ANTIMICROBIAL EFFICACY OF ERBIUM LASER IN THE ENDODONTIC TREATMENT OF INFECTED ROOT CANALS

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ABSTRACT

Introduction: Chemo-mechanical irrigation for cleaning the infected root canals does not ensure complete removal of microorganisms.

Objectives: The aim of this study was to estimate efficacy of Er:YAG laser compared to conventional methods for the treatment of infected root canals.

Methods: The study included 400 freshly extracted premolar single-rooted teeth. Crowns were cut off obtaining 15 mm long samples of the root canal. The apical part was drilled through. Teeth were divided into four groups inoculated with different microbial strains (*Enterococcus faecalis, Streptococcus sanguinis, Fusobacterium nucleatum* and *Candida albicans*). Three different methods for removal of microorganisms were applied, i.e.: Er:YAG laser radiation, irrigation with 5.20% NaOCl and Qmix. Microbial viability was analyzed by flow cytometry.

Results: All treatment methods effectively eliminated the majority of microbial cells. Statistically significant differences were observed in mean percentage of dead cells between tested organisms ($p<0.05$) for Er:YAG laser (30 and 90 seconds) and 5.20% NaOCl irrigations, but not for Qmix ($p=0.052$). There were significant differences in rates of dead microorganisms when comparing treatment methods ($p<0.001$). The percent of dead cells was significantly higher for *C. albicans* and *S. sanguinis,* than for *E. faecalis* or *F. nucleatum*. Longer duration laser radiation was significantly more efficient compared to 30 seconds radiation ($p<0.001$). Synergic disinfectant efficacy of irradiation in combination with irrigant was over 90% with NaOCl and over 95% with Qmix.

Conclusion: Er:YAG radiation is sufficiently efficient as standard disinfection method in endodontics, and can also serve as adjuvant therapy to standard mechanical and irrigational treatments.

Keywords: root canals, disinfection, Er:YAG, laser, endodontic procedures.

REZUMAT

Introducere: Irigarea chimio-mecanică pentru curățarea canalelor radiculare infectate nu asigură îndepărtarea completă a microorganismelor.

Obiective: Scopul acestui studiu a fost de a estima eficacitatea laserului Er:YAG, comparativ cu metodele convenționale de tratare a canalelor radiculare infectate.

Metode: Studiul a inclus un număr de 400 de dinți premolari cu o singură rădăcină, proaspăt extrași . Coroanele au fost tăiate, obținându-se probe de 15 mm lungime ale canalului rădăcinii. Partea apicală a fost forată. Dinții au fost împărțiți în patru grupuri inoculate cu diferite tulpini microbiene (*Enterococcus faecalis, Streptococcus sanguinis, Fusobacterium nucleatum și Candida albicans*). S-au aplicat trei metode diferite de îndepărtare a microorganismelor, și anume: radiația laser Er:YAG, irigarea cu 5,20% NaOCl și Qmix. Viabilitatea microbiană a fost analizată prin citometrie în flux.

Rezultate: Toate metodele de tratament au eliminat efectiv majoritatea celulelor microbiene. Diferențe semnificative din punct de vedere statistic au fost observate în ceea ce privește procentul mediu al celulelor moarte între organismele testate ($p <0,05$) pentru laserul Er: YAG (30 și 90 secunde) și 5,20% irigare NaOCl, dar nu și pentru Qmix ($p = 0,052$). Au existat diferențe semnificative în privința ratei microorganismelor moarte la compararea metodelor de tratament ($p <0,001$). Procentul de celulele moarte a fost semnificativ mai mare pentru *C. albicans și S. sanguinis*, decât pentru *E. faecalis* sau *F. nucleatum*. O durată mai lungă a radiației laser a fost semnificativ mai eficientă comparativ cu radiația de 30 de secunde ($p <0,001$), ucigând 80% din celulele microbiene. Eficacitatea iradierii în combinație cu irigantul a fost de peste 90% cu NaOCl și cu 95% cu Qmix.

Concluzie: Radiația Er:YAG este suficient de eficientă ca metodă standard de dezinfecție în endodonție și poate servi și ca tratament adjuvant pentru tratamentele standard mecanice și irigare.

Cuvinte-cheie: canale radiculare, dezinfecție, Er: YAG, laser, proceduri endodontice.

