Australian Endodontic Journal
The Journal of the Australian Society of Endodontology Inc., the Australian and New Zealand Academy of Endodontists and the Asian Pacific Endodontic Confederation

Aust Endod J 2014

ORIGINAL RESEARCH

Effectiveness of KTP laser versus 980 nm diode laser to kill *Enterococcus faecalis* in biofilms developed in experimentally infected root canals

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Keywords
BioTimer Assay, endodontic infections, *Enterococcus faecalis* biofilm, laser, root canal disinfection.

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doi:10.1111/aej.12057

Abstract

This study aimed to evaluate the antibacterial action of KTP (potassium-titanyl-phosphate) laser irradiations (compared with 980 nm diode laser), associated with conventional endodontic procedures, on *Enterococcus faecalis* biofilms. Fifty-six dental roots with single canals were prepared with Ni-Ti rotary instruments, autoclaved, inoculated with an *E. faecalis* suspension and incubated for 72 h. They were randomly allocated to control and treatment groups. Laser parameters were as follows: power 2.5 W, Ton 35 ms, Toff 50 ms (KTP laser); power 2.5 W, Ton 30 ms, Toff 30 ms (980 nm diode laser). To evaluate the residual bacterial load, BioTimer Assay was employed. The chemo-mechanical treatment together with laser irradiations (KTP and 980 nm diode lasers) achieved a considerable reduction of bacterial load (higher than 96% and 93%, respectively). Regarding both laser systems, comparisons with conventional endodontic procedures (mortality rate of about 67%) revealed statistically highly significant differences ($P \leq 0.01$). This study confirms that laser systems can provide an additional aid in endodontic disinfection.

Introduction

Pulpal and periapical diseases are primarily due to the action of bacteria and their products, which mainly reach the endodontium owing to the presence of deep carious lesions. Bacterial infections are also responsible for endodontic failures; in fact, microorganisms can persist within root canals or can invade them again after the endodontic obturation (1).

Therefore, two of the major aims of an endodontic treatment are still represented by the optimisation of root canal disinfection and the prevention of reinfection in order to ensure a better long-term prognosis.

Root canal irrigants are usually the most important means by which endodontic microorganisms can be eliminated, even though they are organised in biofilms adhering to dentinal walls of root canals (2–4).

Nevertheless, a complete endodontic sterilisation is hardly achieved by conventional procedures (5), probably due to three main factors: the complex anatomical structure of the endodontium, the biofilm lifestyle of colonising bacteria and the difficulty for irrigating solutions to penetrate deep into dentinal tubules up to the apical portion of root canals. Microorganisms can invade dentinal tubules even up to 1 mm, while irrigating solutions reach a depth of about 100 μm only (6,7). Moreover, it is difficult to obtain a complete sterilisation of the apical third of root canals due to the thin diameter of this portion and to the high surface tension of endodontic irrigants.

Laser technology has been introduced in endodontics to improve the results obtained with conventional procedures. The use of lasers, associated with traditional procedures, can help the operator to overcome the problem of the insufficient penetration depth of disinfectant solutions. In fact, through irradiations, previously inaccessible portions of root canal systems can be reached thanks to the better penetration of lasers into dental tissues (8) and to the use of flexible and thin (Ø = 200 μm) optical fibres.

Several laser systems can be utilised as additional aids in the disinfection of root canals, and many studies have been conducted in order to analyse their interaction with target tissues (8–11). Diode lasers are very common in endodontics and their bactericidal properties are mainly related to the photo-thermal effect (8,10,12–14). Diode lasers are efficient in endodontic disinfection owing to the affinity of their wavelengths for bacterial cells. Moreover, laser irradiations are able to penetrate deep into dentinal tubules because they are not absorbed by dental hard tissues. So a 63% reduction of bacterial population can be achieved at a depth of 750 μm (8,12–14).

