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Effect of ultrasonic activation on the efficacy of antimicrobial photodynamic therapy: Evaluation of penetration depth of photosensitizer and elimination of *Enterococcus faecalis* biofilms

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ABSTRACT

Background: This study aimed to assess the effect of ultrasonic activation of photosensitizer on the efficacy of Antimicrobial Photodynamic Therapy (aPDT) against *Enterococcus faecalis* and penetration depth of photosensitizer.

Materials and methods: In this *ex vivo* study, mature microbial biofilm of *E. faecalis* was formed in the root canals of 58 single-rooted single-canal mandibular incisors following their decoronation. The roots were longitudinally sectioned by a diamond disc and split into halves by a chisel. The *E. faecalis* biofilm was quantified and the penetration depth of photosensitizer was determined by the microbial viability assay and stereomicroscopic analysis in the following three study groups: (1) Ultrasonically activated 5.25% sodium hypochlorite (NaOCl) for 20 s, (2) aPDT using methylene blue (MB) plus 660 nm diode laser with 150 mW power for 1 minute, and (3) ultrasonically activated MB for 20 s followed by aPDT as in group 2. Independent sample *t* test and one way ANOVA were used to compare the dye penetration depth and microbial load, respectively in the apical and coronal regions among the groups.

Results: The penetration depth of photosensitizer in group 3 was significantly greater than that in group 2 ($P < 0.05$). The *E. faecalis* count in all three experimental groups was significantly lower than that in the control group ($P < 0.05$). Groups 1 and 3 were significantly superior to group 2 in terms of reduction in microbial count but the difference between groups 1 and 3 was not significant ($P > 0.05$).

Conclusion: Ultrasonic activation of photosensitizer in aPDT increases the penetration depth of photosensitizer into the dentinal tubules and enhances its antibacterial activity.

Highlight: Ultrasonic activation of photosensitizer in aPDT enhances its penetration depth into dentinal tubules and increases antibacterial efficacy.

There was no significant difference between antibacterial effects of aPDT + ultrasonic and ultrasonic activated NaOCl.

1. Introduction

Efficient disinfection of the root canal system is a prerequisite for a successful endodontic treatment. Complete elimination of microorganisms from the root canal system cannot be achieved by the commonly used techniques such as mechanical instrumentation, root canal irrigation and application of intracanal medicaments [1]. Despite many cases of successful endodontic treatment in presence of residual microorganisms in the root canal system [2], evidence shows that residual bacteria can lead to an adverse outcome in regenerative endodontic

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procedures [3]. Moreover, chemomechanical preparation is challenging in necrotic teeth with weak dentinal walls. Thus, the efficacy of disinfection of the root canal system should be further improved. There is also a clear need for an effective non-invasive disinfection technique for teeth with immature or necrotic pulp [4]. Recently, several in vitro and in vivo studies [5–7] have reported promising results regarding the optimal efficacy of root canal disinfection with Antimicrobial PhotodynamicTherapy (aPDT). However, many researchers believe that aPDT cannot replace the commonly used disinfection techniques but can be used as an excellent adjunct for efficient root canal disinfection [8–10].

Enterococcus faecalis (*E. faecalis*) is a Gram-positive facultative anaerobe and is among the most resistant culprits involved in development of endodontic infections [11]. It has a biofilm-forming capability and can break down dentin in a nutrient-free environment. It can also penetrate deep into dentinal tubules [12].Thus, *E. faecalis* in the form of biofilm was used in this study.

Sodium hypochlorite (NaOCl) is the irrigant of choice for root canal irrigation; however, some concerns exist regarding the weakening effect of NaOCl on dentin structure [13], its toxic effect [14] and risk of hypochlorite accident in immature teeth with a necrotic pulp [15]. Thus, researchers have long been in search of alternative root canal disinfection systems such as laser, ultrasound, the Gentle-Wave system, photon-induced photoacoustic streaming and aPDT [16].

In aPDT, a non-toxic dye, known as a photosensitizer, is activated with a certain wavelength of light to produce reactive oxygen species [17]. Since the duration of activity and the penetration depth of dye are very short (less than 0.04 s and 0.02 µm, respectively) in biological systems, it seems that the antibacterial efficacy of aPDT depends on the penetration depth of photosensitizer into the dentinal tubules [18]. Evidence shows that ultrasonic activation may result in better cleaning of accessory canals in the apical third of the root canal system and can increase the penetration depth of irrigating solutions into the dentinal tubules [19,20].

