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In Vitro Effect of Er:YAG Laser on Different Single and Mixed Microorganisms Being Associated with Endodontic Infections

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Abstract

Objective: The purpose of this *in vitro* study was to evaluate the antimicrobial effect of activated irrigation with different modes of erbium-doped yttrium aluminum garnet (Er:YAG) laser application on microorganisms related to secondary endodontic infection.
Background: Er:YAG laser has been recommended as an adjuvant tool for root canal disinfection during endodontic treatment.
Materials and methods: Laser-activated irrigation (LAI) with 300 or 600 μm tips were tested with or without intermittent irrigation with 0.9% sodium chloride (NaCl) solution against different microorganisms (five single strains and dual species (*Streptococcus gordonii* combined with *Actinomyces oris* or *Fusobacterium nucleatum*) in root canals after 3 days of incubation. In a 21-day infection model, LAI was used together with intermittent rinsing with sodium hypochlorite (NaOCl) against the dual-species mixtures; here the incidence of microbial regrowth after up to 7 days was monitored.
Results: In the 3-day root infection model, LAI protocols did not show any significant reduction of the microbial load when compared with manual irrigation with saline solution. In the 21-day infection, *S. gordonii* combined with *A. oris* were not detectable anymore after applying the LAI protocol with a 600 μm tip (30 mJ/10 pps) up to 7 days after treatment.
Conclusions: Application of LAI with a 600 μm tip by using an Er:YAG laser might be advantageous in treatment of endodontic infections.

Keywords: cavitation, endodontics, irrigation, *Enterococcus*, *Candida*, disinfection

Introduction

Successful endodontic therapy requires, besides a full debridement of pulpal tissue and dentin debris, an effective elimination of microorganisms from the infected root to prevent its reinfection. For this purpose, the irrigation with disinfecting agents is strongly recommended as an additional step to remove and kill pathogens inside dentinal tubules.[1,2] Sodium hypochlorite (NaOCl) is the most commonly used endodontic irrigating solution in the dental practice owing to its antimicrobial and proteolytic properties and its excellent ability to dissolve organic tissue.[3] Despite these favorable properties, the irrigation with NaOCl as an adjuvant to root canal preparation cannot completely remove microorganisms from the dentine and consequently allows for persistent endodontic infections.[4]

In case of post-treatment infections, the presence of persistent microorganisms is regularly reported. *Enterococcus faecalis* is frequently found because of its resistance to some disinfecting agents.[5] In addition, *Candida* spp. and *Actinomyces* spp. have been isolated from secondary infections as well as *Fusobacterium nucleatum*, which is more commonly isolated from root canals with periapical lesions.[6,7]

To achieve better success rates in endodontic treatments, several laser systems have been introduced to remove the smear layer and, apparently, to enhance the disinfection

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efficacy in root canals. Erbium-doped yttrium aluminum garnet (Er:YAG) laser has been reported to be the most appropriate for endodontic use owing to the proximity of its wavelength to the absorption maximum of hydroxyapatite and its higher absorption by water, with minimal thermal side effects.[8,9] The activation of endodontic irrigants has been proven to enhance irrigant effectiveness with respect to the cleaning and disinfection of root canals.[10] The activity of NaOCl, for example, can be improved by increase of the temperature: at 45°C (i.e., 8°C above body temperature) NaOCl was described to be 100× more effective than at 20°C.[11] However, an intracanal temperature increment of <2.5°C was observed when using an Er:YAG laser.[12] Therefore, the main mode of action is probably due to cavitation effects that have been observed through the application of Er:YAG lasers during endodontic irrigation, thereby increasing the removal of dentin debris and microbial cells.[13,14] In addition, an antibacterial activity on *E. faecalis* biofilms was described by application of Er:YAG laser light.[15] In contrast, other authors have stated that the effects of the laser are not superior to the antimicrobial properties of NaOCl alone, but that the use of the Er:YAG laser can improve the effectiveness of this irrigating solution when both are used in combination.[16] Therefore, there is still some uncertainty about the disinfecting capacity of laser light itself.

