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The comparison of intracanal medicaments, diode laser and photodynamic therapy on removing the biofilm of *Enterococcus faecalis* and *Candida albicans* in the root canal system (ex-vivo study)

Leila Moradi Eslami, Mehdi Vatanpour*, Neda Aminzadeh, Payman Mehrvarzfar, Soudabeh Taheri

Dental School of Tehran Azad University, Neyestan 9th, Pasdaran, Tehran, Iran

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ABSTRACT

Background and aim: Long term success in endodontic therapy is attributed to removal and debridement of intracanal microorganisms. The aim of this study was to compare the antimicrobial effects of calcium hydroxide ($Ca(OH)_2$), triple antibiotic paste (TAP), photodynamic therapy(PDT), toluidine blue(TOL), light emitting diode (LED) and 940 nm diode laser (DL) on the biofilm of *Enterococcus faecalis* and *Candida albicans* in the root canal system of ex-vivo human teeth.

Materials & Methods: 84 intact, uniradicular, extracted human premolar teeth were prepared and the apical foramina were sealed with glass ionomer. The samples were transformed to microtubes containing sterile BHI and were sterilized. After incubation of *E. faecalis* and *C. albicans* into the canals, the samples were kept in an incubator for 8 weeks to form the biofilm. Then the samples were randomly divided into 7 groups of 12, including: control and groups treated with $Ca(OH)_2$,TAP, TOl, LED, PDT, and DL. Then the samples were fixed, gold coated and observed under scanning electron microscope (SEM).

Result: Significant reductions in biofilm thickness were noted in TAP,PDT and LED ($P < 0.05$). The greatest reduction in biofilm thickness was seen in TAP; however, the differences between TAP and PDT and LED were not significant ($P > 0.05$).

Conclusion: Since one of the main purposes in root canal therapy is to eliminate the bacteria, this study showed that the application of TAP, PDT, and LED exposure lead to least biofilm thickness.

1. Introduction

One of the major challenges in endodontic therapy is to remove pulpal debris and bacterial populations from the root canal system. Although biomechanical preparation may eliminate the microorganisms effectively, more than 50% of the walls may remain intact during instrumentation, due to the anatomical complexity of the root canal system [1–3]. Studies show that *E. faecalis* and *C. albicans* are common and important microorganisms found in teeth with refractory endodontic infection [4,5]. *E. faecalis* is a gram-positive anaerobic cocci which can resist to endodontic treatment via biofilm formation [6]. Besides, *C. albicans* has been identified in 21% of primary endodontic infections [4,7]. Biofilm is a microbial proliferation process, in which the cells stick irreversibly to each other and also a solid surface, therefore they resist and respond to peripheral changes as one unit.

During root canal treatment, several medicaments with antimicrobial effects are used, among which $Ca(OH)_2$ and TAP are the most common.

Antimicrobial activity of $Ca(OH)_2$ is related to release of hydroxyl ions which leads to protein denaturation by destruction of cytoplasmic membrane and DNA of the bacteria [8].

TAP consists of metronidazole, ciprofloxacin and minocycline, which has efficient bactericidal effect to remove the microorganisms of infected dentinal and pulpal tissues [9,10]. Due to adverse effects of these medicaments on physical properties of dentin and bacterial resistance [11,12], modern technologies, such as photodynamic therapy (PDT) and high level laser (HLL) have been introduced to the market in order to disinfect the root canal system.The benefits of PDT include immediate action, access to complex areas such as pits and furcation, prevention of bacteremia in immunocompromised patients, and patient relief. [13,14]. The key feature of the photosensitizer is the ability of selective accumulation in tissues, which interferes with biological

* Corresponding author.
E-mail address: Drvatanpour@gmail.com (M. Vatanpour).

