Photoactivation of curcumin and sodium hypochlorite to enhance antibiofilm efficacy in root canal dentin

Prasanna Neelakantan a,*, Cheng Qing Cheng a, Vinoddhine Ravichandran a, Teresa Mao a, Priyanka Sriraman a, Swetha Sridharan a, Chandana Subbarao a, Subash Sharma a, Anil Kishen b

a Biofilm Research Cluster and Department of Conservative Dentistry and Endodontics, Saveetha Dental College and Hospitals, Saveetha University, Chennai, India
b Discipline of Endodontics, Faculty of Dentistry, University of Toronto, Toronto, Canada

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KEYWORDS
Biofilm; Confocal microscopy; Curcumin; Enterococcus faecalis; Photosensitizer; Sodium hypochlorite

Summary
Background: To test the effect of ultrasonic or light activated curcumin and sodium hypochlorite against Enterococcus faecalis biofilms in vitro.
Methods: E. faecalis biofilms were grown within root canals (n = 175) and divided into 7 groups (n = 25). Group 1, sterile saline; group 2, 3% sodium hypochlorite; group 3, 3% sodium hypochlorite activated with ultrasonic files (30s cycles for 4 min); group 4, 3% sodium hypochlorite irradiated with blue light (1200 mw/cm² for 4 min); group 5, curcumin (2.5 mg/mL); group 6, curcumin (2.5 mg/mL) activated with ultrasonic files (30s cycles for 4 min); group 7, curcumin (2.5 mg/mL) irradiated with blue light. The biofilms’ ultrastructure was examined using scanning electron microscopy. Bacterial viability was assessed by confocal microscopy. Data were analyzed by one-way ANOVA and Student–Newman–Keuls test (P = 0.05). The quantitative analysis of the colony-forming units was carried out from dentinal shaving and analyzed by One-way ANOVA and Tukey multiple comparison test (P = 0.05).
Results: All treatment groups showed a significantly higher percentage of dead bacteria than the saline control (P < 0.05). The percentage of dead bacteria was significantly higher when light activated curcumin was used (P < 0.05). At both depths (200 and 400 microns), light activated curcumin showed no growth of bacteria.
Conclusions: Light activation produced significantly higher antibacterial efficacy than ultrasonic agitation, with light activated curcumin producing the maximum elimination of biofilm bacteria within the root canal lumen and dentinal tubules.

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* Corresponding author at: Plot 1500, 16th Main Road, Anna Nagar West, Chennai, Tamil Nadu, India. Tel.: +91 98847 54914; fax: +91 044 2616 3639.
E-mail address: prasanna_neelakantan@yahoo.com (P. Neelakantan).

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Introduction

Photodynamic therapy has shown great potential in the treatment of localized bacterial infections [1]. It involves the photoactivation of a photosensitizer with low-energy coherent or non-coherent light, in the presence of oxygen to produce reactive oxygen species, such as hydroxyl radicals, superoxides and singlet oxygen. These reactive oxygen species act on multiple targets in a bacterial cell, resulting in instantaneous killing. Since photodynamic therapy results in the instantaneous killing of bacteria, generation of photodynamic therapy-resistant bacteria is highly unlikely [2]. Though this method has been shown to be effective against both gram-positive and gram-negative bacteria, they are more effective against the gram-positive species [2].

Chemomechanical preparation of the root canal system aims at complete eradication of intracanal microbial biofilms, which is unachievable with the current materials and techniques [3,4]. The root canal anatomy poses several challenges in disinfection owing to the complexities that prevent irrigant penetration into the anatomical complexities within the root canal. Therefore, the role of irrigant activation becomes important to potentiate the antimicrobial efficacy of an endodontic irrigant within the root canals. Of late, research has been focused on the use of sonics, ultrasonics and laser/light energy for the intracanal activation of irrigants [5–7]. The primary goal of combining a suitable endodontic irrigant with activation methods is not only to achieve marked reduction of bacterial biofilms from the main canal but also from the anatomical complexities and dentinal tubules [5]. Photodynamic therapy is known to be less toxic to mammalian cells and less damaging to dentin ultrastructure [8].

