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Research Article

Intratubular Antibacterial Effect of Polyethyleneimine Nanoparticles: An *Ex Vivo* Study in Human Teeth

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Enterococcus faecalis is a facultative gram positive bacterium which can remain in the teeth root canals and cause refractory or persistent periapical diseases. *E. faecalis* bacteria that penetrate the dentinal tubules can be the source of intracanal infection and endodontic disease. Quaternary ammonium polyethyleneimine (QPEI) nanopolymers were shown to have long lasting antibacterial activity against gram positive and gram negative bacteria. The present study evaluated the intratubular antibacterial effect of an epoxy resin sealer incorporating 1% QPEI against *E. faecalis* in a human dentin model. Root canals of extracted teeth were inoculated with *E. faecalis* for 7 days prior to standard endodontic treatment. The antibacterial effect of an epoxy-amine resin endodontic sealer was tested at concentration of 0% or 1% (wt/wt) added QPEI nanoparticles. Reduction in bacterial viability ($p < 0.01$) was depicted in the dentinal tubules of the root canals obturated with the sealer incorporating QPEI nanoparticles. In conclusion, QPEI nanoparticles when incorporated in a small percentage into epoxy-resin based sealer may target *E. faecalis* in the dentinal tubules, producing a potent antibacterial effect that reduces significantly bacterial viability.

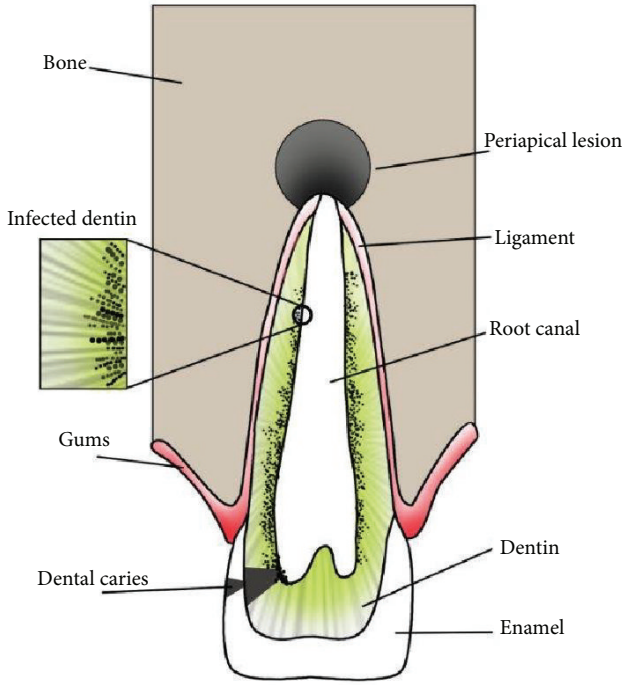
1. Introduction

Intracanal infection in teeth is the main reason of endodontic disease [1]. Bacteria may reside in planktonic or biofilm state [2] in the main canal (Scheme 1). However, they may also penetrate the dentinal tubules [3]. Mechanochemical preparation is the common strategy to eradicate bacteria from the infected root canal and the dentin. One of the disadvantages of this strategy is that it cannot prevent root canal late reinfection which may reoriginate from the previously infected dentinal tubules. *Ex vivo* and clinical studies showed that in spite of temporary absence of bacteria following chemomechanical preparation bacteria reappear following successive endodontic appointments. Antiseptic rinsing or antibacterial dressing does not eliminate the infecting bacteria [4, 5] suggesting that intratubular bacteria may serve as a reservoir out of reach of endodontic preparation. Such scenario calls for a development of better sealers that will possess long term antibacterial properties. Contemporary sealers lack antibacterial effect, leaving the remaining bacteria unchallenged within a short time following obturation. This effect is attributed to solubility or setting reaction of the sealer [6].

Enterococcus faecalis is a facultative gram positive bacterium which can remain in the root canals and cause refractory or persistent periapical diseases (Scheme 1). *E. faecalis* can adhere to dentin collagen (main organic component of dentine), invade the dentinal tubules, and therefore withstand root canal debridement [7].

