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Laser Assisted Deposition of Silver Nanoparticles into Dentinal Tubules

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Abstract Decontamination of infected root canals and dentinal tubules is a crucial step of endodontic treatment. The aim of this study was to test antibacterial effect of silver nanoparticles, their possibility to penetrate dentinal tubules and to transport antimicrobial activities in deeper parts of root dentin.

Silver microparticles and nanoparticles were used for detection of antibacterial activity against the typical endodontic pathogen *Enterococcus faecalis* (ATTC 29212). Interaction between silver micro- and nanoparticles and root canal walls as well as dentinal tubules was detected using laser confocal microscope and scanning electron microscope. Moreover SEM/Energy Dispersive X-ray Spectrometry was used for depiction of distribution of silver ions in the course of dentinal tubules. Root canals of experimental extracted teeth were pretreated by chemicals and 2780 nm laser. After filling of root canals by micro- and nanoparticles, laser shock waves initiating hydrodynamic effect were used for improvement of penetration of particles into tubules.

Antibacterial effect tested on *Enterococcus faecalis* culture revealed 180 CFU/dish for microparticles and 70 CFU/dish for nanoparticles in comparison with 520 CFU/dish of control cultivation without silver treatment. Laser and chemical pretreatment of root canal showed clean surface without smear layer and very good conditions for penetration of nanoparticles into dentinal tubules. According to the SEM examination, only nanoparticles were able to penetrate into dentinal tubules and they were mostly completely fulfilled by nanomaterial to the depth around 60 μm. Afterwards, EDS point analysis was used to get more details about dentinal tubules infiltration, which was detectable up to the depth of 120 μm.

Within the limits of present study, it may be concluded that nanosilver can be succesfully incorporated into antibacterial strategy against *E. faecalis* and its combination with laser shock waves is promissing method for killing bacteria in deeper parts of dentinal tubules.

***Keywords*:** silver nanoparticles, endodontic infection, laser treatment, dentinal tubules, antibacterial test

Introduction

Throughout history, silver and its compounds have been used extensively for many applications as a result of their useful properties. It is believed that silver was known and used longer than what is recorded in history. Archeological evidence suggests that civilizations have been using silver since at least 3000 B.C. Ancient Egyptians and Persians used silver vessels to keep their water clean and safe. Romans and Greeks knew its powerful bactericidal effect and used it for healing wounds. Since soluble silver compounds are toxic to some bacteria, viruses, algae and fungi, various applications have emerged based on the strong germicidal impacts of silver compounds.

Silver nanomaterials are fine particles of metallic silver that have at least one dimension less than 100 nm. Nanosilver is not a new discovery; it has been known for over 100 years. Previously, nanosilver or suspensions of nanosilver were referred to as colloidal silver.

Nanosilver, when in contact with bacteria, adversely affects the cellular metabolism of the electron transfer systems, and the transport of substrate in the microbial cell membrane. Nanosilver also inhibits multiplication and growth of those bacteria which caused infection, odor, itchiness and sores. Very important statement by Lok et al. [1] was that most important difference between silver nanoparticles and silver ions is in their antibacterial capacity: nanomolar concentrations in the case of nanoparticles and micromolar ranges in the case of silver ions.

Nanosilver is an effective killing agent against a broad spectrum of Gram-negative and Gram-positive bacteria [2,3,4] including antibiotic-resistant strains [5,6]. Gram-negative bacteria include genera such as Acinetobacter, Escherichia, Pseudomonas, Salmonella, and Vibrio.

Gram-positive bacteria include many well-known genera such as Bacillus, Clostridium, Enterococcus, Listeria, Staphylococcus, and Streptococcus. Antibiotic-resistant bacteria include strains such as methicillin-resistant and vancomycin-resistant Staphylococcus aureus, and Enterococcus faecium. A beneficial feature of nanosilver is inhibitory effect on the formation of biofilms [5].

