

# Bactericidal effect of Er:YAG laser combined with sodium hypochlorite irrigation against *Enterococcus faecalis* deep inside dentinal tubules in experimentally infected root canals

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This study evaluated the bactericidal effect of Er:YAG laser radiation combined with sodium hypochlorite (NaOCl) irrigation in the treatment of *Enterococcus faecalis* deep inside dentinal tubules. The Er:YAG laser was activated, respectively, at 0.3, 0.5 and 1.0 W for either 20 or 30 s; 52.5 g l<sup>-1</sup> NaOCl and normal saline were used for the control groups. Root canals before and after treatments were examined using scanning electron microscopy (SEM). Bacterial reductions both on the root canal walls and at 100, 200, 300, 400 and 500 µm inside the dentinal tubules were analysed using a one-way analysis of variance. SEM results showed that the Er:YAG laser combined with NaOCl disinfected the dentinal tubules from 200 to over 500 µm depth as irradiation power and time increased. This combination killed significantly more bacteria than both the negative control group at each level tested and the positive control group at 300, 400 and 500 µm inside the dentinal tubules. It reached 100 % in all experimental groups, both on the root canal walls and at 100 and 200 µm inside the dentinal tubules. However, at 300, 400 and 500 µm inside the dentinal tubules, only the groups treated with 0.5 and 1.0 W for 30 s exhibited no bacterial growth. Of the two groups in which no bacteria were detected at all tested depths, Er:YAG laser irradiation at 0.5 W for 30 s combined with NaOCl irrigation was preferable because of the lower emission power and shorter irradiation time, and may serve as a new option for effective root canal disinfection.

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## INTRODUCTION

The primary goal of endodontic treatment is to optimize the disinfection of an infected root canal and to prevent its reinfection (Seal *et al.*, 2002). In addition to mechanical preparation, chemical irrigation is also necessary to eliminate the pathogenic micro-organisms inside infected root canals. Presently, sodium hypochlorite (NaOCl) is the most commonly used root canal irrigant owing to its properties such as tissue dissolution and effective disinfection (Siqueira *et al.*, 1997). However, NaOCl alone cannot eliminate bacteria located inside dentinal tubules beyond 130 µm, which might cause reinfection of the

endodontically treated root canal (Berutti *et al.*, 1997; Peters *et al.*, 2001).

In the past decades, passive ultrasonic irrigation (PUI) has been widely used for root canal irrigation. Studies have shown that PUI with NaOCl could not only effectively remove the smear layer inside infected root canals but also significantly improve the cleaning of biofilm-infected dentine (George *et al.*, 2008; De Moor *et al.*, 2010; Ordinola-Zapata *et al.*, 2014). For PUI, however, the apical third of a root canal should be enlarged to at least an International Organization for Standardization (ISO) size of 35–40 to allow needle placement to within 1–2 mm of the apical seat (Huang *et al.*, 2008; Peeters & Gutknecht, 2014), which might limit its application.

In recent years, various laser systems have received considerable attention in the field of laser-assisted endodontic therapy (Stabholz *et al.*, 2004; Williams *et al.*, 2006). The Er:YAG laser has conventionally been used for removal of

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Abbreviations: ISO, International Organization for Standardization; PIPS, photon-induced photoacoustic streaming; PUI, passive ultrasonic irrigation; SEM, scanning electron microscopy.

carious dentine, preparation of the cavity, and treatment of dentine hypersensitivity (Yonemoto *et al.*, 2006; Yilmaz *et al.*, 2011). However, it has gained increasing attention for its possible application to endodontic therapy because of its antibacterial effect, and particularly for its anti-biofilm effect (Schoop *et al.*, 2002; Meire *et al.*, 2012). Previously, we found that the Er:YAG laser combined with NaOCl showed a superior bactericidal effect over other laser systems (Nd:YAG laser, Er,Cr:YSGG laser, and antimicrobial photodynamic therapy) and NaOCl alone (Cheng *et al.*, 2012). However, at the same time, Er:YAG laser treatment combined with NaOCl showed limited bactericidal effect on *Enterococcus faecalis* deep ( $> 300 \mu\text{m}$ ) inside dentinal tubules using current irradiation protocols. We hypothesized that the optimization of laser irradiation parameters would produce a better bactericidal effect on micro-organisms deep ( $> 300 \mu\text{m}$ ) inside dentinal tubules. In the present study, we evaluated the bactericidal effect of Er:YAG laser irradiation combined with NaOCl irrigation on *E. faecalis* deep inside dentinal tubules under different irradiation power and time settings.