Quaternary ammonium polyethyleneimine (QPEI) are nanopolymers that were proven to have long lasting antibacterial activity against gram positive and gram negative bacteria. Unlike antibacterial components of common root canal such as calcium hydroxide based materials [8], QPEI are chemically stable, nonsoluble, and biocompatible [9].

The aim of the present study was to evaluate the intratubular antibacterial effect of an epoxy resin sealer incorporating 1% QPEI against *E. faecalis* in a human dentin model.



Scheme 1: Schematic representation of a tooth and surrounding tissues following carries exposure of the pulp to bacteria. The left rectangle represents an enlargement of the infected dentinal tubules adjacent to the root canal.

2. Materials and Methods

2.1. Quaternary Ammonium Polyethyleneimine Nanoparticle Synthesis. Synthesis was as previously described [10]. Briefly, nanosized particles were prepared by dissolving PEI in ethanol that was reacted with dibromopentane under reflux for 24 hrs. N-alkylation was conducted using octyl. Alkylation was carried out under reflux for 48 hrs followed by 24 hrs neutralization with sodium bicarbonate. Then N-methylation, using methyl iodide, was conducted at 42°C for 48 hrs followed by 24 hrs neutralization with sodium bicarbonate. The supernatant obtained was decanted and precipitated in double distilled water (DDW), washed with hexane and DDW, and then freeze-dried. The average yield was ≥85% (mol/mol). Then the particles were washed with a 2% solution of N-lauryl-sarcosine surfactant (NLS). Prepared QPEI nanoparticles (20 g) were placed in a Buchner funnel using a paper filter and a vacuum source. A volume of 200 mL of NLS solution was passed through the nanoparticles under vacuum conditions.

AH plus (Dentsply DeTrey, Konstanz, Germany), a two-paste epoxy-amine resin endodontic sealer, was used. QPEI nanoparticles were added to the paste at concentrations of 0% or 1% (wt/wt). AH plus with or without nanoparticles was manually mixed according to the manufacturer's instructions and placed in a 37°C incubator until fully set.

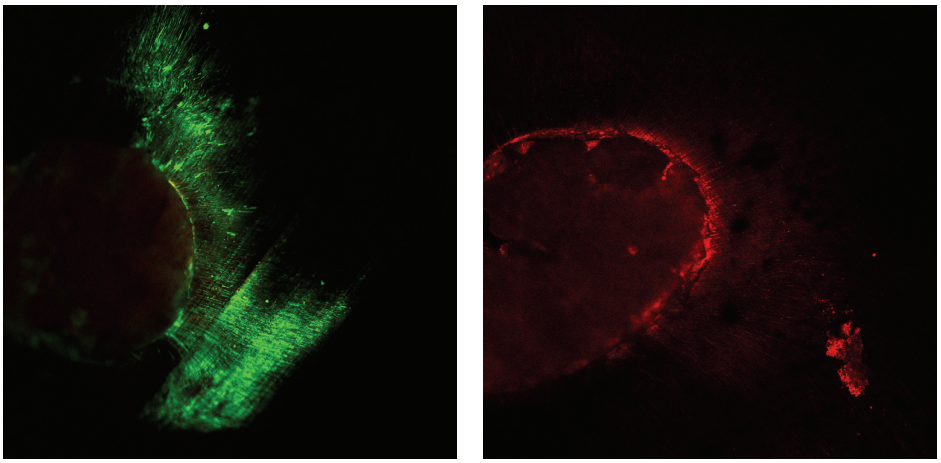
2.2. Teeth Selection. Twenty human single rooted teeth extracted for periodontal or prosthodontic reasons, with no previous endodontic treatment caries, coronal restorations, signs of resorption, or cracks, were selected from a pool of extracted teeth stored in a 0.5% sodium azide solution. The crown of each tooth was resected horizontally below the cementoenamel junction. A standard endodontic access cavity was prepared and the coronal third was flared with Gates Glidden burs (Mani Inc., Takanezawa, Japan) sizes 3, 2, and 1 in a step-down preparation with a maximum insertion depth of 3 mm. Apical patency was established by introducing a number 10 K-file (Mani Inc.) into each canal until the tip of the file became visible at the apical foramen. WL was determined by subtracting 1 mm from that length. Working length (WL) was adjusted to 15 mm.

