EVALUATION OF ANTIMICROBIAL EFFECT OF PHOTO-ACTIVATED DISINFECTION ON *Enterococcus Faecalis* and *Candida Albicans*

FOTO-AKTİF DEZENFEKSİYONUN *Enterococcus faecalis* ve *Candida albicans* ÜZERİNDEKİ ANTİMİKROBİYAL ETKİSİNİN DEĞERLENDİRİLMESİ

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Abstract

In order to provide disinfection in the root canal system, it is necessary to clean root walls and root canal with antimicrobial agents in addition to effective instrumentation. Aim of this *in vitro* study was compare antimicrobial effects of photo-activated disinfection (PAD) and calcium hydroxide (CH) on *Enterococcus faecalis* (*E. faecalis)* and *Candida albicans* (*C. albicans).*

Microbial strains used in the study were obtained from Ankara Refik Saydam Hıfzıssıhha Institute Culture Collection. In study, *C. Albicans* and *E. faecalis* microorganisms were used. PAD and CH were used as antimicrobial agent. Six petri dishes were prepared for each group (negative control, PAD and CH groups). A cavity was formed on the agar in each petri dish to be 5 mm in diameter and 2 mm in depth. PAD and CH were applied to agar plates of each microorganism at sterile conditions. Prepared 18 petri dishes were incubated at 37 °C for 24 hours. Then, microbial inhibition diameters of the groups were measured. Statistical analysis of data was evaluated with t-test. Significance level (*p*) was considered to be 0.05 in the application of the test.

For *E. faecalis* and *C. albicans* the highest inhibition zone diameters were measured in PAD group. PAD showed higher antimicrobial effect than CH against *E. faecalis* and *C. albicans* ($p<0.001$). Antimicrobial effect of CH and PAD against *C. albicans* was found statistically higher than *E. faecalis* ($p<0.001$).

Within the limitations of this *in vitro* study, it was found that antimicrobial effect of PAD against *C. albicans* and *E. faecalis* is higher than CH.

Keywords: *Candida albicans*, Calcium hydroxide, *Enterococcus faecalis*, Photo-activated disinfection.

Özet

Kök kanal sisteminde dezenfeksiyon sağlamak için, etki enstrümantasyona ek olarak kök duvarlarının ve kök kanalının antimikrobiyal ajanlarla temizlenmesi gerekir. Bu *in vitro* çalışmanın amacı, foto-aktif dezenfeksiyon (PAD) ve kalsiyum hidroksitin (CH) *Enterococcus faecalis (E. faecalis)* ve *Candida albicans (C. albicans)* üzerindeki antibakteriyel etkilerini karşılaştırmaktır.

Araştırmada kullanılan mikrobiyal suşlar Ankara Refik Saydam Hıfzıssıhha Enstitüsü Kültür Koleksiyonundan elde edildi. Çalışmada *C. Albicans* ve *E. faecalis* mikroorganizmaları kullanıldı.

Antimikrobiyal ajan olarak PAD ve CH kullanıldı. Her grup için altı petri kabı hazırlandı (negatif kontrol, PAD ve CH grupları). Her petri kutusundaki agar üzerinde 5 mm çapında ve 2 mm derinliğinde bir boşluk oluşturuldu. Steril koşullarda her mikroorganizmaya ait agar plakalarına PAD ve CH uygulandı. Hazırlanan 18 petri kabı, 37 ° C'de 24 saat süreyle inkübe edildi. Daha sonra grupların mikrobiyal inhibisyon çapları ölçüldü. Verilerin istatistiksel analizi t-testi ile değerlendirildi. Test uygulamasında anlamlılık düzeyi (p) 0.05 olarak kabul edildi.