## METHODS

**Tooth preparation.** One hundred and fifty-five caries-free, intact, single-rooted, freshly extracted for orthodontic treatment, permanent human teeth with straight root canals were selected radiologically from the School of Stomatology, Fourth Military Medical University. Teeth with previous coronal restorations or root canal treatment were excluded. The experimental protocol was approved by the ethics committee of the institutional review board (IRB-REV-2013024) of the university, and written informed consents were obtained from all the donors. The tooth preparation was similar to that in our previous study, which was a modification of the preparation described by Haapasalo & Orstavik (1987). In brief, the selected teeth were sterilized and stored in  $5 \text{ g l}^{-1}$  NaOCl for 24 h, then decoronated to a standard 12 mm root segment with a rotary diamond saw (Isomet low speed saw; Buehler) at 700 r.p.m. under water-cooling. The root canals were enlarged at both ends using an ISO 021 round bur at slow speed under water-cooling;  $5 \text{ g l}^{-1}$  NaOCl irrigation was adopted during the instrumentation. The shaped canals were then individually treated with  $52.5 \text{ g l}^{-1}$  NaOCl and  $170 \text{ g l}^{-1}$  EDTA (pH 7.2) for 4 min sequentially. Thereafter, they were treated with sodium thiosulfate for 5 min and were then sterilized by autoclaving ( $121^\circ\text{C}$ , 15 min) in distilled water, and five specimens were randomly selected to ensure that the smear layer and micro-organisms had been eliminated by examining them under scanning electron microscopy (SEM).

**Culture of *E. faecalis* and specimen inoculation.** *E. faecalis* (ATCC 4083), thawed from a frozen stock, was streaked onto brain-heart infusion (BHI; Difco) agar plates and cultured for 24 h at  $37^\circ\text{C}$ . Single colonies were inoculated into BHI broth and incubated at  $37^\circ\text{C}$  for 24 h. The *E. faecalis* suspension was then adjusted spectrophotometrically to approximately  $10^8$  cells  $\text{ml}^{-1}$ . Before incubation, the root surfaces were sealed with nail polish. The individual specimens were then incubated in sterile centrifuge tubes with 1 ml adjusted *E. faecalis* suspension at  $37^\circ\text{C}$  under anaerobic conditions for 4 weeks. The medium was refreshed every 3 days; each time, samples of the replaced medium were selected randomly and identified using 16S rRNA gene sequencing to ensure their purity. Five randomly selected specimens were examined by SEM at 7, 14, 21 and

28 days each to ensure the presence of *E. faecalis* and to quantify how deeply they invaded the dentinal tubules.

**Preparation for SEM examination and quantification of invasion depth.** Specimens selected for SEM examination were processed as follows. Longitudinal grooves were carved onto the root surfaces with high-speed diamond burs (Brasseler) without invading the inner parts of the root canals. They were then split with an acuminate chisel and hammer. Thereafter, the specimens were fixed in  $25 \text{ ml l}^{-1}$  glutaraldehyde for 24 h, dehydrated in graded series of acetonitrile solutions (500, 700, 800, 900 and  $1000 \text{ ml l}^{-1}$  twice) for 20 min each, dried in a lyophilizer (ES-2030; Hitachi), sputter-coated with platinum (Ion Sputter E-1045; Hitachi), and observed by SEM (S-4800; Hitachi). To quantify the depth to which the bacteria had invaded dentinal tubules, one microscopic field was selected every 1 mm starting from one side of the specimen ( $n=5$ , 12 mm length), making 60 fields in all (12 fields per root). In each microscopic field, each whole dentinal tubule was checked for the presence of bacteria. The distance between the orifice of a dentinal tubule and the deepest site to where the bacteria had invaded was measured (SEM, S-4800; Hitachi). This distance was defined as the invasion depth. The results were calculated as mean values.

