Antibacterial potential of nano-particulate intracanal medications on a mature E. faecalis biofilm in an ex vivo model

ABSTRACT

Aim: To investigate and compare the antibacterial and antibiofilm activity of Chlorhexidine Digluconate functionalized Hydroxyapatite Nanoparticles and silver Nanoparticles as intracanal medicaments on a mature E. faecalis biofilm model in root canals of extracted teeth.

Methodology: Sixty-eight human maxillary central incisors were mechanically prepared, sterilized, infected with E. faecalis, and then incubated for 28 days under anaerobic conditions to develop a mature E. faecalis biofilm. Eight teeth were used to monitor biofilm formation and maturation over the incubation period with field emission scanning electron microscopy (Fe SEM), while the other 60 teeth were divided into two experimental groups (n=20) in which 2% chlorhexidine digluconate functionalized hydroxyapatite nanoparticles (Group A) and 0.02% silver nanoparticles (group B) were used as intra canal medications, and two control groups (n=10). The positive control group (Group C) was used to check for bacterial viability throughout the experiment, while the negative control group (Group D) was used to check for sterility of the procedures. Finally, bacterial samples were collected and analyzed quantitively by culture counts and qualitatively by real time PCR (RTQ-PCR).

Results: RTQ-PCR detected E. faecalis DNA in all groups except for the negative control group. The mean values of E. faecalis DNA detected in groups A, B and C were 99.6 ng/mL, 67.1 ng/mL, 2797.4 ng/mL respectively. Statistical analysis of these results showed that both treatment groups presented statistically significantly lower mean values of E. faecalis DNA quantities compared to the positive control group. However, there was no statistically significant difference between them (P<.05).

Conclusions: Chlorhexidine Digluconate functionalized Hydroxyapatite Nanoparticles and silver nanoparticles demonstrated an effective antimicrobial activity against mature E. faecalis biofilms.

KEYWORDS antibacterial potential, intracanal medications, E. faecalis biofilm, nanoparticles

Sherif Al-Hassan Abdo Heidar3
Shehab Eldin Saber1,2*
Somaya Abdel-Latif Abdel-Aziz Eissa4
Salma Hassan El-Ashry4

1Department of Endodontics, Ain Shams University, Cairo, Egypt
2Department of Endodontics, The British University, Cairo, Egypt
3Department of Endodontics, South Valley University, Qena, Egypt
4Department of Medical Microbiology and Immunology, Cairo University, Giza, Egypt

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Introduction

Microorganisms harboring within the root canal system are the primary cause of apical periodontitis (1). They populate dentinal tubules, apical ramifications, lateral canals and isthmuses, so they are less accessible to chemo-mechanical approach of disinfection (2, 3). Several anti-bacterial strategies have been employed to reduce the number of microorganisms in the infected root canal systems, including the use of various irrigation regimens and intracanal medicaments in combination with root canal instrumentation (4). Although systemic antibiotics appear to be an effective adjunct in certain surgical and non-surgical endodontic procedures, their administration is not without the potential risk of adverse systemic effects, such as allergic reactions, toxicity and the development of resistant strains of microbes (5). Furthermore, a necrotic root canal is a secluded cavity inaccessible to the local immune system (6), and the concentration of drug that reaches the canal space after systemic administration of antibiotics is minimal and unlikely to inhibit bacterial growth (7).

Intracanal medicaments in the Nano-form can achieve optimal therapeutic activity through its interaction with the human body at both the sub-cellular and molecular levels. Nanoparticles exclusive features include smaller sizes, increased surface area to volume ratio, and higher chemical reactivity, compared with their bulk counterparts (8). Silver is among the historical antimicrobials used since Hippocrates’ early suggestions (9). Silver nanoparticles have several antibacterial effects such as interaction with bacterial DNA sulfhydril groups, unwinding bacterial DNA, interference with cell-wall synthesis/cell division and production of reactive oxygen species (8). These collective effects account for their broad-spectrum bactericidal activity and the rare existence of bacterial resistance to it (10).