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substances and results in releasing singlet oxygen and free radicals.Therefore, PDT can specifically target the microorganisms without damage to host tissues. PDT can be accomplished by light emitting diode (LED) or laser. Several studies have proven the antibacterial effect of toluidine blue (TOL) on oral pathogens [13,15]. It has been identified that high power diode laser has satisfactory results in disinfecting the root canal system and has the ability to reach the certain complex anatomical structures, where other conventional instruments and approaches are incapable [16]. High power lasers function based on the heat produced by selective dose [13]. Since there are few studies on the effect of intracanal medicaments in comparison with lasers on the biofilm of *E. faecalis* and *C. albicans*, the purpose of this research is to compare the effect of PDT, TOL, LED irradiation, DL, Ca(OH)2 and TAP on the biofilms of *E. faecalis* and *C. albicans* in the root canal system. This research has been carried out in dental school of Azad University of Tehran in 2017-18.

2. Methods & materials

2.1. Tooth preparation

84 intact, uniradicular, extracted human premolar teeth with a single canal were collected and cut at the CEJ in order to get the equal working length of 12 mm in all samples by introducing a K-file #15 (Maillefer/Dentsply, Switzerland) in the canal until its tip was visualized at the apical foramen. Then the canals were subsequently prepared up to master file size #30 with Protaper rotary file #F3 (Maillefer/Dentsply, Switzerland), according to the manufacturer's instruction. After each file, the canals were irrigated with 2 ml 5.25% sodium hypochlorite (NaOCl) (Barf, Paksan Co, Tehran, Iran) using 1-ml insulin syringe and 30-G needle (Soha Co, Tehran, Iran). In order to remove the smear layer from dentinal tubules, EDTA 17%, 5.25% NaOCl and normal saline for 1 min were used respectively. The apical foramina were sealed with glass Ionomer (Fuji II LC/GC, Japan) [4]. In order to interrupt the intracanal connection with outer root surface, all the roots were sealed with a nail varnish. Then a horizontal groove was made by a diamond disc at the 5 mm of the root coronally (Fig. 1).

2.2. Microbial culturing

Each tooth was transformed to an autocavable microtube containing sterile Brain Heart Infusion Broth medium (BHI) (Merck, Darmstadt, Germany) which was immersed 0.5 mm deep under the broth, then, the teeth were sterilized in autoclave (Hirayama, Tokyo, Japan) for 30 min at 121 °C and 15 Psi pressure.

Suspension of *E. faecalis* (ATCC29212) and *C. albicans* each equivalent to a 1 Mc Farland standard (3 × 10 [8] concentration) were inoculated into the canals with the ratio 1:1. First 5 teeth were randomly selected as pilot group, incubated in incubator (Pars Azma, Tehran, Iran) at 37 °C for 2 weeks while were placed on a shaker with 20 rpm. The samples were visually checked every 72 h in order to inject extra sterile broth to reach the primary standard level, if any decrease was observed.

Fig. 1. A sample of sectioned teeth which is coated with nail varnish.

After two weeks, the teeth were removed from the incubator and washed with distilled water. After fixation and observing under SEM, Since the biofilm thickness was not adequate enough to be evaluated by SEM, and according to Zand [17], the incubation time was considered 8 weeks.

Aforementioned, after keeping 84 samples in the incubator for 8 weeks, washing with distilled water to remove the culture medium and free cells, Then the samples were randomly (according to the table of random numbers) divided into 1 control group and 6 experimental groups of 12, including:

1 Control group: positive control (10 samples with no antibacterial treatment) and negative control (2 samples with no microbial inoculation and no antimicrobial treatment)
2 $Ca(OH)_2$ group: $Ca(OH)_2$(Merck/Darmstadt, Germany) with creamy consistency of 1.5/1 (powder/liquid) [6] was inserted into root canal system for one week.
3 TAP group: ciprofloxacin (TEMAD, Karaj, Iran), minocycline (COA, China), and metronidazole (Tehran Chemie, Tehran, Iran), with proportion of 1:1:1 in 1 mg/ml were mixed with saline and placed into the canals.
4 LED group: The canals were irradiated with a 630 nm LED(FotoSan/Copenhagen, Denmark) (15 W), twice for 30 s, at speed of 2 mm/seconds. Fiber tip (diameter: 200 μ) entered into root canals 1 mm shorter than the apex.
5 TOL group: 1 mg/ml TLB(Merck/Darmstadt, Germany) for 5 min was placed in canals, then the samples were washed.
6 PDT group: 1 mg/ml TOL (Merck/Darmstadt, Germany) for 5 min was placed into the canals, and subsequently 630 nm LED exposure (FotoSan/Copenhagen, Denmark) (15 W), twice for 30 s, at speed of 2 mm/seconds was applied. Fiber tip (diameter: 200 μ) entered into root canals 1 mm shorter than the apex.
7 DL group: 940 nm DL (Biolase/California, US)(1 W) and speed of 2 mm/s, four times for 10 s with 15 s intervals was applied. The system was coupled to an optical fiber with a diameter of 200 μ which entered into canals 1 mm shorter than the apex.