**Laser system.** An Er:YAG laser (Fotona) emitting a wavelength of 2940 nm with a photon-induced photoacoustic streaming (PIPS) tip (Fotona) with a diameter of 300  $\mu\text{m}$  was used.

**Grouping.** After incubation, the specimens were divided randomly into nine groups, and each one individually received the treatment specified in Table 1. The protocols can be described as follows.

Untreated group: 10 root canals were left untreated.

Normal saline group (negative control): 15 root canals were irrigated with 5 ml normal saline for 60 s with a 27-G side-vented needle (Patterson Dental Supply).

NaOCl group (positive control): 15 root canals were irrigated with  $5 \text{ ml } 52.5 \text{ g l}^{-1}$  NaOCl for 60 s with a 27-G side-vented needle (Patterson Dental Supply), followed by 5 ml sodium thiosulfate for 60 s and 5 ml normal saline for 60 s to deactivate any residual NaOCl.

Er:YAG laser combined with NaOCl irrigation: six groups, each containing 15 root canals, were first filled with  $52.5 \text{ g l}^{-1}$  NaOCl, and each individual group was irradiated with the Er:YAG laser at an output power of 0.3, 0.5 and 1.0 W for either 20 or 30 s, then successively filled with normal saline and distilled water and radiated

**Table 1.** Treatment protocols

Group	<i>n</i>	Pulse energy (mJ)	Pulse mode	Repetition rate (Hz)	Power (W)	Irrigant	Time (s)
Untreated	10	—	—	—	—	—	—
NS	15	—	—	—	—	NS	60
NaOCl	15	—	—	—	—	NaOCl	60
0.3 W, 20 s	15	20	SSP	15	0.3	NaOCl	20
0.3 W, 30 s	15	20	SSP	15	0.3	NaOCl	30
0.5 W, 20 s	15	20	SSP	25	0.5	NaOCl	20
0.5 W, 30 s	15	20	SSP	25	0.5	NaOCl	30
1.0 W, 20 s	15	20	SSP	50	1.0	NaOCl	20
1.0 W, 30 s	15	20	SSP	50	1.0	NaOCl	30

NS, Normal saline; NaOCl,  $52.5 \text{ g l}^{-1}$  NaOCl; SSP, super-short pulse with 50  $\mu\text{s}$ .

with the Er:YAG laser at the corresponding output power for 20 s. The optical tip (PIPS) was placed at the orifice of the root canal and activated at 15 s intervals. During the irradiation, 52.5 g l<sup>-1</sup> NaOCl, normal saline and distilled water, as used to fill the root canals initially, were injected into the root canals by means of a syringe.

**Estimation of c.f.u. and evaluation of bacterial reduction.** The 10 untreated root canals and 10 of the 15 treated root canals in each group were used to measure the number of viable bacteria before and after treatments, respectively. The samples on root canal walls were collected using three sterile paper points per canal, with the paper points saturated with normal saline and kept in the canals for 60 s. The paper points were transferred immediately to a sterile centrifuge tube containing 1 ml normal saline and vortexed for 10 s. Dentine shavings from different depths (100, 200, 300, 400 and 500 µm) inside the dentinal tubules were removed from the canals using round, sequential, sterile, low-speed burs with increasing diameters (ISO sizes 023, 025, 027, 029 and 031); each removed approximately 0.1 mm of dentine around the canal. Powder dentine samples were transferred immediately to 1 ml normal saline and vortexed for 10 s. The suspensions were diluted 10-fold, and 100 µl of each dilution was spread onto BHI agar plates and incubated for 24 h at 37 °C under anaerobic conditions. The c.f.u. were recorded. The numbers of c.f.u. multiplied by the corresponding dilution ratios represent a close estimate of viable bacteria on the root canal walls and at different test depths inside the dentinal tubules before (*M*) and after (*N*) treatments, respectively. The bacterial reduction is equal to  $(M-N)/M \times 100\%$ .

**SEM examination after treatment.** The remaining five treated root canals in each group were subjected to SEM examination as described above.

**Statistical analysis.** The invasion depth of *E. faecalis*, the number of viable bacteria before treatment, and the bacterial reduction after treatment were expressed as means  $\pm$  SD and analysed using a one-way analysis of variance followed by the least significant difference test using the SPSS statistics package for Windows (version 13.0). The statistical significance level was set at  $\alpha=0.05$ .

