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Comparison of the Antimicrobial Efficacy of Calcium Hydroxide and Photodynamic Therapy Against *Enterococcus faecalis* and *Candida albicans* in Teeth With Periapical Lesions; An In Vivo Study

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Abstract

Introduction: Elimination of pathological microflora of root canal systems is a major goal in endodontic treatment. This study aimed to compare the antimicrobial efficacy of calcium hydroxide as an intracanal medication and antibacterial photodynamic therapy (aPDT) against *Enterococcus faecalis* and *Candida albicans* in teeth with periapical (PA) lesions.

Methods: This in vivo study was conducted on 20 patients with single-rooted mandibular premolar with previously failed endodontic treatment. This study was performed as a clinical trial (IRCTID: IRCT2016090429686N1). After conventional chemo-mechanical root canal preparation (hand and rotary instruments and 2.5% NaOCl), microbiological samples were obtained using sterile paper points, then stored in thioglycolate solution and transferred to a microbiology laboratory. Group 1 (n = 10) specimens underwent aPDT (diode laser 808 nm + 50 mg/mL methylene blue), while creamy calcium hydroxide paste was used in group 2 for a duration of 1 week. A control sample was taken with sterile paper points and F3 Protaper rotary file. The samples were dispersed in transport medium, serially diluted, and cultured on selective mediums to determine the number of colony forming units (CFUs). Data were analyzed by Mann-Whitney U test at 5% significance level. The significance level for all analyses was set at $P<0.05$.

Results: Number of CFU significantly decreased in both groups after the interventions ($P<0.001$); however, there was no significant difference in the colony count between the 2 groups.

Conclusion: aPDT and calcium hydroxide therapy showed the same antimicrobial efficacy on *E. faecalis* and *C. albicans*.

Keywords: Antimicrobial efficacy; Calcium hydroxide; *Enterococcus faecalis*; Photodynamic therapy; Root canal treatment.

Introduction

Microbial infection plays an important role in the development of periapical (PA) lesions.[1] Elimination of the pathological microflora of the root canal system is a major goal in endodontic treatment. Inadequate disinfection of the root canal system may lead to treatment failure and development of PA lesions.[2] Killing the bacteria of root canal system is not always achievable with current root canal treatment (RCT) techniques (mechanical debridement associated with chemical irrigation).[3,4] The anatomical complexity of the root canal system makes it almost impossible to completely eliminate the bacteria using conventional mechanical and chemical techniques, even with the highest technical standards.[5]

A 94% treatment success rate has been reported in cases with a negative culture before the obturation of root canal system. This rate decreases to 68% in cases undergoing root canal filling despite the cultures being positive.[2]

There is a challenge for the clinician in treatment of teeth with PA lesions, all efforts and attempts have been made to eliminate irritating agents from the root canal system in order to provide healing in the periradicular tissues.[5]

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A current method which is used to eliminate intracanal bacteria is dressing of canals with medicaments. The most commonly used inter appointment medicine is calcium hydroxide.

Literature shows that calcium hydroxide has an excellent effect on intracanal microorganisms. The antibacterial effect of calcium hydroxide is based on a high pH (approximately 12.5-12.8). Resistant microorganisms such as *Enterococcus faecalis*, gram-negative facultative anaerobic rods and *Pseudomonas* play a prominent role in the development of infections in root canal systems.[6,7]

Evidence shows that fungi are often present in infections resistant to conventional RCT and are partially responsible for treatment failure of PA lesions despite the debridement and irrigation of the root canal system.[8] *Candida albicans* and some other microorganisms have been isolated from root canals of teeth with and without PA lesions.[9]

Many studies reported that these organisms can resist the antibacterial effect of calcium hydroxide.[10] Furthermore, the use of intracanal dressing forces the clinician to have multiple treatment visits and this may cause inter appointment microbial recontamination, as well as being cost and time consuming for both patients and clinicians. Laser irradiation is a new approach for disinfection of the root canal system and easier access to the hard-to-reach areas such as the tubular network. This is due to the ability of the high power laser to better penetrate into the tooth structure compared to the irrigating solutions.[11]