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INTRODUCTION

Oral microorganisms represent a major problem in dentistry [1, 2]. Because of the complex structure of the root canal system, infecting microorganisms exhibit resistance to irrigation and mechanical cleaning of root canals, making impossible the complete elimination of microorganisms [3]. The effectiveness of the chemo-mechanical treatment methods depends largely on the individual anatomy of the tooth [4]. Although during endodontic treatment, irrigation with NaOCl removes the majority of infecting microorganisms, reinfection is still possible because of the smear layers that reduce effectiveness of disinfecting agents [2]. The methods using chelator substances such as ethylenediaminetetraacetic acid (EDTA) or Qmix, which remove smear layer, have been suggested to exhibit an enhanced disinfectant activity [5].

In addition to irrigants, more modern methods, such as laser radiation were introduced. Currently, there are various equipment radiation lasers available (Er:YAG, Er, Cr:YSGG, Nd:YAG, diode, CO_2), which are appropriate for using in periodontology and endodontics, each with its special advantages. Er:YAG lasers are effective in removing residuals and detoxifying radicular cement [6-8]. Er:YAG laser is a device that allows control of laser radiation with a feedback system that selectively detects subgingival calculus or plaque. Fluorescence-controlled Er:YAG radiation led to clinical improvements after 3 to 6 months, the efficacy being similar to that of ultrasonic debridement [9]. Antimicrobial effect of laser-activated irrigation with Er:YAG on *Enterococcus faecalis* and *Streptococcus mutans* biofilms in root canals proved a significant decrease in viable counts, compared to irrigation alone [10, 11].

There are a lot of studies which deal with the performance and the disinfecting effects of Er:YAG laser [12, 13], but lasers are still not used routinely in dental practice. More laboratory and clinical evaluations are required to become convinced that lasers could represent novel tools for endodontists. Thus, the objectives of this study were to check the efficacy of Er:YAG laser radiation as antibacterial method for treatment of infected root canal, and compare the effectiveness to standard irrigants and chelators used for elimination of intracanal bacterial population.

MATERIALS AND METHODS

Samples

A number of 400 freshly extracted premolar single-rooted teeth were included in the study, that was performed at the Faculty of Prishtina in 2017. The crown of each tooth was cut off using a water-cooled diamond blade in a low speed saw Isomet 1000 (Buehler GmbH, Germany) obtaining 15 mm long root specimens. Only teeth with round canals were chosen for further analysis. To determine the working length, a #10 Kerr file (Maillefer Instruments SA, Switzerland) was used. The canals were then enlarged to an apical size of #35 (F3) using Protaper files (Maillefer Instruments, Switzerland). Between each file copious irrigation with 2.5% NaOCl was performed. After root canal instrumentation, teeth were rinsed with 17% EDTA and then sterilized. The canals were dried with paper points (Dentsply Maillefer) and finally root apex was closed with composite material. Confirmation of sterilization was microbiologically tested on blood agar for 24 hours at 37 °C. When the growth of any microorganism was observed, the sample followed above mentioned procedure of sterilization once again, until no bacterial of fungal contamination was observed on blood agar plates.

Teeth were equally divided into four groups of 100 specimens, depending on the inoculating strain, respectively *Enterococcus faecalis, Streptococcus sanguinis, Fusobacterium nucleatum* and *Candida albican*s. The groups were further divided into seven subgroups, depending on the disinfection method (Er:YAG laser, NaOCl, Qmix, combination of laser and NaOCl, combination of laser and Qmix, positive, negative control).

Bacterial suspension and biofilm growth

The ATCC (American Type Culture Collection) *Enterococcus faecalis* (ATCC 29212), *Streptococcus sanguinis* (ATCC 10556), *Fusobacterium nucleatum* (ATCC 25586) and *Candida albican*s (ATCC 10231) microbial strains were refreshed by inoculation from frozen vials

on blood agar plates, which were incubated for 24 hours in an aerobic atmosphere at 37 °C. Growth of monospecific biofilms was based on the inoculation of sterilized root with microbial inoculum of very high densities corresponding to 5 McFarland ($1.5x10^9$ cells/mL), obtained in thioglycolate (TIO) broth. Teeth were inoculated with the suspension and incubated for seven days at 37 °C in an anaerobic atmosphere generated by Anoxomat™ Mark II (Mart Microbiology B.V, Netherlands). Every second day 30 μL of fresh bacterial or fungal suspension of the same density in TIO broth was added.