In the groups of Ca(OH)2 and TAP, the samples were incubated for one more week at 37 °C and humidity of 100% and 5–10% CO_2. Subsequently, the samples of both groups were gone under fixation in order to evaluate the biofilm thickness.

2.3. Fixation

Firstly, the roots were cut at the groove into halves by a chisel. The coronal part of each section was observed under the SEM, due to its flat surface. The root sections were carefully washed with a 0.5 M potassium phosphate buffer at 5 °C and pH = 7.2. The roots were fixed in glutaraldehyde solution 2%, at 5 °C for 20 h, then, were washed with phosphate-buffered saline (PBS) for 15 min, and were fixed again in osmium tetroxide 1% at 5 °C for 12 h. PBS was used as the final irrigant. Then the acetone with increasing concentrations (30%, 60%, 80%, and 100%) were respectively used, each for 10 min, to dehydrate the samples. Afterwards, the roots were dried in the oven at 37 °C for 5 h.

2.4. SEM evaluation

After drying the fixed samples in desktop sputtering system (Technics Hummer, USA), they were gold coated (Fig. 2), and observed under scanning electron microscope (SEM) (Fig. 3). All sections were analyzed by two blinded observers and the maximum thickness in each section was selected for measurement.

After collecting raw data, samples' distribution was normal

Fig. 2. Gold coated teeth.

according to Kolmogorov-Smirnof statistic test. To determine the statistically significant differences between independent groups, one-way analysis of variance (ANOVA) was used. In addition, Tamhane test was used to compare the groups two by two.

3. Results

The mean and standard deviation of biofilm thickness is shown in Table 1.There was a significant difference between PDT with control group (pvalue = 0.002), $Ca(OH)_2$(pvalue = 0.042), and TOL (pvalue = 0.004). In addition, a significant difference was observed between TAP with control group (pvalue = 0.00), Ca(OH)2 (pvalue = 0.001), TOL (pvalue = 0.00), and HLL (pvalue = 0.006). The difference between LED with control group (pvalue = 0.016), and TOL (pvalue = 0.042) was significant.

Treatment by LED, PDT, and TAP were significantly effective in reduction of the biofilm thickness, whereas the least biofilm thickness was seen in TAP group with no significant difference with LED and PDT (Chart 1).

4. Discussion

A successful root canal therapy is attributed to complete removal of intracanal microorganisms. Antimicrobial irrigants are not adequate enough for complete canal disinfection; hence, antimicrobial medicaments are suggested between sessions [18]. Owing to the fact that effective debridement and removal of microorganisms are the key factors to a successful endodontic treatment, the purpose of this study is to evaluate and compare the effect of 6 different disinfection methods on the biofilm of *E. faecalis* and *Candida albicans* in the root canal system.

E. faecalis and *C. albicans* are the major microorganisms found in infected root canal systems and are more prevalent in secondary and persistent infections compared with primary infections. The biofilm model was used instead of the planktonic to simulate the clinical scenario and in vivo situation [2,19]. The result showed that these species were capable of forming a biofilm with 89.64 μ thickness. On the other hand, in a research done by Pei et al. [20], after 7-day incubation, the biofilm thickness caused by *E. faecalis* was 25.5 μ. This difference may be due to different combination of cultured microorganisms, their synergic effects and different incubation time.

