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Antibacterial Effect of Nano-based Intra-canal Medicaments against Enterococcus Faecalis

Mohamed Riad Elgarhy[1], Kariem Mostafa El Batouty[2], Mohamed M. Nagy[2,3]

1 Assistant lecturer of Endodontics, Faculty of Dentistry, Badr University in Cairo, Cairo, Egypt
2 Professor of Endodontics, Faculty of Dentistry, Ain Shams University, Cairo, Egypt
3 Professor of Endodontics, Faculty of Dentistry, Galala University, Suez, Egypt

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Abstract

Introduction: Intracanal medicaments have been thought as an important step in killing the bacteria in root canals. The application of nanoparticles in the medication applied between visits was supposed to improve its antibacterial effect. With the introduction of nanotechnology in dentistry, intracanal medicaments could become more effective against bacteria.

Objectives: The purpose of this study was to evaluate the antibacterial efficacy of silver nanoparticles incorporated with Calcium hydroxide and Nano Chitosan intracanal medicaments against Enterococcus faecalis biofilms formed on root dentin.

Materials and methods: 40 extracted human single rooted permanent teeth were selected; samples were randomly divided into three equal experimental groups eight samples each according to the intracanal medicament used. Group 1 (n=8) Calcium hydroxide with nano silver intracanal medicament was used, group 2 (n=8) Nano chitosan intracanal medicament was used, group 3 (n=8) Nano conventional Calcium hydroxide intracanal medicament was used and two control groups eight samples each (n=8). After inoculation with Enterococcus Faecalis, teeth were injected different intracanal medicaments for 7 days then evaluation of dead and live bacteria percentage was done using Confocal LASER Microscope.

Results: Reduction of Enterococcus faecalis mean percentages were significantly higher in groups 1 and 2 than group 3 regardless the root level and different root regions except the coronal region.

Conclusion: Nano silver and nano Chitosan provide promising antibacterial effect which was proved by Confocal LASER Microscope

Introduction

Endodontic failure as one of the most challenging situations is commonly due to inappropriate mechanical debridement, persistence of bacteria in the canals and apex, poor obturation quality and coronal leakage. In most of the cases, the endodontic failure results from persistent or secondary intraradicular infection. Despite the high success rate of endodontic treatment, failures do occur in many cases and most of the times can be attributed to the already stated causes.

Biofilm was found to be responsible for chronicity of the disease, as it makes bacteria within it resistant to antibiotics, enable it to evade immune system as it doesn't induce immune response due to bacterial coverage with normal substances as polysaccharide that is considered normal component for the body immune system and also enables genetic material exchange between different microorganisms which results in the increase in the virulence of the microbiota.

The success of endodontic retreatment is directly influenced by the elimination of microorganisms in infected root canals. Intracanal medicaments supplement this to eliminate the persistent bacteria and prevent their regrowth, thereby making root canal environment conducive for periapical tissue repair and act as a barrier against leakage from the temporary filling. Calcium hydroxide is one of the most commonly used intracanal medicaments having antibacterial activity on a wide range of microflora present in the root canal.

Intracanal medicaments have been thought as an important step in killing the bacteria in root canals. The application of nanoparticles in the medication applied between visits was supposed to improve its antibacterial effect. With the introduction of nanotechnology in dentistry, intracanal medicaments could become more effective against bacteria regarding antibacterial effect, depth of penetration, debris neutralization and post operative pain.

Recently nanotechnology was found to improve the antibacterial efficacy of intracanal medicaments. Nano-sized particles can increase the contact surface area of medicaments to bacterial biofilms and increase the pH of $Ca(OH)_2$. A smaller size means better penetration inside the bacterial cells and an easier delivery to contaminated areas. Nanotechnology paved the way for the use of chitosan (CH) which has an excellent antiviral, antifungal and antibacterial properties. It also allowed the use of silver (Ag) nanoparticles which are known for their ability to destabilize the bacterial cell membrane and increase its permeability which leads to bacterial death. Hence this study was designed to evaluate the antimicrobial efficiency of nanosized intracanal medicaments against the E.faecalis biofilm.

Objectives

The aim of this study was to evaluate the antibacterial effect of nano based intracanal medicaments comparing to conventional Calcium hydroxide.