Based on studies that show that silver nanoparticles can penetrate the cell wall of Gram-negative bacteria [7,8], it is reasonable to suggest that the resultant structural change in the cell membrane could cause an increase in cell permeability, leading to an uncontrolled transport through the cytoplasmic membrane, and ultimately cell death. It has also been proposed that the antibacterial mechanism of silver nanoparticles is related to the formation of free radicals and subsequent free radical-induced membrane damage [9,10]. Hwang et al. [11] performed a study of stress-specific bioluminescent bacteria, based on a synergistic toxic effect of the silver nanoparticles and the silver ions that they produce.

Nanosilver dressings as well as nanosilver-derived solutions proved to have anti-inflammatory activity [12]. In animal models, nanosilver alters the expression of matrix metallo-proteinases [13], suppresses the expression of tumor necrosis factor (TNF)-α, interleukin (IL)-12, and IL-1, and induces apoptosis of inflammatory cells [14,15]. Silver nanoparticles (diameter 14 ± 9.8 nm) modulate cytokines involved in wound healing [16].

Silver nanoparticles are extraordinarily efficient at absorbing and scattering light and, unlike many dyes and pigments, have a color that depends upon the size and the shape of the particle. The strong interaction of the silver nanoparticles with light occurs because the conduction electrons on the metal surface undergo a collective oscillation when excited by light at specific wavelengths. Known as a surface plasmon resonance (SPR), this oscillation results in unusually strong scattering and absorption properties. In fact, silver nanoparticles can have effective extinction (scattering + absorption) cross sections up to ten times larger than their physical cross section. The strong scattering cross section allows for sub 100 nm nanoparticles to be easily visualized with a conventional microscope. When 60 nm silver nanoparticles are illuminated with white light they appear as bright blue point source scatterers under a dark field microscope (Fig.1).

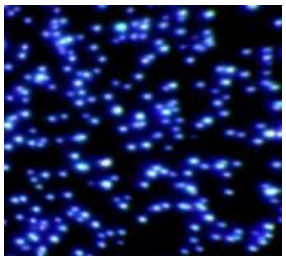

Fig.1: 60 nm silver nanoparticles illuminated with white light and stimulated blue light emission.

When nanoparticles are in solution, molecules associate with the nanoparticle surface to establish a double layer of charge that stabilizes the particles and prevents aggregation. The basic problem of treatment of infected root canals is elimination of bacterial infection which is localized not only directly within root canals but also in dentinal tubules [17,18]. Anatomical and structural parameters of dentinal tubules are suitable for bacterial colonization but typical problems are connected with their decontamination. Bacteria penetrate dentinal tubules up to a depth of 200 – 1000 μm and antibacterial activities of routinely used solutions are very limited. Fig.2 demonstrates spontaneous penetration of erythrosin solution inside dentinal tubules of root dentin. Nanoparticles infiltrate fine anatomical structures much more easily than materials in the micrometer scale.

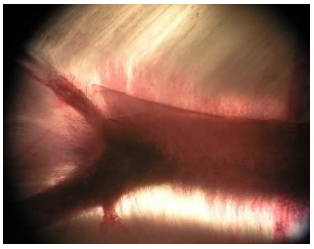

Fig.2: Splitted dental root after intracanal application of erythrosin solution. Red color as indicator of penetration into dentinal tubules.

The aim of this study was to test antibacterial effect of silver nanoparticles and their possibility to penetrate dentinal tubules and to transport antimicrobial activities in deeper parts of root dentin.

Material and methods

Silver microparticles and nanoparticles were used for detection of antibacterial activity against the typical endodontic pathogen *Enterococcus faecalis* which is a well-recognized pathogen associated with persistent apical periodontitis in endodontically treated teeth. In addition it can penetrate dentinal tubules and its cultivation is easy and rapid. Generator of microparticles Craftgen KSG4-CB3 (Craftgen, Czech Republic) was used for preparation of silver microparticles, silver nanoparticles (60 nm particle size) in aqueous buffer containing sodium citrate as stabilizer was supplied from Sigma- Aldrich, St. Louis, USA. Interaction between silver micro- and nanoparticles and root canal walls as well as dentinal tubules was detected using laser confocal microscope Olympus LEXT OLS 3000 (Olympus, Japan). The microscope allows the observation of materials and material surfaces with top-quality resolution of up to 120 nm in axes x and y and up to 50 nm in axis z. Details of relationships between root dentin wall and micro- and nanoparticles were observed using scanning electron microscope Vega Tescan (Tescan, Brno, Czech Republic) (SEM). Moreover SEM/Energy Dispersive X-ray Spectrometer (Quantax EDS, Bruker, USA) (EDS) was used for depiction of distribution of silver ions in the course of dentinal tubules.