2.3. Root Canal Instrumentation. All root canal preparations were completed by one operator (D. W) proficient in both systems. RC-Prep (Premier Dental Products, Plymouth Meeting, PA) was used in all canal preparations as a lubricant. The root canal was irrigated with 1 mL 2.5% sodium hypochlorite solution after each instrument change. Each instrument was discarded after 4 canals. The canals were prepared with PTU system with a torque-limited electric motor (X-Smart, Dentsply Maillefer, Ballaigues, Switzerland) according to the manufacturer's guidelines up to F3 (#30/.09). Apical size was adjusted to size #40 utilizing K-files (Mani Inc., Takanezawa, Japan). Radiographs from Bucco-Lingual (B-L) and Mesio-Distal (M-D) directions were obtained prior and following instrumentation. Each apical foramen was sealed with an epoxy resin applied on the outer surface of the apex. The root canal space was measured volumetrically and the mean volume was 10 (±1.2) μL. The prepared roots were autoclaved and stored at 4°C and 100% humidity until used. Roots were embedded, apices down, in an Eppendorf tube to a level 3 mm short of the cut surface. From this point, strict asepsis was applied, and all procedures were carried out in a bacteriologic hood.

2.4. Bacterial Strain and Culture Conditions. *E. faecalis* strain (ATCC V583) was grown overnight in brain-heart infusion (BHI) broth (Difco, Detroit, MI, USA) at 37°C under aerobic conditions. The top 4 mL was transferred to a fresh test tube and centrifuged for 10 min at 4,165 ×g. The supernatant was discarded and the bacteria were resuspended in 5 mL of PBS and vortexed gently for 10 sec. Two hundred μL was used for each experiment (~1×10^9 bacteria per mL).

2.5. Root Canal Infection. Each root canal was filled with the freshly inoculated broth and incubated at 37°C and 100% humidity for 1 week. The canal content was gently replaced with a fresh, similarly inoculated, culture broth every 24 h and was further incubated. Thus, *E. faecalis* was allowed to grow in the root canals for 7 days as previously described [11] ensuring total colonization of the root canal tubules by *E. faecalis*.

2.6. Root Canal Filling and Cutting. After the final 24 hours of *E. faecalis* inoculation the root canals were washed to remove all unattached bacteria. The remaining attached bacteria were subjected to disinfection (stage a) by 5 cc of 2.5% NaOCl and further circumferential filling to size #40 as described earlier; then canals were sampled for viable bacteria. In group A root



(a) (b)

Figure 1: Sealer incorporating QPEI nanoparticles inhibit *E. faecalis* growth. Representative confocal laser scanning microscopy of obturated root canal with a nonmodified sealer (a) and with a sealer incorporating QPEI nanoparticles (b). *E. faecalis* cells stained using BacLight LIVE/DEAD viability stain.

canals were then obturated with Gutta Percha (Dentsply DeTrey) and AH Plus (Dentsply DeTrey) in group B roots were obturated with AH Plus that was mixed with QPEI nanoparticles. After 14 days of incubation, the roots were sectioned by a low-speed, diamond-saw, sectioning machine (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water cooling. To prepare the dentin slabs of 1 mm thickness, cuts were made perpendicular to the long axis of the tooth. One dentin slab was obtained from each tooth.

2.7. Confocal Laser Scanning Microscopy (CLSM). Samples were stained using live-dead staining (Molecular Probes, Invitrogen Detection Technologies Eugene, OR, USA), according to the manufacturer's instructions. Images were observed using an Olympus IX70 (Olympus Corporation of the Americas, NY, USA), lens 10, zoom 3.5, and analyzed using Image pro software 7 (Media Cybernetics, Inc. Rockville, MD, USA).

2.8. Statistical Analysis. Statistical analysis was performed using the Paired Student's t-test, with significance level set at 0.05.