Material and methods

Samples selection and preparation

Forty extracted human single rooted permanent teeth were selected with the following inclusion criteria: caries free, single rooted teeth, no cracks, no internal or external resorption,

canal curvature between 0 and 10 o. The teeth were stored in distilled water at room temperature till time of use to avoid dehydration. Crowns were sectioned to obtain a standardized length 16mm using a safe sided diamond disk under coolant. Length was confirmed using digital caliper.

All samples (n=40) were randomly divided into five equal experimental groups eight samples each (n=8) according to the intracanal medicament used.

One positive control group was inoculated with live microorganisms (n=8). One negative control group was inoculated with microorganisms and autoclaved to show dead microorganisms (n=8).

Teeth were decoronated, and root length was standardized to 15mm by using a diamond disc operated at low speed under copious amount of coolant. After decoronation, #10 K-file was used to negotiate canals and create a glide path. Protaper universal files were used to prepare the canals till F4 file. Root canal instrumentation was performed in strict accordance with the manufacturers' recommendations using a crown down technique. All files were operated by a 16:1 gear reduction handpiece powered by an electric torque control motor. Shaping files S1 and S2 were used to flare the coronal two third of the canal then the canals were irrigated with NaOCl and canal patency was maintained by #10 K file extended 0.5 mm beyond the apex. Then K-file # 10 was inserted and measured until the tip of the file was just visible from the apical foramen, then the working length was established by subtracting 1.0 mm from this length. S1/S2 files were used with a brushing outstroke action until the working length was reached, then the finishing file F1 which has a tip size equal #20 mm and taper 7% for the 1st 3 mm then has a regressive taper 5.5% along the rest of the cutting blades used in a pecking motion until the working length was reached, F2 (0.25/8%), F3 (30/6%) and F4 (40/5%) used also to finish the canal preparation to the full working length in a speed of 250 rpm. Canals were irrigated with 5 ml of 2.5% NaOCl using 30G needle at each exchange of file. At the end of instrumentation, irrigation was done with 5 ml of 2.5% NaOCl for 1 minute followed by 2 ml of 17% Ethylene Diamine Tetra Acetic Acid (EDTA) solution for 1 minute then 3 ml saline as a final flash. Canals were dried with paper point size #40. Finally, teeth were autoclaved at 121□ for 20 minutes to ensure complete sterilization of the root canals.

Preparation of the nano particles

1. Preparation of silver nano particles

Silver nanoparticles (AgNPs) were prepared by chemical reduction method, as described by Xinping et al[(1)]. A solution of silver nitrate (AgNO3) was used as Ag1+ ions precursor. Sodium citrate was used as a mild reducing and stabilising agent. The colour of the solution slowly turned a greyish yellow, indicating the reduction of the Ag1+ ions to Ag nanoparticles. Propylene glycol (PG) was used as a vehicle to improve the consistency.

Characterization of silver nanoparticles

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 (Cary 5000, Agilent Technologies, CA, USA). The particle size and shape were determined using a transmission electron microscope (TEM) (JEOL JEM-2011, JEOL, Tokyo, Japan), which was performed on JEOL JEM-2100 high.

The prepared silver NPs show that UV-Vis absorption spectrum exhibited a single plasmon band at ~410 nm. Indicative to formation of spherical silver NPs.

TEM micrographs: The TEM micrographs clearly show that the prepared silver NPs has spherical shape and size is ranging between 20 ± 3 nm.

2. **Preparation on chitosan nano particles**

CSNPs were prepared based on the ionotropic gelation of CS with tripolyphosphate (TPP)26. Low molecular weight CS (Nanotech for Photo Electronics, Cairo, Egypt) with a 75% to 85% degree of deacetylation, was dissolved in 1% (v/v) acetic acid at a concentration of 2% (w/v). TPP (EMD Millipore, Billerica, MA, USA) was dissolved in sterile distilled water to obtain a solution of 0.5% (w/v). CSNPs were spontaneously formed upon incorporation of TPP solution to the CS solution in acetic acid, in a ratio of 1:4 TPP:CS, by magnetic stirring (1000 rpm) at room temperature. CSNPs were separated by centrifugation at 60,000 rpm for 15 minutes. The supernatant was discarded and CSNPs were extensively rinsed with deionised water and then freeze-dried.

