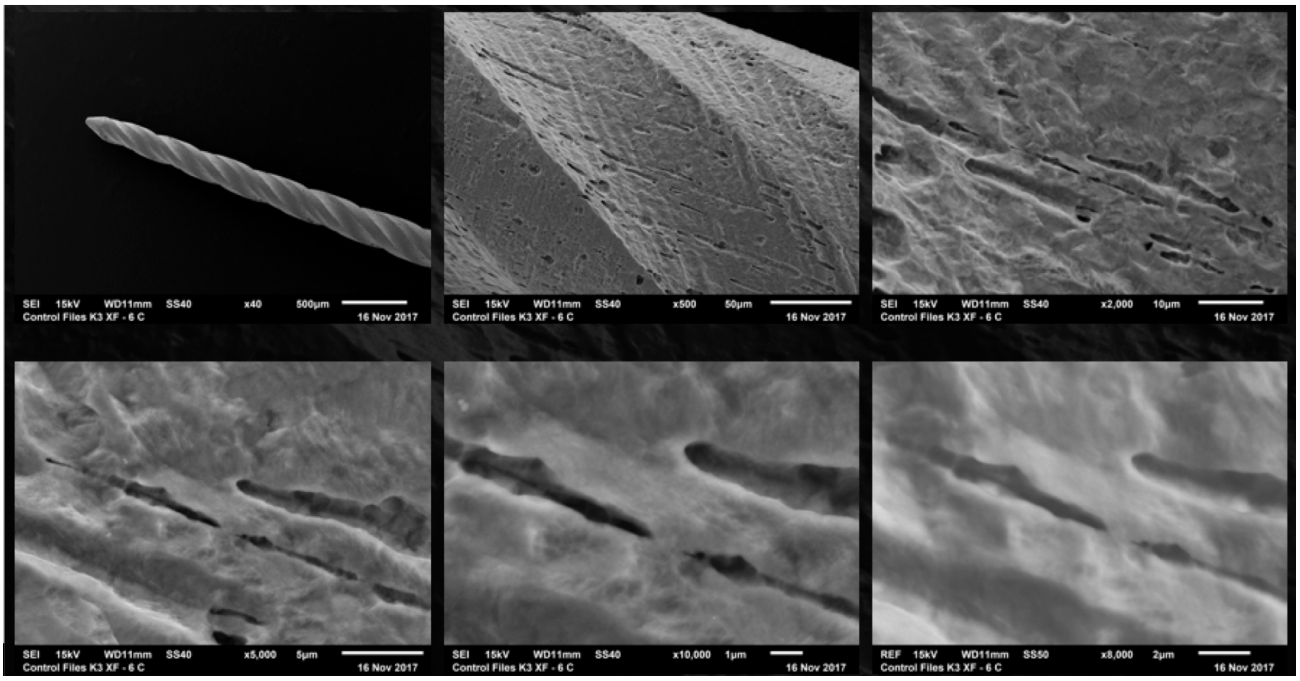

Fig. 2. SEM images at x40, x500, x2000, x5000, x10,000 in secondary detector mode (SEI) and at x8,000 using the backscattering detector (BSE).

particularly the 1:20 dilution of the as-prepared nanoparticles allowed the achieving of adherence to the file and low agglomeration (Fig. 3b). Figures 3c and 3d show files modified using washed nanoparticles. The immersion process produces files with apparently a good adherence to the files, but much more aggregation, as shown in the Figures.

Antibacterial properties

Figure 4a shows a Gram stain smear of the bacterial structures observed under a stereoscopic microscope. All the files were tested during the antibacterial studies, and particularly the antibacterial ability of the AgNPs-modified files was evaluated. To test the capability of the modified files to inhibit general growth of microorganism in the culture broth, the files were immersed and preserved in the broth for 80 days, where 80% of the broth was changed every 2 days and replaced for fresh one. Figure 4b shows a contaminated control file at5000x, it can be observed that the file is covered by a large number of microorganisms, with the development of bands of hyphae that form the biofilm layer.