3. Results

Specimens were subjected to CLSM examination. Reduction in bacterial viability ($p < 0.01$) was depicted in the dentinal tubules of the root canals obturated with the sealer incorporating QPEI nanoparticles.

In both tested groups staining was evident in the tubules surrounding the root canal. Live bacteria, stained green, were more numerous in the dentinal tubules of the control group (Figure 1(a)) than in the test group depicting mostly red stained cells (Figure 1(b)).

Data analysis revealed that in the root canals obturated with the conventional sealer the percentage of live cells in the control group (77% ± 15%) was higher than in the test group incorporating QPEI nanoparticles (45% ± 13%) (Figure 2). Furthermore, statistical analysis showed that the reduction in live bacteria was observed in the teeth obturated with the sealer incorporating QPEI was significant ($p < 0.01$).

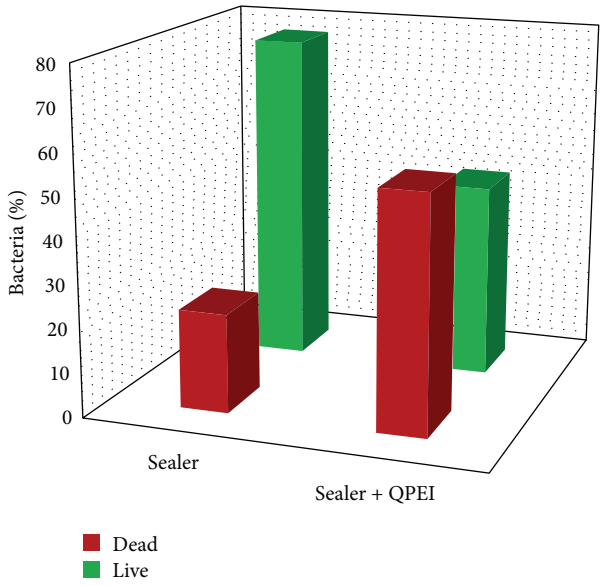


Figure 2: The average bacterial number (percentage) of live bacteria (green) and dead bacteria (red). Root canals obturated ($n = 10$ teeth) with the sealer incorporating QPEI nanoparticles show significantly ($p < 0.05$) less live cells (green) when compared to the root canals ($n = 10$ teeth) obturated with the nonmodified sealer.

4. Discussion

Antibacterial activity of epoxy-amine resin endodontic sealer containing small percentage of QPEI nanoparticles reduces

significantly *E. faecalis* viability in dentinal tubules of human teeth. The QPEI nanoparticles target *E. faecalis* in the dentinal tubules exhibiting a potent and prolonged antibacterial activity and thus are of potential therapeutic use.

Endodontic therapy aims to prevent and manage diseases of the pulp and periapical tissues (American Association of Endodontists, Glossary of Endodontic Terms, http://www.aae.org/). Normally, the dental pulp is sterile and is responsible for the production of dentin and immune response. Dentin formation by the peripheral pulp odontoblast cells is obtained by initial layering of dentin matrix that later mineralizes. Upon dentin formation the odontoblast withdraws towards the pulp center thus forming a tubule that elongates as the dentinogenesis progresses [12]. This tubule is 2-3 μ at the pulp side and 0.9 μ at the initial point of formation. Furthermore the tubules density is 60,000 to 80,000 per square millimeter. In health the odontoblast extension maintains dentin maturation and acts as the spearhead of the immune system that reacts to bacterial invasion following decay, trauma, or attrition. This reaction includes an increase in plasma proteins, activation of the innate immune systems, increased intratubular dentin formation, and an extracellular buffering effect that mediate the formation of caries crystals [13]. All these mechanisms are aimed at slowing bacterial penetration into the pulp. However if the bacterial assault is not arrested either by medical intervention or by the abovementioned mechanisms and some environmental changes (arrested caries), the odontoblast will die leaving the empty hosting affected tubule as a potential avenue for pulp infection [14]. Occasionally undifferentiated cells emerge from the pulp, replace the dead odontoblasts, and seal the breach in the pulp edge of the tubule. Eventually bacteria infect the pulp and lead to necrosis and subsequent destruction of all the odontoblastic processes. Clinically this process is asymptomatic in most cases. Hence, without notice the dentinal tubules may become a safe haven for bacteria.

