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Evaluation of Antimicrobial Efficacy of Silver Nanoparticles and Chitosan Nanoparticles against Enterococcus Faecalis and Candida Albicans Biofilm (An in vitro Study)

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1- Abstract

Aim: antibacterial activities of silver nanoparticles and chitosan nanoparticles against Enterococcus Faecalis and Candida Albicans biofilm in an in- vitro model **Material and methods:**

Forty extracted human single-rooted permanent teeth were included in the present study. All samples were prepared up to size Revo S (AS 40/0.06), and autoclaved for 20 minutes at 121°C then divided into 5 equal groups (n=8); negative control (no further treatment was preformed) and positive control (only microbial inoculation was preformed) , all tested groups were inoculated then further treated with irrigation solutions group I (treated with

nanosilver particles) , group II (treated with chitosan nanoparticles) and group III (treated NaOCl/ EDTA). For all specimens microbial analysis was performed.

Results: The higher median value of bacterial count in Log CFU ml -1 in Positive control was 6.20 (5.99-6.55), followed by Group I (Nano- silver) was 1.38 (0.51-1.88), then Group II (Nano-chitosan) was 1.38 (1.04-1.55), followed by Group III (Sodium hypochlorite) was 0.73 (0.34-1.37), then Negative control was 0 (0-0). Kruskal Wallis test revealed a statistically significant difference between groups (P=0.00). Mann-Whitney test revealed no significant difference between groups I and II, with p-value (p>0.05).

Conclusion: Nano silver and nano chitosan particles are effective microbial agents against E. faecalis and Candida albicans however; sodium hypochlorite has a significantly superior antimicrobial efficacy.

Keywords: Silver Nanoparticles, Chitosan Nanoparticles, Enterococcus Faecalis, Candida Albicans, Biofilm.

2- Introduction

Endodontic infection is largely acknowledged to be polymicrobial in nature, comprising both anaerobic and fungal species.[1]As a consequence, our objective is to create a germ-free root canal system in order to promote optimum recovery. Because complete eradication of microorganisms is impossible due to the complexities of the root canal system, lowering bacterial load with different irrigating solutions may be the answer. Obturation also wraps any resistant germs that remain.[2] Primary endodontic infections are often caused by a diverse range of species, with gram-negative anaerobic rods predominating, while subsequent infections are typically caused by one or a few bacterial species.[3]It was discovered that the instrumentation method used left 35% or more of the root canal surfaces untouchable. As a consequence, irrigation is essential in root canal cleaning.

As a consequence, removing germs from the root canal system is crucial to long-term endodontic treatment success.[4]Despite advances in endodontic treatment devices and procedures, the success rate of the therapy remains a problem. The colonization of many kinds of bacteria in biofilm, the formation of a smear layer during instrumentation, the intricate topology of the root canal system, and the persistence of microorganisms in dentinal tubules are the major reasons of failure and reinfection in endodontic treatment.[5] Postoperative pain is common in endodontic therapy, with rates ranging from 3% to 58% in single and multiple visit treatment. It might be caused by periapical tissue microbiological, mechanical, or chemical damage to periapical tissues, with one of the most common reasons of pain being irrigated beyond the apex.[6] A slew of irrigation therapies have been developed to enhance and supplement mechanical debridement and alleviate post-operative pain. Canal irrigation solutions should be non-toxic, have low surface tension, lubrication, long-lasting antibacterial effect, be easily accessible, have an acceptable odor, and be inexpensive. Chlorhexidine, sodium hypochlorite, EDTA, MTAD or tetracycline isomer, phenol and alcohol derivatives, iodide potassium iodine, and formocresol are some of the most often used root canal irrigation solutions.[7]Sodium hypochlorite has a broad antibacterial spectrum and may kill a wide range of microorganisms. It also has disadvantages such as toxicity and the risk of tissue damage, an unpleasant taste, the inability to eliminate all germs present in infected canals, and the likelihood of physically modifying the structure of dentinal canal walls.[8] The use of nanomaterials has led in the creation of new capabilities in a wide range of scientific fields.

