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Araştırma/ Research Article

ANTIMICROBIAL EFFECTIVENESS OF PHOTON-İNDUCED PHOTOACOUSTIC STREAMING, PHOTOACTIVATED DISINFECTION AND SODIUM HYPOCHLORITE IRRIGATION *IN* INFECTED ROOT CANALS

FOTON İLE İNDÜKLENEN FOTOAKUSTİK DALGALANMA TEKNİĞİ, IŞIKLA AKTİVE EDİLEN DEZENFEKSİYON VE SODYUM HİPOKLORİTİN ENFEKTE KÖK KANALLARINDAKİ ANTİMİKROBİYAL ETKİLERİ

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ABSTRACT

Aim: This study aimed to evaluate the antibacterial and antifungal effects of different disinfection methods for infected root canals with *Enterococcus faecalis* or *Candida albicans in vitro*.

Material and Methods: Hundred extracted, single-rooted human teeth with straight root canals were selected. After chemo-mechanical preparation of the root canals, except of those in the negative control group, contamination was performed using *Enterococcus faecalis* or *Candida albicans* for 7 days. The infected teeth were divided into three subgroups (n=15) according to the disinfection method applied: photo-activated disinfection, photon-induced photoacoustic streaming and 2.5% sodium hypochlorite irrigation. Microbial samples were collected from the root canals before and after disinfection. A hundred colony forming units were counted, and data were statistically analysed.

Results: All experimental groups showed significant reduction of *Enterococcus faecalis* and *Candida albicans.* Photon-induced photoacoustic streaming and sodium hypochlorite irrigation had similar antimicrobial effect, which was higher than photo-activated disinfection for both microorganisms ($p<0.05$).

Conclusion: None of the testing methods for root canal disinfection was able to achieve complete elimination of microorganisms. However, the results of this study state that photon-induced photoacoustic streaming, photo-activated disinfection and 2.5% NaOCl has significant antimicrobial effect against *Enterococcus faecalis* and *Candida albicans* biofilms in root canals.

Key words: *Candida albicans*, *Enterococcus faecalis*, Photodynamic Therapy, Root Canal, Sodium Hypochlorite

ÖZ

Amaç: Bu çalışmada, *Enterococcus faecalis* veya *Candida albicans* ile enfekte edilmiş kök kanallarında farklı dezenfeksiyon yöntemlerinin antibakteriyel ve antifungal etkilerinin in vitro olarak incelenmesi amaçlanmıştır.

Gereç ve yöntem: Çalışmada yüz adet çekilmiş, tek köklü, düz kanallı diş kullanıldı. Kök kanallarının kemo-mekanik preparasyonunun ardından, negatif kontrol grubu haricindeki dişler 7 gün boyunca *Enterococcus faecalis* veya *Candida albicans* ile enfekte edildi. Enfekte edilen dişler uygulanan dezenfeksiyon yöntemine göre ışıkla aktive edilen dezenfeksiyon, fotonla indüklenen fotoakustik dalgalanma tekniği veya 2.5% sodyum hipoklorit irigasyonu uygulanmak üzere üç alt gruba (n=15) ayrıldı. Dezenfeksiyon öncesi ve sonrasında kök kanallarından mikrobiyal örnekler alındı. Koloni oluşturan birimler sayıldı ve veriler istatistiksel olarak analiz edildi.

Bulgular: Tüm deney gruplarında, *Enterococcus faecalis* ve *Candida albicans* mikroorganizmalarında önemli ölçüde azalma gözlendi. Fotonla indüklenen fotoakustik dalgalanma tekniği ve sodyum hipoklorit irrigasyonu benzer antimikrobiyal etkiye sahip bulunurken ($p>0.05$), her iki grubun gösterdiği antimikrobiyal etki ışıkla aktive edilen dezenfeksiyondan iki mikrooganizma türü için de daha yüksek bulundu ($p<0.05$).

Sonuç: Uygulanan dezenfeksiyon yöntemlerinin hiçbiri ile mikroorganizmaların tümünün elimine edilmesi mümkün olmadı. Bununla birlikte, bu çalışmanın sonuçları; foton ile indüklenen fotoakustik dalgalanma tekniği, ışıkla aktive edilen dezenfeksiyon ve 2.5% sodyum hipokloritin kök kanallarındaki *Enterococcus faecalis* ve *Candida albicans* biofilmlerinde önemli ölçüde antimikrobiyal etkinliğe sahip olduğunu göstermektedir.