Functionalized nanoparticles, refers to conjugation of chemicals including drugs or proteins on nanoparticle surfaces to enhance their biological properties (11). Hydroxyapatite [molecular formula: Ca_{10}(PO_4)_6(OH)_2] is a calcium phosphate bioceramic used for drug delivery, due to its chemical ability to incorporate molecules and drugs. In addition to its osteo-conductive potential they are chemically and structurally similar to the mineral phase of bones and teeth (12, 13). Chlorhexidine is a lipophilic cation that interacts with the negatively charged phosphate group of the bacterial cell wall, resulting in altered osmotic equilibrium and increased permeability of the bacterial cell (5). The aim of the current study was to investigate and compare the antibacterial potential of chlorhexidine digluconate functionalized hydroxyapatite nanoparticles and silver nanoparticles as intracanal medicaments on a mature E. faecalis biofilm model in root canals of extracted teeth.

Materials and Methods

Selection and preparation of the samples
Ethics committee approval (number 01062017) was granted for all procedures from the review board of Ain-Shams dental school, Cairo, Egypt. Sixty-eight recently extracted human maxillary central incisors with mature apices and single root canals were selected for use in this study. All teeth were stored in 0.5% thymol solution at 4 °C before use. Following periapical radiographs, the crowns were reduced using a safe sided diamond disc (NTI diamond disc, Axis Dental, USA) mounted on a high-speed contra-angle with water coolant, and the tooth length was standardized to 18 mm from the root apex to the coronal border. Cleaning and shaping of samples were performed using the Protaper rotary nickel titanium system (Dentsply Maillefer, Ballaigues, Switzerland) in the presence of 2.5%NaOCl (Calix, Miami, USA) irrigation until finishing F5 (50/0.4). Following preparation, all canals received a final irrigation sequence of 5ml of 17% EDTA (Calix, Miami, USA) followed by 5ml of 2.5% NaOCl, and 5ml of sterile water (Baxter sterile water, Dealmed Medical supplies, USA) to remove the smear layer.
The teeth were then air-dried and steam autoclaved at 121 °C for 30 minutes. The entire root surface, including the apical foramen of each sample, was coated with two layers of nail polish (Max Factor, Cosmetics and Fragrances, London, UK).

**Grouping of the samples**

Table 1 presents the distribution of the selected samples (n=68) among the groups. Samples were divided into the following groups.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>N=</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP A</td>
<td>20</td>
<td>Medicated with nano-hydroxyapatite functionalized with 2% chlorhexidine digluconate gel</td>
</tr>
<tr>
<td>GROUP B</td>
<td>20</td>
<td>Medicated with 0.02% silver nanoparticles gel</td>
</tr>
<tr>
<td>GROUP C</td>
<td>10</td>
<td>The positive control group used to check for bacterial viability throughout the experiment</td>
</tr>
<tr>
<td>GROUP D</td>
<td>10</td>
<td>The negative control group used to check for sterility of the procedures</td>
</tr>
<tr>
<td>GROUP E</td>
<td>8</td>
<td>Used to monitor biofilm maturation by SEM</td>
</tr>
</tbody>
</table>

*Development of E. faecalis biofilm*

Under strict aseptic conditions, *E. faecalis* American type culture collection (ATCC 29212) reference strain was inoculated in brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) and adjusted to bacterial count concentration of 0.5 McFarland (1.5x10⁶ cfu/mL). All samples, except for the negative control group, were each transferred to centrifuge tubes filled with 1.5 mL *E. faecalis* BHI suspension, and incubated under anaerobic conditions at 37 °C for 4 weeks. Broth was replenished by fresh BHI regularly every second day to clear dead cells and to ensure bacterial viability. Samples of the negative control group followed exactly similar procedures except for inoculation of sterile BHI broth.