## RESULTS

### Tooth preparation

As shown in Fig. 1, unlike the root canals immediately after instrumental preparation (a, b), the smear layer and micro-organisms were eliminated after treatment with 52.5 g l<sup>-1</sup> NaOCl, 170 g l<sup>-1</sup> EDTA, and autoclaving (c–e). The orifices of the dentinal tubules had been completely opened (e). In addition, the dentinal tubules were clear and unimpeded (f–h).

### Specimen inoculation and quantification of invasion depth

After 4-week bacterial incubation, biofilm-like structures (Fig. 2a–c) could be found on the root canal walls, and the bacterial cells exhibited a smooth, uniform and bright surface (Fig. 2c). In addition, the depth of *E. faecalis* in the dentinal tubules increased with incubation time from about 104.1 µm (90–115 µm) after 1 week to approximately 331.2 µm (302–366 µm) at the end of 4 weeks

(Fig. 3). However, the SEM results showed that the number of bacteria decreased as the invasion depth increased. The spread of *E. faecalis* inside the dentinal tubules reached a depth of approximately 102 µm (Fig. 2d–f) and 173 µm (Fig. 2g–i) after 1 and 2 weeks, respectively. Even fewer *E. faecalis* were found at approximately 286 (Fig. 2j–l) and 343 µm (Fig. 2m–o) after 3 and 4 weeks, respectively. In addition, very few *E. faecalis* invaded the dentinal tubules beyond 500 µm after 4 weeks (Fig. 4), and the greatest invasion depth was observed to be more than 1350 µm (Fig. 4d–f).

### Estimation of c.f.u. and evaluation of bacterial reduction

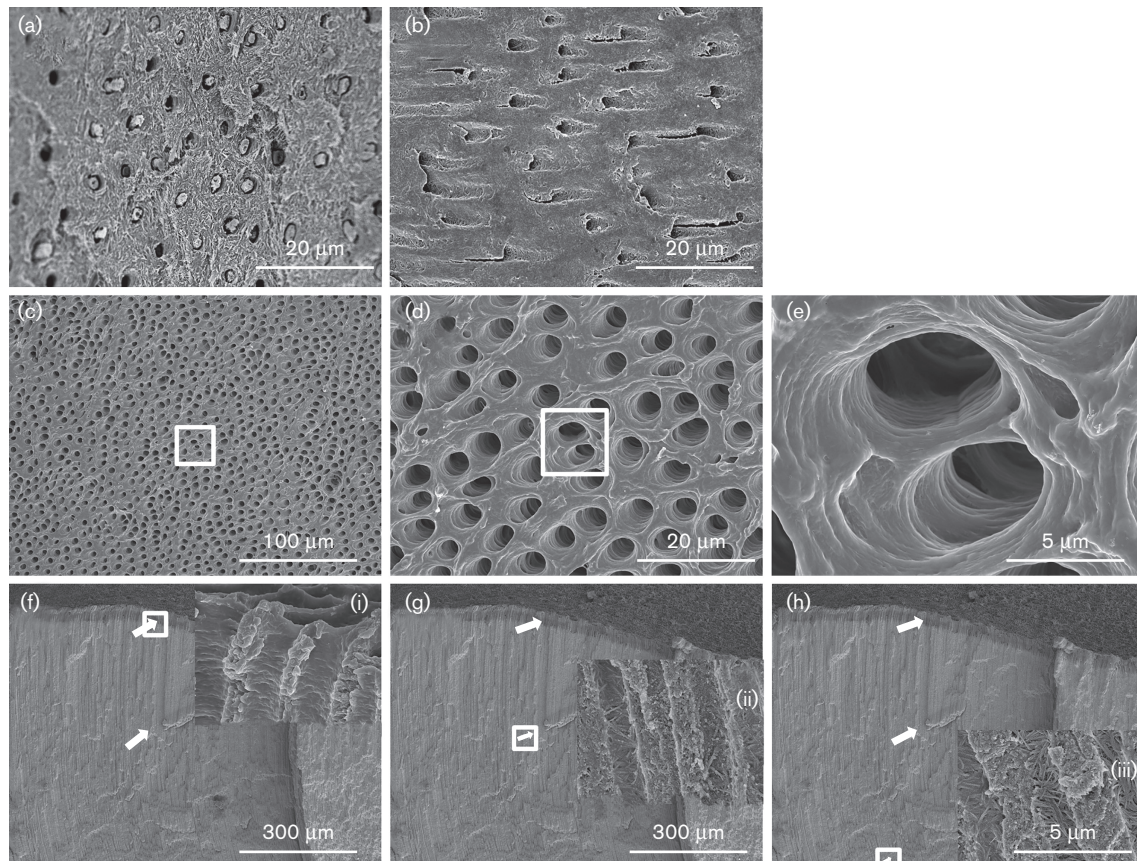
The number of *E. faecalis* before treatment decreased significantly as the depth inside the dentinal tubules increased (Fig. 5). It was revealed that millions of *E. faecalis* bacteria were located on the root canal walls while only a few dozen were found at 500 µm inside the dentinal tubules.