Medicinal plants represent a rich source of antimicrobial agents. They are an important source of many potent and powerful drugs [9]. Wide ranges of medicinal plant extracts are established to possess antioxidant and immune-modulatory properties with minimum toxic/side effects [10]. These agents can find significant application as topical antimicrobials where host cells are located in the vicinity. Currently, there has been a growing trend to seek natural agents for dental treatment. Curcumin is such an antimicrobial agent, which can be used as a photosensitizer [11]. Thus, curcumin may not only exhibit antimicrobial properties but also produce photodynamic effects to further potentiate its antimicrobial efficacy.

Enterococcus faecalis, a facultative anaerobic gram-positive coccus, is implicated in the re-infection of the root-filled teeth, owing primarily to their ability to adhere to the root dentin, resist some of the antimicrobials used within root canals, survive hard environmental conditions, develop antibiotic resistant strains, and form communities organized in biofilm, thereby enabling it to become highly resistant to phagocytosis, antibodies, and antimicrobials than non-biofilm-producing organisms [12,13]. In addition, they show an exceptional ability to form robust biofilms on root dentin with homogeneous deposition of extracellular polymeric matrix. Therefore this microorganism has been utilized as a model organism to compare the antibiofilm efficacy of different antimicrobials in endodontics [6–8,10,11,13].

The current study was undertaken to examine the antibiofilm efficacy of ultrasonically agitated and light activated curcumin and sodium hypochlorite irrigation on matured monospecies E. faecalis biofilms grown on root dentin in vitro. The null hypothesis of the study was that the ultrasonic agitation or light activation did not improve the antibiofilm efficacy of curcumin and sodium hypochlorite irrigation in root dentin.

Materials and methods

Sample preparation

Single-rooted mandibular premolars with a closed apex[n = 175] were used in the study based on a protocol approved by the Institutional Review Board and Ethics Committee of the University. The teeth were collected in 0.01% sodium hypochlorite solution and maintained hydrated until use. The crowns were sectioned and the root lengths were standardized to 15 mm. The working length was defined as 1 mm short of the apical foramen. The root canals were prepared with Mtwo rotary nickel titanium instruments [VDW GmbH, Munich, Germany] to an apical size of 35/0.04 taper using 3% sodium hypochlorite as the irrigant.

The biofilm tooth model employed in this study was adapted from Lin et al. [14]. Buccal and lingual grooves were made to split the tooth longitudinally. The split halves were reapproximated using utility wax placed over the root tip to simulate an in vivo closed apical system that provided resistance to irrigant flow by creating an apical vapor lock effect [14,15]. Smear layer was removed by placing the sections in an ultrasonic bath of 5.25% sodium hypochlorite and 17% ethylene diamine tetraacetic acid for 4 min each, rinsed in sterile water for 1 min and autoclaved (20 min at 121°C).

Bacterial inoculation and biofilm generation

E. faecalis [ATCC 29212] was plated on brain heart infusion broth supplemented with 1.5% [wt/vol] agar and incubated anaerobically at 37°C for 24 h. A single colony of E. faecalis was collected from the agar plate and suspended in sterile brain heart infusion broth. Root specimens were placed in sterile centrifuge tubes containing 3 mL E. faecalis suspension [1 × 10^8 mL⁻¹] and incubated under anaerobic conditions at 37°C for 4 weeks. Fresh broth was replaced every second day to remove dead cells and to ensure bacterial viability. After incubation, the specimens were removed from the tubes aseptically and rinsed with sterile phosphate-buffered saline to remove the culture medium and nonadherent bacteria. Dentin sections [n = 4] were observed by a field emission scanning electron microscope [FE-SEM; JSM-7500F, JEOL Ltd., Tokyo, Japan] to verify the presence of E. faecalis biofilms on the dentin surfaces [Figure 1].