*Monitoring of E. faecalis biofilm maturation*

During the incubation period, 8 samples were submitted for Field emission scanning electron microscope (Fe SEM: Model Quanta 250 FEG, Netherlands) examination to detect the progress of *E. faecalis* biofilm development and maturation after 3 days, 7 days and 28 days as described previously (14). Briefly samples were split with a hammer and chisel into two halves, gently rinsed with 5 mL sterile phosphate-buffered saline (PBS) to remove the culture medium and nonadherent bacteria. Then submerged in 4% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, dehydrated through ascending grades of ethanol, dried by critical point dryer, and sputter-coated with gold in a vacuum evaporator (Emitech K550X sputter coater, England).

*Preparation of nano-particulate intracanal medications*

Nano-hydroxyapatite (Nanotech Egypt photo-electronics, Cairo, Egypt) was functionalized with chlorhexidine digluconate (Sigma Aldrich, St Louis, USA) according to the method described by Soriano-Souza *et al.* 2015 (13). Briefly, 100 mg of nano-hydroxyapatite were added to 4 ml solution of 20% chlorhexidine digluconate. The mixture was sonicated at 37 °C for 24 hours. Then centrifuged at 600 rpm for 5 minutes. This was followed by washing one time with water and finally drying to collect the purified material.

This preparation was verified by high resolution Transmission Electron Microscope (JEM-2100, JOEL, USA), as well as by Field Emission Scanning Electron Microscope (Fe SEM: Model Quanta 250 FEG, Netherlands) equipped with Energy Dispersive X-ray (EDX) unit. The EDX analysis was done to detect the elements in the loaded nanoparticles to confirm the incorporation of chlorhexidine digluconate compositional elements in the hydroxyapatite nanoparticles. Ultraviolet spectrophotometry (Ocean Optics USB 2000, Winter Park, USA) was also used for further confirmation of structural change via detecting shift of the absorbance band.
between nano-hydroxyapatite functionalized with chlorhexidine digluconate and its precursors; nano-hydroxyapatite and chlorhexidine digluconate. A 2% concentration was prepared by mixing 2 grams of nano-chlorhexidine with 100 ml distilled water under very well stirring, then 0.72 grams sodium chloride (Sigma Aldrich, MO, USA) were added under stirring, then 2.7 grams methyl cellulose (Sigma Aldrich, MO, USA) was added portion-wise under stirring for 1 hour, till a gel consistency was obtained. In order to prepare silver nanoparticles gel, 100 ml of 0.02% silver nanoparticles (Nanotech Egypt photo-electronics, Cairo, Egypt) was added to 0.72 grams Sodium Chloride and stirred. Then, 2.7 gm of Methyl Cellulose was added portion-wise under stirring for 1 hour, till a gel consistency was obtained. A plain gel containing Sodium Chloride, Methyl Cellulose mixed with distilled water was also prepared for application into samples of the positive and negative control groups.

Application of intracanal medication in root canals
Following an incubation period of 28 days, a standard volume (15 µL) of intracanal medications were introduced into the samples using microbiologic micropipettes, Group A was medicated with nano-hydroxyapatite functionalized with 2% chlorhexidine digluconate gel, Group B was medicated with 0.02% silver nanoparticles gel, while Groups C and D which represent the positive and negative control groups respectively were medicated with a similar volume of the plain gel. The coronal access of each root canal was dressed with sterile cotton pellet then sealed with Cavit (3M ESPE, Germany). Finally, samples were incubated anaerobically at 37 °C for 7 days in a 100% humid environment.

Root canal re-entry
Following seven days of incubation, Cavit was removed aseptically by a sterile excavator. Each sample was washed with 5 mL sterile Phosphate Buffered Saline (Thermo Fisher Scientific, Waltham, MA, USA) to remove the root canal contents and then submitted for detection. Four samples from each group were assessed for biofilm disruption by Fe SEM. The remaining samples underwent bacterial quantification by RTQ-PCR supplemented with culture evaluation of bacterial counts.