Antimicrobial agents based on nanoparticles are an important class of nanomaterials for biological applications. Novel disinfection and microbial control strategies benefit this family of nanomaterials. Nanoparticles have acquired popularity as antibacterial agents due to their wide range of action and biocompatibility. Nanoparticles have a bigger surface area and charge density due to their polycationic/polyanionic composition, resulting in more interaction with bacterial cells.

Silver nanoparticles have unique physicochemical and biological activities in addition to antibacterial capabilities.[9] Antibacterial activities of zinc oxide nanoparticles have also been established. They impact both gram-positive and gram-negative bacteria. They even have antibacterial activity against heat and pressure resistant spores. Based on this, it was established that assessing and comparing a new irrigation solution including nanosilver and nanozinc-oxide particles in addition to sodium hypochlorite would be helpful.

Furthermore, Chitosan Nanoparticles are projected to have a greater antibacterial effect than regular sized Chitosan by entering and damaging microbial cell membranes.[10].

Aim of the study is evaluate antibacterial activity of Silver Nanoparticles and Chitosan Nanoparticles, against Enterococcus Faecalis and Candida Albicans biofilm in an in- vitro. **3- Material and methods**

I.1. Trial design

In-vitro comparative controlled study.

I.2. Ethical considerations

Before commencing the study; it was approved by the ethical committee of Faculty of Dentistry, Minia University. Under no.(RHDIRB2017122004) (438). Patients or Parents of the patients signed a written informed consent for using their teeth in the study. Following completion of the study teeth were collected in hazardous waste container and disposed by incineration.

I.3. Sample size calculation:

Based on a previous study by Baumgartner JC et al.[8], sample size for antibacterial action as primary outcome was calculated using power of G. it was found that the appropriate sample size for the study was a total of 40 teeth. Group I, II, III (8 teeth each) and 8 teeth as negative control and 8 teeth as positive control. The power is 80% and α error probability =0.05.[8].

Based on the irrigating solution to be used, a total of 40 samples were randomly divided into five equal experimental groups, each with eight samples (n=8). The first three groups received silver nanoparticles, chitosan nanoparticles, NaOCl, and EDTA as final irrigation procedures, in that sequence. The last two groups (positive and negative control groups) received live microbe injections (n=8) and sterile from microbes respectively. **I.4. sample collection and preparation**

I.4.1. Sample collection:

Minia University's Department of Oral Surgery, Faculty of Dentistry, provided forty extracted human permanent teeth. As inclusion criteria, caries-free, mature, Single-rooted, Permanent teeth with Single canal, with no fractures, and no internal or external root resorption were selected.

All teeth were decoronated with a double coated diamond disc placed on a low-speed hand piece to reduce coronal interference and standardize the length to 16mm. **I.4. 3. Sample Preparation:**

All samples were prepared crown down using a Saeshin Cordless Traus Endo Motor using a Revo S rotary device up to size AS 40 (40/0.06)with a speed range of 250 to 400 rpm

and torque of 2.6N/cm. the root canals were irrigated with 5 ml of 2.5% NaOCl as an irrigating solution Using a 30 gauge side perforated needle (1 ml irrigating solution in between each file at a flow rate of 1 ml every 10 seconds) at a depth of 2-3mm short of the working length. The samples were then autoclaved for 20 minutes at 121°C ; To guarantee sterility prior to bacterial inoculation, the samples were kept in a sterile bag until use. **I.4. 4. Sample grouping:**

Based on the irrigating solution employed, all samples (n=40) were randomly divided into five equal experimental groups of eight samples each (n=8); negative control (only autoclaved) and positive control (only microbial inoculation was preformed following autoclaving), all tested groups were autoclaved, inoculated then further treated with irrigation solutions group I (treated with nanosilver particles), group II (treated with chitosan nanoparticles) and group III (treated with NaOCl/ EDTA)

I.5. Microbial preparation and inoculation: I.5.1.