Incorrect usage of high power laser may have high thermal effects on the adjacent tissues and its application takes longer period of time, while photodynamic therapy (PDT) is easier to apply and releases no heat.[12]

Antimicrobial photodynamic therapy (aPDT) is based on the application of a nontoxic photosensitizer,[13] a light source, and oxygen for inducing damage on bacteria.[14]

Different light sources can be used in endodontic aPDT, such as LED or lasers.[15] The PDT wavelength ranges from 600-1200 nm, and all the studies used a wavelength within this spectrum. Currently, specific wavelength mostly applied in PDT belongs to helium-neon lasers (633 nm), gallium-aluminum-arsenide diode lasers (630-690-830- or 906 nm) and argon lasers (488-514 nm).[16] In an in vitro study in aqueous suspension, aPAD with Nd-YAG laser (1024) + Toluidine Blue O and NaOCl resulted in a significant reduction in the colonies of *E. faecalis* cells.[17]

Oral bacteria are sensitive to PDT.[13] The antimicrobial effects of PDT on root canal microorganisms have been evaluated in several in vitro[13,18,19] and in vivo[12,20,21] studies. The majority of these studies have confirmed the efficacy of PDT as an adjunct to standard endodontic therapy. Based on a review,[22] the application of PDT for additional reduction of microbial load of root canal system seems promising, but more works should be performed to strengthen the currently available level of evidence for its use. In addition, this technique provides the possibility to have a single visit treatment.

Controversy exists regarding the selection of single-visit or multiple-visits RCT for infected teeth. Although no significant difference has been found in terms of healing rate between the single-visit and multiple-visit RCT, the prevalence of post-obturation pain has been reported to be lower following single-visit treatment.[23]

In all of the laser assisted treatments, application of suitable wavelengths, together with conventional methods, can effectively kill bacteria in the canal and dentinal tubule.[11] It would be beneficial to identify the ideal combination of photosensitizer and light wavelength via clinical studies in order to investigate the effect of PDT on root canal disinfection.[22]

This study aimed to compare the antibacterial efficacy of calcium hydroxide therapy as a standard method and aPDT by diode laser with a wave length of 810 nm + methylene blue (MB) on root canal disinfection of teeth with PA lesions.

Methods

This experimental and in vivo study was conducted on 20 patients with previously treated mandibular premolars who had referred to the Department of Endodontics of Shahid Beheshti University of Medical Sciences, Dental School, for endodontic re-treatment. After 2 years of their primary endodontic treatment, these patients still had periradicular lesions in their first or second mandibular premolars. Because of the complex anatomy of the root canal system and a higher number of normal variations in mandibular premolars, these teeth were selected for this study (Figure 1).

Power and sample size calculation software version 2.1.31 (Department of Biostatistics, Vanderbilt University) was used for sample size calculation. Considering $P < 0.05$ level of significance, 80% power (20% false negative results) and equal number of specimens in the 2 groups, 16 samples (n = 8 in each group) were required in order to detect 6.0×102 CFU/mL with a standard deviation of 4.0×102 CFU/mL.

Optimal general health, and having a mandibular premolar with a PA lesion on the primary radiography 2 years after primary endodontic treatment were the inclusion criteria. Those with broken tooth, ledge, over-filling, transportation, root resorption or perforation were excluded. The patients were also selected and treated by 2 different endodontics while microbiological sampling was performed by a third practitioner.

Written consent was taken from each patient. After ensuring the presence of PA lesion 2 years after the primary RCT on the initial radiograph, the tooth was isolated with rubber dam and access cavity was prepared. The tooth crown and adjacent areas were rinsed with 2.5% sodium hypochlorite solution to minimize the risk of contamination. Coronal restoration was entirely removed and coronal gutta percha was extracted using # 2 and 3 Gates Glidden drills (Dentsply Maillefer, Switzerland). The remaining gutta percha was extracted using H file hand instruments (Dentsply Maillefer, Switzerland) and chloroform solvent.

