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Assessment of Antimicrobial Efficacy of Silver Nanoparticles Versus Calcium Hydroxide as Intra Canal Medication (*In vivo* Study)

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Abstract: The aim of the present study was to prepare and characterize silver nanoparticles AgNPs paste as intra canal medication and compare its antimicrobial activity against calcium hydroxide $Ca(OH)_2$ paste. Twenty patients with necrotic pulp tissues were divided into 2 groups according to the type of medicament used. Two microbiological samples were taken, one after access cavity preparation and the second after removal of intra canal medication which was inserted for one week after mechanical preparation. The colonies were identified by culture characteristics, microscopic examination, biochemical reactions and polymerase chain reaction (PCR). Colony forming units CFUs were counted and data were analyzed. There was a statistically significant decrease in CFUs between before and after treatment with $Ca(OH)_2$ group and 0.03 mg/ml AgNPs group with 17.95 % and 42.86 % percentage reduction respectively. But in AgNPs group, there was no statistically significant decrease in number of CFUs for *Candida* between before and after treatment. AgNPs paste showed good antibacterial effect and less antifungal effect.

Key words: Intracanal medication · Calcium hydroxide · Silver nanoparticles · Necrotic pulp

INTRODUCTION

Microorganisms are the main causative factors in the development of necrosis of the dental pulp and the formation of periapical lesions. Although we try to clean and irrigate the root canal system properly, some bacteria still remain entrapped in the dentinal tubules. It has been shown that, if the canal is not dressed with a disinfectant between visits, microorganisms will multiply rapidly within days to near the original numbers [1].

Several studies demonstrated that, some of microorganisms were resistant to the most commonly used intra canal medication calcium hydroxide $Ca(OH)_2$ [2]. Recent studies have focused on using nanoparticles to disinfect root canals. Silver is an effective antimicrobial agent with low toxicity and broad spectrum activity Among the various options available.

AgNPs which are one of the most commercialized nanomaterials. They are widely applied as biocides for their strong antimicrobial activity [3]. It seems valuable to evaluate the antimicrobial efficacy of AgNPs as intra canal medication and compare it with the commonly used intra canal medication, $Ca(OH)_2$.

MATERIALS AND METHODS

Preparation of Silver Nanoparticles: Silver nanoparticles (AgNPs) have been prepared by chemical reduction method as reported by Turkevich *et al.* [4] and, Lee and Meisel [5]. A solution of silver nitrate (AgNO3) has been used as Ag^{1+} ions precursor. The color of the solution slowly turned into grayish yellow, indicating the reduction of the Ag^{1+} ions to Ag nanoparticles.

Patient Selection and Classification: Twenty patients medically free with necrotic pulp tissues of single rooted teeth with no periapical lesion, their age ranges from 20 to 50 years of both sexes were selected. Patients were

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randomly divided into two groups according to type of medicament used. The medicaments used were $Ca(OH)_2$ paste and 0.03 mg/ml AgNPs paste.

Endodontic Treatment Was Done in Two Visits.

The First Visit: After approval of the ethics committee of Minia University, access cavities were prepared using sharp round bur mounted on a high-speed hand piece. The first microbiological samples were taken using H-files size 30. The files were inserted into a sterile Wasserman tubes containing 1 ml of sterile brain heart infusion (BHI) broth. The tubes were incubated at 37°C for one day before culturing. Working length was determined by periapical radiographs using bisecting angel technique. Mechanical preparations were completed using REVO-S * rotary system mounted in endodontic motor **. Irrigation was done using normal saline at all procedure steps. AgNPs was placed inside the canals using sterile plastic syringe. The access cavities were closed using sterile cotton pellet and temporary filling.

The Second Visit: After seven days, Intra canal medicaments were removed by irrigation with 10ml of sterile normal saline. The second microbiological samples were taken and cultured. After confirmation of the length of the master cone by periapical radiographic X ray; obturation was done. The obturation was done using Revo-S gutta percha points.

* MICRO MEGA.

** Motor X smart E-Cube, Korea.

Antimicrobial Effect Evaluation: After incubation of microbiological samples, BHI broth was spread on different types of agar plates. After culturing, the blood and Sabouraud agar plates were incubated aerobically at 37° C for two days. The Wilkins agar plates were incubated anaerobically at 37°C for five days. The colonies were identified by Culture characteristics [6], Microscopic examination, Biochemical reactions (Catalase test) [7] and Polymerase chain reaction (PCR).

PCR: DNA extraction was done to the samples according to Qiagen DNA extraction protocol for bacteria (DNeasy blood and tissue handbook 2016) followed by PCR amplification with specific primers using hot start master mix method [8]. Detection of amplified products was done using Agarose gel electrophoresis [9] and Real time curve analysis [10].

Counting of the Types of Microorganisms: After incubation, serial dilution was done to 1/100000 for all specimens. Colonies of each microorganism were counted from the plates and the colony forming units CFUs /ml were calculated according to the following equation: - CFUs / ml = number of colonies × dilution × volume of infected BHI broth [11].