Microbial preparation

In the present study, clinical isolates of Enterococcus faecalis and Candida albicans were collected from the Microbiology laboratory (Central labs, Ministry of Health, Egypt) and used to create biofilm. The microbial strains were cultivated in Brain Heart Infusion broth (BHI; Difco Laboratories, Detroit, MI, USA) for 24 hours at 37oC. The experimental suspensions were created by growing the biological marker on the surface of Brain Heart Infusion Agar (BHIA; Difco Laboratories) and incubating it under identical conditions. The microbial cells were re-suspended in saline to a final concentration of around 3 x 108 cells /mL, and then infected the samples using the No. 1 Mac Farland turbidity standard. **I.5.2. Microbial inoculation and biofilm development:**

Microbial incubation was performed for (positive control group and groups I, II, III) 21 days to allow for biofilm growth and maturation. The teeth were handled using sterile gloves. The root canals of each sample were filled with a 24-hour pure culture solution of E. faecalis and Candida albicans cultivated in Brain Heart Infusion (BHI) broth using a sterile 1ml insulin syringe. All samples were cultured in sealed sterile vials with 100% humidity for 21 days at 37°C. Every 72 hours, the intracanal fluids were replenished with freshly prepared 0.9% physiologic saline solution calibrated to the No. 1 MacFarland turbidity standard.

For the Negative control the samples are autoclaved and don't receive any bacterial inoculation, to make sure that all the samples that come out of the autoclave before any procedure are free of bacteria or contamination to confirm they are sterilized. **I.6. Preparation of the irrigating solutions and irrigation procedure :**

I.6.1. Preparation of Nanosilver suspension: The particle size of nanosilver (NS) in liquid (L) form was 25 nm. NS was produced in two phases, as per the manufacturer's specifications. Nanoparticles were first generated using a catalytic chemical vapour deposition technique, then combined with distilled water. The mixture was prepared using an ultrasonic homogenizer.

I.6.2. Preparation of Chitosan nanoparticles: Chitosan nanoparticles (CNPs) were created by ionic gelation. 0.5 gm of Chitosan was dissolved in 1000 ml of 2% acetic acid and magnetically stirred at room temperature for 30 minutes. The solution was then agitated for 2 hours using a Polytron homogenizer at 5,000 rpm before being centrifuged at high speed utilising a drop-wise addition of 40 ml of tripolyphosphate (TPP) as a cross linking agent. The nanoparticles' formation exhibited an opalescent suspension zone. The solution was then examined further in order to characterise nanoparticles. Particle size was determined using

transmission electron microscopy (TEM), and particle charge was determined using a Zeta potential analyzer. A zeta potential of at least 30 mV is required for electrostatically stabilized nano suspensions. The particle size ranged from 70 to 100 nm.

1.7. Evaluation and assessment

I.7.1. Antimicrobial activity of irrigating solution:

Three paper points were placed independently in each root canal for one minute. 25 microliters of the suspension were pipetted onto a BHI agar plate using a micro pipette. All of the plates were incubated for 24 hours at 37oC in an anaerobic jar before the colonies were counted with a Reichert-Jung colony counter. The technique was done twice for each group sample, and the average number of CFUs in each specimen was calculated. **I.8.Statistical analysis:**

Recorded data were analyzed using the statistical package for social sciences, version 23.0 (SPSS Inc., Chicago, Illinois, USA). The quantitative data were presented as median with inter-quartile range (IQR) was (non-parametric data). Data were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk Test. ***The following tests were done: Kruskall Wallis test***: for multiple-group comparisons in non-parametric data & ***Mann Whitney U test***: for twogroup comparisons in non-parametric data. ***The confidence interval was set to 95%*** and the margin of error accepted was set to 5%. So, the p-value was considered significant as the following: ***Probability*** P-value <0.05 was considered significant. **4- Results**

Table (1): Median and IQR of bacterial count in Log CFU ml -1 for each group and Kruskal Wallis test for non-parametric quantitative data between the fie groups followed by Mann Whitney test between each two group.