Characterisation of chitosan nanoparticles

CSNPs' size and morphology were determined under a high-resolution transmission electron microscopy (TEM) (JEOL JEM-2100, JEOL, Tokyo, Japan) at an accelerating voltage of 200 kV. The overall charge of the synthesised nanoparticles was evaluated by measurement of the zeta potential using the dynamic light scattering technique via a zeta potential analyser (Particle Sizing Systems, Santa Barbara, California, USA).

Preparation of the medicaments

A digital scale was used in the powder calibration and a 3 cm plastic syringe was used in liquid calibration. In group (1) & group (3) Ca(OH)2 powder (PREVEST DENPRO LTD, Jammu, India.) was mixed with either the NS or the distilled water in the form of paste using glass slab and a spatula.

For group (2) methylcellulose was used and under aseptic conditions 20 mg/ml of methylcellulose based CSNPs gel was formed and used as intracanal medicament.

Microbial preparation and inoculation

Three to four colonies were obtained from an overnight culture growth of Enterococcus Faecalis (ATCC ® 29212 ™) and suspended in 4ml of sterile phosphate buffered saline. Mac Fairland turbidity tube method was used to adjust Bacterial count to 1 x108. Spectrophotometer was used to detect the degree of turbidity. Bacterial suspension for the test was prepared by using 0.5 ml of the above suspension diluted with 10 ml Brain Heart Infusion media. The material was ready to be added to a microtiter plate that is formed of wells arranged in eight rows and twelve columns. The suspension for inoculation was used within 15 minutes to avoid further growth. Teeth were handled by sterile gloves. The root canal of each sample was filled with a 24-hour pure culture suspension of E. faecalis grown in Brain Heart Infusion (BHI) broth using sterile 1ml insulin syringe.

All teeth were incubated at 37°C in sealed sterile vials for 21 days in 100% humidity. Then, replacing the intracanal fluids with freshly prepared 0.9% physiologic saline solution adjusted

to No. 1 MacFarland turbidity standard was done every 72 hours. All procedures were done under aseptic conditions.

After 3 weeks of microbial incubation, samples were removed from the inoculation tubes and procedures were done according to each group.

Negative control group of 8 teeth were autoclaved with no further treatment to show the dead microorganisms.

Positive control group of 8 teeth didn't receive canal disinfection to confirm the biofilm formation.

For the experimental groups, the canals were disinfected by using 3 different intracanal medicaments. In all the samples, the prepared medicaments were injected in the root canals and completely filled. The canals were then sealed and samples were stored in clean sterile Eppendorf containing saline for 7 days.

Group 1: Calcium hydroxide with nano silver medicament was used.

Group 2: Chitosan nanoparticles medicament was used.

Group 3: Calcium hydroxide medicament was used.

Evaluation and analysis using Confocal Laser Scanning Microscope (CLSM):

Teeth splitting:

Teeth were returned into clean sterile Eppendorf containing distilled water and splitting started immediately after treatment to avoid further bacterial growth. Samples were fixed into a wooden block by glue then Isomet 4000 was used to make a groove in the external root surface from one side then another groove was made opposite to it. Chisel used to separate the samples into two halves. Then, one-millimetre longitudinal section was obtained from each half to be stable during scanning. During sectioning, copious coolant was used to avoid thermal damage and bacteria killing coolant was used to avoid thermal damage and bacteria killing.

Method of evaluation:

Confocal laser scanning microscopy (CLSM)

Confocal Laser Scanning Microscope was used to directly visualize live/dead bacteria in the dentinal tubules, so it is easy to evaluate the effect of each treatment in bacteria killing. Confocal laser scanning microscope can detect the fluorescence effect emitted from the stained bacteria.

LIVE/DEAD Backlight Bacterial Viability kit permit clear separation of living and dead cells based on different cellular characteristics. The LIVE/DEAD Backlight Bacterial Viability Kit is convenient and easy-to-use for monitoring the viability of bacterial populations as a function of the membrane integrity of the cell. Cells with a compromised membrane that are considered to be dead or dying will stain red, whereas cells with an intact membrane will stain green.