The growth of biofilm was examined in one representative specimen of *E. faecalis* by scanning electron microscopy (JEOL JSM-840A Scanning Electron Microscope**,** JEOL, USA) and confirmed with microbiological methods based on cultivation on agar plates. Root canals were washed with 1X phosphate buffer (PBS) with pH = 8.3. The washed cell suspension was inoculated and smeared on blood agar plates, which were incubated 24 hours at 37 °C. The next day growth of bacteria on the plates was assessed and confirmed when >300 white bacterial colonies (CFU/mL) were grown on a single plate.

Irrigation with 5.20% NaOCl and Qmix, respectively

After one week from inoculation, teeth were rinsed for 20 seconds with 3 mL of 5.2% NaOCl. After flushing the root canal with NaOCl, dental root canals were additionally washed with 2 mL of 10X PBS. At the end, 5 mL of suspension were collected, of which 500 μL were pipetted to prepare a sample for measuring cell viability by flow cytometry.

Er:YAG laser application

Laser application was performed according to publication by De Meyer *et al*. [10] using a Er:YAG laser as disinfecting method alone and in combination with NaOCl or Qmix as irrigants. Fotona's Er:YAG laser TwinLight® Endodontic Treatment (TET) (Fotona, Ljubljana, Slovenia) was used. Laser-activated radiation with 2940 nm Er:YAG laser with pulse activated irradiation (power output 15 W, 20 Hz, pulsing rate of 50 μs, 1500 mJ, conical fibre tip) was performed. The laser beam was completely inserted through root canal with optical fibres of 200 μm and then laser irradiation for 30 or 90 seconds was performed. After laser radiation, followed rinsing of damaged biofilm from the root canals with 5 mL of 1x PBS with 1mM EDTA, pH 8.3. Between rinses, the walls of the root canal were lightly drawn to wash out as many cells as possible. A volume of 5 mL of washed liquid with cells was collected, which was used for further analysis by flow cytometry.

Application of treatment based on Er:YAG laser – irrigation combination

Testing the effectiveness of combined methodology - laser with NaOCl or Qmix irrigation- was as follows: in the first step of the treatment process, a revolutionary photon-induced photoacoustic streaming method was employed, using the power of the Er:YAG laser to create non-thermal photoacoustic shock waves in the canal for 10 seconds. Following this photoacoustic treatment, the canals and subcanals were left for irrigation with NaOCl or Qmix for 10 seconds. After treatment, the damaged biofilm was collected as described above.

Measurement of bacterial viability by flow cytometry

Cell suspensions were obtained after the application of different treatment methods, and 500 μL of suspension have been subtracted for flow cytometry analyses. The cell viability was checked using "The Cell Viability Kit with Liquid Counting Beads" (BD Biosciences). The assay includes the use of a pair of fluorescent dyes, i.e. thiazole orange (TO) and propidium iodide (PI). TO enters living as well as dead cells, but to varying degrees. The living cell membrane is impermeable to PI, which can enter only cells with damaged membrane. The combination of these dyes allows the flow cytometer to differentiate between live and dead cells and thus establish their viability. The procedure was followed according to the manufacturer's instructions. A volume of 500 μL of suspension was placed in a cytometer test tube. To the pipetted suspension 5 μL of TO

and PI dyes and 50 μL of BD Liquid Counting Beads were added. The test tube was well stirred and incubated for 10 minutes at room temperature and in darkness. After incubation, the sample was analyzed on the flow cytometer BD FACSCanto II.

Control procedures

Positive controls were represented by teeth infected with microbial strains collected without application of any treatment methods. Inoculated teeth were washed with 5 mL of 1X PBS with 1 mM 17% EDTA, from which 500 μL for the cytometry viability and 100 μL for inoculation on the blood agar were collected. The growth of microorganisms on the agar plates incubated for 24 hours at 37 °C confirmed the viability of microbial suspension washed from the root canals. A number of >300 CFU/mL was considered as successful growth. One sample of infected root canal was sent for SEM analysis.