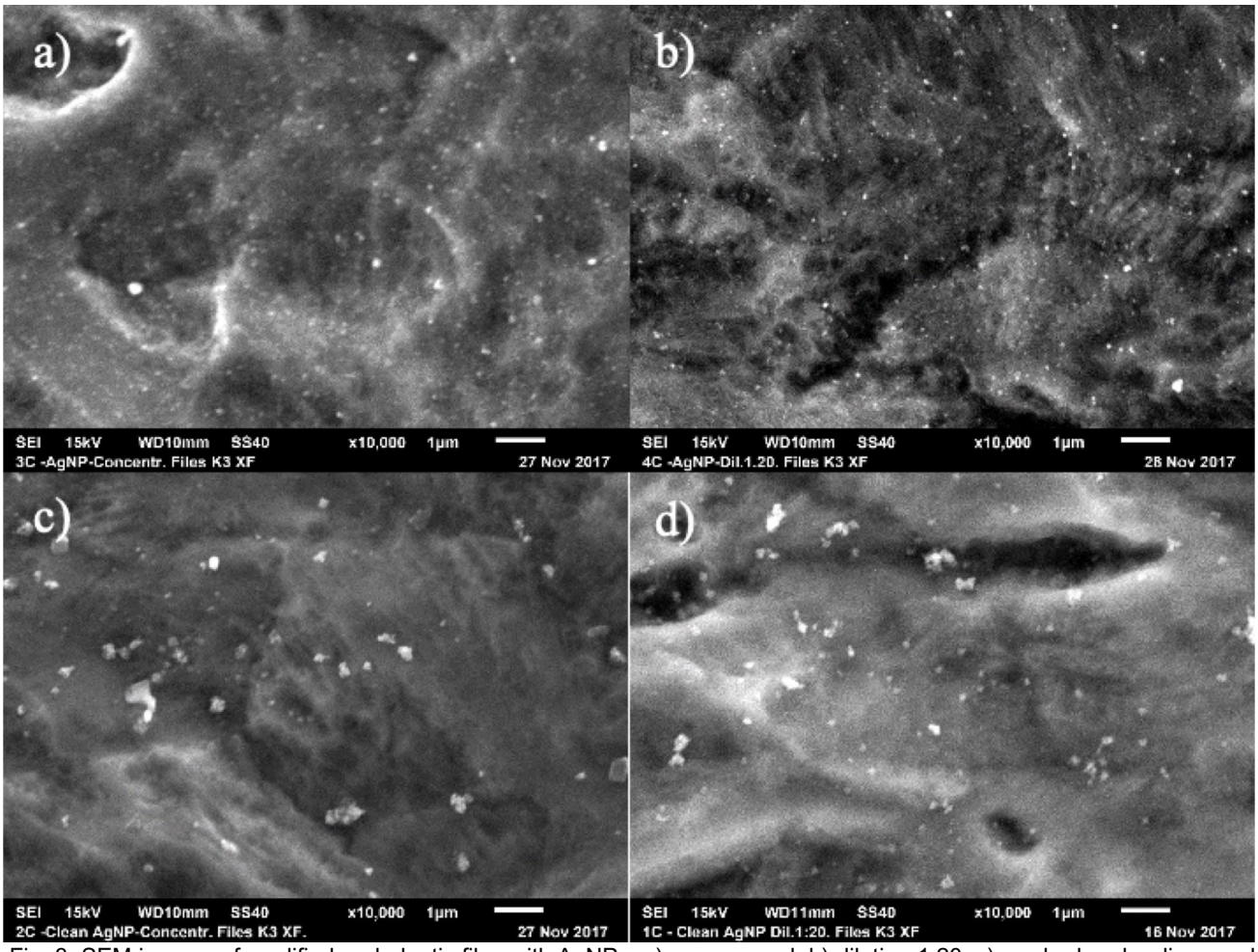

Fig. 3. SEM images of modified endodontic files with AgNPs, a) as-prepared, b) dilution 1:20, c) washed and redispersed particles, d) washed and redispersed particles, dilution 1:20.