The working length was determined by taking 2

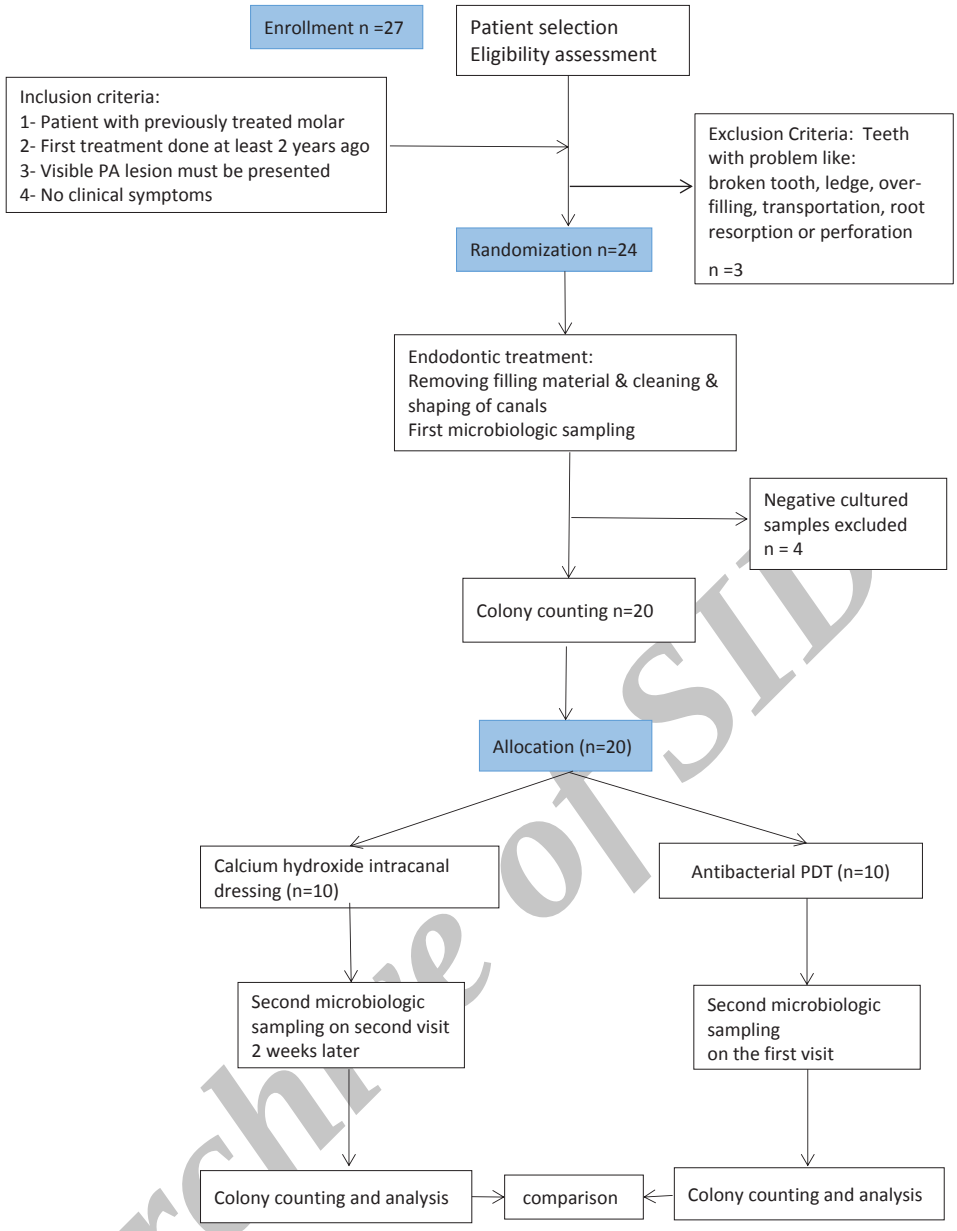

Figure 1. CONSORT Flow Diagram of Experimental Stages and Group Distribution.