There are different ways to apply the Er:YAG laser energy during endodontic irrigation. Although formerly a laser-activated irrigation (LAI) protocol was proposed using a thin and long sapphire tip with conical shape that is used inside the root canal in vertical movements, more recently a so-called photon-induced photoacoustic streaming (PIPS) protocol using lower energies and a large and short tip inserted just in the access cavity was suggested.[17,18]

A recent study reported that LAI using the PIPS protocol resulted in a higher bacterial removal compared with Endo activator or ultrasonically activated irrigation.[19] Furthermore, Korkut et al.[20] illustrated that Er:YAG laser with PIPS activation resulted in cleaner root canal walls compared with Nd:YAG and diode laser groups. Furthermore, the additional use of 5% NaOCl to the PIPS protocol was proposed due to its higher eradication of biofilm and smear layer.[21]

In this context, this *in vitro* study aimed at evaluating the antimicrobial activity of Er:YAG LAI on several microorganisms associated with persistent endodontic infection in instrumented root canals of extracted teeth. The specific aim was to test a recently marketed 600 μm tip against a conventional 300 μm tip for differences in colony forming units (CFU) counts using short- and long-term infection models.

Materials and Methods

Er:YAG laser

The Er:YAG laser, a 2940-nm device (AdvErL Evo Er:YAG Laser—model MEY-1-A; J. Morita Corporation, Kyoto, Japan) was used with conical tips with 300 or 600 μm diameter (R300T or R600T; J. Morita Corporation). Details of the laser settings are given in Table 1.

Microorganisms

The following microbial strains were included in the experiments: *E. faecalis* ATCC 29212, *Streptococcus gordonii* ATCC 10558, *Actinomyces oris* MG1, *F. nucleatum* ATCC 25586, and *Candida albicans* ATCC 76615. Strains were cultivated on trypticase soy agar (TSA) plates (Oxoid, Basingstoke, GB) with 5% of sheep blood for 24 h at 37°C. *A. oris* MG1 and *F. nucleatum* ATCC 10558 and mixtures were always incubated in anaerobic conditions. Thereafter microorganisms were suspended in 0.9% w/v sodium chloride (NaCl) solution (OD600 nm = 1), which was mixed with nutrient broth 1:100, *C. albicans* 1:50. In case of the dual-species infection, suspensions of the microorganisms were mixed 1:1, before adding to the nutrient broth (brain heart infusion broth; Oxoid).

Root canal preparation and contamination

Premolars or incisors extracted by dental practitioners in the Canton of Bern (Switzerland) and stored in 1% chloramine solution were used. Before the donation, patients were informed about the use of their teeth for research purposes and gave their oral consent. This study was in accordance with the approved guidelines and regulations of the local ethical committee. Since each tooth was selected from a pool of extracted teeth, the specimens are categorized as ''irreversibly anonymized.''

After decoronation, roots were prepared up to size 40.02 using EndoWave instruments (J. Morita Corporation) and the Dentaport OTR motor (J. Morita Corporation) at 450 rpm. The final irrigation was performed with 5 mL of 3% NaOCl solution followed by 2 mL of 17% ethylenediamine tetraacetic acid (EDTA) solution. Root canal curvature was measured using radiographic examination according to the method described by Schneider.[22] After stratification, straight and curved roots were equally distributed among the groups.

