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Photodynamic Inactivation of Endopathogenic Microbiota Using Curcumin- mediated Antimicrobial Photodynamic Therapy

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Abstract

Root canal disinfection is one of the main factors governing success of endodontic therapy. Antimicrobial photodynamic therapy (aPDT) is presented as a promising antimicrobial therapy that can eliminate microbiota present in infected root canal systems. In this study, a series of experiments investigated the effects of aPDT on cell viability and biofilm degradation ability of endopathogenic microbiota. *Enterococcus faecalis* and *Pseudomonas aeruginosa* strains as the versatile pathogenic microbiota in endodontic infections were used as the tested strains. Curcumin (CUR) and light-emitting diode (LED) were used as the photosensitizer and light source, respectively. The antimicrobial and anti-biofilm effects of CUR-aPDT were assessed by colony forming unit (CFU), and crystal violet assays. CUR-aPDT significantly decreased the CFU/mL count of E. faecalis and P. aeruginosa compared to the control group ($P<0.05$). The killing percentage of aPDT was achieved 95.0% for *E. faecalis* and 81.1% for *P. aeruginosa*. In addition, the biofilm degradation ability of *E. faecalis* and *P. aeruginosa* significantly decreased to 41.2% and 30.2%, respectively ($P<0.05$). According to the results, neither CUR nor LED showed the antimicrobial and anti-biofilm effects when used alone. In conclusion, the results of this study indicated that CUR-aPDT has an appreciable effect on endopathogenic microbiota upon photoactivation.

Keywords: Antimicrobial photodynamic therapy; Biofilm degradation; Curcumin, *Enterococcus faecalis*; *Pseudomonas aeruginosa*.

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Introduction

The prevention and treatment of the dental pulp and periapical tissues diseases is the main objective of endodontic therapy [1]. This goal can be achieved via preventive and treatment procedures based on a detailed understanding of the etiology and pathogenesis of endodontic infections and a complete knowledge of the effective treatment approaches [2].

Enterococcus faecalis is a versatile pathogenic microbiota that plays a key role in the etiology of treated and untreated root canals and is highly associated with treatment failures of endodontic infections [3]. Also, *Pseudomonas aeruginosa* an endo-periopathogenic bacterium has been detected in the pulpo-periapical diseases. This might show the potential of this bacterium to pervade barriers like dentin [4]. Regardless of the entrance of colonization, it is at least an evidence of the aptitude of this bacterium to promptly inhabit structures as untreated or accessory root canals with altered pulps [4].

Accordingly, treatment of endodontic infections involves chemomechanical procedures using antimicrobial agents [5, 6]. Although the irrigating solutions such as sodium hypochlorite (NaOCl) and chlorhexidine gluconate (CHX) are the important agents in microbial elimination and removal of necrotic tissue and pupls from the infected root canal system, the major disadvantages of NaOCl refer to its corrosiveness to metals, high toxicity, discoloration effect on the tooth, inactivation by organic matter, and relative stability [34]. Also, the functionality of CHX, is dependent on pH [7, 8].

Antimicrobial photodynamic therapy (aPDT), also known as photo-activated disinfection (PAD) or photochemotherapy, is known to be an effective adjunctive therapy, along with the traditional root canal irrigants and antimicrobial agents, in eliminating the intracanal microbiota [9]. This non-invasive technique is based on the degradation of free radicals with high cytotoxicity, such as superoxides and singlet oxygen produced by nontoxic PS irradiated under appropriate wavelength causing serious damage to the microbial cell components [9, 10]. However, it has been shown that aPDT not only can be used for killing bacteria, but it can also be used to reduce the impact of bacterial virulence factors and biofilm degradation and prevent the colonization of bacteria in the root canal [10, 11].

Nowdays, there is a trend on the use of natural photosensitizers in aPDT, due to their outstanding featurs including cost-effectiveness, exposure to the lower concentrations leading less toxicity, and less discoloration of the teeth [12]. Recent investigation showed that curcumin (CUR) is pointed out as a potent photoactivatable substance that exhibits a variety of pharmacological properties such as antimicrobial activiries and anti-inflammatory [12, 13]. Although some studies have reported photoinactivation of cariogenic microorganism with CUR, but there is insufficient indegradation on the effect of this approach on bacteria involved in endodontic infection [14]. This study aimed to assess the effectiveness of aPDT using CUR as a photosensitizer against cell viability and biofilm degradation ability of *E. faecalis* and *P. aeruginosa* as the endopathogenic strains.