In the present study, ultrasonically activated NaOCl was used as the gold standard for root canal disinfection, based on previous studies, that confirms penetration depth of NaOCl into dentinal tubules is enhanced by ultrasonic activation and subsequently its antimicrobial efficacy is increased [21]; the other experimental groups were compared to this group. Studies on the effect of ultrasonic activation of photosensitizer on its penetration depth into dentinal tubules are limited. This study aimed to assess the effect of ultrasonic activation on penetration depth of methylene blue (MB) photosensitizer into dentinal tubules and its association with the antibacterial activity of aPDT against *E. faecalis* biofilm in comparison to NaOCl as the gold standard irrigant in endodontic therapy.

2. Materials and methods

2.1. Sample preparation

A total of 58 single-rooted, single-canal mandibular incisors with straight canals extracted for periodontal reasons were collected and disinfected in NaOCl. They were then stored in sterile saline. The study was approved in the ethics committee of Tehran University of Medical Sciences (IR.TUMS.DENTISTRY.REC.1397.046).

The teeth were decoronated using a diamond disc to standardize the root length at 12 mm. A #15 K-file was introduced into the canal until its tip was visible at the apical foramen. The working length was determined by subtracting 0.5 mm from this length. The roots were instrumented by the single-length technique using Mtwo files up to size 30 with 6% taper to the working length. During filing, root canal was irrigated with 10 mL of 5.25% NaOCl. The external root surface was coated with two layers of nail varnish to prevent external contamination. The apical region was sealed with composite resin.

The instrumented canals were rinsed with 5.25% NaOCl for 1 min followed by 17% EDTA for 1 minute and saline for smear layer removal. The samples were then gamma-sterilized. Next, five samples were randomly selected and incubated in brain heart infusion broth at 37 °C for 48 hours to ensure absence of microbial contamination. The clear culture medium devoid of bacteria ensured sterility after incubation [22].

2.2. Culture and inoculum preparation

E. faecalis (ATCC 29212) strain (Iranian Biological Resource Center, Thran, Iran) was cultured in brain heart infusion agar (Merck KGaA, Darmstadt, Germany) under aerobic conditions and incubated at 37 °C for 24 hours. The *E. faecalis* colonies were then cultured in brain heart infusion broth (Merck KGaA, Darmstadt, Germany) to obtain 0.5 McFarland standard concentration with 1.5×10^8 colony forming units per milliliter (CFUs/mL) using a spectrophotometer (optical density 600: 0.08–0.13). The roots were inoculated with *E. faecalis* suspension using a 3.0 mL syringe with a 27-gauge needle. The teeth were then separately placed in sterile tubes and incubated at 37 °C for 21 days. The bacterial suspension was refreshed weekly to achieve mature *E. faecalis* biofilm [23].

2.3. Photosensitizer and light source

The MB stock solution (Sigma-Aldrich, Steinheim, Germany) at 100 µg/mL concentration was prepared using sterile 0.9% wt/vol NaCl. The stock was kept in the dark before the procedure. Diode laser with 660 nm wavelength and 150 mW output power (DX61, Konftec, Taiwan) was irradiated by a three-dimensional diffuser tip (WF-354, Konftec, Taiwan) to activate the MB for 1 min. The output power of laser was checked by a power meter (Coherent, USA).

2.4. Ultrasonic activation

Ultrasonic irrigation was carried out using Various 970 with level 5 power and E5 tip for 20 s.

2.5. Study design

The teeth were randomly divided into three experimental groups (n = 16) using the random allocation software.

Experimental groups:

Group A. The root canals inoculated with *E. faecalis* biofilm were filled with 10 µL of 5.25% NaOCl, which was activated ultrasonically for 20 s using E5 tip of Various 970 (NSK, Japan).

Group B. The root canals inoculated with *E. faecalis* biofilm were filled with 10 µL of 100 µg/mL MB and incubated in the dark for 5 min. Next, the root canals were exposed to diode laser irradiation with 660 nm wavelength for 60 s (150 mW, 0.75 mm tip diameter with 80% transmission with total energy of 7.2 J). The power density and energy density were 0.27 w/cm^2 and 16.2 J/cm^2, respectively.

Group C. The root canals inoculated with *E. faecalis* biofilm were filled with MB and subjected to ultrasonic activation as in group A. Then, the root canals were exposed to diode laser irradiation as in group B.

Positive control: The root canals inoculated with *E. faecalis* biofilm were filled with 10 µL of sterile saline with no intervention.