Treatment of biofilms

The sectioned teeth were reassembled and placed in a stone casing [14] after which they were randomly divided into 7 treatment groups [n = 25]: group 1, sterile saline; group
from the pulp–dentin junction toward the cementum to determine how effectively the irrigant activation methods disinfected the dentinal tubules of the root canal system. Statistical analysis of the data was done using one-way analysis of variance and Student–Newman–Keuls. The alpha error was set at \( P = 0.05 \).

**Dentin powder analysis**

Dentin debris from the root samples \( n = 20 \) sections was harvested at 2 depths (200 and 400 microns) using Gates Glidden drills nos. 4 and 5 [Mani Inc., Tochigi-Ken, Japan], respectively [17], and collected in 1 mL of sterile brain heart infusion broth and incubated in an anaerobic environment at 37 °C for 24 h. The content of each microcentrifuge tube was serially diluted, 100 \( \mu \)L of broth in 100 \( \mu \)L of normal saline for 5 times. Five microliters of this sample was plated on brain heart infusion agar plates and incubated for 24 h. The microbial colony-forming units count [CFU/mL] was counted and the data were statistically analyzed with one-way ANOVA followed by Tukey multiple comparison \( P = 0.05 \).

**Results**

Confocal laser scanning microscopic [CLSM] analysis of biovolume and viable/dead cells in biofilm structure:

The data obtained from the confocal microscopic analysis of biofilms is tabulated [Table 1]. The mean biofilm height in the control group was 428.32 ± 72.16 \( \mu \)m. Figure 2A–F shows the three-dimensional reconstruction of biofilm structures obtained from different groups. The biofilms in the root canal lumen were completely destroyed in all the experimental groups [groups 2–7]. Consequently, only the percentage of live and dead bacteria within the dentinal tubules was calculated. The percentage of dead bacteria was significantly higher when light activated curcumin was used [group 7, Figure 2F], while the least percentage of dead bacteria was found in the saline treated group \( P < 0.05 \). The superficial layer [200 microns] demonstrated the least amount of live bacteria in all the test irrigation groups, which was significantly lesser than the control group \( P < 0.05 \). At 400 microns, there was no significant difference in the proportion of dead cells present in the groups 2–6 \( P > 0.05 \).

**Dentin powder analysis for the quantitative assessment of the viable biofilm bacteria**

Data from this analysis showed a significant reduction of viable bacteria in all groups compared to the control \( P < 0.05 \). At 200 microns depth, all experimental groups except group 5 and the control saline group showed a 7 log reduction of bacteria [no growth]. Light activated curcumin showed no growth [7 log reduction] of bacteria at 400 microns and the colony-forming units/mL was significantly less than the other groups \( P < 0.05 \).
Table 1  Percent of apparently dead bacterial cells in the overall biofilm biomass and bacterial colony-forming units (CFU/mL) within the dentinal tubules at 200 and 400 microns depth, assessed by confocal laser microscopy and microbial culture analysis after different treatment regimes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Biofilm mass at 200 microns depth</th>
<th>Dead cells % at 200 microns depth</th>
<th>Dead cells % at 400 microns depth</th>
<th>CFU/mL at 200 microns depth</th>
<th>CFU/mL at 400 microns depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (Group 1)</td>
<td>1.975 ± 0.944a</td>
<td>2.86 ± 0.878a</td>
<td>0.94 ± 0.074a</td>
<td>1.5 × 10⁹ ± 0.72 × 10⁹a</td>
<td>1.9 × 10⁹ ± 0.36 × 10⁹a</td>
</tr>
<tr>
<td>Sodium hypochlorite (Group 2)</td>
<td>58.57 ± 5.622a</td>
<td>64.79 ± 3.18b</td>
<td>43.27 ± 3.88b</td>
<td>No growthb</td>
<td>3.2 × 10⁵b</td>
</tr>
<tr>
<td>Sodium hypochlorite – ultrasonic (Group 3)</td>
<td>62.34 ± 4.178b</td>
<td>73.65 ± 2.31b</td>
<td>55.91 ± 3.74b</td>
<td>No growthb</td>
<td>1.5 × 10⁴b</td>
</tr>
<tr>
<td>Sodium hypochlorite – light (Group 4)</td>
<td>74.87 ± 5.872b</td>
<td>81.86 ± 3.29b</td>
<td>62.65 ± 2.86b</td>
<td>No growthb</td>
<td>1 × 10⁴b</td>
</tr>
<tr>
<td>Curcumin (Group 5)</td>
<td>31.6 ± 3.123c</td>
<td>37.16 ± 2.97c</td>
<td>20.15 ± 3.17c</td>
<td>4.2 × 10⁴ ± 0.81 × 10⁴c</td>
<td>4.8 × 10⁴ ± 0.43 × 10⁴b</td>
</tr>
<tr>
<td>Curcumin – ultrasonic (Group 6)</td>
<td>38.96 ± 4.64c</td>
<td>42.81 ± 2.48c</td>
<td>27.32 ± 1.87c</td>
<td>No growthb</td>
<td>3.0 × 10⁴b</td>
</tr>
<tr>
<td>Curcumin – light (Group 7)</td>
<td>97.32 ± 3.29d</td>
<td>98.61 ± 2.1d</td>
<td>86.35 ± 3.92d</td>
<td>No growthb</td>
<td>No growthc</td>
</tr>
</tbody>
</table>