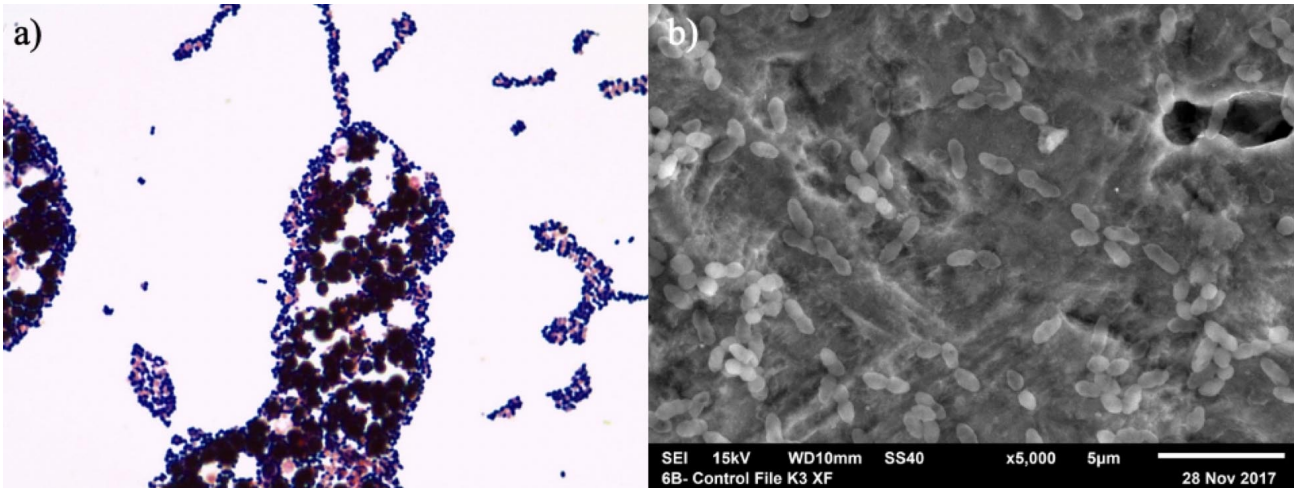

Fig. 4. Growth of *Enterococcus faecalis* and *Candida albicans.*

Figure 5 shows contaminated AgNPs-coated files, the presence of microorganisms was observed but not the presence of bacterial biofilm. More bacteria are observed on the file modified with the as-prepared concentrated colloid, probably because the presence of the stabilizer PVP promotes the proliferation of microorganism, as the organic compound could act as a source of organic carbon. Using the diluted as-prepared colloids to modify the files reduces the carbon supply, and the same applies when the washed nanoparticles, where the excess of PVP is removed, are used to confer the files the antibacterial properties. At higher magnifications (Fig. 6, 25,000x), the alteration of the characteristic form of *Enterococcus* was observed, which could be interpreted as the rupture of the bacterial cell wall, giving a hint of the cell death.