Follow up and Prognosis: Follow up was done for all patients after 1month and 3 months by clinical examination and radiographic evaluation.

Statistical Analysis: Data were presented as mean, standard deviation (SD), frequency and percentage. Dependent t-test used to compare between before and after intra canal medication for Log^{10} CFU/ml. Wilcoxon singed rank test was used for detection of each microorganism.

RESULTS

Nanoparticles Characterization Results

Determination of Size and Shape of Silver Nanoparticles

AgNPs: The particle size and shape were determined using Transmission electron microscope (TEM) which was performed on JEOL JEM-2100 at an accelerating voltage of 200 kV. The average size of the particles was 50 ± 5 nano microns (nm) and the particles were spherical in shape.

Determination of Optical Properties of AgNPs: The optical properties of the prepared nanoparticles were characterized using UV-Vis absorption spectra which were obtained on an Ocean Optics USB2000+VIS-NIR Fiber optics spectrophotometer. Optical Properties (Abs.): ëmax = 401 nm.

***In vivo* Antimicrobial Effect of the Tested Intra Canal Medication:**

By Colony Forming Units CFUs: In $Ca(OH)_2$ group: There was a statistically significant decrease in number of colony forming units CFUs between before and after treatment with $Ca(OH)_2$ with 17.95 % percentage reduction. In cocci, bacilli and *candida*; there was a statistically significant decrease in number of CFUs between before and after treatment with 23.29, 21.13 and 23.90 % percentage reduction, respectively (Table 1, Fig. 1).

Table 1: Mean and standard deviation (SD) for colony forming units (log^{10} CFUs/ml) for different microorganisms

		Time					
		Pre-treatment		Post-treatment			
		Mean	SD	Mean	SD	% of reduction	p-value
Cocci	Group I ($Ca(OH)_2$)	7.3	0.95	5.6	2.41	-23.29%	0.025*
	Group II (AgNPs)	6.8	1.4	1.3	1.77	-80.88%	≤0.001*
Bacilli	Group I ($Ca(OH)_2$)	7.1	1.29	5.6	1.58	-21.13%	0.003*
	Group II (AgNPs)	7.1	1.45	3.2	1.4	-54.93%	≤0.001*
Candida	Group I ($Ca(OH)_2$)	6.57	1.9	5	2.1	-23.90%	0.026*
	Group II (AgNPs)	5.5	1.38	3.29	1.25	-40.18%	0.195 NS
Total	Group I ($Ca(OH)_2$)	7.8	1.23	6.4	1.51	-17.95%	0.01*
	Group II (AgNPs)	7	1.89	4	1.25	-42.86%	≤0.001*

*=significant, NS= Non-Significant

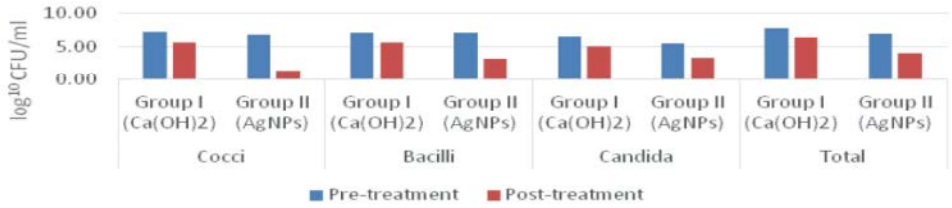

Fig. 1: Bar chart showing the mean colony forming units (log^{10} CFUs/ml) for different microorganisms

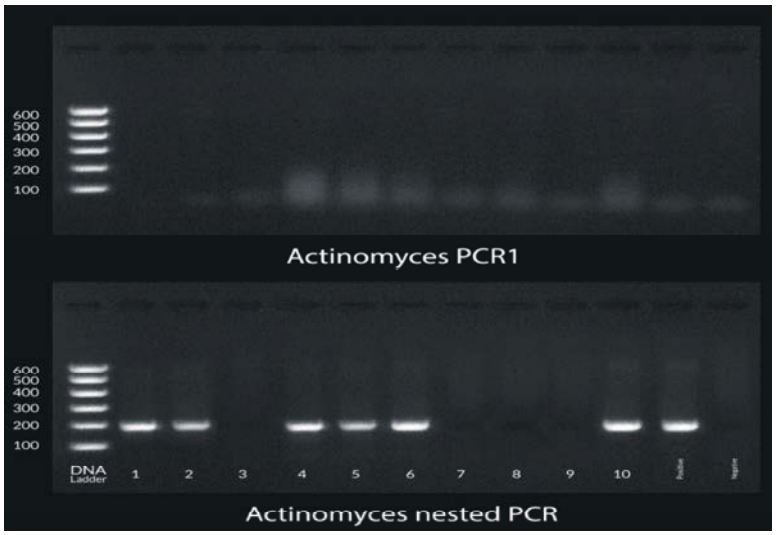

Fig. 2: Detection of the sequence of the primers of *Actinomyces* at 100 nm to 600 nm

In 0.03 mg/ml AgNPs: There was a statistically significant decrease in number of colony forming units CFUs between before and after treatment with 0.03 mg/ml AgNPs with 42.86 % percentage reduction. In cocci and bacilli; there was a statistically significant decrease in number of CFUs between before and after treatment with 80.88 and 54.93 % percentage reduction, respectively. But in *Candida*, there was no statistically significant decrease in number of CFUs between before and after treatment with 40.18 % percentage reduction (Table 1, Fig. 1).