Discussion

Pulp and periapical pathoses are recognized as host responses to biofilm-mediated infection [18]. Therefore, potent antimicrobial strategies that can eliminate bacterial biofilm are an important requisite in endodontic therapy. The core objective of an antimicrobial strategy in endodontics is to bring about destruction of biofilm structure within the root canal system [18,19]. Sodium hypochlorite is the most commonly recommended root canal irrigant. It is a potent antimicrobial agent with an ability to bring about destruction of root canal biofilms [20]. However, its proteolytic nature has a detrimental effect on dentin microhardness, ultrastructural integrity, modulus of elasticity and flexural strength [21]. It is cytotoxic to the periapical tissues in the event of inadvertent extrusion into the periradicular region [22]. Irrigant activation/agitation is an approach to enhance the penetration of the antibacterial irrigant so as to increase the degree of bacterial killing within these locations [19]. The inability of the current root canal irrigation strategies to efficiently eliminate bacterial biofilms from the anatomical complexities and dentinal tubules still remains to be a major challenge [18]. This signifies the need for new irrigants that would have the positive attributes of sodium hypochlorite and potential for enhancement.

The effectiveness of antimicrobial photodynamic therapy in root canal disinfection has been demonstrated by several in vitro and in vivo studies [11,12,23,24]. The highly reactive oxygen species produced during photoactivation interact with the amino acids molecules in dentin collagen to promote cross-linking [25,26]. This increase in the number of intermolecular collagen bonds has been found to enhance the resistance of dentin collagen to bacteria-mediated enzymatic degradation and improve the fracture toughness of dentin [25–27]. Low-energy level red and near infrared light is known to penetrate dentin tissue/dentinal tubules without inducing untoward effects on dentin matrix [27]. Therefore, the current study examined the efficacy of photodynamically activated curcumin to eliminate biofilm and disinfect dentin tissue in vitro. Few studies have focused on the antibiofilm activity of photodynamic activation of curcumin [27], but no study till date has investigated the effect of photodynamic activation on antibiofilm activity of curcumin used as a root canal irrigant. The findings from this study showed that photodynamic activation enhanced the antibiofilm activity of curcumin on the root canal lumen and the dentinal tubules when compared to the controls. Hence, the null hypothesis needs to be rejected. The superior antibiofilm efficacy and significant bacterial killing from the dentinal tubules was exemplified by the colony-forming unit analysis of the dentin powder.