Roots were stored in 0.9% w/v NaCl for at least 48 h. Before starting the experiments, roots were exposed for 20 min to moist heat steam at 121°C to ensure sterility. In the 3 days experiments, root canals were infected with single

Table 1. Erbium-Doped Yttrium Aluminum Garnet Specifications

Er:YAG laser parameters	*Wavelength: 2,940 nm* / *Tip*	*Time*	*Frequency*	*Energy*	*Power density*	*Energy density*	*Frequency of treatment*	*Cumulative dose given*
LAI 300	300 μm (tapered)	20s	20 pps	70 mJ	5 W/cm^2	100 J/cm^2	3	15 W/cm^2 300 J/cm^2
LAI 600	600 μm (tapered)	20s	10 pps	30 mJ	0.5 W/cm^2	10 J/cm^2	3	1.5 W/cm^2 30 J/cm^2

ErYAG, erbium-doped yttrium aluminum garnet; LAI, laser-activated irrigation.

species each and the dual mixtures of *S. gordonii* ATCC 10558 with *A. oris* MG1 or *S. gordonii* ATCC 10558 with *F. nucleatum* ATCCC 25586, in the 21 days experiment, only the dual species mixtures were applied. Ten microliters of the microbial suspension were pipetted inside the root canal and incubated for 3 and 21 days, respectively. Nutrient broth was exchanged twice a day (3 days experiments) or twice a week (21 days experiments).

Root infection model with 3 days of incubation

In this study, the terms ''LAI 300'' for the LAI protocol with a 300 μm tip and the term ''LAI 600'' for the modified PIPS protocol using a recently marketed 600 μm tip with and without intermittent irrigation were used.

After 3 days of incubation, different treatments were applied: (1) control: no treatment; (2) manual irrigation with 3 mL of 0.9% w/v NaCl; (3) LAI 300: Er:YAG laser application inside the root canal for three times during 20 sec (70 mJ, 20 pps, 5 W/cm^2, 100 J/cm^2, minimal water cooling outflow), with an R300T laser tip, followed by a final manual irrigation with 1 mL of 0.9% w/v NaCl; (4) LAI 300 with rinsing: Er:YAG laser 70 mJ application inside the root canal for three times, during 20 sec (70 mJ, 20 pps, 5 W/cm^2, 100 J/cm^2, minimal water cooling outflow) with intermittent manual irrigation with 1 mL NaCl followed by a final rinse with 1 mL NaCl; (5) LAI 600: Er:YAG laser application at the entrance of the root canal for three times during 20 sec (30 mJ, 10 pps, 0.5 W/cm^2, 10 J/cm^2, minimal water cooling outflow), with an R600T laser tip, followed by a final manual irrigation with 1 mL of 0.9% w/v NaCl; (6) LAI 600 with rinsing: Er:YAG laser 30 mJ application at the entrance of the root canal for three times, during 20 sec (30 mJ, 10 pps, 0.5 W/cm^2, 10 J/cm^2, minimal water cooling outflow) with intermittent manual irrigation with 1 mL NaCl followed by a final rinse with 1 mL NaCl).

For manual irrigations, a side vented needle (30G; Ultradent, South Jordan, UT) and a Luer lock syringe were used. The laser application in treatments (3) and (4) was performed by inserting the laser tip in the root canal at $\sim$2 mm from the apex and slowly moving it up and down.[23] Immediately after treatments, samples were collected with two sterile paper points (ISO 40), which were inserted in the root canal for 20 sec and placed in 1 mL of 0.9% w/v NaCl. Thereafter, a dilution series was made and each 25 μL was spread on TSA plates with 5% sheep blood. After an incubation time of 48 h (*A. oris* MG1, *F. nucleatum* ATCC 25586 5d) and the respective conditions, CFUs were determined.