By PCR:

Effect of Intra Canal Medication Type on Identified Microorganisms: In both intra canal medication groups, there was no statistically significant difference in types of microorganisms before and after treatment.

DISCUSSION

A new silver nanotechnology has become favourable due to its effects against biofilms [12]. The

AgNPs interact with multiple targets in the microbial cell, such as cell membrane, enzymes and plasmids, simultaneously providing the bacteria least capacity to gain resistance [13]. The size of AgNPs was 50 nm as the size of the particle was related to the antimicrobial activity; the smaller particles give more bactericidal effects than larger particles [14- 16]. Concentration of AgNPs used in this study was 0.03 mg/ml. As reported by Xinping [3] this concentration had the most effective antimicrobial properties with least toxicity. $Ca(OH)_2$ was chosen as the base line because of its prevalent clinical application [17, 18]. Despite the limitations of $Ca(OH)_2$, however it´s antimicrobial action as intra canal medication is well documented [18, 19].

All microbiological steps which were done in this study were done under laminar air flow to prevent contamination [3]. Polymerase chain reaction (PCR) was done for accurate confirmation of the types of microorganisms [20, 21]. The medicaments were removed using saline before bacterial sampling because it has no any antimicrobial effect so it does not affect the results of the study [22, 23]. Counting the colony forming units (CFUs) was performed because it allows the calculation of the quantity of microorganisms within the root canals [24].

The results of the present study showed that the percentage reduction of numbers of microorganisms before and after treatment with 0.03 mg/ml AgNPs paste group was significantly higher than $Ca(OH)_2$ paste group. This may be attributed to high affinity of silver to negatively charged molecules within the bacterial cells which inactivate critical functions of bacterial cells and prevent bacterial growth and biofilm formation. Silver nanoparticles are effective against a broad range of pathogens, which form biofilm, such as *E. coli*, *Strep. pneumoniae*, *S. aureus* and *Actinomyces niger* [25]. The antimicrobial efficacy of $Ca(OH)_2$ is related to hydroxyl ion release in an aqueous environment, thick mixtures paste of $Ca(OH)_2$ may not be ideal as intra canal dressings [26]. Also the possibility of neutralizing the high alkalinity of $Ca(OH)_2$ by the dentin and biofilm matrix of bacteria, which lead to reduced antibacterial effect [27, 28]. This result is in accordance with the results of Javidi [29] who stated that $Ca(OH)_2$ alone was unable to eliminate *E. faecalis* sufficiently even after 1 week. However, combining $Ca(OH)_2$ with silver nanoparticles effectively eliminated *E. faecalis* after only one day. Also Sadeghi [30] stated that nanosilver solution had antimicrobial properties against *Actinomyces viscosus* and *Streptococcus sanguinis*. Zhang [31] concluded that AgNPs alone or with $Ca(OH)_2$ had an obvious inhibitory effect on the biofilm of *E. faecalis*. The result of this study is in contrast with the results of Mozayeni [32] who concluded that Nanosilver gel was not effective against *E. Faecalis*. This may be attributed to the different synthesis procedure of nanosilver particles and the fact that the added gel may have inhibited the release of nanoparticle ions.

But for *Candida*, there was no statistically significant decrease in number of CFUs between before and after treatment in AgNPs paste group. This result is in accordance with the results of Mozayeni [33] who concluded that antifungal efficacy of $Ca(OH)_2$ and 2% CHX gels was significantly higher than that of nanosilver gel in all tested samples. And with the results of Ballal [34] who concluded that $Ca(OH)_2$ showed higher efficacy at the first 24 hours against *Candida albicans*. The result of this study is in contrast with the results of Baker [14] who stated that 8 mg/cm2 AgNPs had antimicrobial activity against *Candida albicans*. This may be due to using different concentration of AgNPs.

There was no statistically significant difference in types of microorganisms before and after treatment except for *Enterococcus* which appeared after treatment significantly in 0.03 mg/ml AgNPs group. This may be attributed to the detection of a bacterial species after treatment by molecular methods may result from the detection of DNA molecules still present in the specimen, which does not mean cell viability [35].

CONCLUSIONS

- The antimicrobial effect of 0.03mg/ml AgNPs paste was better than $Ca(OH)_2$ paste.
- AgNPs paste seems to be a valuable option in the battle against different types of bacteria.

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