After the interventions, the teeth were gently rinsed with saline and the canals were dried with sterile #30 paper points. The roots were then split into halves with a sterile stainless steel chisel to assess the microbial load and penetration depth of MB.

2.5.1. Microbial sampling of the canals

To determine the *E. faecalis* colony count (CFUs/mL), half of the roots were used in each group; 0.01 ± 0.002 g dentin chips were collected from the apical and coronal thirds of the root canal walls separately using a sterile low-speed endodontic handpiece (X-Smart,

Dentsply Maillefer, Ballaigues, Switzerland) with ISO 012 round tungsten carbide bur. Dentin chips were collected into sterile tubes containing 1 mL of sterile saline, and the microbial viability assay was performed according to a previous study [23]. Finally, the colony count in the experimental groups (CFUs/mL) was calculated using the Miles and Misra method [24].

2.5.2. Assessing the penetration depth

In this study, a stereomicroscope (SMZ1500; Nikon, Japan) was used to measure the penetration depth of MB photosensitizer into the dentinal tubules. Specimens in all groups were longitudinally grooved with a diamond disc (Brasseler USA,) and split into halves with a stainless steel chisel. One half was chosen to measure the penetration depth of dye into the dentinal tubules. All measurements were made by one operator who was blinded to the study using a stereomicroscope. The measurements were made at ×20 magnification in two zones of each specimen: Apical region (6 mm) and coronal region (6 mm).

Four measurements were made in each zone and the mean value was calculated as the penetration depth at the respective site. The microscope was equipped with Support Tools Ver1.2.1 software and all measurements were made using the software program [22].

2.6. Statistical analysis

The data were analyzed using descriptive statistics via SPSS version 25. Independent sample *t*-test was used to compare the dye penetration depth in the apical and coronal regions among the groups. One-way ANOVA was used to compare the microbial load in the apical and coronal regions among the groups. The Tamhane's post-hoc test was applied for pairwise comparisons (due to non-homogeneity of variances). Also, the correlation between the dye penetration depth and the microbial load in each region was analyzed using the Pearson's correlation test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Confirmation of E. faecalis biofilm formation

As shown in Fig. 1a , *E. faecalis* biofilm was formed on the root canal walls and penetrated into the dentinal tubules at 3 weeks after inoculation.

3.2. Quantitative evaluation of E. faecalis biofilm (CFUs/mL)

According to the results presented in Tables 1 and 2, NaOCl + ultrasonic (U), aPDT, and aPDT + U significantly decreased the percentage of viable *E. faecalis* both in the apical and coronal regions compared to the control group ($P < 0.05$).

The reduction in bacterial count in the apical part was 90.7%, 98.6% and 99.58% in aPDT, aPDT + U and NaOCl + U groups, respectively compared to the control group (Table 1).

The reduction in bacterial count in aPDT, aPDT + U and NaOCl + U groups was 56%, 98.3% and 99.53%, respectively in the coronal part in comparison with the control group (Table 2).

The reduction in *E. faecalis* count in aPDT + U and NaOCl groups was significantly greater than that in the aPDT group, and there was no significant difference between aPDT + U and NaOCl + U groups, neither in the apical nor in the coronal region ($P > 0.05$).

3.3. Evaluation of penetration depth

As shown in Fig 1b, c and Table 3, the penetration depth into dentinal tubules in aPDT + U group was significantly greater than that in aPDT group both in the apical and coronal regions ($P < 0.05$).

A correlation was noted between the penetration depth of MB into the dentinal tubules and the percentage of bacterial reduction in the