Bacterial quantification
For each root canal, a size 40 sterile absorbent paper point (Diadent, South Korea) was placed inside the root canal for 1 minute, then size 40 H-file (Mani Inc, Japan) was used to vigorously cut circumferential dentin, followed by another size 40 paper point. Finally, the H-file and paper points used in each root canal were transferred aseptically to a labeled vial containing 1 ml sterile saline solution. The vials were vortexed for 15 seconds, then form each vial 0.5 mL was collected and freezeed for RTQ-PCR analysis and 0.5 mL was collected for culture analysis.

Polymerase chain reaction (PCR) amplification assay
E. faecalis DNA was extracted by Qiagen DNA mini Kit (Qiagen, Hamburg, Germany) in accordance with manufacturer’s instructions for Gram-positive bacteria DNA extraction (i.e. including a lysozyme bacterial wall lysis). DNA was eluted in 200 µL of the elution buffer included in the kit. DNA was stored at (-20 °C) until used for RTQ-PCR (15).

RTQ-PCR amplification and detection was done using Real Time PCR ViiA 7 system (Applied Biosystems, Foster city, USA) using a 96-well format. Reactions were set-up in a PCR-specific hood under aseptic laboratory conditions. To quantify E. faecalis, RTQ-PCR reactions were performed in a total volume of 20 µL. Briefly, 20 µL total reactions consisted of 10 µL SybrGreen PCR Mastermix, forward and reverse primers 1 µL from each primer (10 moles/µL) and 8 µL of extracted DNA. The primer sequences of E. faecalis have been used according to Williams et al. 2006 description (16).

The published sequences of the forward and reverse primer respectively were:
5’-CGCTTTCTTCTCTCCGCAGT-3’
5’-GCCATGCGGCATAAAGGTG-3’
Temperature cycles included an initial
enzyme activation and DNA denaturation step at 95 °C for 15 min, followed by 50 cycles of amplification including a denaturation step for 15 s at 94 °C, 60 °C annealing for 30 s and extension 30 seconds at 70 °C. Cycle threshold (CT) values were calculated using the RTQ-PCR Sequence Detection Software. Melting curve profile was used to evaluate specificity of PCR reactions. Putative PCR artifacts (primer-dimmer) or contaminating nonspecific DNA will be presented as multiple peaks in melting curve profile representing artifacts (primer-dimmer). RTQ-PCR sensitivity was verified via the construction of standard curve, the curve was derived via known quantities of *E. faecalis* reference strain, which was verified by Nanodrop weighing.

**Culture based analysis**
Each sample was serially diluted into suspensions equivalent to 10^2, 10^3, 10^4 and 10^5 CFU/mL. For detection of *E. faecalis*, 0.1 ml from every dilution was seeded on plates containing Bile Aesculin agar (Oxoid Basingstoke, UK). Plates were incubated for 24 hours at 37 °C. Following incubation, colonies were counted, and the number of colony-forming units (CFU/ml) were calculated.

**Results**

**Hydroxyapatite functionalization with Chlorhexidine Digluconate characterization**
High resolution TEM images of nano-hydroxyapatite functionalized with chlorhexidine digluconate (Figure 1) revealed highly electron-dense regions demarcated by dark colors representing nano-hydroxyapatite, overlapped with chlorhexidine digluconate that appeared as lighter regions due to its less electron-dense composition. Fe SEM imaging (Figure 2) revealed white nano-metric plate-like particles representing hydroxyapatite nanoparticles overlapping the grey organic chlorhexidine digluconate surface, indicating their successful conjugation.