Statistical analysis

Statistical analysis was performed using the statistical software SPSS 20 (IBM, New York, USA). Two-Way ANOVA statistical test with post-hoc Tukey test were used with the percentage of dead microbial cells as a dependent variable. Type of microorganism and disinfecting method were factor variables. Statistical significance was set at $p<0.05$.

RESULTS

In the final analysis 400 samples of the root canals were included. SEM showed the surface of root canal wall and confirmed the growth of microbial cells. Examination of the root canals after incubation showed the formation of a thick biofilm on the wall of the root canal (Fig. 1). Table 1 represents results of efficacy for laser application and irrigation on infected root canals. Results are presented as average percentage of dead cells that were detected by flow cytometry in each sample. In total, combination of two treatments (irrigation with NaOCl or Qmix and laser application) induced the highest percentage of dead microbial cells from infected root canals and consequently showed the best disinfection efficacy.

The analysis of positive controls revealed that even with no treatment, more than 30% of all microbial cells were dead. In four specimens of negative controls no microbial cells were detected, respectively.

Table 1: Percent of dead microbial cells from 400 treated root canals after application of different treatment methods, measured by flow cytometry

	% of dead microbial cells detected by flow cytometry				
	Enterococcus faecalis (N=100)	*Candida albicans* (N=100)	*Streptococcus sanguinis* (N=100)	*Fusobacterium nucleatum* (N=100)	***p-value***
Er:YAG					
30 sec (N=44)*	74.48	79.02	82.19	72.46	0.049
90 sec (N=44)*	80.47	89.09	88.38	78.18	0.034
5,20% NaOCl (N=44)*	59.54	66.57	72.22	72.61	0.022
Qmix (N=44)*	75.21	79.65	84.13	70.15	0.052
Er:YAG+NaOCl					
30 sec (N=44)*	81.15	84.63	94.76	80.04	0.041
90 sec (N=44)*	90.35	92.47	92.81	89.35	0.152
Er:YAG+Qmix					
30 sec (N=44)*	90.95	90.48	92.14	88.86	0.112
90 sec (N=44)*	95.21	96.69	96.79	91.48	0.687
p-value	<0.001	<0.001	<0.001	<0.001	
Positive control (N=44)*	38.80	36.46	35.54	33.59	0.741

**each testing group consisted of 11 specimens; each negative group consisted of only 1 specimen*
N = number of specimen

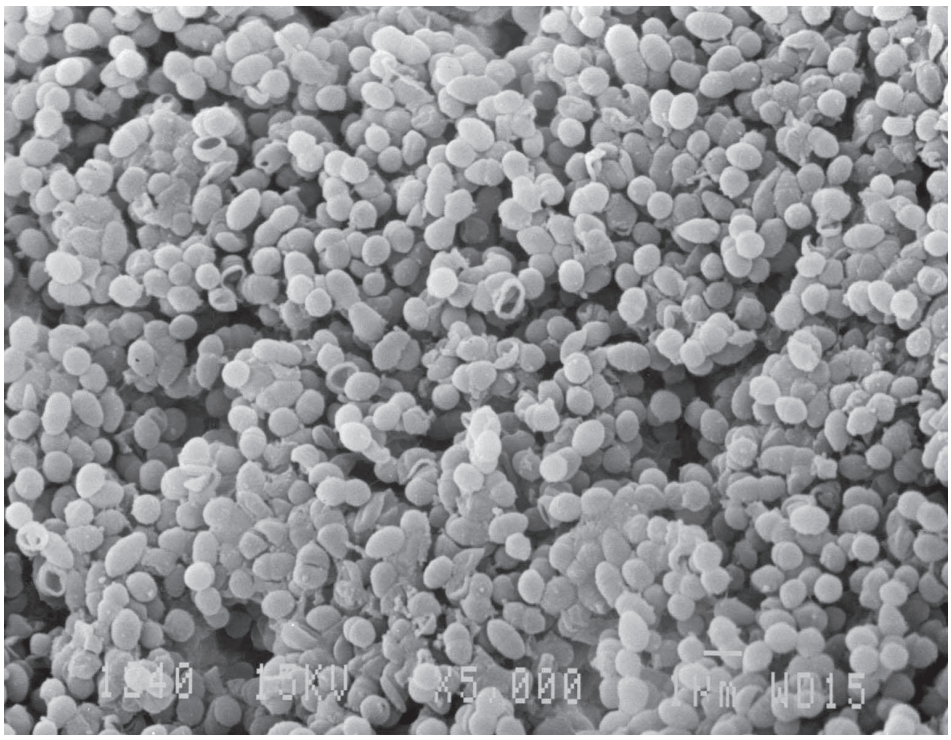

Fig. 1: SEM analysis of positive colonization and the formation of *E. faecalis* biofilm.