Scanning laser confocal microscopy is considered a valid tool to evaluate bacterial viability in various treatment modalities. In the present study, we used CLSM to evaluate *E. faecalis* penetration into dentinal tubules of extracted human teeth. Variable patterns of bacterial penetration into the dentinal tubules were observed demonstrating bacterial penetration of up to 100–400 μm into the tubules. These findings coincide with previous findings that demonstrate the extent of bacterial penetration [15, 16]. Endodontic treatment aims to eradicate bacteria from root canal and dentin tubules by mechanical removal of infected tissues and concomitant chemical treatment with antiseptic solution such as sodium hypochlorite and chlorhexidine (mechanochemical preparation). Histological studies showed that bacterial contamination in tubules is not eradicated following irrigation with sodium hypochlorite [17, 18]. Intracanal application of antiseptic and antibiotic materials is often used as an auxiliary therapeutic measure aimed at promoting bacterial eradication. Medicaments such as calcium hydroxide or antibiotic pasts are used to further improve bacterial control before obturation. A microbiological study that examined the viability of *E. faecalis* in dental tubules following root canal treatment revealed the existence of viable *E. faecalis* cells 60 days following treatment regardless of the use of calcium hydroxide [19]. Recent CSLM investigations confirm these results showing that 29–50% of bacteria in the tubules survive calcium hydroxide treatment as compared to 83–98% that survive standard treatment prior to root canal filling [11].

It should be emphasized that following mechanochemical preparation, the last line of antibacterial defense is the filling materials. Unfortunately, sealers were reported to have a short and decreasing antibacterial effect that lasts not more than 7 days following obturation [6].

Cationic polymers represent a large group of potent antimicrobial. Their advantage compared to conventional antibiotics is their nonspecific mode of action [20–23]. In particular, QPEI have been shown to attain potent and long lasting antibacterial surface properties *in vitro* [24–26] and *in vivo* when incorporated into dental resin-composite materials [24]. Unfortunately, the exact mechanism of quaternary ammonium compounds is at most theoretic and is not fully understood. It has been suggested that electrostatic interaction between the polycationic structure and the predominantly anionic components of the microorganisms play a fundamental role in antibacterial activity. The lethal action of quaternary ammonium compounds is considered to be through adsorption and penetration into the bacterial cell wall. Presumingly, these compounds combine with the protein and analogous fatty layer of the cell membrane, block the normal exchange of ions and substances, and cause leakage of intracellular contents, leading to cell death [27]. Interestingly, herein modified epoxy-resin based sealer incorporating QPEI nanoparticles were able to target bacteria in the dentinal tubules and reduce significantly bacterial viability.

E. faecalis is known to be a highly recalcitrant bacterium due to its ability to withstand alkaline and glucose starvation, and thus it is prone to cause persistent infections [28, 29]. Peters et al. [30] argued that bacteria in dentinal tubules are tumbled beneath the root canal filling and will eventually die. However, microbiological [31] and histological [31, 32] studies demonstrated the growth of isolated islands of biofilms between an existing root canal filling and dentin walls. Unfortunately, as discussed above, the current intratubular infection control techniques fall short from the desired effectiveness to prevent infection of persistent infection.

In the present study we evaluated a standard epoxy resin which was modified by incorporating 1% QPEI nanoparticles. Although only a small percentage of QPEI were added the total bacterial population in the tubules was reduced by almost 50%. In conclusion, QPEI nanoparticles when incorporated in a small percentage into epoxy-resin based sealer may target *E. faecalis* in the dentinal tubules, producing a potent antibacterial effect that reduces significantly bacterial viability. Our results show that incorporation of QPEI nanoparticles into endodontic sealers may offer a potential therapeutic solution to prolong the antibacterial activity of the sealers and thus target recalcitrant bacterial infection.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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