Materials and Methods

Preparation of microorganisms

The suspensions of *E. faecalis* ATCC 29212 strain and *P. aeruginosa* ATCC 9027 strain were inoculated in brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) and incubated aerobically at 37 °C to reach to an optical density of approximately 1.0×10^6 colony forming units (CFUs)/mL.

Light source and photosensitizer

A light-emitting diode (LED; DY400-4, Denjoy, China) with a power intensity of 1000–1400 mW/cm^2 at an absorption spectrum with a central wavelength at 450 ± 30 nm was used and the irradiance tested was 72 J/cm^2. CUR (Sigma-Aldrich, St Louis, MO, USA), as photosensitizing agent, in concentration of 40 µM solved in dimethyl sulfoxide (DMSO) solution (Sigma-Aldrich, St Louis, MO, USA) at the final concentration of 0.5%.

Study design

The experimental groups were treated in accordance with the following experimental conditions:

A: CUR group (at concentration of 40 µM)

B: LED group (irradiation at an influence rate of 72 J/cm^2)

C: aPDT (CUR at concentration of 40 µM + irradiation at an energy density of 72 J/cm^2)

D: Positive control (only bacterial suspension)

E: Negative control (only BHI broth)

Colony count assessment in treated endopathogenic strains

The antibacterial effect of CUR-mediated aPDT against *E. faecalis* and *P. aeruginosa* was evaluated based on the previous study [15]. In short, 100 µL of each microbial suspension (1.0×10^6 CFU/mL) was added separately to the wells of a 96-well flat-bottomed sterile polystyrene microplates (TPP; Trasadingen,

Switzerland). 100 µL of CUR poured separately in designated wells. After that, the microplates stayed in the dark for 5 min in the specified conditions depending on the type of microorganism. The wells were exposed to LED according to the groups. Group A was not exposed to radiation, and the control group did not receive any treatment. The CFU/mL values of the test wells were determined as previously explained [15].

Assessment of biofilm degradation ability in treated endopathogenic strains by crystal violet assay

Evaluation of biofilm degradation ability of the treated *E. faecalis* and *P. aeruginosa* was done according to previous study [15]. Breifly, after incubation of 1.5×10^8 CFU/mL of each suspension in a flat-bottomed sterile polystyrene microplate for 24 h, the wells were exposed to the CUR, LED, and aPDT. The plates gently washed with phosphate-buffered saline (PBS), stained with 0.1% (wt/vol) crystal violet, and de-stained with ethanol (96%). The wells were then filled with 33% (vol/vol) acetic acid and biofilm degradation was quantified by measuring the absorbance of the solution at 570 nm using a microplate reader (Thermo Fisher Scientific, US).

Statistical analysis

In order to verify the differences between the groups and assess the effects of the treatments, the data were statistically analyzed by ANOVA and Tukey's test using software SPSS V. 22.0. All experiments were performed in triplicate. The results were expressed as mean values ± standard deviations (mean ± SD) and were considered significant at the level of $P < 0.05$.

Results

The effect of treatments on planktonic forms of endopathogenic strains

Based on the results shown in Figure 1, only CUR-aPDT group could significantly reduce the count of *E. faecalis* and *P. aeruginosa* to 95.0% and 81.1%, respectively, in comparison with the control group ($P<0.05$). As seen, there was significant difference between CUR-aPDT with CUR and LED groups ($P<0.05$), whereas, it found interestingly that there was no considerable difference between CUR and LED groups ($P>0.05$).

The effect of treatments on endopathogenic strains biofilms

According to the results, CUR and LED groups could not significantly reduce the biofilm forms of *E. faecalis* and *P. aeruginosa* compared to the control group

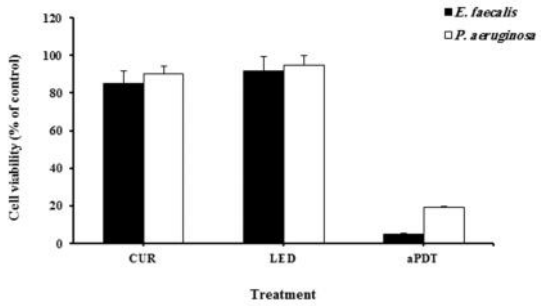

Figure 1. Effect of treatments on planktonic forms of *Enterococcus faecalis* and *Pseudomonas aeruginosa* strains. *represents $p<0.05$ as compared to corresponding control.