A Zeiss Confocal Laser Scanning Microscope 710 Axio Observer confocal microscope was set at the excitation wavelength 543 nm and emission wavelengths of 561 nm and 16 Bit depth to inspect the tooth samples (×20 with an additional ×2 zoom).

Results

A. Effect of using different intracanal medicaments regarding their antimicrobial effect in different root levels:

Regarding coronal root level of the selected samples, maximum antimicrobial mean values of percentage of dead bacteria were revealed in -ve control group (93.5±2.87), followed by group 1 (Calcium hydroxide with silver nano particles) (66.1±4.25), group 2 (Chitosan nano particles) (62.5±2.54) and finally group 3 (Conventional Calcium hydroxide) (58.4±4.06). While for middle root level, maximum antimicrobial values were revealed in -ve control group (94.8±1.87), followed by group I (62.6±3.37), group 2 (60.3±2.54) and finally group 3 (56.1±3.87). For apical root level, maximum antimicrobial values were revealed in -ve control group (95±1.88), followed by group I (60.4±2.84), group 2 (57.4±4.55) and finally group 3 (57.4±4.55), as listed in table (1) and showed in figure (2).

For significance evaluation between different groups, one way analysis of variance (One Way ANOVA) test was performed followed by Tukey`s post hoc test for multiple comparisons which revealed overall significant difference between studied groups as P-value < 0.05 with insignificant multiple comparisons difference between group 1, 2 and 3 as P-value > 0.05, listed in table (1).

While for significance evaluation between different root levels for each group, one way analysis of variance (One Way ANOVA) test was performed followed by Tukey`s post hoc test for multiple comparisons which revealed overall significant difference between for all groups except +ve and -ve groups as P-value < 0.05 with significant multiple comparisons difference for group 1, 2 and 3 between middle and apical root level as P-value < 0.05, listed in table (1).

M; Mean, SD; Standard Deviation, P; Probability Level
Means with same Uppercase superscript letter in the same row were insignificant different using Tukey's post hoc test
Means with same Lowercase superscript letter in the same column were insignificant different using Tukey's post hoc test
***; Significant Difference using One Way ANOVA**
NS; Insignificant Difference using One Way ANOVA

Table 1 ne Way ANOVA and Multiple Comparisons of mean percentages of dead bacteria in the different groups

	Coronal	Middle	Apical	P-value
+ve Control	2.9±1.79^{aA}	2.9±1.52^{aA}	2.6±1.5^{aA}	0.9118 (ns)
-ve Control	93.5±2.87^{bA}	94.8±1.87^{bA}	95±1.88^{bA}	0.3701 (ns)
(Group 1)	66.1±4.25^{cA}	62.6±3.37^{cA}	60.4±2.84^{cB}	0.0138*
(Group 2)	62.5±2.54^{cA}	60.3±2.54^{cA}	57.4±4.55^{cB}	0.0210*
(Group 3)	58.4±4.06^{cA}	56.1±3.87^{dA}	53±3.09d^{dB}	0.0273*
P-value	<0.0001*	<0.0001*	<0.0001*	