Anahtar Kelimeler: *Candida albicans*, *Enterococcus faecalis*, Fotodinamik Terapi, Kök Kanalı, Sodyum Hipoklorit

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INTRODUCTION

The main objective of endodontic treatment is the elimination of endodontic pathogens from the infected root canals.[1] Effective disinfection of the root canal system plays an important role in the long-term success of endodontic therapy.[2,3] However, total elimination of microorganisms is still a challenge in endodontics.

Enterococcus faecalis is the predominant bacterial species in persistent endodontic infections[4]. It has been frequently isolated from root canals with pulpal and/or secondary periapical inflammations.[5] In addition to bacteria, the frequency of fungal infections in endodontically treated teeth with persistent periapical pathology ranges from 3.7% to 10%.[6] Among the fungi, *Candida albicans* is the most common species[7] and has been associated with endodontic failures.[8]

Sodium hypochlorite (NaOCl) is the gold standard endodontic irrigant, particularly because of its antibacterial and organic tissue dissolution capabilities.[9,10] However, an unpleasant taste, cytotoxic effect, inability to remove the smear layer and insufficient elimination of all bacteria from the root canals are the primary disadvantages of NaOCl.[11] Thus, there is an urgent need to determine new disinfection agents and systems as an alternative to NaOCl for use in endodontic treatment. Several novel disinfection methods including photoactivated disinfection[11], laser activated irrigation[12] and new instruments such as XP-Endo finisher[13] have been tested to improve chemomechanical preparation of root canals.

Recently, DiVito et al. reported a technique known as photon-induced photoacoustic streaming (PIPS), which works on the principle of transferring pulsed energy to activate irrigation solutions, enhancing their debriding and cleaning efficiencies.[12] PIPS uses 2,940-nm erbium laser and a newly designed radial and stripped tip, with specific minimally ablative laser settings [low energy (20 mJ), pulse repetition rate (15 Hz) and very short pulse duration (50 µs)].[14] PIPS propagates strong photoacoustic shock waves that causes three-dimensional irrigant streaming throughout the root canal without mediating a thermal effect of direct laser irradiation on the dentin tissue.[15,16]

Photoactivated disinfection (PAD) is a novel disinfection method that has been used in endodontics.[11] PAD is alternatively known as lightactivated disinfection, photodynamic therapy, photodynamic antimicrobial chemotherapy, lethal photosensitization or photodynamic antimicrobial chemotherapy.[17] In PAD, following activation by light of a specific wavelength, photosensitising molecules attach to the bacterial or fungal membrane. Highly reactive oxygen species that are released following the photoactivation of photosensitiser kill the microorganisms.[1,18] Reportedly, photoactivated disinfection is not only effective against bacteria but also against other microorganism such as fungi and viruses. Moreover, toxicological tests have revealed that PAD has no negative side effects[11] and that the components of PAD are nontoxic to vital tissues.[1]

The use of different laser lights in combination with photosensitisers has been stated in numerous studies on PAD.[19,20] However, to prevent the periapical tissues from thermal damage in those studies, the lasers characterised by limited energy outputs have been used. This situation renders the PAD to be a relatively time-consuming procedure. Furthermore, the high cost of laser devices hinders their extensive use among the clinicians. Conventional light sources might solve this problem.[18] A system that involves a light-emitting diode (LED) lamp in the red spectrum, with a power peak at 628 nm and a photosensitiser, has been produced for PAD.[11]

Most of the studies on endodontic disinfection have been conducted to investigate the removal of *E. faecalis* from the root canals. To the best of our knowledge, there are only few studies that have investigated the effect of different disinfection protocols on *C. albicans*. Furthermore, various researchers have examined the antibacterial effect of PAD, PIPS and NaOCl, but none of them have compared their antibacterial and antifungal effects.