Root infection model with 21 days of incubation

For this long-term experiment only two combinations of microorganisms (*S. gordonii*+*A. oris* and *S. gordonii*+*F. nucleatum*) were selected. After 21 days of incubation, treatments were applied as before, however, using NaOCl solution for irrigation: (1) control: no treatment; (2) NaOCl: manual irrigation with 3 mL 1.5% NaOCl (w/v) followed by a final irrigation with 2 mL of 0.9% w/v NaCl solution; (3) LAI 300: Er:YAG laser application inside the root canal for 3 $\times$ 20 sec (70 mJ, 20 pps, 5 W/cm^2, 100 J/cm^2, minimal water cooling outflow) with an R300T laser tip and intermittent manual irrigations with 1 mL 1.5% NaOCl followed by a final rinse with 2 mL 0.9% w/v NaCl; (4) LAI 600: Er:YAG laser application at the entrance of the root canal for 3 $\times$ 20 sec (30 mJ, 10 pps, 0.5 W/cm^2, 10 J/cm^2, minimal water cooling outflow) with an R600T laser tip and intermittent manual irrigations with 1 mL 1.5% NaOCl followed by a final rinse with 2 mL of 0.9% w/v NaCl. Samples were collected and plated as described earlier. After this procedure and thereafter every other day, fresh nutrient broth was again added to the root canals and samples were again collected after 2, 5, and 7 days. This procedure allows multiplication and thereafter determination of the persistent microorganisms.[24]

Statistical analysis

In all experiments, values of 10 independent results per group entered statistical analysis. Microbial counts were expressed as log10 CFU. One-way analysis of variance, *post hoc* Tukey test or Kruskal–Wallis, and *post hoc* Dunn test were used, depending on the distribution of the data. Data were analyzed by SPSS 24.0 program (IBM, Chicago, IL). Fisher exact test and Benjamini–Hochberg correction were used to compare percentages of contaminated roots. The level of significance was set at 5%.

Results

Activity on microorganisms in the root canal infection model after 3 days of incubation

In the short-term experiment with single species infection the reduction of CFU was between 0.50 log10 (*A. oris*) and 2.23 log10 (*S. gordonii*) with manual irrigation. There was no clear pattern of CFU reduction with laser irradiation (LAI 300 and LAI 600) with or without additional rinsing. Compared with the negative control, the activation in both laser groups without and with intermittent manual irrigation led most to a statistically significant reduction. Compared with manual irrigation, only two experiments resulted in a statistically significant reduction of CFU counts (Fig. 1a, c).

The pattern of CFU reduction of dual-species mixtures depended on the microorganisms that were used. When root canals were infected with *A. oris*/*S. gordonii* a CFU reduction of 5 log10 CFU for the LAI 600 application, and a reduction of CFUs below the detection limit for LAI 600 application with intermittent manual irrigation were observed (Fig. 2). When the root canals were infected with *F. nucleatum*/*S. gordonii*, the reduction of bacterial counts was 1.1 log10 CFU at the highest when applying LAI 600 with intermittent manual irrigation with 0.9% w/v NaCl (Fig. 2).

Activity on microorganisms in the root canal infection model after 21 days of incubation

In the long-term experiment the root canals were infected with dual species for 21 days, and the irrigation solution was NaOCl. The combination of *A. oris*/*S. gordonii* was susceptible to the LAI 600 application, and no bacterial regrowth could be detected even after 7 days of reincubation (Fig. 3). For the combination of *F. nucleatum*/*S. gordonii*, no cultivable bacteria were found immediately after LAI 300 application, but after reincubation for 4 and 7 days a regrowth of bacteria could be detected in half of the roots (Fig. 3). The manual irrigation with NaOCl led to an incomplete eradication of bacteria immediately after irrigation and, subsequently, after reincubation (Fig. 3).