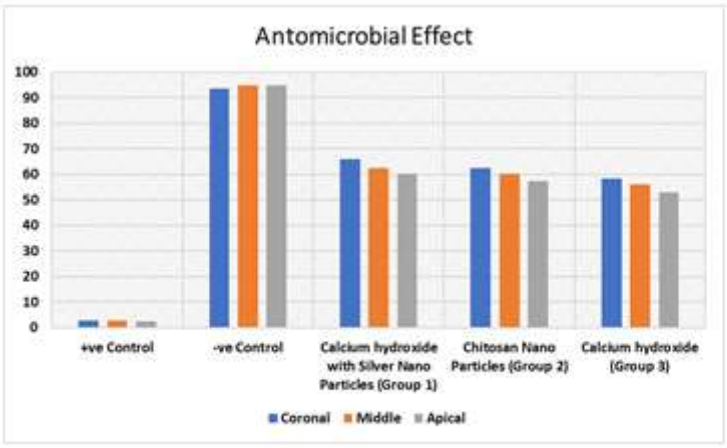

Figure 1 Bar Chart revealing percentage of dead bacteria in all groups

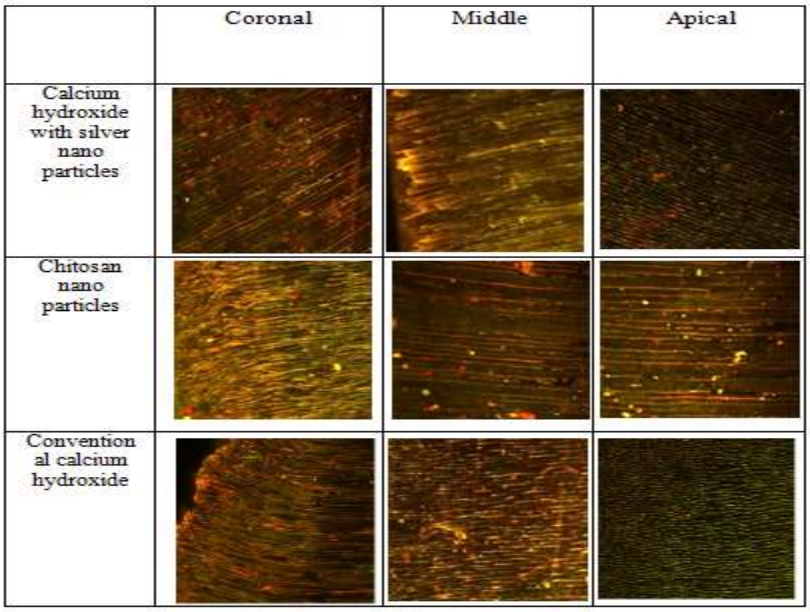

Figure 2 shows live and dead bacteria in coronal, middle and apical third of Calcium hydroxide with nano silver, chitosan nano particles and conventional calcium hydroxide samples

B. Comparisons between mean values of dead bacteria percentage in the three groups regardless the Root Level:

Regardless root level of the selected samples, maximum antimicrobial values were revealed in -ve control group (90.8±3.15), followed by group I (64.6±4.65), group 2 (61.9±3.98) and finally group 3 (55.8±4.02)., as listed in table (2) and showed in figure (3).

For significance evaluation between different groups, one way analysis of variance (One Way ANOVA) test was performed followed by Tukey`s post hoc test for multiple comparisons which revealed overall significant difference between studied groups as P-value < 0.05 with insignificant multiple comparisons difference between group 1 and 2 as P-value > 0.05, listed in table (2).

(Group 1)	(Group 2)	(Group 3)	+ve Control	-ve Control	P-value
64.6±4.65[a]	61.9±3.98[a]	55.8±4.02[b]	10.1±4.89[c]	90.8±3.15[d]	<0.0001*

Table 1 mean, standard deviation (SD) values and results of repeated measures ANOVA test for comparison between percentages of dead bacteria with different intracanal medicaments regardless of root level

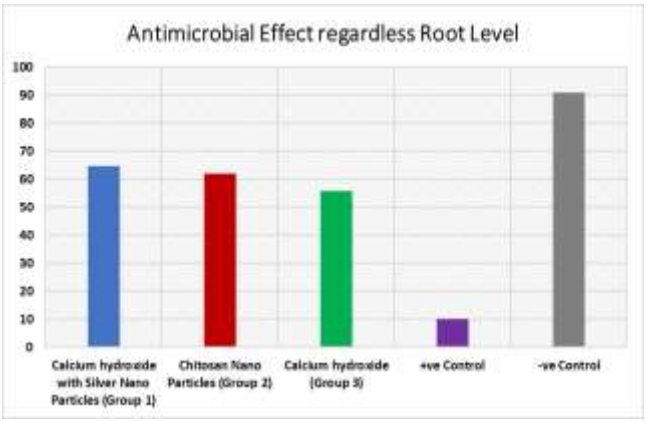

Figure 1 Bar chart representing mean and standard deviation values for percentage of dead bacteria with different intracanal medicaments regardless of root level

DISCUSSION

The present study was carried out in an attempt to assess and compare different new intracanal medicaments and their antibacterial effect against Enterococcus Faecalis under confocal LASER microscope.