The aim of this study was to determine and compare the antimicrobial effect of 2.5% NaOCl irrigation, PAD and PIPS in root canals infected with *E. faecalis* or *C. albicans*. The null hypothesis to be tested was that there were no differences in the antimicrobial effectiveness among the groups, and the PAD and PIPS would be alternative to NaOCl irrigation for disinfection of the root canals.

MATERIALS AND METHODS

Tooth sample preparation

The approval for this study was granted by the Ethics Committee of the Health Sciences Institute of University of Marmara, Turkey (21.12.2012-10). A hundred extracted single-rooted human teeth were collected. Teeth were decoronated with a water-cooled diamond fissure bur (Intensiv SA, Grancia, Switzerland) #16 to obtain roots in an equal length (15 mm). The canal patency was confirmed by using a size #10 K-file (Mani, Matsutain Seisakusho Co., Tochigi-Ken, Japan) and the working length was considered 1 mm shorter than the root canal length. Root canals were instrumented with the Protaper rotary files (Dentsply Maillefer, Ballaigues, Switzerland) up to size F3, to achieve a K-file #35 size in the apical region. Copious amounts of 2.5% NaOCl solution (Wizard**,** Rehber Chemistry, Istanbul, Turkey) were used for irrigation during root canal instrumentation. To remove the smear layer, root canals were filled with 1 ml of 17% EDTA (Eudent Ed-Sol; Intermed S.A., Greece) and rinsed with 1 ml of 2.5% NaOCl, sequentially for 2 min each. A 0.9% sterile saline solution was used as a final irrigant and the root canals were dried using sterile size F3 Protaper paper points (Dentsply Maillefer, Ballaigues, Switzerland). The apical foramina of the samples were sealed with composite resin restorative material (Paradigm Z250, 3M ESPE; St Paul, MN, USA), and the external root surfaces were covered with two layers of nail varnish to prevent bacterial leakage. Each root was placed into a block of acrylic resin (Dentsply, Degudent GmbH, Hanau, Germany). All the prepared samples were autoclaved at 121°C for 20 min.

Microbiological Procedures

The microorganisms, *E. faecalis* and *C. albicans,* were cultured for 24 h at 37°C in Brain-Heart Infusion agar (BHI agar; Merck, Darmstadt, Germany) and Sabouraud Dextrose agar (SD agar; Merck, Darmstadt, Germany), respectively. A cell suspension of 2×10^8 cells/mL was prepared in BHI broth for *E. faecalis,* and another cell suspension of 2×10^6 cells/mL was prepared in SD broth for *C. albicans* (equivalent to 0.5 McFarland standard).

Forty-five of the teeth were contaminated with 10 µl of the suspension of *E. faecalis*, and the other 45 teeth with 10 µl of the suspension of *C. albicans,* using a syringe system with 30-gauge needle (NaviTip FX; Ultradent Products Inc, South. Jordan, UT), up to the working length. The infected teeth were incubated at 37°C for a week and the culture medium (the *C. albicans* suspension or the *E. faecalis* suspension) was replenished every 48 h for each tooth in the *C. albicans* and *E. faecalis* groups.

Testing Procedures

The sample size was determined by using power analysis. Ninety teeth were randomly divided into two main experimental groups, Group A for the teeth infected with *E. faecalis* and Group B for the teeth infected with *C. albicans***,** subjected to three different disinfection protocols. Thus, the teeth in Group A and Group B were further divided into three subgroups of 15 teeth each (n = 15) (Group A1, A2 and A3 and Group B1, B2 and B3), depending on the disinfection protocol applied.

In Group A1 and B1 (the NaOCl groups), the root canals were irrigated with 5 mL of 2.5% NaOCl solution for 2 min using a 30-gauge needle.