The antibacterial effects of the micro- and nanoparticles were tested on *Enterococcus faecalis* (ATTC 29212 (Thermo Scientific, USA). *E. faecalis* was cultivated on liquid medium of anaerobe basal broth (Oxoid, GB) at 37° C for 24 hours. 1 ml suspension containing $1x10^4$ colony-forming units was mixed with 10 µl of silver micro- and nanoparticles and incubated at 37°C for 24 hours. Then 10 µl of the inoculums was plated on brain heart infusion broth agar (Beckton Dickinson, USA) and incubated at 37°C for 24 hours. Bacteria inoculum without nanoparticles was used as the negative control. The test was performed 6 times for each group and CFU of *E. faecalis* were counted for group comparison. The data were analyzed using pairwise comparisons of Mann-Whitney U-test. Statistical significance was preset at P=0,05.

Parallelly, the suspension of *E. faecalis* was dispersed on the surface of Slanetz Bartley agar (Oxoid, GB) and a specified field of medium was covered with one drop of silver micro- or nanoparticles. After 48 hours of incubation at 37°C, CFU of *E. faecalis* were compared between untreated and treated (nanoparticles) parts of cultivation medium.

20 vital, intact premolars extracted for orthodontic reasons were used in the next part of study. After extraction, the soft tissues adhering to the root surface were removed with scalers and all teeth were stored in 0,12% chlorhexidine at +8°C. In the beginning of experiments, the teeth were trepaned from oclussion by diamond bur in high speed driling machine under continual water cooling. After finishing of access into root canals the rest of dental pulp was exstirpated and the length of root canals was measured by insertion of K-file ISO 10 (Maillefer, Ballaigues, Switzerland). Mechanical root canal preparation was performed by Hedstroem files (Maillefer, Ballaigues, Switzerland) and Hedstroem files were used for root canals enlargement up to ISO 40. Root apexes were closed by glassionomer fillings. The root canals were irrigated with saline solution using irrigation canula and subsequently the root dentin walls were prepared using 2780 nm Er,Cr:YSGG laser Waterlase iPlus (Biolase, Irvine, CA, USA). Radially firing tip (RFT3) was used with settings 1,25W, 50 Hz, H mode, 34% air, 0% watter. The tip was always inserted by apical movement close to the apex and slow spiral active movement of the tip was used in opposite direction. This manoeuvre was repeated 4 times. After drying, root canals were filled with 17% solution of EDTA for 3 minutes and subsequently with 0,25% trypsin-EDTA solution (Sigma- Aldrich, St. Louis, USA) for 10 minutes to remove rests of organic substances and to enable better penetration of nanoparticles into dentinal tubules. After following rinsing with saline solution, the root canals were dried with absorbent paper points. Further, the root canals of 10 teeth were filled with silver microparticles (0,1 mg/ml) and in next 10 teeth with nanoparticles (60 nm, 0,02 mg/ml). The same laser equipment was used for generation of shock waves of laser hydrodynamic effect. Settings of iPlus laser were slightly different: 0,75W, 20 Hz, H mode, 10% air, 0% water, RFT3 tip.

All teeth were splitted along their long axis and prepared for examination using confocal laser microscope and scanning electron microscope.

Results

Antibacterial activity of silver micro- and nanoparticles against *E. faecalis* was initially tested during growth of this bacteria on solid medium of brain heart infusion broth agar. Fig.3 represents cultivation results of untreated inoculum. Significant reduction of colony-forming units (CFU) on the surface of cultivation medium was visible when silver microparticles were included into bacterial suspension (Fig.4). Antibacterial effect of silver nanoparticles against *E. faecalis* was more efficient than in the case of microparticles. Remarkable reduction of colony-forming units is visible in Fig. 5.