Two new intracanal medicaments were used Calcium hydroxide combined with silver nanoparticles, and nano chitosan medicaments. While conventional calcium hydroxide was used as control group.

The introduction of nanotechnology to the field of endodontics aimed to improve the antimicrobial efficacy, mechanical integrity of previously diseased dentine matrix[(2)]. Nanoparticles can be defined as microscopic particles with at least one dimension in the range of 1 to 100 nm, this very small size offers them unique physicochemical properties as large surface area to volume ratio with higher penetration power than their bulk counterparts of the same concentration and also offers them high chemical reactivity[(3, 4)].

Antimicrobial advantage of nano based materials was shown by many studies as that systematic review conducted by Sherstha and Kishen [(5)] who found that the application of nanoparticles in irrigation or medication resulted in improving its antibiofilm efficacy, Kishen et al[(6)] who found significant reduction of E. faecalis adherence to nanoparticulates treated dentine and Barros et al[(7)] who found that incorporation of nanoparticles in sealers improved the killing ability against biofilm,

The materials were selected in this study for the following reasons: Ca(OH)2 which is the most used intracanal medication owing to its antibacterial properties which was able to eliminate bacteria surviving chemo-mechanical preparation according to Sjogren et al[8] and limit their regrowth inside the root canal system Peters et al[9]. The mechanism of action of calcium hydroxide depend on its ionic dissociation into calcium and hydroxyl ions on contact with an aqueous solution, and the main actions of $Ca(OH)_2$ are attributed to the effect of these ions on vital tissues, such as inducing hard tissue deposition and being antibacterial, also, $Ca(OH)_2$ has a high pH (12.5 - 12.8) and is chemically classified as a strong base Kim and Kim[10], 2014, When maintained in the root canal for at least 1 week, it decrease the percentage of the microorganisms up to 92.5 % Shuping et al[11].

Current studies indicated that combination of calcium hydroxide with other vehicles such as chlorhexidine or silver nanoparticles improves its antibacterial activity Singh et al[12], Javidi et al[13], Pourhashemi et al[14] and Zhang et al[15].

AgNPs have excellent antibacterial activity on microorganisms as it has a small particle size (1-100nm), which leads to a greater contact surface area and charge density than bulky powders. So, it has a significantly greater degree of interaction and contact between the positively charged nanoparticles and the negatively charged bacterial cell surface Mohammadi et al[16].

Moreover, AgNPs' small size allows them to readily penetrate and extend its action into dentin microporosities and areas of the root canal that are typically inaccessible to the commonly used endodontic irrigants ElKateb et al[17]. Also it has far lower propensity to induce microbial resistance than antibiotics Krishnan et al [18] and with low toxicity to mammalian cells(i.e. biocompatible) Kim et al[19].

Chitosan is a non-toxic cationic biopolymer usually obtained by alkaline deacetylation from chitin, which is the principal component of crustacean exoskeletons[20]. The covalent immobilization of chitosan on dentinal collagen has been proposed to induce the remineralization of the exposed and demineralized dentin structure because its functional phosphate groups might bind to calcium ions to form a favorable surface for crystal nucleation, resulting in the formation of a calcium phosphate layer[21]. Chitosan treatment improves the resistance of the dentinal surface to degradation by collagenase[22]. Furthermore, chitosan presents with biocompatibility, chelating capacity, and also antimicrobial effects against a broad range of gram-positive and gram-negative bacteria as well as fungi[6, 23, 24].

Previous in vitro studies have demonstrated the significant antibiofilm efficacy of chitosan nanoparticles (CNPs)[6, 23]. Chitosan nano particles have the tendency to reduce biofilm-forming bacteria and disrupt biofilm structure[6]. The higher antibacterial activity of NCS could be attributed to its higher surface area, leading to better interaction with the bacterial cell membrane. However, testing the efficacy of these nanoparticles on biofilms formed in situ would provide a stronger correlation with the findings from the in vivo studies since coronal leakage of saliva is one of the main factors that allows for bacterial recolonization in root-filled teeth[25]. Further in vivo studies are necessary to substantiate the findings of this research.