In Group A2 and B2 (the PIPS groups), the PIPS protocol was performed by an erbium-doped yttrium aluminium garnet laser (the Er:YAG laser), with 2,940-nm wavelength (Light walker, Fotona, Ljubljana, Slovenia) and a proprietary designed 12-mm-long 400 µm endodontic quartz tip. Laser operating parameters were 15-Hz, 20-mJ per pulse, 0.3-W power, and a 50-µs pulse duration. The co-axial water spray feature of the handpiece was set to "off." The tip was placed into the coronal reservoir only, and sterile bi-distilled water was continuously deposited in the canal by a 30-gauge needle, ensuring the presence of irrigant in the pulp chamber throughout the 20 sec duration of laser activation.[21]

In Group A3 and B3 (the PAD-FotoSan groups), an LED lamp-FotoSan (CMS Dental, Copenhagen, Denmark) in the red spectrum, with 628 nm wavelength, was used for the PAD protocol. A 0.25 mL Toluidine blue O solution (TBO; Sigma-Aldrich, St. Louis, MO) was injected into the canals using a 30-gauge needle. The TBO was agitated with a K-file for 60 sec to permit the photosensitiser to attach to the target microorganisms in the canal. The endodontic tip of FotoSan was inserted into the canals, 3 mm short of the working length, and light was activated for 30 sec.[22]

10 teeth received no treatment to test the sterility during the experiment as the negative control group. Each contaminated tooth served as its positive control before performing the test procedures. The

positive controls were used to check bacterial viability, and the negative controls were used to test sterility throughout the experiment.

In order to standardise all the experimental groups, root canals were rinsed with 1 mL of 0.9% sodium chloride solution (using a 30-gauge needle), which remained in the root canals for 60 sec. Bacteriological samples were collected with three paper points of ISO #25 before and after the disinfection procedures from all the teeth, including the canals of the negative controls. Paper points were placed into Eppendorf tubes with 1 mL of BHI broth for *E. faecalis* or 1 mL of SD broth for *C. albicans,* and then vortexed for 1 min. After 10-fold serial dilutions, aliquots of 10 µl were plated onto the BHI agar (for *E. faecalis* groups) and SD agar (for *C. albicans* groups) plates and incubated for 48 h at 37°C. Viable bacteria and fungi were quantified by determining the number of colony forming units per millilitre (CFUs/mL) on all the plates. All the tests were performed in duplicate and the averages of duplicate counts was determined.

Statistical Analysis

Statistical analyses were performed using the SPSS 17 software. Percentages of reduction of microorganisms were calculated, and percentage reduction data were stated as mean and median values[23]. To verify the distribution of the parameters between the research groups, Kolmogorov–Smirnov and Shapiro–Wilk tests were applied. Because of non-normal distribution of the data, statistical calculations were based on the nonparametric Mann–Whitney U-test and Kruskal–Wallis test. Wilcoxon signed-rank test was used for the intragroup comparative analysis. The p values<0.05 were considered statistically significant.

RESULTS

High microbial growth was determined in all the initial samples (positive controls), which demonstrated that the contamination was effective in all the root canals of the testing groups. Moreover, negative controls did not show any microbial growth. The NaOCl irrigation groups had the greatest percentage of microbial reduction among all the experimental groups. All disinfection protocols showed significant reduction in the number of CFUs after all the experimental procedures ($p<0.05$). Table 1 presents the distribution of the results (mean, median and range) before and after the disinfection protocols, the reduction rates of CFUs and the number of negative cultures.

In Group A, the disinfecting efficacy of NaOCl (99.99%) and PIPS (99.88%) did not show statistically significant difference ($p=0.114$), while exhibiting a higher antibacterial effect than the PAD (97.62%) ($p=0.001$). Likewise, in Group B, the disinfecting efficacy of NaOCl (99.96%) and PIPS (99.96%) did not show statistically significant difference ($p=0.372$), while exhibiting a higher antibacterial effect than the PAD (99.08%) ($p=0.001$).

Table 2 presents the assessment of the percentage reduction of microorganisms depending on the type of microorganism used in the testing groups. There was no statistically significant difference in the reduction of microorganisms between Group A1 and B1 ($p=0.79$) and between A3 and B3 ($p=0.868$). However, Group A2 showed a significantly higher microbial reduction when compared with Group B2 ($p=0.003$).