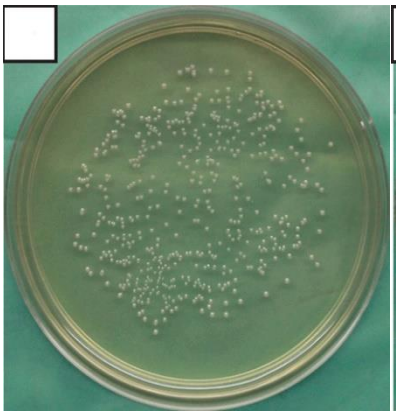

Fig.3: Spontaneous growth of *E.f.* on solid medium of brain heart infussion broth agar.

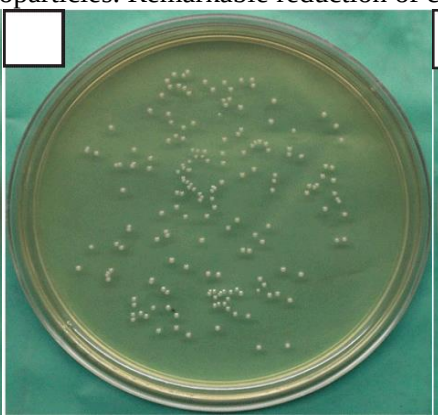

Fig.4: Significant reduction of CFU after inclusion of silver microparticles.

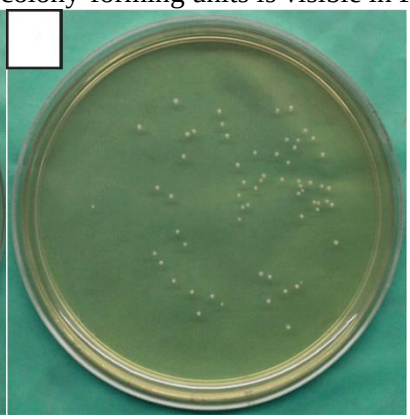

Fig.5: Inhibitory effect of silver nanoparticles on the growth of *E.f.* colonies.

Significance of differences between particular types of treatment is noticeable in Fig. 6. While the average number of CFU/dish of untreated bacterial suspension reached 520 colonies, significant decrease (180/dish) was detectable after interaction with silver microparticles. More significant ($p<0,05$) was reduction of CFU/dish in the group of nanoparticles with the average number of 70 colonies. Fig.7 represents antibacterial activity of silver nanoparticles against *E. faecalis* growing on Slanetz Bartley medium. Circular areas located on the right side of cultivation medium were determined as control and left side areas were treated by nanoparticles. Six times lower average number of CFU was recorded in areas influenced by silver nanoparticles in comparison with controls.

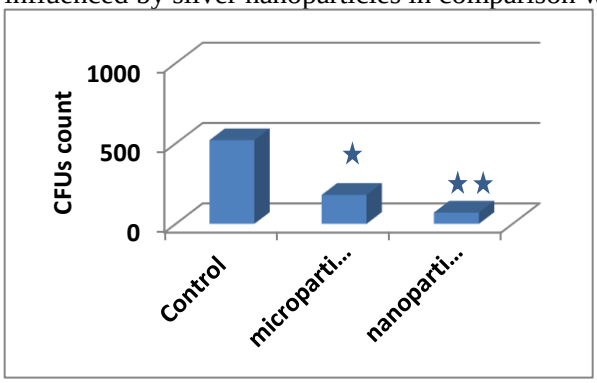

Fig.6: CFUs count among different groups lower average number of CFU on (columns) designated with different asterisk numbers are significantly different from each other ($p<0,05$).

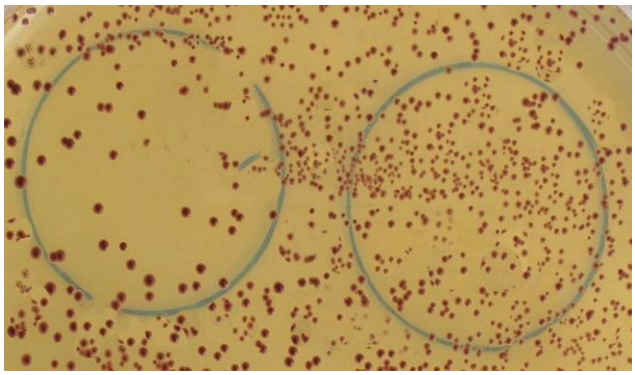

Fig.7: Six times solid medium was recorded in areas influenced by silver nanoparticles (left circle) in comparison with control area (right circle).