The reductions in bacterial number in each group at different depths inside the dentinal tubules are presented in Table 2. These reductions in the experimental groups were significantly greater than those in the negative control group at all the tested depths ( $P<0.001$ ). The reductions were also significantly greater than those of the positive control groups at 300, 400 and 500 µm inside the dentinal tubules ( $P<0.05$ ). In addition, reductions in all the experimental groups reached 100% both on the root canal walls and 100 and 200 µm inside the dentinal tubules. At 300 µm inside the dentinal tubules, the differences between the experimental groups were not statistically significant ( $P>0.05$ ). The reduction in the bacterial number caused by irradiation at 0.3 W for 20 s at 400 µm inside the dentinal tubules was significantly smaller than that of the other experimental groups ( $P<0.05$ ). The reductions in the bacterial number in groups irradiated at 0.3 W were significantly smaller than those in groups irradiated at 0.5 and 1.0 W at 500 µm inside the dentinal tubules ( $P<0.05$ ). Furthermore, only the groups treated with 0.5 W for 30 s and 1.0 W for 30 s showed no bacterial growth (100% loss of bacteria) at 300, 400 and 500 µm inside dentinal tubules.

### SEM examination after treatment

In the negative control group, reduction in the bacterial number could be observed only on the root canal walls (Fig. 6a–d). The surface of bacteria remaining both on the root canal walls and inside the dentinal tubules was still smooth, uniform and bright (Fig. 6d, h). There were almost no bacteria on the root canal walls in the positive control group (Fig. 7a–d) except for a few with shrunken surfaces (Fig. 7d), indicated by rough, shrunken and fractured bacterial cells left inside the dentinal tubules (Fig. 7e–h). Some intact bacterial cells persisted at around 200 µm (Fig. 7i–l). However, no bacteria were found on the root canal walls in the experimental





**Fig. 1.** Uninoculated root canals. After treatment with NaOCl and EDTA, and autoclaving, smear layer and micro-organisms inside the instrumentally prepared root canals were eliminated (c–e). The orifices of the dentinal tubules were completely opened (e). The dentinal tubules were clear and unimpeded (f–h). (a, b) Root canals immediately after instrumental preparation; (c–h), root canals treated with 52.5 g l<sup>-1</sup> NaOCl, 170 g l<sup>-1</sup> EDTA, and autoclaving.

groups (Fig. 8a–d) and even fewer bacterial cells remained inside the dentinal tubules (Fig. 8e–t) compared with those in the positive control group. Er:YAG laser irradiation combined with NaOCl disinfected the dentinal tubules from 200 to more than 500 µm depth as the irradiation power and time increased. While in the group treated at 0.3 W for 20 s the dentinal tubules were disinfected up to 300 µm in depth [244 µm, for example, in Fig. 8(e)], the group treated at 0.3 W for 30 s and the groups treated at 0.5 and 1.0 W for 20 s experienced the bactericidal effect up to around 300 µm [314 µm, for example, in Fig. 8(i)] and 400 µm [411 µm, for example, in Fig. 8(m)], respectively. In the groups irradiated at 0.5 and 1.0 W for 30 s the dentinal tubules were disinfected to more than 500 µm deep [533 µm, for example, in Fig. 8(q)].