E. faecalis ve C. albicans için en yüksek inhibisyon bölgesi çapları PAD grubunda ölçüldü. PAD, *E. faecalis* ve *C. albicans*' a karşı CH' den daha yüksek antimikrobiyal etki gösterdi ($p < 0.001$). CH ve PAD' nin *C. albicans*' a karşı antimikrobiyal etkisi *E. faecalis*' den istatistiksel olarak daha yüksek bulundu ($p < 0.001$).

Bu *in vitro* çalışmanın sınırlamaları dâhilinde, PAD' nin *C. albicans* ve *E. faecalis* üzerindeki antimikrobiyal etkisinin CH' den daha yüksek olduğu bulunmuştur.

Anahtar Kelimeler: *Candida albicans*, *Enterococcus faecalis*, Foto-aktif dezenfeksiyon, Kalsiyum hidroksit.

1. INTRODUCTION

The success rate in endodontic treatment depends on various factors such as complicated anatomy of the root canal system, dentin structure and composition, biofilm structure, antimicrobial resistance of microflora (Siqueira, 2001). Root canal disinfection requires effective removal of microorganisms and smear layer, breakup of the biofilm layer (Orstavik & Haapasalo, 1990; Siqueira, Rocas, Favieri & Lima, 2000). Microorganisms and their products found in the infected dental root canal cause many adverse conditions from pulp necrosis to periapical lesion (Silva Garcez et al., 2006). In order to provide disinfection in the root canal system, it is necessary to clean root walls and root canal with antimicrobial agents in addition to effective instrumentation (Bonsor, Nichol, Reid & Pearson, 2006). The presence of residual microorganisms in the canal after chemo-mechanical preparation may cause to fail the treatment. For successful treatment, it is important to minimize the number of residual microorganisms, as well as to tightly fill the root canal (Hancock, Sigurdsson, Trope & Moiseiwitsch, 2001; Sundqvist, Figdor, Persson & Sjogren, 1998).

Irrigation solutions and intracanal medicaments are used in the disinfection process (Bonsor et al., 2006). Calcium hydroxide (CH) is a commonly used intracanal medicament due to its antimicrobial activity, inducing hard tissue formation and tissue dissolving properties (Nerwich, Figdor & Messer, 1993). It performs the antibacterial activity by the decomposition of calcium and hydroxyl ions. Alkaline structure allows it to reach deep of dentin (Alacam, Yoldas & Gulen, 1998). The most frequently microorganism isolated from persistent infections after of endodontic treatment is *Enterococcus faecalis* (*E. faecalis*). Likewise, *Candida albicans* (*C. albicans*) are associated with teeth with pulp necrosis (Schlafer, Vaeth, Horsted-Bindslev & Frandsen, 2010). Elimination of these microorganisms is clinically important. However, some studies have shown that calcium hydroxide is insufficient to eliminate *E. faecalis* (Haapasalo & Orstavik, 1987; Safavi, Spangberg & Langeland, 1990; Siqueira & de Uzeda, 1996).

In recent years, an alternative disinfection method called Photo-Activated Disinfection (PAD) has been used to eliminate biofilm-mediated microbial infections (Hamblin & Hasan, 2004). PAD is based on light activation of the photosensitive molecule that selectively accumulates in the microbial cell at a suitable wavelength. Activation of the photosensitizer in the presence of oxygen produces reactive oxygen species (single oxygen, superoxide, hydroxyl radicals), leading to the death of microorganisms (Jori & Brown, 2004). PAD application is minimally invasive and does not cause tissue toxicity. PAD is used for disinfection of deep carious lesions and periodontal pockets in clinic. It is not known exactly how effective it is in root canal disinfection (Bergmans et al., 2008). However, it has been reported that it can be used successfully in the destruction of multidrug resistant pathogens (Garcez, Nunez, Hamblim, Suzuki & Ribeiro, 2010).

Aim of this in vitro study was evaluate antimicrobial effects of PAD and CH on *E. faecalis* and *C. albicans*.