Fe SEM imaging (Figure 2) revealed white nano-metric plate-like particles representing hydroxyapatite nanoparticles overlapping the grey organic chlorhexidine digluconate surface, indicating their successful conjugation. Elemental analysis by EDX (Figure 3) revealed the presence of calcium and phosphate originating from hydroxy-
UV spectroscopy recorded a shift in the absorbance band of nano-hydroxyapatite functionalized with chlorhexidine digluconate to 301 nm in comparison with its precursors chlorhexidine digluconate and hydroxyapatite nanoparticles that recorded absorbance bands at 262 and 296 nm, respectively (Figure 4). Moreover, the high intensity of the absorbance recorded at 301 nm indicates that many CHX aggregates were successfully conjugated to the hydroxyapatite nanoparticles.

Fe SEM images of infected root canals (Figure 5A-D) showed that *E. faecalis* consistently adhered to collagen structures, colonized dentin surfaces, progressed toward the dentinal tubules and formed a thick mature biofilm at end of incubation period.

**Bacterial Quantification**
The specificity of RTQ-PCR was confirmed by the melting curve (Figure 6) which exhibited a single sharp peak at 80.9 °C that represents the target gene only, while the sensitivity of RTQ-PCR was confirmed by the standard curve (Figure 7) that displayed a linear regression of DNA mass after serial dilutions.

*E. faecalis* counts were expressed as *E. faecalis* weight in nano-grams per milliliter (ng/mL). RTQ-PCR detected *E. faecalis* DNA in all groups except for the negative control group. The mean values of *E. faecalis* DNA detected in groups A, B and C were 99.6 ng/mL, 67.1 ng/mL, 2797.4 ng/mL respectively (Table 2).

Statistical analysis of these results showed that both treatment groups presented statistically significantly lower mean values of *E. faecalis* DNA quantities compared to the positive control group (p<0.001). However, there was no statistically significant difference between them (p>0.05).

**Culture results**
All specimens exhibited CFU on culture plates except for the negative control group. Logarithmic transformation (Log10 transformation) of CFU count was performed due to the high range of bacterial counts. Mean values in groups A, B and C were 2.5 ± 0.3, 2.4 ± 0.3 and 4.2 ± 0.3 respectively (Table 3).
Statistical analysis of these results showed that both treatment groups showed statistically significantly lower CFU values of *E. faecalis* compared to the positive control group (p<0.001). However, there was no statistically significant difference between them (p>0.05). As regards to the negative control group, all samples showed no growth in culture plates.

**Discussion**

Nanoparticle medications are capable of reducing biofilm bacteria, disrupting biofilm constitution and furthermore, retaining a sustained antibacterial effect (10). This is provided through their higher surface area, charge density, and greater degree of interaction with cells (3, 17). Therefore, this study sought to investigate and compare the antibacterial potential of chlorhexidine digluconate functionalized hydroxyapatite nanoparticles and silver nanoparticles as intracanal medicaments on a mature *E. faecalis* biofilm model in root canals of extracted teeth.

Nano-hydroxyapatite functionalized with chlorhexidine digluconate and silver nanoparticles were selected as the test medications. The antibacterial potential of chlorhexidine and silver nanoparticles is well documented (8, 18, 19).

Adsorption of chlorhexidine digluconate onto the hydroxyapatite nanoparticles was confirmed by HR TEM, Fe SEM, EDX and UV Spectroscopy. This finding agreed with Soriano-Souza et al. 2015 (13).

Fe SEM monitoring for *E. faecalis* biofilm displayed that the bacteria formed the three configurations; single, pair and short chain (20). Fe SEM examination also presented some of *E. faecalis* virulence mechanisms such as aggregation, invasion of dentinal tubules and biofilm formation (21). At the end of the incubation period, it was evident that *E. faecalis* formed a mature endodontic biofilm that entirely covered the whole dentin surface as described by Saber and El-Hady 2012 (4).