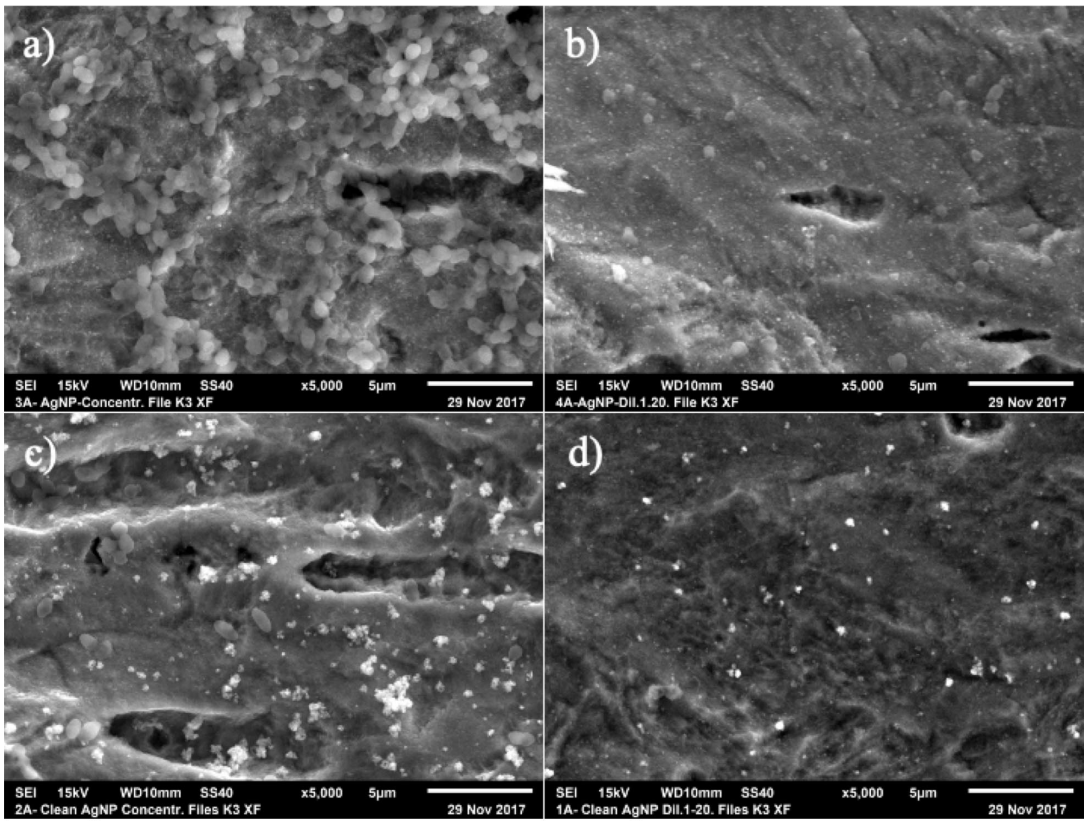

Fig. 5. SE Micrographs at x5,000, a) AgNPs as-prepared, where bacteria can be observed; b) dilution 1:20, c) washed and redispersed particles, d) washed and redispersed particles, dilution 1:20, where presence of a reduced number of bacteria and absence of biofilm formation is noticeable.

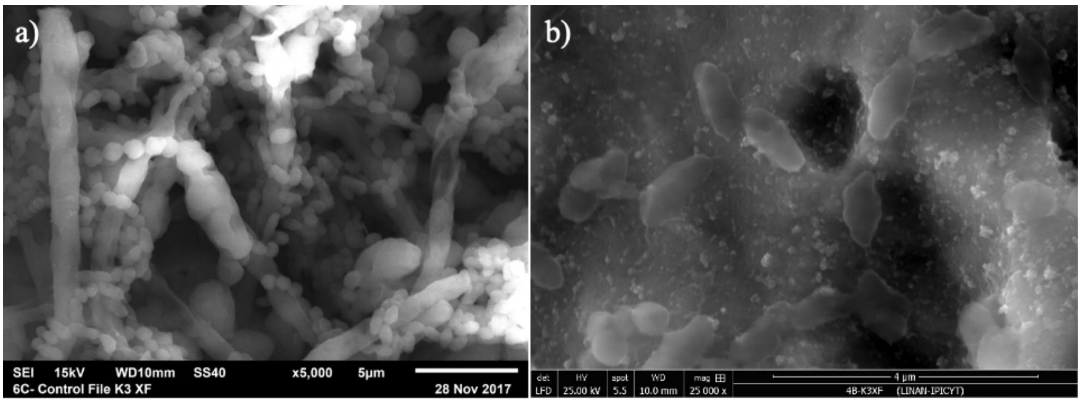

Fig. 6. SEM image, a) at x5,000, with bacteria, hyphae and biofilm formation, b) SEM image at higher magnification (25,000x), with alteration of bacterial wall on file with silver nanoparticles.

DISCUSSION

The main objective of endodontic treatments is to reduce the bacterial load through irrigation and biomechanical preparation using endodontic files. Several investigations show that *Enterococcus faecalis* and *Candida albicans* are microorganisms that are considered opportunistic pathogens involved in the persistence of infection, influencing the prognosis of treatment failure (Kayaoglu & Ørstavik, 2004). In addition, they are resistant to the action of numerous irrigating substances, and their permanence in the root canal is associated, on the one hand, to the ability to survive in hostile environments and, on the other, to the formation of a biofilm, together with other microorganisms (Kayaoglu & Ørstavik, 2004; Siqueira & Sen, 2004). Endodontic microbiological investigations have shown that the prevalence of Enterococcus ranges from 67% to 77% (Vidana *et al*., 2011), and fungis can be isolated from 1 - 17 % in persistent infections of the root canal (Sen *et al*., 2003). Several authors have demonstrated the bactericidal effect of silver nanoparticles on *Enterococcus faecalis*, as well as against *Candida albicans*, although the results on this microorganism have not yet been conclusive (Chandra *et al*., 2017).