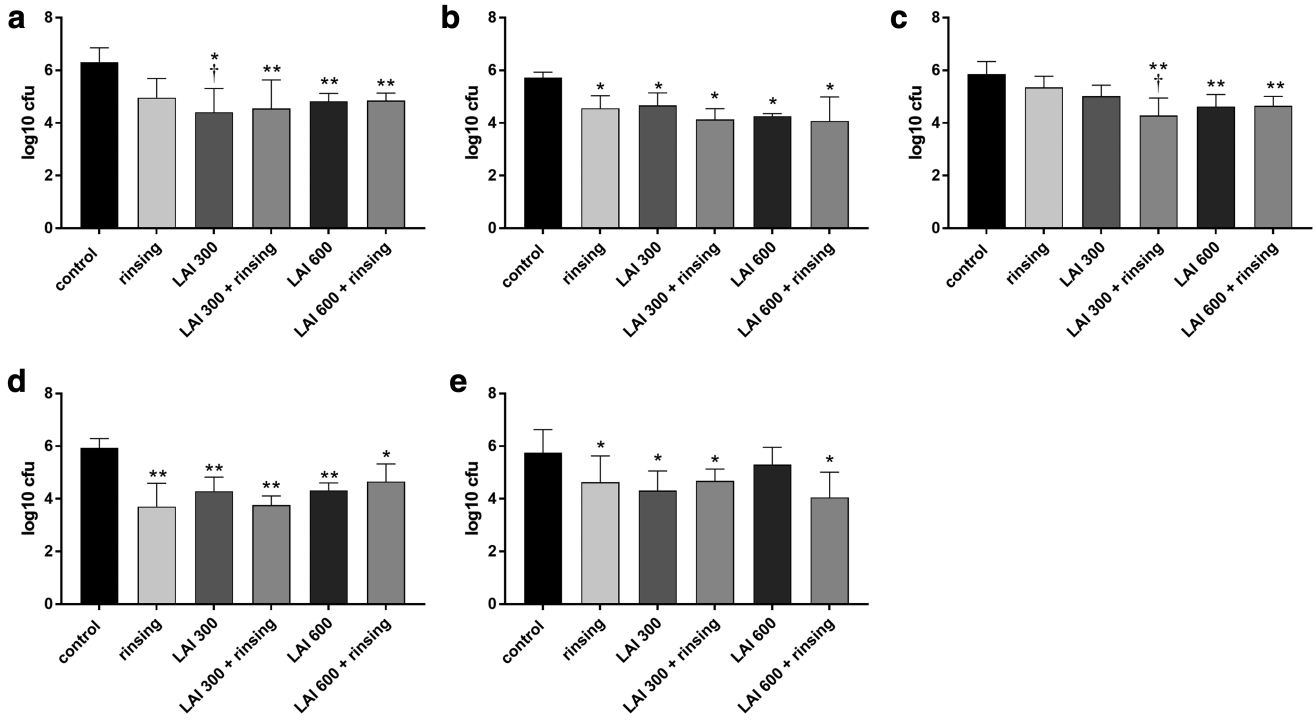

FIG. 1. Antimicrobial activity of rinsing with 0.9% w/v NaCl (rinsing), LAI protocol with a 300 or 600 μm tip (LAI 300 and LAI 600, respectively) without and with rinsing against 3-day single-species cultures of *Enterococcus faecalis* **(a)**, *Candida albicans* **(b)**, *Actinomyces oris* **(c)**, *Streptococcus gordonii* **(d)**, and *Fusobacterium nucleatum* **(e)** grown inside root canals. $^{*}p<0.05$, $^{**}p<0.01$ compared with control group. $^{\dagger}p<0.05$ compared with rinsing group (ANOVA/Tukey). ANOVA, analysis of variance; LAI, laser-activated irrigation; NaCl, sodium chloride.

Discussion

A short-term infection model to test the effect of different LAI protocols (LAI 300 and LAI 600) on endodontic pathogens compared with manual irrigation with 0.9% w/v NaCl was used. Furthermore, the influence of additional irrigation between the laser activation periods was tested. In the long-term infection model, roots were incubated for 21 days to achieve a predictable invasion of bacteria into the dentinal tubules. We tested if the applied laser activation protocols had different impacts on root canal disinfection with NaOCl. The microorganisms used in this study were typical strains associated with endodontic infection.[4–6]