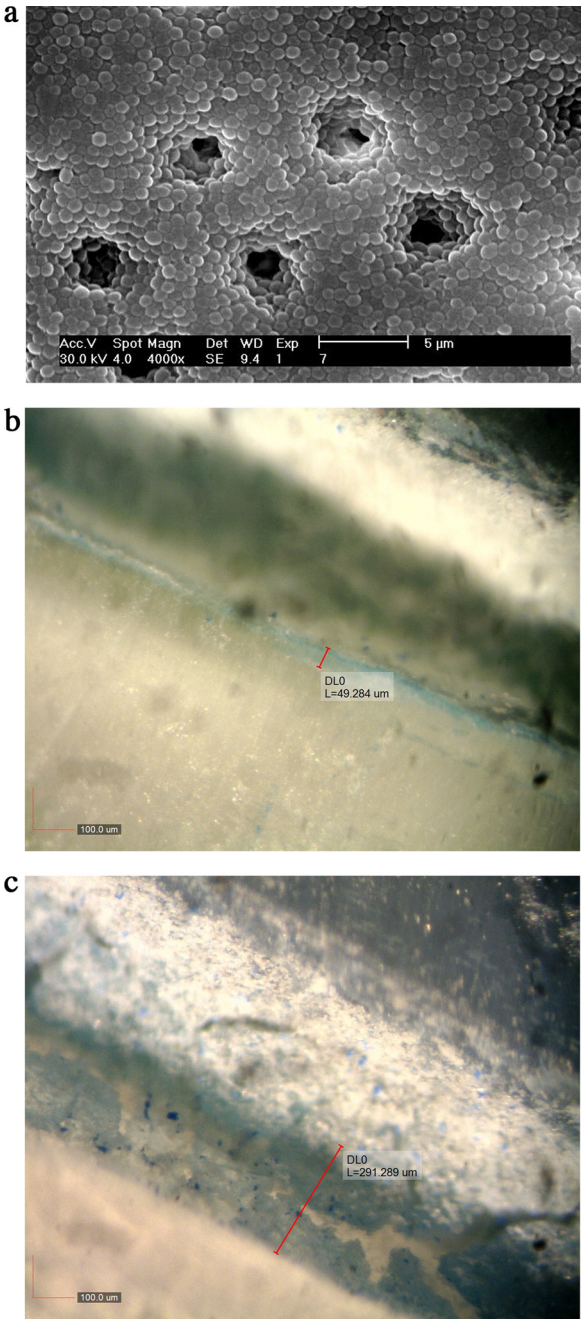

Fig. 1. a. Scanning electron microscope images of *E. faecalis* biofilm in root canal, b. Penetration depth of MB in aPDT group, c. Penetration depth of MB in aPDT + U group.

Table 1
E. faecalis CFU in experimental and control groups in apical part.

Group	Experimental group	Reduction percentage of bacterial count (%)	P.value
Control	aPDT	90.7	0.000
	aPDT + U	98.6	0.000
	NaOCl + U	99.58	0.000
aPDT	aPDT + U	7.9	0.004
	NaOCl + U	8.88	0.001
aPDT + U	NaOCl + U	0.98	0.393

Table 2
E. faecalis CFU in experimental and control groups in coronal part.

Group	Experimental group	Reduction percentage of bacterial count (%)	P.value
Control	aPDT	56	0.000
	aPDT + U	98.3	0.000
	NaOCl + U	99.53	0.000
aPDT	aPDT + U	42.3	0.000
	NaOCl + U	43.53	0.000
aPDT + U	NaOCl + U	1.23	0.211

Table 3
Penetration depth of MB in dentinal tubules(μm).

Group	Region	Mean (μm)	Std. error of mean(μm)
group A: aPDT	Apical	32.78	3.83
	Coronal	54.99	4.05
group B :aPDT + U	Apical	325.87	25.69
	Coronal	456.84	9.64

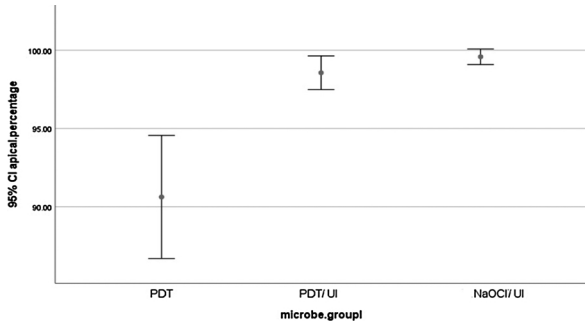

Fig. 2. Error bar of mean & 95% confidence interval of microbial colony reduction in experimental groups in apical.

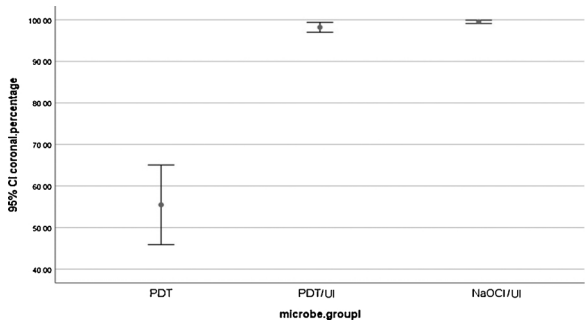

Fig. 3. Error bar of mean & 95% confidence interval of microbial colony reduction in experimental groups in coronal.

experimental groups in both the apical (intraclass correlation coefficient = 0.993) and coronal (intraclass correlation coefficient = 0.999) regions (Figs. 2 and 3).