This study compared the antibiofilm activity of curcumin and sodium hypochlorite subjected to photoactivation or ultrasonic activation, using two methods: Confocal laser scanning microscopy and bacterial culture based technique. Confocal microscopy provides valuable information on the ability of a material to kill microorganisms. Its ability to render data in a three-dimensional fashion helps in obtaining information about the biofilm structure following treatment [28]. Colony-forming units analysis of the dentin powder aided in determining quantitatively, the degree of bacterial reduction within the dentinal tubules. Confocal microscopy was used to determine the percentage of dead and live bacteria, and is a more valid method to study the efficacy of antimicrobial agents on bacterial biofilm structure [20,28].

The resistance of biofilm bacteria to antimicrobial agents could be several times more than their planktonic counterparts due to the relative impenetrability of the antimicrobials into biofilms [29], emphasizing the need for activation methods. Ultrasonically activated irrigation involves the transmission of acoustic energy from the oscillating file to the irrigant to allow movement of irrigants laterally into the anatomical eccentricities of the root canal system [30]. This study showed that ultrasonic activation of curcumin and sodium hypochlorite resulted in higher percentage of dead bacteria than the control groups.
This finding could be attributed to the higher rate of catalytic decomposition or degassing of hypochlorite [31]. Consequently, application of a fresh solution of sodium hypochlorite is important to exploit this property [32]. Ultrasonically activated curcumin showed significantly lower percentage of dead bacteria than ultrasonically activated hypochlorite. This finding highlighted that unlike in the case of sodium hypochlorite, ultrasonic activation was ineffective in enhancing the antibacterial properties of curcumin. Previous reports on the antibiofilm efficacy of ultrasonically activated hypochlorite are not conclusive [33,34]. This difference could be attributed to the methodological variables used in the studies such as: biofilm model, concentration of sodium hypochlorite used and the method of evaluation.

Conventional photoactivated disinfection in root canal treatment employs one of the two photosensitizers (methylene blue or toluidene blue) after the use of sodium hypochlorite. These photosensitizers need light within the wavelength range of 620—660 nm to elicit their effect, which needed dedicated light sources [35]. A recent study demonstrated that use of conventional photosensitizers after
chemomechanical preparation of root canals with sodium hypochlorite did not offer significant effect in the reduction of E. faecalis [35]. Hence, novel photosensitizers need to be evaluated for better clinical performance of photoactivated disinfection.

The present study demonstrated that photodynamic activation of curcumin was able to bring about the highest percentage of bacterial killing [97.32 ± 3.29%]. Photokilling by curcumin has been demonstrated previously [13]. However, this appears to be the first report on the application of curcumin as a photosensitizer for root canal disinfection. The basic structure of curcumin with the presence of two highly conjugated electron systems renders it as an effective photosensitizer. On irradiation with light, active oxygen species are produced. Curcumin exhibits strong absorption of light within the wavelength of 420–430 nm. Furthermore, it forms inter and intramolecular hydrogen bonds which influence the photophysical properties both in the normal state and on excitation by light [36, 37]. It has been proposed that on illumination, curcumin produces hydrogen peroxide as an intermediate, which is toxic to bacterial cells [13]. Another important moiety in curcumin is the keto-enol form, which is considered primarily responsible for the phototherapeutic effects [38, 39]. An important advantage of curcumin is its ability to exhibit its lethal effects without binding or being in close proximity to bacteria [13]. Nevertheless, binding of the PS to the cell wall of bacteria potentially increases the sensitivity of the microbe to light [38]. The usefulness of curcumin as a potential inter-appointment intracanal medicament is being investigated. Future research should also be directed to elucidate the exact mechanisms of photokilling by curcumin in the endodontic environment. Furthermore, the efficacy of photoactivated curcumin after chemomechanical root canal preparation with sodium hypochlorite needs to be assessed.

In conclusion, light activated disinfection of root canal systems using curcumin as photosensitizer showed superior antibiofilm activity than ultrasonic activation. Photoactivated curcumin demonstrated significantly higher antibacterial activity than sodium hypochlorite.

Conflict of interest

The authors declare no conflicts of interest.

References


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