DISCUSSION

To the best of our knowledge, this is the first *in vitro* study that researched the antibacterial and antifungal effects of PAD, PIPS and NaOCl. The PAD has fundamental advantages over the conventional root canal chemical irrigants. Firstly, the antimicrobial effect of PAD is limited to the areas that have been subjected to the photosensitiser and light of a specific wavelength, without injuring the host tissues. The resistance to PAD does not depend on its multitarget mode of action as well.[24] Moreover, PAD, with its components, represents a safe treatment, which is one of the important aspects of root canal disinfection.[1,25] In the present study, we used TBO in PAD as the photosensitising agent, which has an additional antimicrobial activity due to its chemical and physical properties and can be used with specific wavelength laser photonic energy. Also, TBO can be used for reduction of both gram-positive and gram-negative endopathogenic bacteria.[24] A previous study suggested that *E. faecalis* was sensitive to TBO alone, without any exposure.[22] In photoactivated therapy, LED light is a safer alternative light source, because it does not create notable heat.[22] The FotoSan that we used in this study has been recently developed based on this objective and has a nebulously toxic influence and perfect biocompatibility.[11] Rios ve ark.[22] reported that FotoSan showed 97% *E. faecalis* elimination.

Table 1. Microbiologic counts before and after root canal disinfection with NaOCl, PIPS and PAD (n=15 each group)

	Microorganism counts (x10^3)						%Reduction (Median)	Negative Culture
	Counts before			Counts After				
	Mean	Median	Range	Mean	Median	Range		
Group A1	2976.7	100	10-10000	0.02	0	0-0.2	100	11/15
Group A2	10000	10000	10000-10000	11.8	10	1-100	99.9	0/15
Group A3	2914.7	1000	20-10000	202.8	1	0.002-3000	99.8	0/15
Group B1	702.0	100	10-3000	0.02	0	0-0.2	100	11/15
Group B2	768.0	1000	10-2000	0.02	0.01	1-100	99.99	0/15
Group B3	4049.3	300	40-40000	6.32	1	0.002-3000	99.87	0/15
p		**0.001***			**0.001***		**0.001***	

Kruskal Wallis Test **p<0.05*

Table 2. Percentage reduction of microorganisms according to Microorganism type in Experimental groups.

	E. feacalis	*C. albicans*	p
	Mean ±SD (median)	**Mean±SD (median)**	
NaOCl	99,99±0,01 (100)	99,96±0,1 (100)	**0,790**
PAD	97,64±7,67 (99,8)	99,08±2,06 (99,88)	**0,868**
PIPS	99,88±0,25 (99,9)	99,96±0,1 (99,99)	**0,003****

Mann-Whitney U test **p<0.05* ***p<0.01*
SD, standard deviation; NaOCl, sodium hypoclorite; PAD, photoactivated disinfection; PIPS, Photon-İnduced Photoacoustic Streaming

Similarly, in our study, FotoSan eliminated 97.64% of the *E. faecalis* bacteria.

Based on their role in endodontic failures and clinical importance[26], *E. faecalis* and *C. albicans* were the selected microorganisms in this experiment. We used paper point technique to collect the microbial samples from the root canals in the present study. This technique has the advantage that it can be carried out *in vitro* and *in vivo*. Nevertheless, microbial sampling was limited, because only the microorganisms in the main canal could be collected, and the ones inside the dentinal tubules were unapproachable.[26]

Because of the water content of the biofilms and high absorption of Er:YAG laser beam in water, the Er:YAG laser is quickly absorbed in the biofilms on dental hard tissues.[27] Bacteria are killed directly by the laser energy, along with a synergistic bactericidal effect rendered by the activation of the irrigant solutions. The laser-activated irrigation mechanism might be based on a rapid fluid motion, caused by the implosion and extension of laser-induced bubbles. This results into the dissolution and removal of the root canal surface tissue.[28] PIPS, a recent irrigation method, can clean, debride and disinfect the root canals even with sterile water, activated by a photomechanical phenomenon.[21,27] The PIPS has certain headstarts comparing with chemical disinfectant agents with hand irrigation methods. Pulsed Er:YAG laser has a non-thermal bactericidal effect, avoiding the undesirable impacts of thermal energy.[29] Furthermore, it has been asserted that one of the benefits of the PIPS technique is the minimal invasive root canal instrumentation required. [30] In this experiment, root canals were prepared to an apical size of #35 K-file. Alternatively, canals could have been shaped to an apical size #20, for minimally invasive preparation.[23] However, it was considered that this size would be too small for syringe irrigation to be efficient for the NaOCl groups.