Bacterial count in Log CFU ml -1	**Negative control**	**Positive control**	**Group I (Nano-silver)**	**Group II (Nanochitosan)**	**Group III (Sodium hypochlorite)**	**p-value**
Median (IQR)	0 (0-0)	6.20 (5.996.55)	1.38 (0.511.88)	1.38 (1.041.55)	0.73 (0.34-1.37)	<0.001*
Mean±SD	0.00±0.00	5.51±2.24	1.24±0.77	1.37±0. 35	0.84±0.57	
Range	0-0	0-6.71	0.23-2.38	1.01-2	0.23-1.77	
P value (between each two groups)						
Negative control		<0.001*	<0.001*	<0.001*	<0.001*	
Positive control			0.012*	0.012*	0.009*	
Group I (Nano-silver)				0.833	0.045*	
Group II (Nanochitosan)					0.039*	

*IQR: interquartile range *: Significant level at P value < 0.05*

This table shows that the higher median value of bacterial count in Log CFU ml -1 in Positive control was 6.20 (5.99-6.55), followed by Group I (Nano- silver) was 1.38 (0.511.88), then Group II (Nano-chitosan) was 1.38 (1.04-1.55), followed by Group III (Sodium hypochlorite) was 0.73 (0.34-1.37), then Negative control was 0 (0-0). Kruskal Wallis test revealed a statistically significant difference between groups (P=0.00). Mann-Whitney test revealed no significant difference between groups I and II, with p-value (p>0.05).

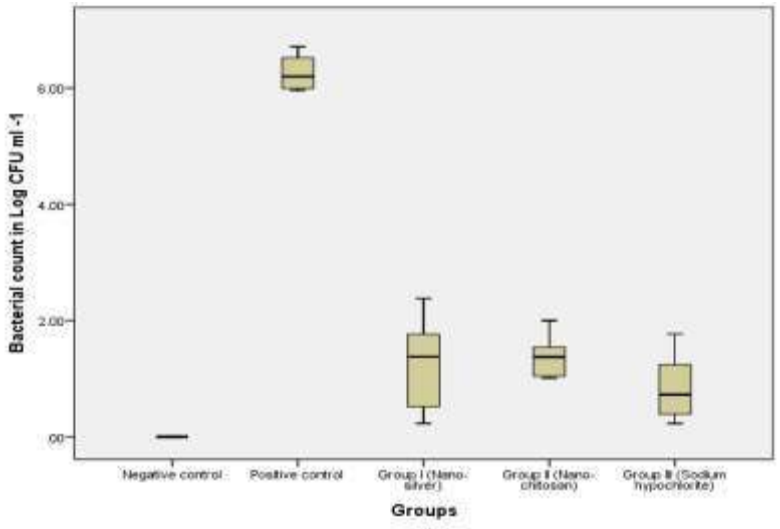

Fig. (1): Box plot between groups according to bacterial count in Log CFU ml^{-1}.

5- Discussion

The root canal system is intricate due to the presence of isthmuses, ramifications, fins, apical delta, lateral canals, dentinal tubules, and other features. These environments promote the formation of bacterial biofilms and influence the outcome of endodontic treatment. (11) Bacteria in biofilm are very resilient to severe growth and environmental conditions. As a consequence, combining instrumentation with an effective antibacterial irrigant is advantageous in achieving acceptable bacterial load reduction. (12, 13)

Bacteria cause the development of all pulp and periapical disorders. If the root canal system is thoroughly debrided and the root canal gap is effectively sealed, endodontic treatment may be successful for a long period. (14)