In dentistry KTP (potassium-titanyl-phosphate) laser has been used primarily for surgical and tooth bleaching procedures (KTP laser irradiations are selectively absorbed by blood and pigmented tissues) (15,16), while its use in endodontics is recent (17,18). Bactericidal properties of KTP laser are chiefly related to the photo-thermal effect. As well as for other laser systems (for instance, diode lasers), the most likely mechanism involved in endodontic disinfection seems to be laser-induced localised heating of the bacterial microenvironment, resulting in lethal temperatures. Gram-negative bacteria are more sensitive to laser irradiations than Gram-positive bacteria, which are more resistant owing to their cell wall composition and structure (18).

The aim of the study was to evaluate the antibacterial activity of KTP laser, compared with 980 nm diode laser, in association with conventional procedures of chemo-mechanical preparation of root canals. The study was carried out on root canals of extracted teeth experimentally infected with *Enterococcus faecalis* biofilms. To evaluate the *E. faecalis* population, an innovative method, known as *BioTimer Assay* (BTA), was used.

Materials and methods

Preliminary treatment of dental roots

A total of 56 single-rooted teeth (with one root canal), with roots of equal length, extracted for orthodontic or periodontal reasons, was selected. Patients were informed about and agreed to the use of their teeth in this experimental study.

To remove the crown, each tooth was sectioned at the cementoenamel junction through a friction grip diamond-coated cylindrical bur under continuous water irrigation. Afterwards all the roots underwent a preliminary treatment, as detailed below:

- Root canal shaping through Ni-Ti rotary instruments, in accordance with ProTaper® systematic (Dentsply Maillefer, Ballaigues, Switzerland), until F1 instrument (Ø = 0.20 mm); irrigations with saline solution (0.9% NaCl) were performed to promote instrumentation procedures (17). Saline solution was chosen as the irrigant in the preliminary treatment in order to verify the antibacterial action of laser irradiations alone, without the influence of any other device (for instance, sodium hypochlorite), in the following treatment of root canals (L1 and L2 groups – see below);
- Sealing of the apical foramen through light-curing composite resin (Z100™ MP, 3M ESPE, Pioltello, Italy), in order to facilitate the infection of the root canal.

After preliminary treatment, dental roots were placed in 1.5 mL Eppendorf tubes in upright position and sterilised by autoclaving at 121°C for 15 min.

Experimental infection of dental roots

E. faecalis CCM2541 was streaked onto brain–heart infusion (BHI) (Oxoid Ltd., Hampshire, UK) agar plate and incubated at 37°C for 24 h. Then a total of 10 isolated colonies of *E. faecalis* was inoculated in BHI broth and incubated overnight at 37°C. A 1:50 dilution of the overnight broth was then prepared in BHI broth and incubated for 3 h at 37°C to obtain a suspension of about $5 \pm 1.3 \times 10^8$ colony-forming units CFU/mL. A total of 5 μL of this dilution, containing about 10^6 CFU, was used to inoculate each root canal. Then infected roots were placed in 1.5 mL sterile Eppendorf tubes in upright position and incubated in a humid atmosphere at 37°C for 72 h, in order to allow bacteria to form biofilms within each root canal. Preliminary experiments showed that a contamination of the outer part of the roots did not take place in these conditions (data not shown).

Treatment of experimentally infected root canals

The 56 roots were randomly subdivided in six groups as follows:

1. CTR (11 roots): no treatment
2. CTR T (11 roots): mechanical instrumentation and irrigations with 5% sodium hypochlorite
3. L1 (8 roots): irradiations with KTP laser
4. L2 (8 roots): irradiations with 980 nm diode laser
5. T + L1 (9 roots): mechanical instrumentation, irrigations with 5% sodium hypochlorite and irradiations with KTP laser
6. T + L2 (9 roots): mechanical instrumentation, irrigations with 5% sodium hypochlorite and irradiations with 980 nm diode laser

The chemo-mechanical treatment (CTR T, T + L1 and T + L2 groups) was performed as follows: first irrigation with 5% sodium hypochlorite, treatment with ProTaper® F2 instrument (Ø = 0.25 mm), second irrigation with 5% sodium hypochlorite for 1 min, removal of the irrigant, washing with 5% sodium thiosulfate (in order to inactivate sodium hypochlorite) and final washing with sterile saline solution.