First, it has to be noted that the Er:YAG laser itself in general did not have an additional direct antimicrobial effect. Our own pretests (results not shown) failed to prove a direct bactericidal effect of Er:YAG laser irradiation. This is in line with Sahar-Helft et al.[25] who reported that Er:YAG LAI did not improve CFUs of *E. faecalis* biofilm compared with manual dynamic irrigation, and also Ordinola-Zapata et al.[26] reported better antibacterial effects on *E. faecalis* with the use of needle irrigation of NaOCl than the use of

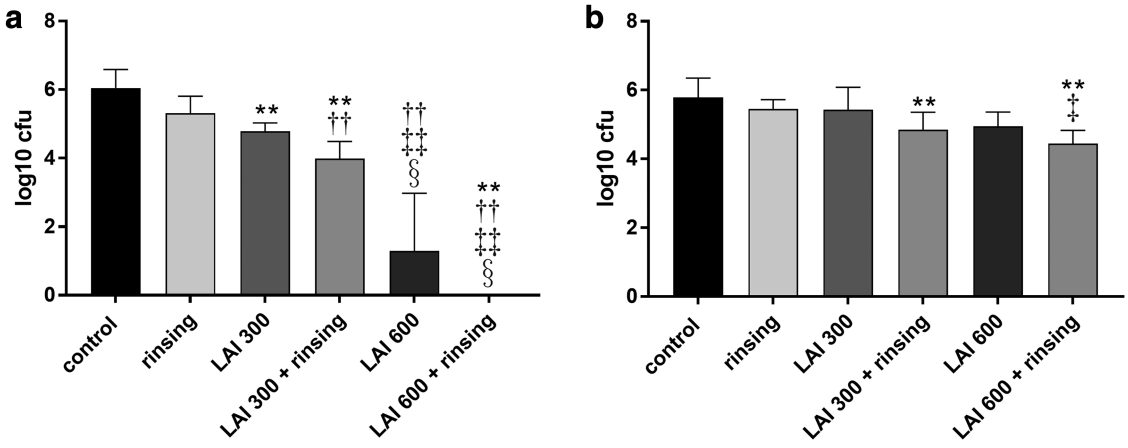

FIG. 2. Antimicrobial activity of rinsing with 0.9% w/v NaCl (rinsing), LAI protocol with a 300 or 600 μm tip (LAI 300 and LAI 600, respectively) without and with rinsing against 3-day dual-species cultures of *S. gordonii* and *A. oris* **(a)** or *S. gordonii* and *F. nucleatum* **(b)** grown inside root canals. $^{**}p<0.01$ compared with control group, $^{\dagger\dagger}p<0.01$ compared with rinsing group, $^{\ddagger}p<0.05$, $^{\ddagger\ddagger}p<0.01$ compared with 70 mJ. $^{\S}p<0.01$ compared with 70 mJ+rinsing (ANOVA/Tukey).