A recent *in vitro* study concluded that PIPS protocol activated with (5%, 3%, 1%) NaOCl solution revealed effective eradication of *E. faecalis* biofilm and removal of smear layer, when bacterial samples were obtained before and right after the treatment.[15] Ozkaya et al.[27] in a scanning electron microscope study, reported that when 1% NaOCl was used with coronal position of PIPS, more effective elimination of *E. faecalis* biofilm at all root levels was evident, than that by the use of saline with PIPS. Many studies showed over 99% bacterial reduction when PIPS was used with different concentrations of NaOCl solution in root canals *in vitro.*[16,23,31] However, some *in vitro* studies indicated that although PIPS reduced the bacteria effectively, it did not increase the antimicrobial effect of NaOCl.[16,25] Balic et al.[16] stated that PIPs with QMix was more efficient than the conventional irrigation with NaOCl (99.998%) and the

PIPS with NaOCl (99.966%). In the current study, no antimicrobial irrigant was used with PIPS, because the present study was planned to estimate the mechanical effects of irrigant activation with PIPS. The present study suggests that the PIPS disinfection is highly effective in eliminating *E. faecalis* and *C. albicans* from the root canals, even with distilled water, probably because of the physical action of rapid movement of irrigant and direct irradiation of the biofilm.[32] Pedulla et al.[31] reported that NaOCl used with PIPS showed 99.8% bacterial reduction, while bi-distilled water with PIPS showed 77.03%.[31] Interestingly, PIPS with bi-distilled water eliminated 99.88% *E. faecalis* in our study. Higher reduction rate in our study than the results obtained by Pedulla et al.[31] may be explained by the different methodologies used in the studies.

According to the results of this study, complete elimination of microorganisms was not achieved. Although negative cultures were obtained in NaOCl groups, the culture method used in the experiment had its limitations. It did not determine the microbial colonies penetrating deep into the dentinal tubules.[33] Many studies showed that different root canal disinfection methods and agents were not able to eliminate the microorganisms completely.[16,17,18,27] Their findings are in agreement with our results.

In a previous study, it was shown that FotoSan eliminated 97% of *E. faecalis,* but it showed less antibacterial effect than 6% NaOCl.[22] Our results are consistent with this study, and FotoSan showed 97.64% bacterial reduction of *E. faecalis,* which has provided less antibacterial effect than 2.5% NaOCl. Tuncay et al.[11] concluded that root canals disinfected with FotoSan revealed 91% reduction of *E. faecalis,* whereas 2.5% NaOCl showed a total bacterial elimination. Although methodology of their study is very similar with ours, this finding is contrary to our results. The difference between the results may be because of variation in the sample size or the studied specimens.

Dumani et al.[34] found that 5 mL 2.5% NaOCl irrigation for 2 min reduced 99.68% of *E. faecalis,* which is consistent with our results in NaOCl groups. In our study, 2.5% NaOCl eliminated 99.99% of *E. faecalis* with the use of the same amount and time as Dumani et al.[34] did. Some researchers found that total elimination of *E. faecalis* was achieved with NaOCl when used in a high concentration (5.25%)[35] or for a relatively long time such as 10 min.[36] However, in our study, we found that PIPs, PAD and 2.5% NaOCl disinfected greater part of the microorganisms in 20 sec, 90 sec and 2 min respectively.

CONCLUSIONS

It can be stated that the alternative disinfection techniques like PAD and PIPS are able to disinfect the root canals without using NaOCl irrigation, based on the results of the present study. Furthermore, they are more biocompatible and time saving. In NaOCl and PAD groups, there was no difference between the *E. faecalis* and *C. albicans* reduction in the root canals. On the other hand, PIPS seemed to be more effective in eliminating *C. albicans* than *E. faecalis*. PIPS has been shown to be as efficient as 2.5% NaOCl irrigation in eradicating both *E. faecalis* and *C. albicans.* PAD and PIPS can be recommended as efficient disinfection methods in the root canals. Further investigations should be undertaken *in vivo* to support the results of this study.

Authorship declaration
All authors have contributed significantly, and are in agreement with the manuscript.

Disclosure Statement
The authors have nothing to disclose. There is no conflict of interest related to this study.

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