L1 and T + L1 groups were treated with three irradiations of 5″ with intervals of 5″ using KTP laser (Smartlite®, Deka, Calenzano, Italy; wavelength: 532 nm). Power 2.5 W, Ton 35 ms and Toff 50 ms values were according to manufacturer's parameters for endodontic disinfection.

L2 and T + L2 groups were treated with three irradiations of 5″ with intervals of 5″ using diode laser (Wiser®, Doctor Smile, Brendola, Italy; wavelength: 980 nm). Power 2.5 W, Ton 30 ms and Toff 30 ms values were according to manufacturer's parameters for endodontic disinfection.

Using a laser power meter (Nova®, Ophir-Spiricon, North Logan, UT, USA), we evaluated the actual average powers emitted by the two laser devices (the actual outputs were checked before each experiment). For both laser systems we noticed that there was a good correspondence between the actual average power measured and the manufacturer's average power.

Cylindrical optical fibres (Ø = 200 μm) with plain tips were used. According to the protocols existing in literature (17), optical fibres were placed at a distance of 1 mm from the bottom of each root canal. During the emission of the laser beam, rapid spiral movements from the apex to the coronal third irradiating the whole canal were realised (L1, L2, T + L1 and T + L2 groups).

In T + L1 and T + L2 groups laser treatment was performed in the presence of 5% sodium hypochlorite (second irrigation – see above for details).

Evaluation of *Enterococcus faecalis* CCM2541 population

To evaluate the *E. faecalis* CCM2541 population in biofilms adherent to the inner walls of root canals, BTA protocol was used (19–21). BTA method that employs BioTimer-Phenol Red (BT-PR) medium measures microbial metabolism: the time required for colour switch of BT-PR medium (red to yellow), due to bacterial metabolism, is correlated to the initial bacterial concentration. Therefore, the time required for colour switch determines the number of the bacteria present in the sample at Time 0 through a specific correlation line (19–21).

To draw the correlation line specific for *E. faecalis*, 0.2 mL of BHI overnight broth cultures were mixed with 1.8 mL of BT-PR medium. Serial twofold dilutions in 1 mL of BT-PR medium were performed in 24-well plates (Becton Dickinson Italia, Milan, Italy) and counted simultaneously using CFU method. Incubation was performed at 37°C without shaking. The colour of the inoculated BT-PR medium was checked at regular time intervals. For each twofold dilution, the time required for colour switch of BT-PR medium was recorded and plotted versus the $\log_{10}$ of CFU. The correlation line to count *E. faecalis* was described by the following linear equation:

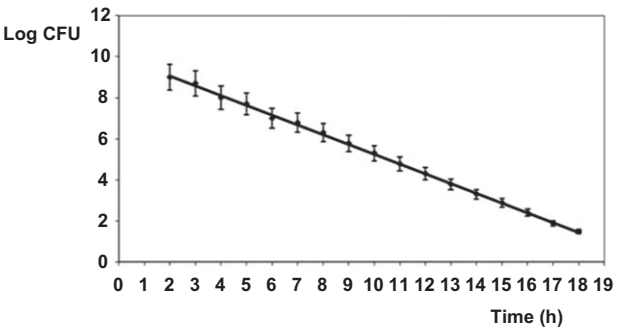

Figure 1 Correlation line relating the time required for colour switch of *BioTimer-Phenol Red* reagent to the number of bacterial cells of *Enterococcus faecalis* CCM2541. The correlation line was described by the following linear equation: $y = -0.4767x + 10.022$.

$y = -0.4767x + 10.022$, where y corresponds to $\log_{10}$(CFU/mL), whereas x corresponds to the time required for colour switch (h) (Fig. 1).