radiographs of different angles and using an apex locator (Rapex 5, VDW, Germany). The root canal system was then prepared by Protaper NiTi rotary instruments (Dentsply Maillefer, Ballaigues, Switzerland) 300 rpm in crown down action according to the manufacturer's instructions.

The finishing of the canals was performed until F3 files reached full working length. Final instrumentation of canals was done with hand instrument (k-file #35, Dentsply Maillefer).

Abundant irrigation was performed with 5ml of 2.5% NaOCl solution (ChlorCid, Ultradent Products, Inc, South jordan, Utah, USA) and a final rinse with 5ml of sterile salin solution. The instrumented canals were washed using disposable syringes and 30-guage needles. Finally the canals were dried using 3 sterile #30 paper points (Ariadent, Iran) that remained in the canals for one minute. Bacterial biofilm sample was collected by F3 Protaper for 10 sec and then the paper points were stored in thioglycolate solution and transferred to the microbiology laboratory. These samples were considered as the first microbiological samples from the canal.

Group 1: aPDT Group

Half of the specimens were randomly chosen for aPDT and 0.5 mL of 50 mg/mL with 5% concentration MB as the photosensitizer was applied into the canal for 5 minutes. Diode laser (Dr. Smite, Lambda Scientifica, Italy) with 810 nm wavelength and 0.2 W power was irradiated into the canals. Laser beam was guided into the canals using a fiber optic cone with 200 μm diameter corresponding to the tip of a #30 file (ISO). The tip of the fiber optic cone

was placed 1 mm short of the working length and extracted towards the orifice with a helical motion at a speed of 2 mm/s. After 10 seconds of irradiation, the tissue was left to relax for 10 seconds. MB was rinsed off the canal with saline solution and the final microbiological sample was obtained from the canal using 3 sterile #30 paper points with 2% taper that reached the working length. Bacterial biofilm sample was collected by F3 Protaper rotary file for 10 seconds. The paper points were placed in tubes containing thioglycolate and transferred to the microbiology lab. Then the canals were rinsed with NaOCl 2.5% to avoid tooth staining caused by MB during PDT.[16]

Group 2: Calcium Hydroxide Therapy

In group 2, the canals were filled with creamy calcium hydroxide paste and access cavity was temporarily restored with Coltosol (Ariadent, Iran) with a minimum of 4 mm thickness. After a 7-day period, the final rinse was done with 2.0 mL saline, and the final microbiological sample was obtained using 3 sterile #30 paper points that reached the working length and remained in the canal for one minute. Bacterial biofilm sample was collected by F3 Protaper rotary file for 10 seconds and paper points were stored in thioglycolate solution and transferred to the lab. Root canals were filled with gutta percha (Ariadent, Iran) and AH26 sealer (Dentsply Maillefer, Ballaigues, Switzerland) using cold lateral compaction technique. Access cavity was temporarily sealed and patients were referred to the Department of Operative Dentistry or Prosthodontics for treatment to be continued.

In the lab, tubes containing paper points were incubated at 37°C for 24 hours. After that, tubes containing 9 mL of saline solution (8.5% concentration) were prepared. Specific culture media for each bacterium were prepared with bile esculin azide agar (Liofilchem, USA) which is a selective medium for *E. faecalis* and sabouraud dextrose agar (Merck, Germany) which is a selective medium for *C. albicans*. After 24 hours incubation in 37°C, 1 mL of each test tube was transferred to the tube containing saline solution and from the second tube to the third and so on. One milliliter of the final tube was extracted and discarded. The tubes were vortexed (VTX-3000L, Harmony) and 0.1 mL of each tube was transferred to the specific agar culture medium for each bacterium and cultured with an L-shaped bent glass rod using the spread technique (spreading the bacteria over the surface to produce single colonies). Culture media were incubated for 24 hours and colonies were counted. Plates with colonies less than 20 or more than 200 were excluded. After counting the colonies, the colony count in each plate was divided by the dilution factor of that plate and the result was reported in CFU/mL.