Root canal disinfection is difficult due to the intricacy of root canals and the presence of bacteria in biofilms. Root canal shaping only removes 50% of germs from the root canals, while antimicrobial treatments such as sodium hypochlorite remove 80% of bacteria. As a consequence, irrigation is necessary during the chemo-mechanical approach for removing bacteria and cleansing the root canal system. (15)

Enterococcus faecalis is the most common resistant bacteria isolated from endodontically treated teeth with apical periodontitis due to its many virulence factors, including its ability to form calcified biofilm on root dentin in a calcium carbonate and calcium phosphate rich microenvironment with its small diameter.(16, 17) It has a high ability to thrive, adapt, and colonies in harsh environments without the help of other bacteria, it can tolerate starvation, and it is resistant to several intracanal therapies. (18) Enterococcus faecalis was cultivated within the root canals for three weeks in this study to mimic its regular endodontic significance. (19)

Candida albicans is the most common fungus found in root canals. It is most often isolated from necrotic teeth with a long-term endodontic infection. Using culture, molecular methods, and in-situ electron microscopy, fungi were discovered in root canal infections. [20] Candida albicans has been isolated from resistant root canal infections associated with apical periodontitis, and it is regarded to be a powerful pathogen capable of infecting periapical lesions. [21]

According to Egan et al, yeasts were present in 32.7% of saliva and 10% of root canals linked with chronic periapical periodontitis. It was also shown that there was a substantial link between the presence of yeasts in saliva and the formation of root canals. [21]

Candida albicans was identified in 7-18% of infected root canals, the majority of which had chronic apical periodontitis [22]. Candida albicans cells were detected in 40-55% of untreated root canals. [23] It has many virulence characteristics, including the ability to adhere, colonies, and penetrate dentin to form biofilms. It can also adapt to a broad range of pH and withstand extreme environmental conditions, vary gene expression in response to environmental factors, attach to a variety of surfaces, produce proteolytic and degradative enzymes, and change morphologic forms to escape immune detection. It also has a strong affinity for collagen types I and IV, which aids in candida adherence to dentin. [24]

After treatment, endodontic infections are dominated by environmentally resistant bacteria and yeasts, which are more resistant to disinfection and therapeutic treatments than anaerobic bacteria in cases of early apical periodontitis. [25] Because of their ability to tolerate different endodontic treatment procedures and grow in the nutrient-depleted environment of a treated dental root canal, Enterococcus faecalis and Candida albicans were selected for this study. Because bacteria in biofilms grow more resistant to adverse environmental conditions, researchers researched Enterococcus faecalis and Candida albicans in biofilm form rather than planktonic form. They develop resistance to the disinfection chemicals used in root canal treatment, as well as the complexities of the root canal system. [26]

Many studies to examine the efficiency of any endodontic irrigant have used planktonic cultures to develop the microbial strains. Planktonic bacteria, on the other hand, do not typically fit the in vivo growth conditions seen in infected root canals, where bacteria build a biofilm on the dentinal walls. [27]

Bacteria in biofilm have been shown to be 100-1000 times more resistant to antibacterial treatments than planktonic bacteria. As a consequence, the rapid killing of planktonic bacteria by different disinfectants does not completely match the same substance's impact on bacteria in in-vivo biofilms. [28] As a consequence, microorganisms in biofilm form should be included in any study on the therapeutic action of endodontic irrigant.

Microorganisms in biofilm may protect themselves from external threats by enclosing resources that each species need for growth inside internal compartments. They have a synergistic effect on resident cells of the same or other species, and they share genetic resources. [29]. According to Madsen et al., horizontal gene transfer rates in biofilm communities are often higher than in planktonic settings. [27]

As a consequence, the most important role of any root canal irrigant is to help in the eradication of any microbes and biofilm from un-instrumented surfaces, which account for around 30-50% of the root canal walls. The optimum root canal irrigation should be efficient against biofilms, non-toxic, and non-caustic on periodontal tissues. There is always a persistent

need for drugs that have antibacterial activity while causing minimal tissue irritation and having little impact on dentin.