To evaluate the *E. faecalis* CCM2541 population in biofilms, infected roots were placed in 1.5 mL Eppendorf tubes containing 1 mL of BT-PR reagent and incubated at 37°C. The time for colour switch was recorded and used to estimate the bacterial load through the correlation line. Because the correlation line links the time for colour switch of BT-PR medium and the CFU of planktonic bacteria, the number of *E. faecalis* in biofilm was defined as planktonic-equivalent CFU (PE-CFU).

Field Emission Scanning Electron Microscopy (FESEM)

For FESEM observations, dental roots were prepared and inoculated as described earlier. After incubation (72 h), dental roots were treated with 2.0% glutaraldehyde for 1 h at room temperature in the dark. After washing with phosphate solution, dental roots were fixed in 1% osmium tetroxide for 2 h at 4°C in the dark. After post-fixation, the samples were dehydrated in a graded series of ethanol solutions (from 30% to 100%) at 10 min intervals and critical point dried. Then root canals were examined using FESEM. The FESEM images were recorded by the Auriga® 405 (Carl Zeiss AG, Oberkochen, Germany), operating at low extracting voltage (2 kV). The applied angles of dental roots were 60 (tilted).

Statistics

The correlation line was obtained by linear regression analysis, and linear correlation coefficients were calculated from the following equation: $r = (n\Sigma xy - \Sigma x\Sigma y)/\mathrm{sqrt}((n\Sigma x2 - (\Sigma x)2)(n\Sigma y2 - (\Sigma y)2))$. Results were expressed as mean values ± standard deviations obtained from at least three independent experiments. Statistical analysis was performed using the Student's *t*-test, and