In cases with failed root canal treatment, placing an intracanal medicament between chemomechanical preparation step and obturation steps is highly recommended[26] to give

time for adequate disinfection and decrease the bacterial load within the root canals as much as possible.

Microcomputed tomography studies revealed that 35-42% of root canal walls remain untouched by endodontic instruments whatever the instrumentation method used[9] and this high light on the great role of chemicals used in cleaning whether as an irrigant during instrumentation or as intracanal medicament.

Various studies have analyzed the composition of the root canal system of previously treated teeth with a persistent apical lesion. Studies showed different results regarding the predominant species in failed endodontically treated cases. Pinheiro ET et al. emphasize that E. faecalis was the most frequently isolated bacteria from the root canal systems (45.8%) in previously treated cases[27]. Siqueira and Roças and Sedgley et al. reported similar results[7, 28]. They observed the prevalence of E. faecalis was 77% and 79.5%, respectively, using (PCR). Furthermore, Sedgley et al. also detected that the prevalence of E. faecalis was significantly higher in retreatment cases (89.6%) than in primary infection (67.5%)[28].

Regarding antibacterial properties, In the present study, human teeth were selected to simulate the clinical condition that might face any practitioner during root canal treatment, so upper central incisors were used because of the straight and single root canal configuration. They were used to avoid root canal complexities that may hinder the proper delivery of irrigation into apical one third as well as to avoid iatrogenic errors in the form of transportation, zipping and ledges that occasionally occurs during the preparation of other curved canals and can affect the outcome of this study.

All samples were autoclaved to allow safe handling. Then, these samples were stored in distilled water to avoid dehydration.

Teeth were decoronated to a length 15 mm to represent root portion only. Working length was established at 14 mm and preparation was done by using protaper Universal as protaper Universal was considered a gold standard files. Preparation was done up to F4 file to allow standardization of the preparation, ensure presence of progressive taper, increase the efficacy of the irrigation[29] and to allow 30-gauge needle to reach 1 mm of the working length so the apical preparation (0.40 mm tip size - 0.05 taper) must be larger than the needle diameter.

During preparation, NaOCl was used as an irrigant of choice as it is the most widely used irrigant in the chemo-mechanical preparation of root canal system because it has a strong antimicrobial activity and has ability to dissolve organic materials[30], however NaOCl alone cannot effectively remove the smear layer, which may prevent or delay the penetration of antimicrobial agents into the dentinal tubules as well as interfere with the adhesion of root canal sealers to the canal walls thus compromising the quality of obturation[31], so the association of EDTA and NaOCl solutions has proved to be effective in removing smear layer[32]. EDTA acts upon the inorganic components of the smear layer while NaOCl dissolves the collagen, leaving the entrances to the dentinal tubules open.[33]

All samples were autoclaved at 121o C for 20 minutes to ensure proper sterilization before bacteria incubation and ensure presence of only single type of bacteria after incubation.

E. faecalis was chosen for our study as it has been the most commonly isolated bacteria from the root canal system especially in failing endodontic cases[33-35] In addition, E. faecalis can

penetrate into the dentinal tubules and form biofilms[36], which are more resistant to canal disinfection[37, 38].

Samples were left for 21 days because it was a sufficient time to ensure proper inoculation of bacteria inside dentinal tubules and formation of well matured biofilm. Then experimental groups where injected by the 3 intracanal medicaments and sealed with steaky wax and samples were stored in clean sterile Eppendorf containing saline for 7 days.

Following treatment, Splitting was done and in order to fix samples in confocal laser scanning microscope, all samples were sectioned into 1 mm thickness. During sectioning copious amount of coolant was necessary to avoid thermal elevation and bacteria killing.

There are many tools used to detect bacteria like wide field microscope, scanning electron microscope and confocal laser scanning microscope. Confocal Laser Scanning Microscope was a valuable tool to detect live and dead bacteria. It provides detailed information about the presence and distribution of bacteria inside dentinal tubules in the total circumference of the root canal walls at relative low magnification through the use of fluorescent stains[12].