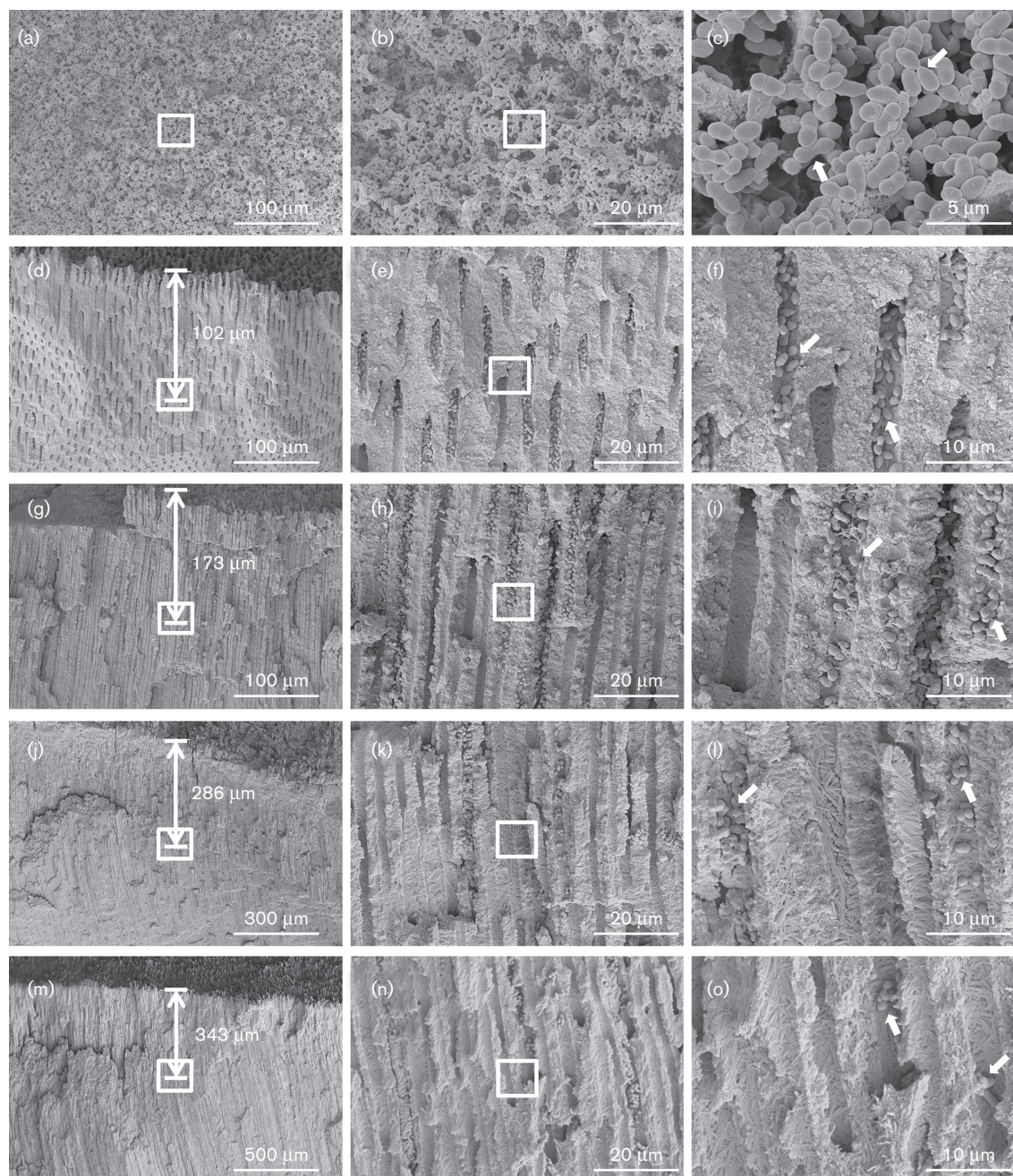
## DISCUSSION

The occurrence and development of pulpal and periapical diseases are primarily associated with bacteria and their products inside the root canals. Endodontic therapy is