2. MATERIAL AND METHOD

Microbial strains used in the study were obtained from Ankara Refik Saydam Hıfzıssıhha Institute Culture Collection. In this study, *C. Albicans* (ATCC 10231) and *E. faecalis* (ATCC 29212) microorganisms were used. Fresh passages of microorganisms were made and culture densities were adjusted to Mcfarland 0.5 standard. 20 microliter (µl) samples taken from *E. faecalis* suspension were transferred to Mueller Hinton Agar. 20 µl of sample taken from *C. albicans* suspension was transferred to RPMI agar. The samples were homogeneously spread over the entire surface of the medium with a sterile stick. Petri dishes were kept at room temperature for 15 minutes. Six petri dishes were prepared for each group (negative control, PAD and CH groups). In each group, three petri dishes were for *E. faecalis* and three petri dishes were for *C. albicans*. Total of 18 petri dishes were prepared. A cavity was formed on the agar in each petri dish to be 5 millimeter (mm) in diameter and 2 mm in depth. Then negative control, PAD and CH groups were applied at sterile conditions.

CH (Ultracal XS; Ultradent Products Inc., South Jordan, USA) was mixed under sterile conditions in a safety cabinet according to the company's instructions. It was placed in round sterile polyethylene molds with 2 mm thickness and 5 mm inner diameter. Polyethylene molds were placed in the cavity on the medium. Fresh medical disc was used for each experiment procedure.

PAD solution provided by the manufacturer was added to in the cavity on the medium. Solution was toluidine blue (TBO; Sigma-Aldrich, St Louis, MO, USA). Irradiation was performed with the FotoSan light (FotoSan; CMS Dental, Copenhagen, Denmark). FotoSan light energy output was measured 1 J/s (wavelength peak at 628 nm). According to the instruction of manufacturer laser light was applied for 30 seconds. The application was repeated for each experiment procedure.

The cavity on the medium was left empty in negative control group. Prepared 18 petri dishes were incubated at 37 °C for 24 hours. Thereafter, microbial inhibition diameter was measured round of the disks containing CH and PAD after 24 hours. It was also measured in the negative control group. Experiment was performed and repeated under strict aseptic conditions. The antimicrobial effect was expressed in terms of the diameter of zone of inhibition (in mm) at the end of incubation period. The same procedures were repeated in every period and measurements were recorded.

2.1. Statistical Analyze

The statistical analysis of the data was analyzed by Statistical Package for Social Sciences Windows Version 16.0 program (SPSS Inc., Chicago, IL, ABD). Statistical analysis of data was evaluated with t-test. Significance level (p) was considered to be 0.05 in the application of the test.

3. RESULTS

After PAD application, the inhibition zone diameters of medium with *E. faecalis* and *C. albicans* were measured as 24 mm and 28 mm, respectively. After CH application, the inhibition zone diameters of medium with *E. faecalis* and *C. albicans* were measured as 8 mm and 23 mm, respectively (Table 1). Inhibition zone formation was not observed in the negative control group. For *E. faecalis* and *C. albicans* the highest inhibition zone diameters were measured in PAD group (Table 1).

Antimicrobial effect of PAD on *E. faecalis* was higher compared to CH. Antimicrobial effect of PAD against *E. faecalis* was found statistically more significant than CH ($p<0.001$). Antimicrobial effects of PAD and CH on *C. albicans* were close to each other. However, statistical analysis indicates that antimicrobial effect of PAD against *C. albicans* was found to be more significant than CH ($p<0.001$).

PAD showed higher antimicrobial effect than CH against *E. faecalis* and *C. albicans* ($p<0.001$) (Table 2).

Antimicrobial effect of CH on *C. albicans* was higher compared to *E. faecalis*. Antimicrobial effect of CH against *C. albicans* was found statistically more significant than *E. faecalis* ($p<0.001$). Antimicrobial effect of PAD on *E. faecalis* and *C. albicans* was close to each other. However, statistical analysis indicates that antimicrobial effect of PAD against *C. albicans* was found to be more significant than *E. faecalis* ($p<0.001$). Antimicrobial effects of CH and PAD on *C. albicans* was found higher than *E. faecalis* (p<0.001) (Table 2).