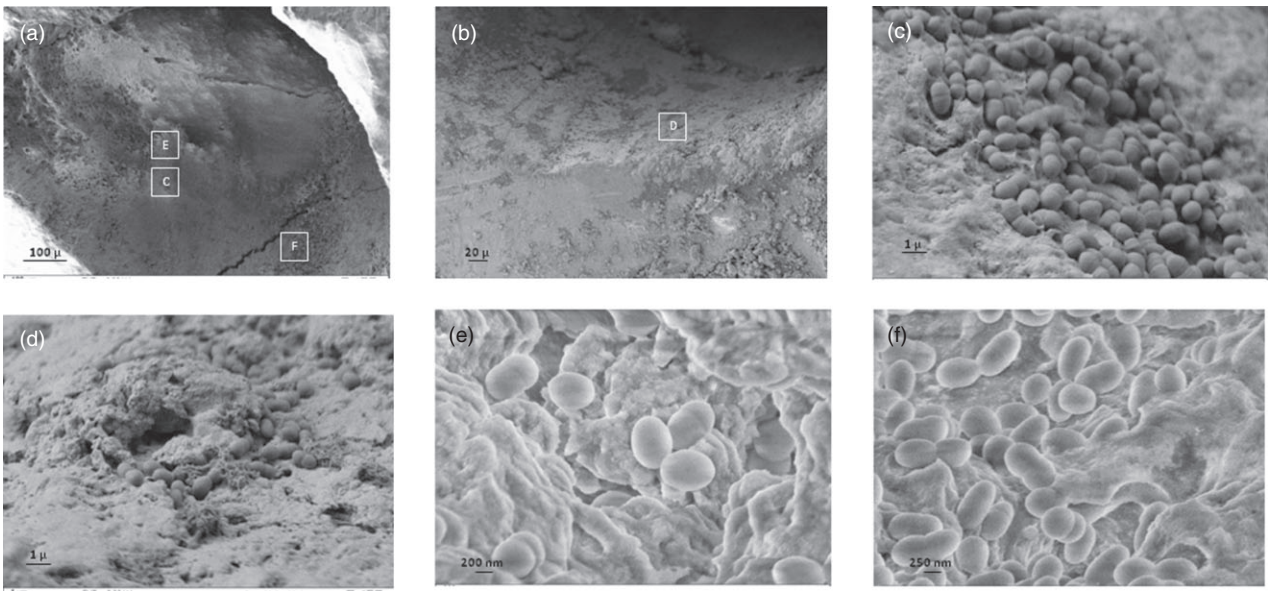

Figure 2 Field emission scanning electron microscopy images: inner walls of root canals shaped with ProTaper® F1 instrument, infected with *Enterococcus faecalis* CCM2541 and incubated for 72 h. The images were taken at the entrance of root canals (cervical third of the roots). (a–b) Low magnification of the inner walls of root canals; (c–f) higher magnifications of delimited areas in panels (a) and (b) showing adherent bacteria immersed in the extracellular matrix (biofilm).

P-values lower than 0.05 ($P \leq 0.05$) were considered significant.

Results

In preliminary experiments, we evaluated the colonisation of dental roots by *E. faecalis* CCM2541 after 72 h of incubation. For this purpose, infected dental roots were examined by FESEM (Fig. 2). Adherent bacteria were shown on the inner walls of root canals (Fig. 2, panels c and d). Moreover, bacteria immersed in biofilm matrix were observed at the highest magnifications (Fig. 2, panels e and f).

Thereafter, we evaluated the ability of BTA to detect bacteria in dental roots. Dental roots of equal length were inoculated with 5 µL of different suspensions in sterile saline of *E. faecalis* broth culture ranging from 1.0×10^4 to 1.0×10^8 CFU/mL, as determined by CFU method. After 1 h of incubation, dental roots were immersed in BT-PR medium and the time for colour switch was detected. The number of bacterial cells was calculated using the previously reported equation of the correlation line. As a 100% agreement was recorded using BTA and CFU protocols (Table 1), BTA was judged reliable to evaluate *E. faecalis* population in dental roots.

In Table 2 and Figure 3 the PE-CFU of adherent *E. faecalis* onto the inner walls of root canals after treatments and the percentages of survival are reported, respectively. The chemo-mechanical treatment alone significantly reduced the number of adherent *E. faecalis* in biofilm with respect to control ($P < 0.01$), and a reduction of about 5 log was observed. Also the treatment with laser alone significantly reduced the bacterial population with respect to control ($P < 0.01$), and a reduction of about 1 log was recorded. Interestingly, KTP laser was more efficient than 980 nm diode laser in reducing *E. faecalis* population ($P < 0.01$). When laser applications were combined with chemo-mechanical treatment, a significant reduction of *E. faecalis* biofilms was observed with respect to both control and chemo-mechanical treatment ($P < 0.01$). Even if the two lasers coupled with chemo-mechanical treatment did not kill the entire population, very low bacterial counts were recorded (2.1 and 3.6 PE-CFU/dental root for KTP and 980 nm diode laser, respectively).

Discussion

The long-term success of an endodontic treatment is strictly related to the effective cleaning and decontamination of root canal system. However, to obtain a thoroughly bacteria-free endodontium is still unlikely (5), so the risk of the development of a treatment-resistant bacterial infection exists (1).

The use of laser systems for endodontic disinfection provides an opportunity to reduce the problems concerning the difficult access of instruments and irrigants to certain areas of root canals, mainly at the apical ramifications.

Table 1 Validation of BioTimer Assay in determining *Enterococcus faecalis* CCM2541 population in experimentally infected root canals

Inoculum (log_{10}(CFU/mL))	Expected switching time (h)	Actual switching time (h)	BTA counts (log_{10}(PE-CFU/mL))	Differences from the expected values (log_{10}(PE-CFU/mL)) (%)
8.1	4.0	4.2	8.04	0.06 0.79
7.3	5.7	5.8	7.24	0.05 0.8
5.9	8.6	8.7	5.89	0.009 0.15
5.1	10.3	10.0	5.26	–0.15 3.0
4.2	12.2	12.7	4.14	0.05 1.3
3.3	14.1	14.2	3.27	0.03 0.9

Dental roots were infected with different concentrations of planktonic *E. faecalis* CCM2541 as counted by colony-forming unit (CFU) protocol. After 1 h of incubation, infected roots were immersed in BioTimer Assay (BTA) reagent. The time of colour switch of the reagent was used to evaluate *E. faecalis* CCM2541 population, expressed as planktonic-equivalent CFU (PE-CFU), employing the specific correlation line ($y = –0.4767x + 10.022$).