Two factor ANOVA showed statistically significant differences in mean percentage of dead cells between tested organisms ($p<0.05$) for laser radiation (for 30 and 90 seconds), irrigation with 5.20% NaOCl, but no statistical significance ($p=0.052$) for Qmix irrigation. There were also statistically significant differences between treatment methods ($p<0.001$) for all microorganisms, as well as in interaction between organisms and treatment methods ($p=0.040$). Percent of dead cells was significantly higher for *C. albicans* and *S. sanguinis* compared to *E. faecalis* or *F. nucleatum* in all treatment groups (Fig. 2).

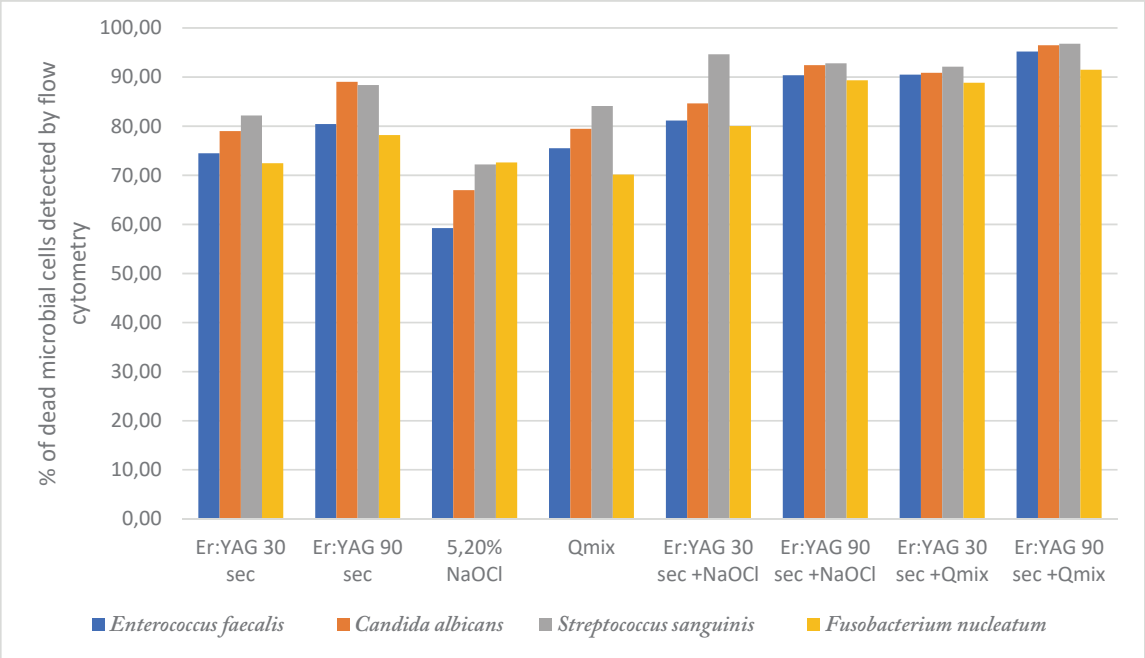

Fig. 2. Comparisons of antimicrobial efficacy of treatment methods among microorganisms that were inoculated into root canal samples.

Meanwhile, when comparing disinfecting methods, combination of laser radiation and irrigation was significantly more effective ($p<0.001$) compared to other groups. While the lowest percent of dead cells was detected in the group treated with 5.20% NaOCl, more accurate analysis showed statistically significant higher percent of dead microbial cells in the group treated by the combination Er:YAG for 90 seconds + Qmix irrigation. There were also no statistical differences among microorganisms, indicating a good disinfecting activity that can handle most infections. Longer duration of laser radiation showed better disinfecting action ($p<0.001$) and achieved over 80% of dead cells. Disinfecting activity of laser radiation in combination with irrigant was over 90% for addition of NaOCl and over 95% for addition of Qmix.