Data Analysis

Considering the dispersion of data and abnormal distribution of the CFU/mL variable, the mean, median, maximum and minimum were calculated for the comparison of CFU/mL before and after the interventions. One-sample Wilcoxon test was used to compare the mean difference before and after the intervention in each group. Mann-Whitney test was applied to compare aPDT and calcium hydroxide therapy. Data were analyzed using SPSS version 18.

Results

Colony forming units (CFUs) decreased after the intervention in both groups. Comparison of the before and after-intervention values was done in each group using the Mann-Whitney test. The mean colony count of *E. faecalis* in the aPDT group was 11 and 4.63 before and after the treatment, respectively. This value for *C. albicans* was 11.89 and 5 before and after the intervention. The mean colony count of *E. faecalis* in the calcium hydroxide group was 12.89 and 7.22 before and after the treatment, respectively. This value for *C. albicans* was 9.35 and 8.11 before and after the intervention. The reduction in the mean colony count of *E. faecalis* ($P=0.000$) and *C. albicans* ($P=0.05$) was statistically significant.

Wilcoxon signed rank test was applied for the comparison of before and after values separately in each group. In the calcium hydroxide group, a significant reduction occurred in both *E. faecalis* ($P=0.005$) and *C. albicans* ($P=0.012$) colony counts. In the aPDT group, significant reductions in *E. faecalis* ($P=0.018$) and *C. albicans* ($P=0.043$) colony counts were also observed (Table 1).

Discussion

Endodontic treatment aims to prevent or treat apical periodontitis by decreasing the intracanal microbial load.

In addition to chemo-mechanical cleaning, the use of intracanal medicaments can help decrease the intracanal microorganisms and prevent microbial colonization in the root canal system in-between the treatment sessions.[24-26]

Calcium hydroxide is the most commonly used intracanal medicament and its antibacterial effect is due to the release of hydroxide ions and to increase the pH of the environment. Moreover, Safavi and Nichols[27] and Barthel et al[28] have shown that calcium hydroxide can induce mono-

Table 1. Colony Counting Before and After Each Treatment

Treatment	Microorganism	Before Treatment	After Treatment	*P* Value
Calcium hydroxide therapy	*E. faecalis*	12.89	7.22	0.005
	C. albicans	9.35	8.11	0.012
aPDT	*E. faecalis*	11	4.63	0.018
	C. albicans	11.89	5	0.043

Abbreviation: aPDT, antibacterial photodynamic therapy.

cytes and trigger the release of tumor necrosis factor α (TNFα), prostaglandin E2 (PGE2) that are responsible for PA tissue destruction and its effects on LPG component of gram-negative bacteria. Although calcium hydroxide can effectively eliminate most of the root canal pathogens, *E. faecalis* and *C. albicans* are resistant to calcium hydroxide.[29,30] *E. faecalis* is a gram-positive bacterium that is usually present in treatment-resistant PA infections and its elimination is very difficult.[31] Its resistance is due to the basic pH of calcium hydroxide. Due to the activity of proton pump, this microorganism acidifies the environment and forms a biofilm; which also confers resistance.[32,33]

Candida albicans is the most commonly isolated fungus from the root canal system [34]. Najzar-Fleger et al showed that 55% of the root canals harbor *C. albicans*.[35]

Candida albicans is also resistant to calcium hydroxide. Its resistance mechanism has yet to be fully understood but it seems that *C. albicans* due to biofilm formation has a strikingly biphasic killing pattern in response to antibacterial agents.[36]