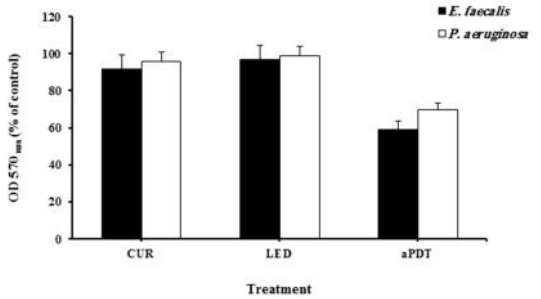

Figure 2. Effect of treatments on *Enterococcus faecalis* and *Pseudomonas aeruginosa* biofilms. *represents $p<0.05$ as compared to corresponding control.

($P>0.05$; Fig. 2). In contrast, a significant difference in reduction of biofilm degradation of endopathogenic strains was observed in CUR-aPDT compared to the untreated bacteria ($P<0.05$). The biofilm forms of *E. faecalis* and *P. aeruginosa* were decreased to 41.2% and 30.2% following CUR-aPDT treatment. As well as, there was a significant difference in biofilm reduction between *E. faecalis* and *P. aeruginosa*, so that, CUR-aPDT was able to reduce *E. faecalis* biofilm 1.4 times more than *P. aeruginosa* biofilm.

Discussion

The utility of the conventional antimicrobial agents for treatment of infected root canals has become limited due to increased rates of resistance coupled with reduced the complete removal of bacteria [16, 17]. Therefore, there is a pressing need for development of alternative antimicrobial methods to fight endodontic infections.

Studies conducted in recent years highlighted that aPDT could be a promising adjunct in antimicrobial endodontic treatment. CUR [1E,6E-1,7-bis(4-hydroxy-

3-methoxyphenyl)-1,6-heptadiene-3,5-dione], isolated from the rootstocks of the plant *Curcuma longa*, has antibacterial and anti-biofilm activities against microorganisms [18]. It has been reported that there is the assembly of a protein-filamenting temperature-sensitive mutant Z (FtsZ). The protofilaments are inhibited by CUR, which leads to an increase in the GTPase activity of FtsZ and causes bacterial cell death [19].

Araújo et al. reported that CUR could be considered as a feasible photosensitizer for aPDT due to its absorption spectrum in the UV/blue wavelength range (300–500 nm with a maximum at 430 nm) [14].

In this study, we investigated the effect of CUR-mediated aPDT aganist *E. faecalis* and *P. aeruginosa*, as the Gram-positive and Gram-negative bacteria involved in endodontic infections, respectively.

As the results of CFU/mL showed, CUR-aPDT could significantly reduce the count of *E. faecalis* approximately 1.2 times more than *P. aeruginosa*. The literatures showed that the microbial reduction by aPDT confronts different challenges when used against Gram-positive and Gram-negative bacteria [20-24]. The cell wall of Gram-positive bacterium is formed by a thick layer of peptidoglycan and lipoteichoic acid that is relatively porous and allows a greater diffusion of the photosensitizer into the bacterium [22-25]. In contrast, the outer membrane of Gram-negative bacteria is formed by a complex composition which acts as an effective barrier limiting the penetration of some substances. The high Mg^{2+} content in the outer membrane of Gram-negative bacteria aids in producing strong lipopolysaccharide LPS links, may result in limitation on the entrance of photosensitizers. Moreover, limiting access or efficiently removing the active biocide by efflux pumps that are encased in the cell envelope of Gram-negative bacteria also contribute to increased levels of resistance [24].

On the other hand, it is known that endodontic infection is resulted of biofilm accumulation of microbiota in root canal system [2]. So, the present investigation evaluated the CUR-aPDT effects on biofilm forms in addition to planktonic phases. According to the results of this study, a significant difference in reducing of biofilm degradation of *E. faecalis* and *P. aeruginosa* strains was observed in CUR-aPDT compared to the untreated bacteria, but this reduction is less than reducing the CFU/mL of bacteria. The previous studies showed that the resistance of microbial biofilms to aPDT is several times more than their planktonic forms due to the impenetrability of photosensitizer into biofilms [26, 27]. According to our results, neither CUR nor LED revealed an antimicrobial effect when used alone. This outcome is in agreement with current photodynamic studies [1, 12, 21, 30]. In fact, these results highlighted the need for photosensitizer-light conjugation to ensure the effectiveness of aPDT therapy. Nevertheless, bacterial reduction is initiated in infected root canals and successful treatment of endodontic will be obtained via selection of appropriate aPDT dosage to remove microbial biofilms [2].

Conclusion

In accordance of our finding, the outcomes of this current investigation demonstrated that CUR-aPDT was able to photoinactivate planktonic suspensions and biofilm forms of *E. faecalis* and *P. aeruginosa* associated with infected root canal systems. Therefore, CUR can be introduced as a proper photosensitizer in aPDT for the treatment of endodontic infections.

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