As in a conventional wide field optical epi-fluorescence microscope, secondary fluorescence emitted by the specimen often occurs through the excited volume and obscures resolution of features that lie in the objective focal plane. The problem was accompanied with thicker specimens which was greater than 2 micro-meters, that usually exhibit a high degree of fluorescence emission that causes loss of the most of the fine details. On the other hand, confocal microscopy provides only a marginal improvement in both axial and lateral optical resolution, and it is able to exclude secondary fluorescence from resulting images. Confocal microscopy offers many other advantages over conventional wide field optical microscopy, as it had the ability to control depth of field, elimination or reduction of background information away from the focal plane that leads to image degradation. Confocal Laser Scanning Microscope had the capability to collect serial optical sections from thick specimens such as teeth sections.

The most important cause for using confocal laser scanning approach was the use of spatial filtering techniques to eliminate out-of-focus light or glare in specimens whose thickness exceeds the immediate plane of focus. In fact, confocal technology is proving to be one of the most important advances ever achieved in optical microscopy.

Regarding antibacterial properties, Calcium hydroxide with nano silver and nano Chitosan intracanal medicaments samples showed higher percentage of dead bacteria throughout all root canal regions and regardless root level. Also, coronal third in all groups was the region with the highest percentage of dead bacteria.

Regardless the root level our study showed that Calcium hydroxide with Nano silver Particles and chitosan groups showed significantly higher percentage of dead bacteria than calcium hydroxide group, a previous study done by Kim et al[10] stated that the antimicrobial effect of Calcium hydroxide results from the release of hydroxyl ions when it comes into contact with aqueous fluids. Calcium hydroxide has a wide range of antimicrobial effects against common endodontic pathogens, but is less effective against Enterococcus faecalis and Candida albicans.

These results are in accordance with studies done by Tülü et al[39] and Yousefshahi et al[40] in which intracanal canal medicaments of silver nano particles added to calcium hydroxide had better antibacterial properties than calcium hydroxide, Previous studies indicated that the inadequate antibacterial capacity of AgNPs used as a root canal irrigant is due to the short interaction time and concentration (94 ppm) of the solution[41]. When 0.1% AgNPs solution was used against E. faecalis biofilms, it was shown that the integrity of the biofilm structure was not destroyed after 2 min contact[42]. Extracellular matrix of the biofilm may have hindered the diffusion of nanoparticles through the bacterial cells that caused an inadequate interaction between nanoparticles and bacterial cells during the root canal irrigation period[42]. Antibiofilm efficacy of AgNPs was found to be significant when it was used as medicament due to the prolonged interaction between positively charged AgNPs and negatively charged biofilm bacteria/structure[42]. This was in agreement with the suggestions that the effectiveness of nanoparticles depended on the concentration and duration of interaction[5, 6].

Also these results were in agreement with nano chitosan previous antibacterial studies[43, 44] and this may be attributed to As a nanoparticle, chitosan has higher penetration rate rather than other antimicrobial agents. After penetrating extracellular matrix of biofilms, nano chitosan as a cationic molecule will interact with anionic particles on the cell surface of microorganisms. Modes of action of nano chitosan as cationic biocide are adsorption on microorganism cells, diffusion through the cell wall, adsorption and destruction of the plasma membrane, cytoplasmic component leakage and cell death[45, 46].

Conclusion

Understanding the virulence factors of microbes is very important to be able to counteract these factors and increase our clinical success rate. Microbial biofilm is one of the most effective virulence factors produced by different types of microbes and proved to be one of the most common causes of microbial resistance toward different disinfecting agents used during endodontic treatment. Enterococcus faecalis species is one of the most biofilm forming bacteria.

Introduction of nanotechnology into different fields of medicine with its well-known antibacterial effect encouraged us to try it in our field of endodontics.

This study was done to evaluate the effect of some intracanal medicaments in their nanoform either clinically on healing of periapical lesions and postoperative pain related to endodontic retreatment and in vitro by using confocal laser microscope to determine antibacterial properties of theses formulas through calculating percentage of dead bacteria in comparison with others in the regular form.

The two new nano formulas showed better results in their antibacterial properties.

This study concluded that new nano trends of intracanal medicaments can be used in cases with failed root canal treatment to help decrease bacterial load along the root canal. Further combinations with other medicament may show better effect and enhance the treatment outcome.

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