According to Zand and Kishen [17,21], as the incubation time for *E. faecalis* prolongs to 6 and 8 weeks, the biofilm ages, and it is likely that bacteria in mature and old biofilms become more resistant due to making the biofilm biomineralized and calcified. In our pilot study, two-week incubation time was not adequate enough to detect the biofilm thickness by SEM. Therefore, 8 weeks was considered as the appropriate time to create a mature biofilm [17].

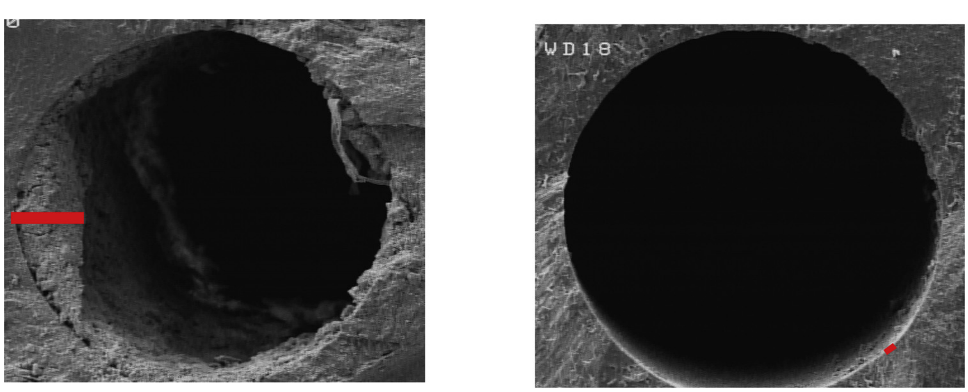
The most common medicaments which are used between sessions, are Ca(OH)2 and TAP. In addition to bactericidal effect, the main features of $Ca(OH)_2$ include dissolving intracanal debris, high pH and mineralization induction [8,22]. According to Mohammadi's research, Ca(OH)2 is a valuable anti-endotoxin agent. However, its effect on microbial biofilms is controversial [23]. In this study, the least biofilm thickness reduction was seen in Ca(OH)2, which was not significantly different from the control group. The least biofilm thickness belonged to TAP, LED, and PDT, which showed statistically significant difference with other groups.

The mechanism of disinfection by TAP is due to its broad spectrum bactericidal effect against gram positive and gram negative bacteria. Besides it can disrupt biofilm structure by its chelating effect [24].

In addition, according to Devaraj et al. [25], TAP brought about complete disruption of *E. faecalis* biofilm structure. However, there was no significant difference on biofilm structure between Ca(OH)2 and control groups. This study confirms our results. Although antibacterial resistance and discoloration due to antibiotics and other advantages of PDT, including: Easy manipulation, and one-visit therapy outweighs the application of PDT.

TOL is a conventional photosensitizer used to deactivate bacteria, viruses and fungi. Its antibacterial and antifungal effect results in deactivation of yeasts, gram positive and negative bacteria, but under the exposure of a light source with certain wavelength [26]. Moreover, a reduction in biofilm thickness was observed in samples treated with TOL without exposure, while its biofilm thickness was not significantly different with control groups [26].

PDT has been introduced as an alternative choice to improve the disinfection, and can be applied in just one visit. PDT consists of three



A B

Fig. 3. Scanning electron microscpe (SEM) images of root canal systems A: Control, B: TAP (Red line shows the maximum biofilm thickness).

Table 1
Mean and standard deviation of biofilm thickness in the control and experimental groups.

Groups	Control	Ca(OH)2	TAP	LED	TOL	PDT	HLL
Mean ± SD(Micrometer)	89.64 ± 29.43	67.65 ± 20.63	31.67 ± 10.20	51.00 ± 9.12	66.22 ± 11.84	41.84 ± 14.46	59.69 ± 18.87