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**Table 2**

Descriptive statistics and results of Kruskal-Wallis test for comparison between RTQ-PCR results in the three groups (nano-gram/mL)

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower bound</td>
<td>Upper bound</td>
</tr>
<tr>
<td>A</td>
<td>99.6B</td>
<td>16.7</td>
<td>96.5</td>
<td>80.0</td>
<td>129.0</td>
<td>87.7</td>
<td>111.5</td>
</tr>
<tr>
<td>B</td>
<td>67.1B</td>
<td>25.0</td>
<td>65.5</td>
<td>32.0</td>
<td>119.0</td>
<td>49.2</td>
<td>85.0</td>
</tr>
<tr>
<td>C</td>
<td>2797.4A</td>
<td>1336.3</td>
<td>2565.5</td>
<td>1172.0</td>
<td>5424.0</td>
<td>1841.5</td>
<td>3753.3</td>
</tr>
</tbody>
</table>

*Significant at P≤0.05. Different superscripts in the same column are statistically significantly different.

**Table 3**

Descriptive statistics and results of one-way ANOVA test for comparison between Log10 CFU in the three groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>SD</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower bound</td>
<td>Upper bound</td>
</tr>
<tr>
<td>A</td>
<td>2.5B</td>
<td>0.3</td>
<td>2.2</td>
<td>2.7</td>
</tr>
<tr>
<td>B</td>
<td>2.4B</td>
<td>0.6</td>
<td>2.0</td>
<td>2.8</td>
</tr>
<tr>
<td>C</td>
<td>4.2A</td>
<td>0.3</td>
<td>4.0</td>
<td>4.4</td>
</tr>
</tbody>
</table>

*Significant at P≤0.05. Different superscripts in the same column are statistically significantly different.
The current study employed specific closed end RTQ-PCR (Fourth generation) assay supplemented with culture-based analysis for microbiologic assessment. The rationale of using molecular methods are higher sensitivity for detecting endodontic pathogens, including cultivable species and as-yet uncultivated (VBNC) state of bacteria (22, 23). Pirani et al (24) detected, using PCR, the clinical presence of *E. faecalis* in root canal teeth affected by primary and secondary periapical lesions to be 7.6% and 39.1% respectively. Bacterial quantification of medicated root canals by RTQ-PCR as well as by culture analysis showed a significant reduction in bacterial DNA counts after the application of nano-hydroxyapatite functionalized with chlorhexidine digluconate and silver nanoparticles (p<0.001) with no difference between them (p>0.05).

Results of the current study displayed the antibacterial and antibiofilm potentials of nanoparticles against mature *E. faecalis* biofilm. Previous studies described by Confocal laser scanning microscopy the potent antibiofilm action of functionalized hydroxyapatite nanoparticles against oral bacterial biofilms and that hydroxyapatite chlorhexidine association inhibited *E. faecalis* growth and adhesion for 6 days (12, 13). This anti-biofilm effect is attributed to the sustained release of chlorhexidine from functionalized hydroxyapatite nanoparticles during experimental assessment, in addition to the higher electrostatic surface charge of cationic chlorhexidine nanoparticles adhering to the negatively charged dentin surface (17).

According to results of the current study, canal medication with silver nanoparticles reduced *E. faecalis* counts and interrupted biofilm integrity. Results of the current...
study is in agreement with results of previous study reported that silver nanoparticles applied for 7 days exhibited an antibiofilm potential, that can be attributed to their positive charge which interact electrostatically with the negatively charged bacterial cells, resulting in leakage of intracellular bacterial components and lysis of bacteria. Moreover, the positively charged silver nanoparticles particles can bind to negatively charged dentin, and inhibit bacterial adherence (25).

Conclusions

Within the limitations of the current study, it can be concluded that nano-hydroxyapatite functionalized with chlorhexidine digluconate and silver nanoparticles demonstrated an effective antimicrobial activity against mature *E. faecalis* biofilms.

Clinical Relevance

Clinical use of nano-particulate intracanal medications should be considered in managing resistant cases of chronic apical periodontitis.

Ethics Statement

The current study was approved from the Research Ethics Committee of Ain Shams University (Cairo, Egypt).

Conflict of Interest

The authors deny any declarations of interest in this study.
Acknowledgements

None.

References