Table 1. Descriptive values of inhibition zone diameters (mm) of the groups are shown (n: number of samples).

		n	Mean	Std. Deviation
CH	***E. faecalis***	3	8	0.20
	C. albicans	3	23	0.20
PAD	***E. faecalis***	3	24	0.15
	C. albicans	3	28	0.10

Table 2. Comparison of inhibition zone diameters (mm) of the groups (T-test, $p<0.05$).

	CH	**PAD**	***p***
E. faecalis	8	24	***< 0.001***
C. albicans	23	28	***< 0.001***
p	***< 0.001***	***< 0.001***	

4. DISCUSSION

The presence of a continuous infection in the root canal system is considered in cases of endodontic failure (Sundqvist, Figdor, Persson & Sjogren, 1998). *E. faecalis* and *C. albicans* were included to study considering studies that reported that they were associated with recurrent infections after endodontic treatment (Love, 2001; Mattigatti, Ratnakar, Moturi, Varma & Rairam, 2012; Waltimo, Orstavik, Siren & Haapasalo, 2000). *E. faecalis* has been shown *in vitro* to resist against antibacterial effect of calcium hydroxide and other medicaments used root canal treatment. *E. faecalis* is usually present in the channel with low numbers, so it can usually be eliminated. However, it is difficult to eliminate once it has settled in the root canal system (Sundqvist, Figdor, Persson & Sjogren, 1998). *E. faecalis* can survive for a long time without nutrients. Thus, it is placed in the dentin tubules and protected from irrigation solutions (Fonseca et al., 2008). Similarly, *C. albicans* has the capacity to grow in the low nutrient medium of the treated canal (Sundqvist et al., 1998). In addition, both types of microorganisms are protected from disinfection by creating advanced biofilms (Mayer, Wilson & Hube, 2013; Wang, Shen & Haapasalo, 2012).

There are many methods to evaluate the antimicrobial activities of the medicaments used in root canal treatment. Although agar diffusion method is used mostly among them, agar diffusion method has limitations such as agar viscosity, selection of suitable medium, incubation time and temperature

(Vianna et al., 2004). Since it allows direct comparison of the antimicrobial effects of the tested materials, agar diffusion method was preferred in this study (Sundqvist, Figdor, Persson & Sjogren, 1998).

In studies evaluating the antimicrobial effect of CH against *C. albicans* and *E. faecalis*, it has been reported that CH can be used alone or mixed with other antimicrobial agents (Ballal, Kundabala, Acharya & Ballal, 2002; Ercan, Dalli & Dulgergil, 2006). Also the effects of contact time with microorganisms on antimicrobial activity were also investigated (Al Mosaid, Sullivan & Coleman, 2003). Ballal et al. (Ballal, Kundabala, Acharya & Ballal, 2002) using agar diffusion test assessed antimicrobial effect of the CH and 2% chlorhexidine gel against *E. faecalis* and *C. albicans*. It has been shown that CH mixed with distilled water is high effect in the first 24 hours against *E. faecalis* and *C. albicans*, but after 72 hours this effect decreased in both the microorganisms. Al Mosaid et al. (Al Mosaid, Sullivan & Coleman, 2003) reported that CH applied on *C. albicans* for 1 hour showed no antibacterial effect. On the other hand *C. albicans* was completely eliminated after 24 and 72 hours of CH application. Similarly, in our study CH was found to be effective against *C. albicans* and *E. faecalis* in 24 hours.