4. Discussion

In this study, the root canals were instrumented with Mtwo rotary system up to size 30-6% in order to create a standard environment for bacterial incubation. Thus, the final shape of the canal allowed the penetration of ultrasonic tip and aPDT instruments into the apical third.

The activating effect of ultrasound on aPDT of the root canal system was first suggested by Ghinzelli *et al.* in 2014 [25]. A previous study showed that ultrasonic activation of photosensitizer significantly decreased the *E. faecalis* count in the root canal system; however, the microorganisms were confined only to the main canal [25]. Thus, we evaluated the count of bacteria lodged deep into the dentinal tubules and main canal.

According to Bumb, aPDT is effective for disinfection of deep dentinal tubules [26]. A previous study used scanning electron microscopy and indicated that *E. faecalis* was not present at 890-900 μm depth of dentinal tubules in aPDT group while the bacteria were present at up to 980 μm depth in the control group [26]. However, no information is available regarding the penetration depth of ultrasonically activated photosensitizer and the antibacterial activity of aPDT in comparison with the use of NaOCl as irrigating solution. According to the results, application of ultrasonic device in aPDT significantly enhanced the penetration depth of photosensitizer and caused greater reduction of *E. faecalis* count.

According to the current results, the ultrasonically activated MB group showed significantly lower mean colony count compared to the group without ultrasonic activation. It seems that deeper penetration of MB into the dentinal tubules is associated with higher antibacterial activity. This finding was in agreement with that of previous studies that reported a significant reduction in microbial count following aPDT and ultrasonic activation of photosensitizer [20,25,27].

NaOCl is the most popular irrigating solution in endodontic treatment of teeth. According to previous studies, NaOCl is effective for elimination of *E. faecalis* from the main canal [28]. However, complete elimination of *E. faecalis* from the dentinal tubules could not be achieved in this study. Tennert et al. demonstrated that aPDT alone was less effective than NaOCl [29]. Also, Nunes *et al.* and Hecker *et al.* indicated that NaOCl resulted in better disinfection than aPDT [30,31]. According to our results, the antibacterial effect of NaOCl + U was significantly greater than that of aPDT while the difference in this respect between NaOCl + U and ultrasonically activated photosensitizer group was not significant. This finding highlights the fact that ultrasonic activation of photosensitizer can increase the antimicrobial efficacy of aPDT.

It appears that acoustic streaming caused by ultrasound leads to better diffusion of photosensitizer and increases its penetration depth into the dentinal tubules [32].The duration of activity and the penetration depth of dye are short in biological systems (less than 0.04 s and 0.02 μm, respectively) [18]. Thus, the photosensitizer has a localized effect on the bacteria. In order to achieve greater antibacterial activity, the penetration depth of photosensitizer into the dentinal tubules should be enhanced.

In the present study, the bacterial count in the coronal region was higher than that in the apical region. This finding can be related to the patency of dentinal tubules in the coronal region. In other words, the bacteria can penetrate easily into the patent dentinal tubules. Furthermore, the penetration depth of MB into the coronal dentinal tubules was greater than that in the apical dentinal tubules. However, this finding did not result in greater reduction in the percentage of bacteria count in the coronal region. These finding are similar to the results of Harrison *et al.* They reported that ultrasonically activated NaOCl resulted in smaller reduction in bacterial count in the coronal region compared to the apical region [33].

In general, considering our findings and those of previous in vitro and in vivo studies, it seems that aPDT can be an effective modality for root canal disinfection in regenerative endodontic procedures when the operator faces challenges in disinfection of the root canal system of necrotic teeth with weak dentinal walls [4]. As mentioned earlier, elimination of microorganisms is imperative for a successful endodontic treatment [3]. Moreover, NaOCl has adverse effects especially on stem cells [14]. Thus, aPDT may serve as an adjunct for root canal disinfection in regenerative treatments. According to Mokhtari and Bolukbasi, aPDT has less cytotoxicity for fibroblasts and osteoblasts compared with NaOCl [34,35].

The current study used dentin chips for microbial assessment. However, it was impossible to collect dentin chips separately from various depths of dentinal tubules. Thus, similar future studies are

required to assess the presence of microorganisms at different depths of dentinal tubules.

5. Conclusion

Our findings suggest that ultrasonic activation of photosensitizer in aPDT can significantly enhance the penetration depth of photosensitizer into the dentinal tubules. It can also increase the antibacterial activity of aPDT against *E. faecalis* biofilm. However, future clinical studies are required to confirm the current in vitro findings.

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