Table 2 Quantitative evaluation of *Enterococcus faecalis* CCM2541 biofilm in experimentally infected root canals

Groups	No. of roots	Log_{10}(PE-CFU/root) (mean ± SD)†	Significance			
			P versus CTR	*P* versus CTR T	*P* versus L1	*P* versus T + L1
CTR (no treatment)	11	8.46 ± 1.25				
CTR T (mechanical treatment + 5% sodium hypochlorite)	11	2.73 ± 2.60	6.2×10^{-6}			
L1 (KTP laser irradiations)	8	7.23 ± 0.38	0.002	0.003		
L2 (980 nm diode laser irradiations)	8	7.68 ± 0.48	0.006	0.002	0.007	
T + L1 (mechanical treatment + 5% sodium hypochlorite + KTP laser irradiations)	9	0.33 ± 0.50	8.8×10^{-7}	4.1×10^{-4}		
T + L2 (mechanical treatment + 5% sodium hypochlorite + 980 nm diode laser irradiations)	9	0.56 ± 0.53	7.1×10^{-7}	0.001		0.142

†The *E. faecalis* population was evaluated using BioTimer Assay. The results are expressed as planktonic-equivalent colony-forming units (PE-CFU) per dental root. KTP, potassium-titanyl-phosphate; SD, standard deviation.

The aim of this study was to evaluate the effectiveness of KTP laser (compared with 980 nm diode laser) in endodontic disinfection, in association with conventional techniques of chemo-mechanical preparation of root canals.

We chose *E. faecalis* as the test organism as it is the bacterial species most frequently associated with persistent endodontic infections, it is difficult to remove owing to its considerable virulence factors and, in particular, because of its ability to grow in biofilm lifestyle (22).

To create an *in vitro* model mimicking the *in vivo* clinical situation, in our experimental study we evaluated the antibacterial activity of the proposed procedures on bacteria grown in biofilm lifestyle. In fact, within root canals bacteria organise themselves in biofilms adhering to dentinal walls and penetrating into dentinal tubules (23).

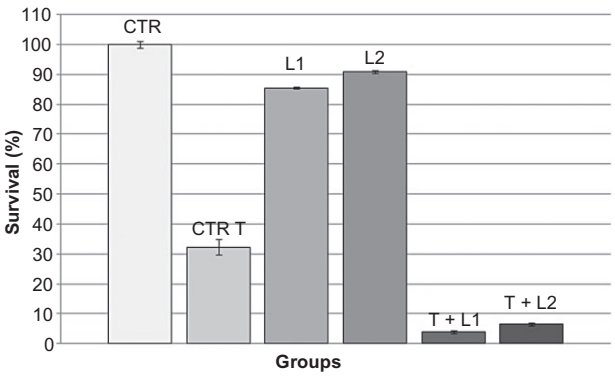

Figure 3 Survival *Enterococcus faecalis* CCM2541 biofilms in experimentally infected root canals after chemo-mechanical and/or laser treatment. Results are expressed as percentage with respect to the untreated infected root canals.