Endodontic files are thin and narrow instruments, with intricated topography. They are non-bactericidal, and can become contaminated with various microorganisms (Roth *et al*., 2006), debris containing necrotic tissue, dentin sludge and blood by-products. Re-use and improper cleaning of these instruments facilitate the exchange of contaminants to other patients, acting as a vector for the transmission of pathogenic microorganisms (Romero *et al*., 2015), so that it has been recommended to immerse the limes in 5.25 % sodium hypochlorite for sterilization (Roth *et al*., 2006). We hoped in this research work, the modification of the surface of the endodontic file with silver nanoparticles would provide a bactericidal effect against the most frequent microorganisms in the infected root canal. Furthermore, it has been reported that the files often fracture inside the duct during biomechanical preparation, which is a potentially serious setback, complicating and compromising the result of endodontic treatment, especially if the fragment hinders the access to the apical part of the infected canal (Spili *et al*., 2005; Shen *et al*., 2009). So, having a file covered with silver nanoparticles could help prevent both the formation of biofilms on its surface and infection within the root canal, avoiding the subsequent treatment failure. Other authors made modifications to titanium brackets and implants with silver nanoparticles through techniques such as electroplating and the sol-gel method, respectively (Secinti *et al*., 2011; Arash *et al*., 2016), however, in the present study only the immersion of the instrument in the solution was attempted, with the aim that some of the silver nanoparticles deposited on the surface of the file can be released when the biomechanical work, for what the files were designed, is performed. In addition, this method would be a much cheaper and easier way to reproduce for the dentist in comparison to those mentioned.

In our study, the synthesis of AgNPs was performed based on the synthesis proposed by Tashdjian *et al*. (2013), with some modifications, achieving a mean size of 10 nm, narrow size distribution and spheroidal shape, which would ensure a significant antibacterial efficacy. It has been demonstrated, that the antibacterial properties of AgNPs depends greatly from the particle size (González-Luna *et al*., 2016). The surface modification of the endodontic files was performed by immersion in as-prepared and diluted dispersions of silver nanoparticles. After the SEM characterization, it was decided to immerse the files in dilution 1:20 of the as-prepared silver nanoparticles dispersion, since this dilution provided the best distribution along the surface of the file.

The SEM images of the files after the antimicrobial tests showed that there was inhibition in the modified files in regard to bacterial and fungal biofilm formation, as well as a clear rupture of the bacteria wall, on the contrary, the observations made for the control files, show the presence of bacteria with an integral wall and bands of hyphae that form the biofilm layer. These results coincide with those found by Secinti *et al*. (2011); they made silver nanoparticle coatings on titanium implants, demonstrating that this coating helped to prevent biofilm formation. Likewise, Arash *et al*. (2016) found that coated brackets with silver nanoparticles, achieving an antibacterial effect against S. mutans for up to 30 days.

Ionized silver is highly reactive, it binds to tissue proteins through various mechanisms that include binding to thiol groups, due to the high affinity of silver for the sulfur-containing ligands, as predicted by the Hard-Soft Acid-Bases Theory, first proposed by Pearson (1963), and brings structural changes in the bacterial cell wall and the nuclear membrane that leads

to cell distortion, lysis and death (Lalley *et al*., 2014). It also binds to DNA and RNA by denaturation and inhibits bacterial replication (Rai *et al*., 2009), and inhibits the enzymes involved in the respiratory process and cellular oxide-reduction causing bacterial death in a few minutes, leaving them uncapable to develop a mechanism of resistance (Shrestha *et al*., 2010).

However, distinguishing between the biocide role of released Ag+ (indirect effect of nanoparticles) and the AgNPs (specific role of the particles) is a challenge until now, due to their co-occurrence during exposure, as most of the antibacterial experiments are conducted under aerobic conditions that promote surface oxidation and therefore a continuous Ag+ release from the nanoparticles surface (Choi *et al*., 2008; Kim *et al*., 2009; Xiu *et al*., 2011; Xiu *et al*., 2012).

The formation of reactive oxidation species (ROS) is a result of exposure of silver nanoparticles to oxygen (Chen *et al*., 2007; He *et al*., 2012; Fauss *et al*., 2014) where ROS act as an oxidizer of bacterial components such as proteins, enzymes, and even to the DNA (Mendis *et al*., 2005). Liu *et al*. (2017) found an elevated antibacterial activity by silver nanoparticles nucleated in situ in the membrane surface. The authors attributed this activity to the oxidation of silver nanoparticles to silver ions. Nevertheless, under the experimental conditions in this work, the formation of ROS is not possible, at least in the last days of the experiments, as the anaerobic conditions do not allow a further oxidation of the nanoparticles, and an important amount of the silver ions released from the oxidized surface are removed as the broth is partially removed and replaced with a fresh one. The synthesis of the nanoparticles is carried out under aerobic conditions, the formation of an oxidation layer on the surface cannot be avoided, but the solubility of the AgNPs is low, and the concentration of Ag+ should be under the experimental conditions very low to act as an antibacterial agent, particularly after the broth was changed several times, where the dissolved silver is removed with the wasted broth. Under this condition, inactivation of thiol-containing proteins could be a more important mechanism, such as proposed by Durán *et al*. (2016).