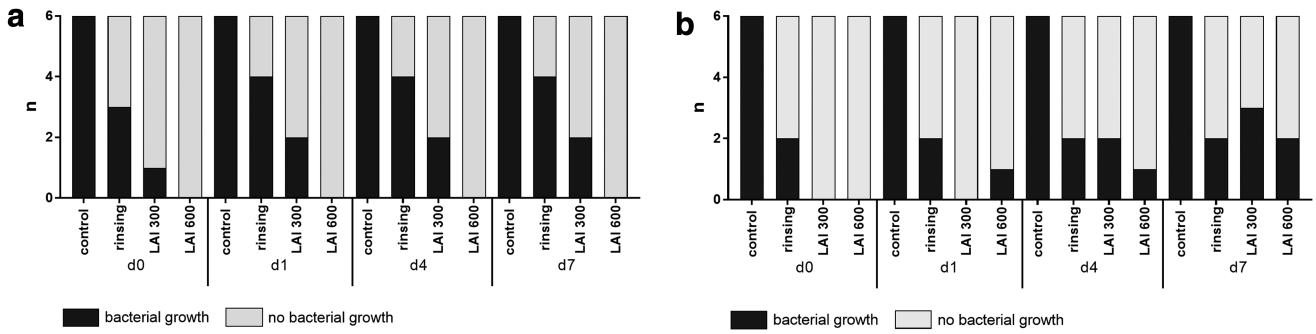

FIG. 3. Samples tested positively for bacterial growth (black) immediately after different irrigation protocols by using 1.5% NaOCl (w/v): rinsing only, with LAI protocol with a 300 or 600 μm tip (LAI 300 and LAI 600, respectively) without and with rinsing up to 7 days after applications. The roots received treatments after a 21-day dual-species infection with *S. gordonii* and *A. oris* **(a)** or *S. gordonii* and *F. nucleatum* **(b)**. NaOCl, sodium hypochlorite.

LAI. The same was reported by Kasic et al.,[27] who found a significant but clinically irrelevant 10% reduction of *E. faecalis* counts in artificially infected root canals. However, it was reported that, using SEM analysis as validation method, Er:YAG irradiation of NaCl solution inside the root canal was able to partially remove the *E. faecalis* biofilm from root canal walls and to expose dentinal tubules.[25]

In the short-term experiments, except for the dual infection with *S. gordonii*/*A. oris*, the CFU reduction did not achieve a 3 log10 units difference, which is considered a significant antimicrobial activity.[28,29] After 3 days of infection no strong invasion of bacteria to the root dentine walls can be expected. Concerning the five different used single strains (*E. faecalis*, *F. nucleatum*, *A. oris*, *S. gordonii,* and *C. albicans*) we consistently found a significant reduction of CFUs of 1–2 log10 units compared with the negative control, but there was in most cases no difference to manual dynamic irrigation with NaCl (Fig. 1). Only for *E. faecalis* the LAI without intermittent rinsing and for *F. nucleatum* the LAI with intermittent rinsing led to a somewhat pronounced elimination of CFUs (1–2 log10 units). In contrast, in the dual-species infection models, especially with *A. oris*/*S. gordonii* a highly significant drop of log10 CFU up to the detection limit was found. This is especially noteworthy because NaCl as irrigation solution itself is not bactericidal. It was recently described that both gram-positive bacteria *A. oris* and *S. gordonii* express enzymes to form disulfide bonds that are needed for pilus assembly, coaggregation, and biofilm formation in case of *A. oris*, and for autolysis, extracellular DNA release, biofilm formation, genetic competence, and bacteriocin production in case of *S. gordonii*.[30] It might be speculated that disulfide bonds are especially sensitive to energy dispersion in fluids as associated with LAI 600. This topic warrants further investigation.

Our findings fit with the observation of Kasic et al.[27] who reported a 10,000-fold reduction of *C. albicans* in the presence of *E. faecalis* using Er:YAG laser irradiation of NaCl inside root canals. The authors did not explain their findings, but it can be speculated that mixing these two strains might lead to different growth and dynamics of the biofilm or to different susceptibility to fluid dynamics.[31] In these experiments, single-strain infections versus dual-strain infections were tested on purpose, because it was anticipated that some bacteria might show different properties regarding invasion into dentinal tubules in the presence of other bacteria.[31] Furthermore, mixed infections are usually present in endodontic failures, and future research should take this into account when disinfection protocols are validated.