Examination of root dentin wall using confocal laser microscope after pretreatment by EDTA, trypsin-EDTA and Er,Cr:YSGG laser revealed clean surface completely without smear layer. Generally, they are evident very good conditions for penetration of nanoparticles into dentinal tubules. Numerous micro- and nanoparticles aggregated around the tubular orifices are visible also by means of SEM. Microparticles form deposits of silver on the surface of dentinal wall, but without possibility to penetrate dentinal tubules (Fig. 8). On the other hand, dimensional relations between diameters of dentinal

tubules and nanoparticles create very good assumptions for their penetration inside. Mutual relationships between colonized root canal and silver nanoparticles enable infiltration of dentinal tubules also between bacterial cells.

First detection of silver nanoparticles was done using confocal laser microscopy. Fig. 9 represents the longitudinal split section of root dentin wall and deposits of nanoparticles within dentinal tubules are visible in the distance around 60 µm from the surface of root canal wall. Dentinal tubules were mostly completely fulfilled by nanomaterial.

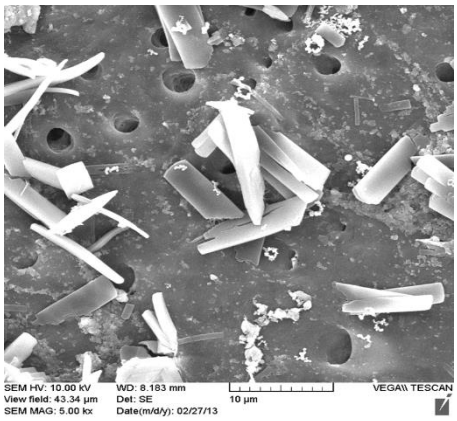

Fig.8: Dimensional relations between diameters of dentinal tubules and micro- and nanoparticles.

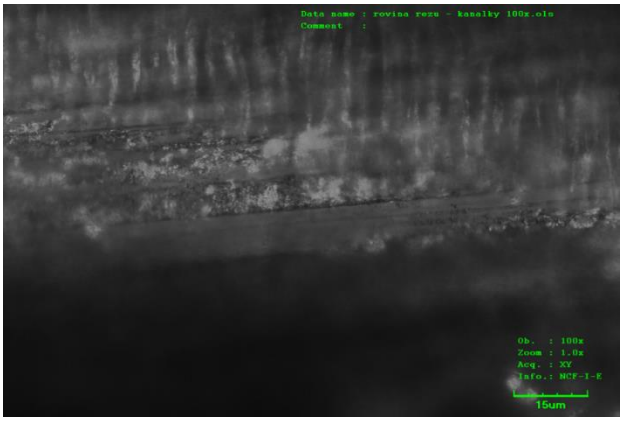

Fig.9: Split section of root dentine wall and deposits of silver nanoparticles mostly fulfilling dentinal tubules (confocal laser microscopy).

Afterwards, EDS point analysis was used to get more details about dentinal tubules infiltration and to confirm findings of confocal laser microscopy. Figure 10 demonstrates positive detection of silver in the depth 120 µm of dentinal tubules after complex treatment of root canals using lateral distribution of laser energy. In the same depth of dentinal tubules of root canals treated without laser stimulation of nanoparticles movement, results of EDS analysis for silver nanoparticles were negative (Fig. 11).