Ercan et al. (Ercan, Dalli & Dulgergil, 2006) in their study on infected extracted human teeth demonstrated that CH mixed with sterile water has a weak antimicrobial effect on *C. albicans* and *E. faecalis*, and the effect has decreased further after 7 day. Mattigatti et al. (Mattigatti, Ratnakar, Moturi, Varma & Rairam, 2012) assessed antimicrobial effect of 2% sodium hypochlorite, 2% chlorhexidine, CH, ethylenediaminetetraacetic acid (EDTA), MTAD and Propolis with agar diffusion test. They reported the lowest antimicrobial effect on *C. albicans* and *E. faecalis* was seen in EDTA and CH groups at the end of 48 hours. In our study was seen low antimicrobial effect against E. faecalis in CH group. The antimicrobial effect of CH against *C. albicans* was higher compared to *E. faecalis*.

PAD was introduced as a new treatment method to eliminate these microorganisms (Fonseca et al., 2008). Since the light used in PAD is not very strong, it does not cause damage to neighbor cells (Biel, Jones, Pedigo, Gibbs & Loebel, 2012). When the studies are examined, it can be said that the disinfection effect and treatment success of PAD varies depending on used paint and laser (Bouillaguet et al., 2010; Fonseca et al., 2008; Soukos et al., 2006; Yao, Zhang & Chu, 2012).

Xhevdet et al. (Xhevdet et al., 2014), *ex vivo* study reported that PAD is a suitable disinfecting agent in extracted teeth contaminated with *E. faecalis* and *C. albicans*. However, they have shown that they cannot completely destroy microorganisms in the root canals. Additionally they have shown that extending PAD application time notably increased the number of killed microorganisms. They recommended longer times of irradiation. Yildirim et al. (Yildirim et al., 2013), sixty human teeth roots were infected with *E. faecalis* suspension. As a result of the study reported that antimicrobial effect of PAD on *E. faecalis* was as effective as using 5% sodium hypochlorite application for 15 minutes. They also showed that 1 minute irradiation time was sufficient to achieve antimicrobial effect in PAD application. In our study, irradiation was applied for 30 seconds according to the manufacturer's recommendation. Similarly, PAD has been found high antimicrobial effect against *E. faecalis* and *C. albicans*.

Silva Garcez et al. (Silva Garcez et al., 2006), their study was used thirty extracted human teeth roots that infected with *E. faecalis*. They indicated that antimicrobial effect of 3 minutes PAD application against *E. faecalis* was more effective than 0.5% sodium hypochlorite application for 30 minutes. They stated that reduction of pathogens in a short time can be achieved by PAD used with conventional endodontic treatment. In another *in vitro* study on extracted human teeth pointed out that FotoSan can be used as a complementary application to kill microorganisms remaining in root canal after endodontic treatment. However, it was stated that a longer irradiation time than the manufacturer's recommendation is required to improve the antimicrobial effect (Poggio et al., 2011). In addition, Schlafer et al. (Schlafer, Vaeth, Horsted-Bindslev & Frandsen, 2010) indicated that photo-activated disinfection using conventional light source (FotoSan) for 30 seconds greatly reduced

alive endodontic pathogens number in root canals and planktonic suspension. Moreover, they said that the irradiation time may be extended in order to increase antimicrobial effect on *C. albicans*. In our study, PAD applied with conventional light source (FotoSan). Irradiation was applied for 30 seconds according to the manufacturer's recommendation.

In the literature, there is no study comparing the antimicrobial effect of PAD and CH against *E. faecalis* and *C. albicans*. In this context, this study will give ideas to other researchers on this subject. Although the study was conducted *in vitro* conditions; it is difficult to exactly predict the reflections of applications in the clinic.

5. CONCLUSION

Within the limitations of this *in vitro* study, it was found that antimicrobial effect of PAD on *C. albicans* and *E. faecalis* is higher than CH. Therefore, we think that use of PAD in conventional endodontic treatment will reduce the residual microorganisms in the root canal. However, we believe that further clinical and *in vitro* studies should be conducted on the subject.

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