Considering the ineffectiveness of calcium hydroxide against these 2 common pathogens, a 2-visit calcium hydroxide treatment is recommended. No statistically significant difference has been reported between the success rate of single-visit and multiple-visit treatments. However, there is always the risk of coronal microleakage and tooth fracture in between the 2 treatment sessions when the restoration is delayed. The duration and cost of treatment should be considered as well. Moreover, due to the complex anatomy of the root canal system, there are always hard-to-reach areas that cannot be cleaned by the conventional chemo-mechanical procedures.

PDT can be used to effectively decrease intracanal microorganisms by accessing hard-to-reach areas. A photosensitizer and low-power laser are used in combination to produce oxygen free radicals and toxic products that can inhibit the growth of microorganisms or kill them.[37]

A study showed that aPDT and diode laser 810 nm irradiations are effective methods for root canal disinfection. This study compared the antibacterial effects of calcium hydroxide and aPDT for root canal disinfection in teeth with a previously failed endodontic treatment and PA lesion.[38] The results showed that both calcium hydroxide and aPDT in addition to RCT effectively decreased colony counts (CFU/mL) and thus, are effective disinfectants. No statistically significant difference was found between the 2 groups in this regard.

Our results were in accordance with those of previous studies by George et al,[39] Garcez et al,[19,21,40] Bonsor et al[20] and Asnaashari et al[11,12,41] regarding the efficacy of PDT using laser or LED as light source.

Only one study conducted by Souza et al reported opposite results. They reported a reduction in intracanal bacterial count but this reduction was not statistically significant. This was may be due to the low oxygen concentration in the canal.[38]

Numerous studies have investigated the safety of PDT. Kashef et al in their study, concluded that PDT with MB/TBO did not have significant cytotoxic effects on human fibroblasts.[42] Xu et al reported that PDT inactivated endodontic pathogens without affecting the viability of host cells.[43]

Considering all the above, aPDT is safe and effective for clinical use and it has the potential to predictably disinfect the canal in one-visit.

One of the advantages of our study was the assessment of the baseline CFUs, because differences in baseline CFUs can compromise the accuracy of the comparison between the 2 interventions. Another advantage was that we used quantitative methods for evaluation of microorganisms. Some studies only rely on the presence or absence of bacteria (positive or negative culture); whereas, we quantitatively assessed the common endodontic pathogens. Moreover, it was an in vivo study so the results are more reliable to be used in clinical settings. Under in vivo conditions, the reflection of scattered beam by the surrounding tissues is higher and consequently more photons are available for the photo reaction.[20] In clinical reality and all of the laser assisted treatments, application of suitable wavelengths is of the most importance. In aPDT, identification of the ideal combination of photosensitizer and light, together is recommended.[12] Based on the results of this study, application of diode laser (810 nm) and 50 mg/mL MB is beneficial to enhance root canal disinfection.

Future studies are recommended to focus on chelating agents in order to increase the efficacy and improve the penetration depth of laser. To increase the accuracy of microbiological analysis, the use of PCR in addition to microbial culture is recommended.

Canal disinfection with aPDT or calcium hydroxide after conventional chemo-mechanical treatment can effectively reduce colony counts (CFU/mL). No significant difference exists between the two mentioned methods and it can be concluded that aPDT with diode laser (810 nm) + MB as photosensitizer may be a suitable alternative to calcium hydroxide when a single-visit treatment is preferred, provided that the results of this study is confirmed by the valid studies in the future. It can result in a fewer number of visits and less chair time for clinicians and patients when root canal therapy is being performed.

Ethical Considerations

The protocol of this study was approved by the Ethics Committee of Medical Sciences at Shahid Beheshti University. This Study was performed as a clinical trial (IRCTID: IRCT2016090429686N1).

Conflict of Interests

The authors have no conflict of interest to declare.

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