always recognized as the most effective treatment for such diseases. However, if not fully removed, bacteria invading deep dentine layers might reinfect an endodontically treated root canal (Nair *et al.*, 1990). Persistent micro-organisms are capable of invading dentine up to a depth of 300–1000 µm (Peters *et al.*, 2001). Our present study showed that *E. faecalis* could invade dentinal tubules to depths of more than 1350 µm. However, only a few dozen *E. faecalis* cells were found at 500 µm inside dentinal tubules. In addition, the majority of the *E. faecalis* were located at less than 400 µm inside dentinal tubules, with only few invading beyond 500 µm. This difference might indicate that disinfecting the dentinal tubules to a depth of 500 µm might produce an ideal disinfection effect during endodontic therapy.

In the present study, there was about a 99.98 % reduction of *E. faecalis* viability on the root canal walls after being treated with 5 ml 52.5 g l<sup>-1</sup> NaOCl for 60 s, which was consistent with a previous study (Wang *et al.*, 2007). However, *E. faecalis* located at 500 µm inside dentinal tubules could only be reduced by approximately 91.99 %. This might





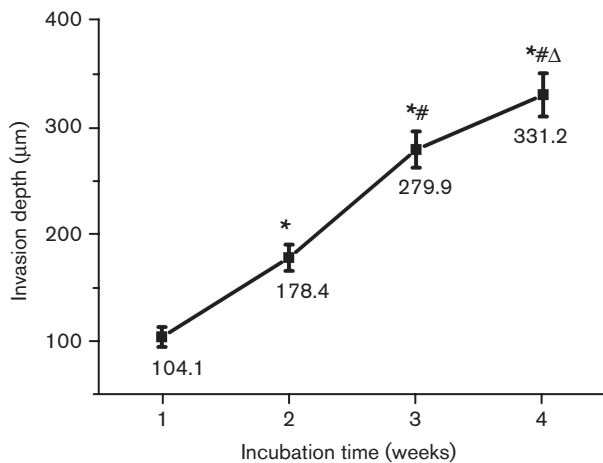
**Fig. 2.** Inoculated root canals. Root canal wall after 4-week bacterial incubation (a–c). Biofilm-like structures formed and bacterial cells displayed a smooth, uniform and bright surface (c). Longitudinally sectioned root canals after 1 (d–f), 2 (g–i), 3 (j–l) and 4 (m–o) weeks of bacterial incubation, respectively. Bacteria were seen inside the dentinal tubules at a depth of approximately 102 (d–f) and 173 µm (g–i). Far fewer bacteria were located inside the dentinal tubules at depths of approximately 286 (j–l) and 343 µm (m–o). White arrows indicate the bacterial cells.

be due to the limited penetration capacity of NaOCl, which requires direct contact to produce its bactericidal effect (Berutti *et al.*, 1997).

The bacterial reduction in number in all experimental groups was greater than that of the NaOCl group at 300,

400 and 500 µm inside dentinal tubules, which indicates that Er:YAG laser treatment might be able to enhance the bactericidal effect of NaOCl by facilitating its penetration into deeper dentine layers. This finding was also confirmed by SEM examination. Viable bacterial cells could be found around 200 µm inside dentinal tubules in the positive





**Fig. 3.** Invasion depth (mean  $\pm$  SD) at different incubation times. The depth *E. faecalis* reached in the dentinal tubules increased as the incubation time increased. \*, Significant difference as compared with 1-week bacterial incubation ( $P < 0.001$ ); #, significant difference as compared with 2-week bacterial incubation ( $P < 0.001$ ); Δ, significant difference as compared with 3-week bacterial incubation ( $P < 0.001$ ).