Post-hoc analysis showed significant differences in the percentages of dead cells between different treatments. Values were comparable for *E. faecalis* and *F. nucleatum* and for *C. albicans* with *S. sangunis*, and proved that laser radiation had greater disinfecting effect ($p < 0.001$, respectively). Efficacy of disinfection also showed differences between time duration of irradiation with laser ($p < 0.001$) for all microorganisms. Qmix was equally effective in killing bacteria as laser and better when compared to 5.20 % NaOCl. Meanwhile, combination of laser irradiation and Qmix irrigation came close to total elimination of cells. Significantly fewer bacteria were killed by the standard method of irrigation with NaOCl.

DISCUSSION

According to the knowledge published in the literature, chemo-mechanical irrigations and instrumentation techniques for cleaning infected root canal do not completely remove infecting microorganisms due to anatomy of the tooth dentin, growth of microorganisms in complex biofilms or their penetration into the dentin tubules, where they are protected from antimicrobial agents, or the treatment method is not sufficiently effective. The microbial strains used in the analysis are commonly isolated and found in teeth in which treatments of root canals have failed, and moreover they are difficult to eradicate from the canals due to formation of biofilms. *E. faecalis* was set as a reference microorganism due its high frequency of isolation from cases of failed endodontic treatment, its resistance to calcium hydroxide treatment and its relative insensitivity to laser irradiation [14-16]. *C. albicans* was chosen based on its various pathogenic characteristics. *C. albicans* has the ability to bind to dentin collagen, invade deep dentin tubules and form biofilms, to activate host defences and to show resistance to different antimicrobial agents used in endodontics. *C. albicans* cells have also been found in the resorption of periapical root surfaces and in periapical granuloma [17].

All treatment methods successfully eliminated majority of microbial cells when compared to control samples. Meanwhile, therapy with Er:YAG laser showed higher percent of dead microbial cells compared to other disinfecting methods ($p < 0.001$). Furthermore, with prolonged irradiation (90 seconds) an even better disinfection rate was observed. More than additional 5% of microbial cells were eliminated due to prolonged radiation. Moreover, that lowest percentage of dead cells was recorded for *F. nucelatum* and *E. faecalis*, *C. albicans* and *S. sanguinis* showed higher percent. Very similar results were also obtained with 5.20% NaOCl or Qmix irrigation. It seems that *F. nucleatum* and *E. faecalis* are very persistent bacteria in root canals. Despite that, NaOCl is still the most widely used root canal irrigant, yet there is no consensus about its optimal concentration [18]. A past study indicated that exposure to high concentrations of NaOCl is the most predictable method for eliminating intracanal bacteria and removing intracanal biofilm [19]. According to the published literature, 5.20% NaOCl was an effective irrigant, but with the lowest rate of dead bacteria. Besides, its unpleasant taste, NaOCl is also highly toxic, may cause severe irritation, and is unable to completely remove the smear layer. For these reasons, the use of Qmix as an irrigant may be a better alternative to NaOCl. However, when 5.20% NaOCl was activated via the Er:YAG laser irradiation, an even greater reduction in bacterial cells was found compared to irrigation or laser alone. The rate of dead microbial cells elevated over 80% for all microorganisms.

Qmix has been employed after the root canal preparation as a rinse to improve root canal cleaning and disinfection already in the past [20, 21]. A previous study [20] employed a 3-minute treatment period to evaluate the antimicrobial effect and it was demonstrated that Qmix promoted additional antimicrobial action, especially when acting for longer periods (>1 minute) [22]. Rinsing with 3 mL of Qmix led to better results (>88%) compared to irrigation with 5.20% NaOCl, as also observed by Gründling *et al*. [23]. Moreover, there were also no statistical differences in disinfection between microorganisms (p=0.687), meaning that Qmix could represent a procedure that could assure the elimination of polymicrobial infections.