In the long-term infection model we wanted to study the effect of LAI combined with NaOCl solution on mature mixed biofilm on the dentine walls and inside the dentinal tubules. The high cell density and the presence of the extracellular matrix as present in a microbial biofilm can render this biofilm less susceptible to laser action.[15] It is a well-known fact that microorganisms in mature biofilms that are at least 3 weeks old are more resistant to antimicrobial therapy than microorganisms in nonmature biofilms.[32] Besides, bacteria can invade dentine tubules up to a depth of 300–1000 μm, disturbing the root canal disinfection process.[33]

In our experiments, NaOCl was not inactivated with sodium thiosulfate because this is not usually performed in the clinical setting. We wanted to adhere to a protocol closely mimicking clinical conditions. Not using sodium thiosulfate has been applied in other studies on root canal disinfection.[34]

In this study it was observed that both laser activation protocols seemed to enhance the antimicrobial effect of 1.5% NaOCl solution. All protocols with laser and NaOCl manual irrigation could decrease substantially the counts of microorganisms from the biofilm, when compared with the contamination control ($p < 0.05$ and a reduction bigger than 3 log stages). These results show the high efficacy of NaOCl as a disinfecting agent for endodontic treatment, also observed in previous studies.[16,35–37] Likewise, former studies have also observed a similar supplementary decontaminating effect of laser on canal irrigation with NaOCl.[9,38,39] Moreover, Golob et al.[21] successfully tested LAI using a modified PIPS protocol and 5% NaOCl in eradication of *E. faecalis* biofilms in root canals. However, this study has shown that the mixture of gram-positive bacteria was remarkably sensitive to the PIPS protocol. This is clinically relevant because mixtures of gram-positive bacteria and facultative anaerobes were found to be predominant in persistent endodontic infections.[40] It seems noteworthy, that the term ''PIPS'' should be strictly linked to the use of the Er:YAG laser energy with continuous irrigation during the laser irradiation. Therefore, protocols using similarly designed tips with similar Er:YAG settings, but using different irrigation protocols should at best be named ''modified PIPS'' to avoid confusion. However, from a clinical perspective, activation of endodontic solutions with a tip that

merely stays at pulp chamber level and does not need to be inserted into the root canal is much more convenient in terms of accessibility, especially when the mouth opening of a patient is restricted.

Our findings can probably be explained by the increased fluid dynamics that lead to biomechanical intracanal cleaning and by an easier penetration of the irrigant into deeper dentine layers caused by Er:YAG laser-induced cavitation effects.[1,9,41] It was shown that the penetration of *A. oris* into the dentinal tubules in the presence of *S. gordonii* was much deeper than in a mono-infection model.[31] The LAI 600 setting was most active in reducing bacteria from dentinal tubules especially for *A. oris*/*S. gordonii*, which were completely eradicated (Figs. 2a and 3). These results can be explained by the dynamics of fluids near the laser tip. With a large tip inserted only up to the entrance of the root canal, with low laser energy settings, the movement of the surrounding fluid is more prominent than when hand irrigation is performed, or when a smaller tip with higher energy is used.[42] It is a matter of speculation why *S. gordonii* in the presence of *A. oris* was much more susceptible to the LAI 600 protocol than in the presence of *F. nucleatum*. Next to the role of disulfide bonds this finding could also be due to the differences in their morphologic factors and cellular arrangements, which lead to different invasion into the dentinal tubules.[43] This observation warrants further investigation, and it stresses the necessity to test not only single strains but also dual- or multiple-strain biofilm models for endodontic disinfection protocols.

However, given the better performance of the LAI 600 activation in our study, it might be hypothesized that LAI 600 by using an Er:YAG laser leads to a deeper penetration of NaOCl into the dentinal tubules, and the specific fluid dynamics associated with a LAI 600 protocol enhance flushing out of bacteria from deeper parts of dentine.

Conclusions

The additional use of Er:YAG laser activation can be recommended to enhance both the rinsing efficacy and the effectiveness of NaOCl. The 30 mJ energy setting with the 600 μm conical tip (''LAI 600'' protocol, 0.5 W/cm^2, 10 J/cm^2) seems to be preferable over the 70 mJ setting with a 300 μm tapered tip (''LAI 300'' protocol, 0.5 W/cm^2, 10 J/cm^2). Intermittent rinsing between laser activation periods might be advantageous.

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Author Disclosure Statement

No competing financial interests exist.

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