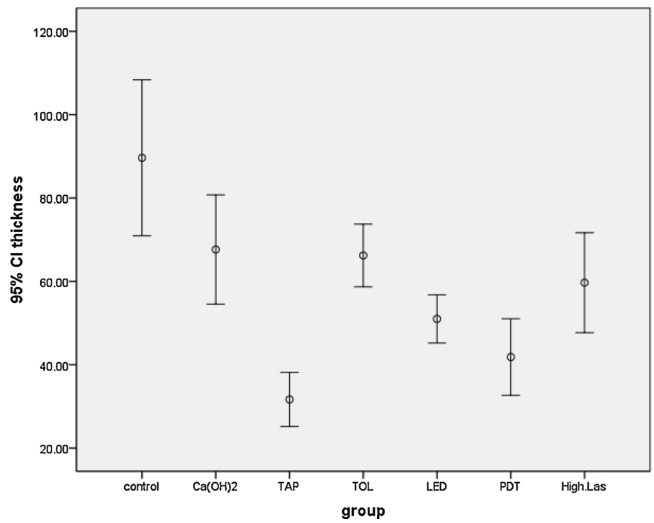


Chart 1. Logarithmic values of biofilm thickness in each group of control and experimental.

elements: 1) Photosensitizer 2) Light source 3) Tissue oxygen [13]. Its mechanism is based on free oxygen radicals. This process may destruct the microorganisms as well as the necessary cellular molecules, such as proteins, membrane lipids and nucleic acids. In this method, the accessibility and penetration into the dentinal tubules can be achieved via application of fiber optic with proper size. This technique can easily removes the periodontal and endodontic microorganism from the host without any side effects, while the bacterial resistance and discoloration of TAP may be harmful to the host [27]. According to Asnaashari's research [13], PDT with a 630 nm LED is more effective against *E. faecalis* than laser 810 nm. This may be due to broader light spectrum of LED. Other benefits of LED can be mentioned as: easy manipulation, access to unavailible areas, safety, cost-effectiveness, painless and by far more easily than high power lasers; e.g. there is no need to wear protective glasses and safety spectacles when working with LED. The LED bulbs generate less heat, and cause less damage to the tissues. That's why the light source in this study was chosen LED [13].

Asnaashari et al. [18] reported a clinical study in which Ca(OH)2 and PDT were significantly effective in reducing *E. faecalis*; however, PDT had better antimicrobial effect than Ca(OH)2. According to our study there was no significant difference between Ca(OH)2 and control group. The different efficacy of Ca(OH)2 maybe due to the type of study; In Asnaashari's research, the antimicrobial effects on planktons of *E. faecalis* had been investigated, on the contrary in this study the changes in biofilm thickness was discussed. In Dexton et al.'s clinical research [28], clinical and radiographic evaluation of three methods of photoactivated disinfection (PAD), TAP, and Ca(OH)2 was surveyed; after 18 months, 15% failure in patients treated with Ca(OH)2 and 5% failure in TAP was observed, while no failure was reported for PDT. In this study, clinical and radiographic evaluations were done; which the successful report on PAD and TAP over Ca(OH)2 is in accordance with our research. Kwan reported that *E. faecalis* has internal pigmentation which can act as endogen photosensitizers. So, there is no need for applying exogen photosensitizer and just LED irradiation can significantly decrease *E. faecalis* biofilm mass. This finding support our results [29]. The bactericidal effect of laser in root canal disinfection is attributed to the type of the laser. However there is still question among researchers on how accurate the laser destroys the microorganisms. High power lasers function based on the heat produced by selective dose. If the parameters are not properly selected, it may lead to complications, such as cementum damage, root resorption and apical necrosis [13]. According to Afkhami et al, high power laser application reduces colony forming units(CFU), but bacteria reduction was significantly less than PDT [27]. In our research, too, the application of 940 nm DL reduced the biofilm thickness, which had significantly more thickness compared to PDT group. If the parameters are not properly selected, it may lead to complications, such as cementum damage, root resorption and apical necrosis [13].

Considering the lack of in vitro studies on biofilm thickness of *E. faecalis* and *C. albicans*, our study focused on this topic. According to our study; TAP, PDT and LED resulted in least biofilm thickness.

5. Conclusion

Treatment with TAP, PDT and LED reduces biofilm thickness in comparison with control and other experimental groups. Since PDT is a one-visit treatment, and does not cause microbial resistance, this method is preferred to TAP. In addition, PDT with LED in comparison with laser benefits more advantages, such as: more efficacy due to broader spectrum, feasibility, safety, and accessibility.

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