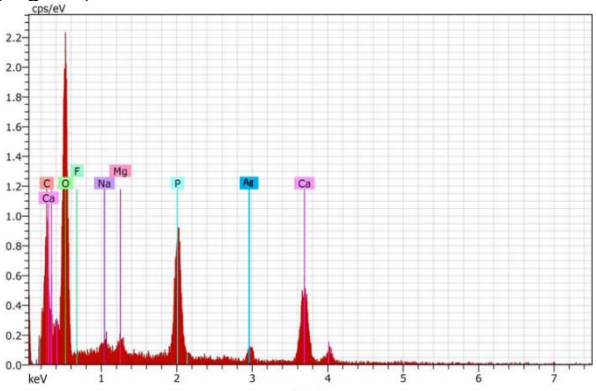

Fig.10: EDS detection of silver in the depth of 120 µm dentinal tubules after laser activation of silver nanoparticles.

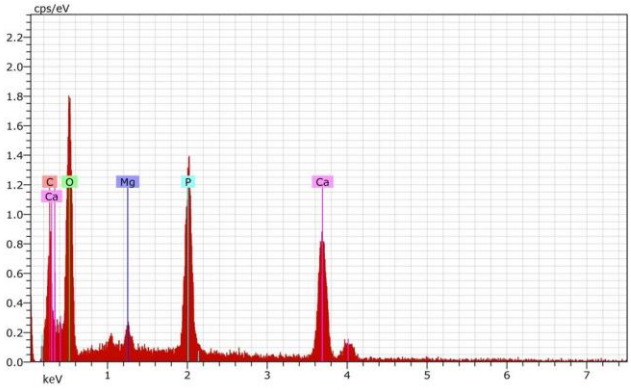

Fig.11: Negative EDS silver detection in dentinal tubules wihout hydrodynamic laser stimulation.

Discussion

The success of endodontic treatment depends upon a number of factors including appropriate instrumentation, irrigation and disinfection of the root-canal system [19]. Dentinal tubules are structures that range in diameter from 2,0 to 3,2 µm at the pulpal wall [20]. For material to penetrate the tubules, the particle size must be smaller then the diameter of the tubule. *Enterococcus faecalis* cells infiltrating significant parts of dentinal tubules are ovoid and 0,5 to 1,0 µm in diameter. They occur singly, in pairs, or in short chains. Mutual relationships between colonized root canals and silver nanoparticles enable infiltration of dentinal tubules also between bacterial cells. On the other side, the most frequently used root canal disinfectants on the basis of calcium hydroxide are normally available in the form of microparticles. This dimensional parameter strongly

limits the possibility to infiltrate dentinal tubules. Significant factor of bacterial decontamination is the depth of penetration of antimicrobial agent into the dentinal tubules. Except viscosity and surface tension which are the two main factors influencing the flow and penetration depth, activation of antimicrobial irrigants is one of many ways to increase their efficacy. Various irrigant activation systems include manual activation with gutta-percha cones, sonic and ultrasonic agitation, agitation with brushes and first results were done with lasers [21]. Necessary condition of liquids or nanoparticles penetration into accessory canals and dentinal tubules is removal of smear layer. In this study, smear layer was removed not only using of 17% EDTA but proteolytic enzyme trypsin was included in the scheme of root dentin walls cleaning. Moreover, its activity is able to destroy protein rests located inside of dentinal tubules.

Antimicrobial activities of nanoparticles are promissing for elimination of bacterial infection in root canals and dentinal tubules. But many studies observed that the diffusion of fluids alone was not able to deliver nanoparticles into dentinal tubules [22]. Relationships between dentinal tubules and smart materials are a frequent topic located on the surface of dental roots in connection with their sensitivity. But the problem of endodontic infection is completely different topic. According to some experiments, it is sufficient for succesfull therapy of intradentinal infection to close orifices of dentinal tubules using nanomaterials, e.g. nanosized hydroxyapatite [23]. But the collapsing cavitation bubbles treatment using high-intensity focused ultrasound resulted in significant penetration (Srestha, 2009). Shock waves induced by laser are more effective for the movement of nanoparticles and moreover energetic potential mediated by radially firing tip is oriented directly against orifices of dentinal tubules.This phenomenon is also of great importance for prevention of nanoparticles aggregation

Within the limits of present study, it may be concluded that nanosilver can be successfully incorporated into antibacterial strategy against *E. faecalis* and its combination with laser shock waves is promissing method for killing bacteria in deeper parts of dentinal tubules.

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