The demonstration of the efficacy of endodontic treatments in killing bacterial population is affected by the difficulties related to the count of bacterial biofilms. The count of bacteria in biofilm is generally achieved by CFU protocol after detaching them from the surface on which they adhere by vortexing or sonication. However, these procedures often do not obtain homogeneous cell suspensions, thus introducing a relevant bias in the results (24). In this study we applied BTA protocol to estimate the *E. faecalis* population after treatments. BTA is an indirect biological method useful to evaluate the viability of bacterial population in biofilm lifestyle without sample manipulations, thus overcoming the aforementioned bias (20,21,25–27). However, we carried out preliminary tests to verify if BTA could be useful to our purpose. We showed that BTA was not affected by the shape and nature of dental roots, so it could be reliably employed in counting *E. faecalis* infecting root canals (Table 1).

The results of this study (Table 2 and Fig. 3) show that laser systems must be used for endodontic disinfection in association with conventional techniques. In fact, even if laser treatment alone showed a significant reduction of bacterial population compared with control group ($P < 0.01$), the residual bacterial load was about 10^7 bacteria. So laser treatment alone could be insufficient to reduce the bacterial population in *in vivo* endodontic infections, even though in the *in vivo* infections the bacterial loads are usually lower than the ones applied in this experimental trial (28,29).

The results of T + L1 and T + L2 groups show a significant reduction of bacterial population both with respect to control group and to chemo-mechanical treatment (Table 2). The combined procedure (chemo-mechanical treatment and laser irradiations) was very effective to kill biofilms: we achieved mortality rates of about 96% and 93% in T + L1 and T + L2 groups, respectively (Fig. 3), and a very little population (<10 bacteria per dental root) survived.

Moreover, in experimental models, the results concerning the disinfectant efficacy of sodium hypochlorite should be considered overvalued. In fact, *in vivo* the presence of organic matter (for instance, inflammatory exudates, pulpal tissue fragments, dentinal collagen and bacterial debris) within root canals consumes sodium hypochlorite and significantly weakens its antibacterial action. This is one of the reasons why a complete endodontic sterilisation is difficult to obtain through conventional techniques (5).

The efficacy of laser systems in endodontic disinfection depends on the specific wavelength and, consequently, on the different interaction with target structures. Nowadays, the understanding of the interaction between laser beams and target tissues is more and more reliable. Therefore, the use of laser systems in endodontic decontamination can be considered a safe procedure, which does not cause adverse effects on dental and periodontal tissues, as long as correct parameters are used. In fact, use parameters also affect the effectiveness of laser systems in endodontic disinfection. This aspect must be mainly considered concerning KTP laser, because the introduction of its use in endodontics is recent (18) (it has been primarily used for surgical and tooth bleaching procedures) (15,16), and in literature there are few studies about its antibacterial activity (30) and its efficacy in endodontic disinfection (17,18).

Considering all the aspects described, it can be concluded that the two wavelengths tested in the present study can provide an additional aid in endodontic disinfection and they can allow the operator to improve the predictability and the prognosis of endodontic treatments. In fact, even if the reduction of bacterial load through conventional chemo-mechanical treatments of root canals is sufficient, in most cases, to obtain a favourable prognosis, the possibility of increasing the bactericidal effect through the use of laser systems must be given serious consideration. With a minimal increase in working time (the steps of laser applications in endodontic disinfection are very simple), a greater reduction of bacterial load in root canals can be obtained. So, although sterility is not achieved, the residual bacterial load becomes negligible and the risk of a persistent endodontic infection decreases. However, in order to further confirm these results and to investigate the laser systems under *in vivo* conditions, clinical studies are necessary.

In the near future, the possibility of increasing the bactericidal action of endodontic techniques through the use of lasers and a more favourable healing process of periapical tissues (owing to the biostimulating effect of laser beams) could represent the reason for carrying out one-visit endodontic treatments, providing significant benefits to patients.

Acknowledgements

Thanks are due to Dr Tiziana Natalizi (Department of Public Health and Infectious Diseases, Sapienza University of Rome, Rome, Italy) and to Dr Francesco Mura (Sapienza Nanoscience and Nanotechnology Laboratories, Department of Fundamental and Applied Sciences for Engineering, Sapienza University of Rome, Rome, Italy) for their technical support in performing microbiological and FESEM experiments, respectively.

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