None of the treatments could remove biofilms nor kill all microbial cells completely. Combination of laser irradiation and irrigation with Qmix was the closest to total elimination of cells. Dissolution ability is mandatory for an appropriate eradication of biofilms attached to dentin. Significantly fewer bacteria were killed in the standard method of irrigation with NaOCl. We believe that longer time of exposure could result in more dead bacteria than in only 30-60 seconds of exposure. The 2940 nm wavelength of the Er:YAG laser was chosen for its high absorption in water. In the current study same conditions and specifications of laser irradiation were used, as were described in the studies by De Meyer *et al.* [10] and Olivi *et al*. [24]. Longer duration of radiation showed better disinfecting action and achieved over 80% of dead cells. Disinfecting activity in combination with irrigant was over 90% for addition of NaOCl and over 95% for addition of Qmix. Since application of Er:YAG effectively eluted smear layer, then killed microorganisms and destroyed attached biofilm, it is probable that further irrigation with NaOCl or Qmix eliminated the rest of viable cells that were not harmed by laser irradiation. The smear layer reduces the effectiveness of disinfecting agents against *E. faecalis* in infected dentin, as this bacteria forms very tough biofilms. Solutions containing NaOCl and/or Qmix could not have high antibacterial activity alone. That is why the combination of two treatment methods achieved more than 90-95% reduction of viable bacterial cells. The effectiveness of laser technique could be explained by the increased consumption of available chlorine ions that occurred after the activation of the irrigant by an Er:YAG laser [25]. Another explanation might be related to the lysing and mechanical breaking up of the bacterial biofilm due to the laser application, after which irrigation with NaOCl killed most of free bacteria. Because the volume of the liquid in the root canal is small, this effect amplifies and improves the removal of bacteria, which has also been confirmed before [26, 27]. The results of the study were similar with those obtained in other *ex vivo* studies, which showed that Er:YAG is the most appropriate laser for intracanal removal of debris and smear layers [28-34].

It appears that lasers, because of their effect on minerals existing in debris and smear layer, can be more effective in removing these two components from the canals. Laser treatment permits the delivery of a non-contact, homogenous, heating effect, independent of the distance of the target tissue from the heat source. This is a major advantage when we consider the irregular surface of the target tissues. Laser light can penetrate area of canals where rinsing solutions have no access, like secondary canals and depth of dentin tubules, and eliminates microorganisms [12, 28, 35, 36]. When Er:YAG is used in combination with NaOCl in canals, better results were obtained [35]. The results of the aforementioned studies clearly state that this laser in combination with a standard root treatment and an appropriate rinsing solution is effective [14, 18, 37]. Results suggest that the Er:YAG laser may be a valuable tool for root canal disinfection when one uses radially emitting laser tips. One benefit of the laser over conventional treatment is that it has the ability to achieve significant disinfection of canals infected with bacteria for which there is evidence that the conventional method is not as effective.

While results did not demonstrate complete elimination of infection from root canals, test system did allow us to quantify the degree of change in bacterial load after laser treatment. We found that an over 90% reduction in viable bacteria could be achieved using the combination of laser and NaOCl or Qmix. Due

to the difficulty in removing microbial biofilm after common disinfecting procedures, the use of auxiliary techniques, such as the use of lasers, may be useful during endodontic treatment. Perhaps this is another economical tool that can be added to the dentists for future applications. However, future studies should be conducted to evaluate the effectiveness of this procedure in clinical trials. Considering that endodontic infections are polymicrobial biofilm-based diseases, evaluating against only one organism represents a limitation to the present study, since the presence of multiple microorganisms might have altered the dynamics demonstrated by the present study. Another limitation is that the study was performed in *in vitro* conditions, thus the results could be totally different in *in vivo* conditions.

CONCLUSIONS

Er:YAG laser appeared to be effective in enhancing the efficacy of irrigation solutions that are commonly used in endodontics. All methods successfully killed microbial cells forming biofilms in the infected root canals, but none of them achieved 100% elimination. Longer duration of laser radiation allows better disinfection, since 90-second radiation showed an average above 80% removal of the microorganisms, while 30-second only above 70%. Combination of the two methods, respectively irrigation and radiation with laser for 90 seconds was effective in eradicating 90-95% of microbial biofilms. The combined use of different methods is therefore necessary to enhance antimicrobial effectiveness. Additional clinical studies are needed to clarify the effect on endodontic treatment outcomes *in vivo*.

Conflict of interests: Authors declare no confict of interests.

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