Silver in its metallic state should be almost inert to oxidation, particularly in anaerobic media such as the one used in this work, but nanoparticles have a higher surface energy and are therefore more reactive and sensitive to moisture, leading to ionization of the surface silver atoms and to a controlled release (depending of the solution pH, complex species, or redox potential) of silver ions. In this sense, the mechanisms of action of the nanoparticles should be different from the one in previous studies (brackets and implants), as the experiments were carried out in aerobic media (Secinti *et al*., 2011; Arash *et al*., 2016).

There is a need to perform investigations in order to find out if the microorganisms are effectively destroyed by the nanoparticles, but the main conclusion by now is that even in the lack of oxygen, the AgNPs act and hinder the growth of the microorganisms, so that the use of modified limes could effectively prevent failure of the endodontic treatment, as the apical region of the tooth is poor in oxygen. Although this assumption is not conclusive, it seems also that the protein content in the broth is not acting as an effective physical barrier to cells-nanoparticles interactions. Another important observation, is that the stabilizing ligand PVP is not acting as a barrier between the particles and the microorganisms, but could act as a nutrient of the latter. The presence of mineral salts in the broth (chlorides or another ions), also does not hinder the antibacterial properties of the AgNPs. Particularly chloride ions can associate with silver ions forming low solubility salts. Future studies can be derived from this research, such as evaluating the amount of AgNPs that are released from the file when friction with the dentinal walls and its mode of action on root canal microorganisms, as well as investigating the behavior of an endodontic file coated with AgNPs that arrives to fracture within the contaminated root canal.

CONCLUSIONS

This study revealed that the coating of silver nanoparticles on the surface of endodontic file manages to act on the morphology of the bacterial wall, causing death and thus inhibiting the formation of microbiological biofilms. Since ROS could not be present under anaerobic conditions, the bactericidal effect of the silver nanoparticles indicates that ROS-promoted oxidative stress is not an important mechanism of action under this system. Inactivation of thiol-containing proteins could be a more important mechanism under absence of oxygen. This simple modification method for NiTi files is promising in order to avoid infections after endodontic treatment.

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RESUMEN: El éxito de los tratamientos de endodoncia está relacionado con la neutralización de las bacterias y sus toxinas, dentro y fuera del sistema de conductos radiculares de un diente. Habitualmente esto se consigue, en condiciones ideales, mediante una acción químico-mecánica (limas endodónticas e irrigación con soluciones adecuadas). Las nanopartículas de plata (AgNPs) son conocidas por sus propiedades antibacterianas, por lo que el objetivo de este trabajo fue modificar la superficie de limas endodónticas de Níquel-Titanio (NiTi) con AgNPs, para conferirles capacidad antimicrobiana y evitar infecciones. Se realizó la preparación y caracterización de coloides estables de AgNPs. Diez limas NiTi para endodoncia (marca K3 XF, Sybron-Endo Keer) se dividieron en dos grupos; 5 para el grupo 1 (control, limas tal como se recibieron) y 5 para el grupo 2 (limas previamente recubiertas con AgNP). Ambos grupos fueron sometidos a la formación de biopelículas utilizando un cultivo compuesto por *Candida albicans* y *Enterococcus faecalis*, en un ambiente anaeróbico. Las limas endodónticas se modificaron exitosamente con nanopartículas de plata y, después de la exposición en condiciones anaeróbicas a los microorganismos seleccionados. El análisis mediante microscopía electrónica de barrido reveló proliferación de microorganismos en la superficie de las limas de control. En contraste, las limas modificadas y recubiertas no presentaron formación de biopelículas, y las bacterias evidenciaron cambios en la morfología de su pared celular, posiblemente asociados a la ruptura de esta y a la consecuente muerte celular. El recubrimiento de nanopartículas de plata en la superficie de las limas cambia la morfología de la pared bacteriana, provocando la muerte y por lo tanto la inhibición en la formación de biopelículas microbiológicas. Dado que es poco probable que estén presentes especies reactivas de oxígeno (ROS) en las condiciones anaeróbicas utilizadas, el efecto microbicida de las nanopartículas indica que el estrés oxidativo promovido por ROS no es un mecanismo de acción importante, y la inactivación de proteínas que contienen tioles podría ser un mecanismo más importante.

PALABRAS CLAVE: actividad antimicrobiana, *Candida albicans***, limas endodónticas,** *Enterococcus faecalis***, nanopartículas de plata.**

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