For group I (treated with silver nanoparticles) showed statistically significantly lower CFU than that of positive control indicating dramatic decrease in microbial count following irrigation with silver nano particles previous studies **(30)** have shown silver nano particles to exhibit simultaneous antibacterial and smear layer removal efficacy. This may be due to the controlled release of silver ions in an aqueous environment. Because of their large surface area and strong reactivity, silver nanoparticles exhibit exceptional physical, chemical, and biological properties when compared to their bulk counterparts. (31) Silver nanoparticles with diameters ranging from 10 to 100 nm have been shown in studies to be bactericidal against both Gram-positive and Gram-negative bacteria. (32) The antibacterial action of silver nanoparticles ;more over; may be due to Ingestion of free silver ions, followed by inhibition of adenosine triphosphate synthesis and DNA replication, production of reactive oxygen species by silver nanoparticles and silver ions, and direct damage to cell membranes are the three most common ways of antibacterial activity recorded. (32)

The results further more come in accordance with Jung et al.(33), who explained that Silver ions may interact with the bacterial cell to generate bactericidal effect. They interact strongly with the peptidoglycan cell wall and plasma membrane, as well as with cytoplasmic DNA and bacterial proteins. These methods will allow this irrigation solution to affect bacteria in the root canal. Silver nanoparticles (NPs) may bind to the bacterial cell wall and subsequently enter it, producing structural changes in the cell membrane such as membrane permeability and cell death. The formation of "pits" on the cell surface occurred, as did the accumulation of nanoparticles.

This is also similar with the findings of Kim et al. (34), who discovered that when Silver NP comes into contact with bacteria, free radicals are produced, and these free radicals have the ability to damage the cell membrane and make it porous, eventually leading to cell death.

For group II (treated with chitosan nano particles) showed statistically significant lower CFU than positive control group which suggests the high antimicrobial efficacy ; this comes in agreement with Sinha et al , Xu et al, Shrestha et al, and Del Carpio-Perochena etal (35, 36, 37,38) whom found that noano chitosan to be a highly antimicrobial agent in root canals. This may be explained that Nano chitosan possesses antibacterial activities against a broad range of bacteria and fungi, both gram-positive and gram-negative. Because of its polycationic nature, it has antibacterial capabilities. Glucosamine's positively charged NH3 + groups interact with the negative charges on the surface of bacteria, generating cell permeability and intracellular component leakage. It may help prevent microorganisms from adhering to dentin. (36)Chitosan nanoparticles may inhibit bacterial enzymatic breakdown, making bacterial penetration less likely. It may also help to enhance the mechanical properties of root dentin. (37)

Group III (NaOCl and EDTA) has significantly less CFU than did group I, II indicating superior antibacterial activity than silver nanoparticles and chitosan nanoparticles ; These findings are consistent with Rodrigues et al.'s findings (39) that NaOCl had significantly greater antimicrobial activity and biofilm elimination ability than Silver NP at time intervals ranging from 5 to 30 minutes,

The results came in accordance with previous studies by Gianrdino et al (40) showed that NaOCl was able to eliminate *E. faecalis* biofilm in 30 seconds, in addition to Dunavant etal (41) demonstrated that NaOCl killed all bacterial colonies within an organized biofilm. This is mainly attributed to its ability to dissolve organic debris due to its proteolytic effect along with

the chlorine release which affects a broad range microbe together with release of oxygen that eradicates anaerobic bacteria.[42]

Whereas in disagreement with the present study Afkhami et al. [43] whom discovered that irrigation with 100 ppm Silver NP had similar antimicrobial efficacy as 2.5% NaOCl; which may be due to the difference in methodology with our study.

6- Conclusion

Under the limitation of the current study the following can be concluded: Nano silver and nano chitosan irrigant are effective microbial agents against E. faecalis and Candida albicans however; sodium hypochlorite has a significantly superior antimicrobial efficacy than nano chitosan.

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