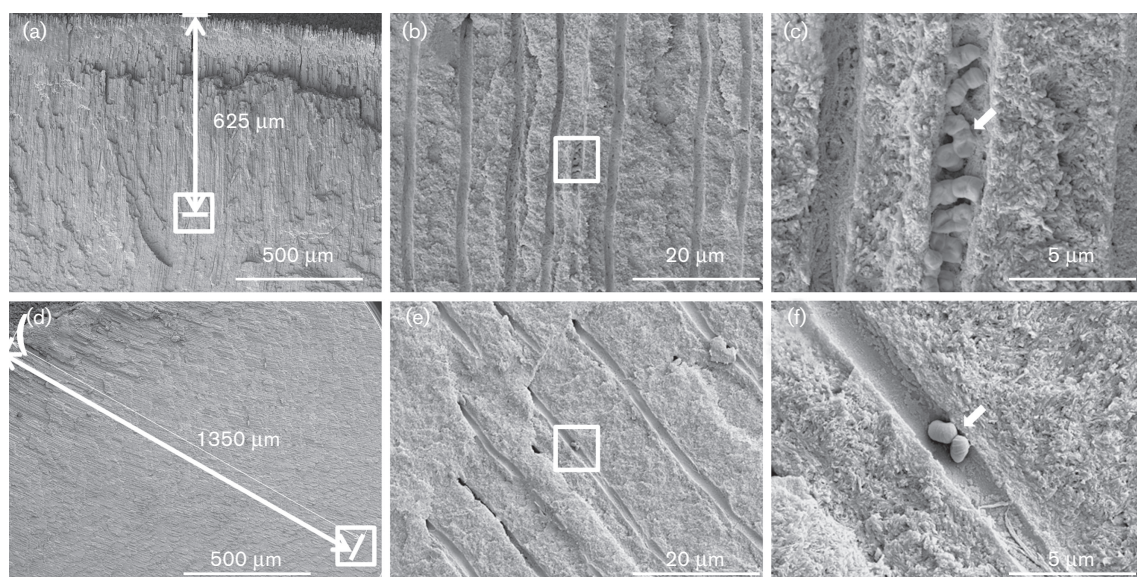
control group. However, the dentinal tubules could be disinfected from 200 to more than 500 µm in the Er:YAG/NaOCl groups by increasing irradiation power and time.

In the present study, the bactericidal effect of Er:YAG laser irradiation combined with NaOCl on the root canal wall was equal to that of PUI with NaOCl as described in a previous study (Chen *et al.*, 2013). However, the

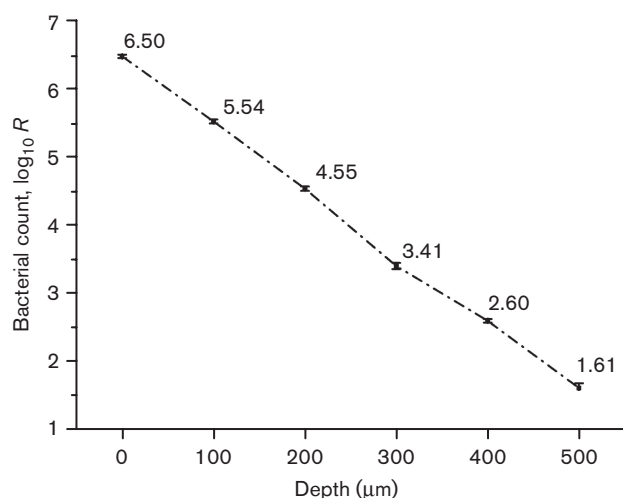
application of PUI might be limited, especially for curved root canals, owing to the fact that the apical third of a root canal needs to be enlarged to at least an ISO size of 35–40 to place the needle within 1–2 mm of the apical seat (Peeters & Gutknecht, 2014). Instead, the optical fibre of the Er:YAG laser was positioned at the orifice of the root canal when used for root canal disinfection, without inserting it inside the root canal, which may be effective for both straight and curved root canals.

The bactericidal potential of Er:YAG laser treatment might be related to the evaporation effect of cellular water, which could expand quickly during the laser pulse and lead to the disintegration of the bacterial cell wall. This might explain the bacterial morphology observed – rough, shrunken and fractured (Aoki *et al.*, 1998). It was found that Er:YAG laser irradiation could dramatically improve the bactericidal effect of NaOCl irrigation (Macedo *et al.*, 2010). Our present study showed that root canals treated with Er:YAG laser irradiation combined with NaOCl were much ‘cleaner’ than those treated with NaOCl alone. This observation might be explained by the cavitation phenomenon, which was thought to be the physical mechanism of laser-assisted irrigation (Blanken *et al.*, 2009). The cleaning effect of laser-assisted irrigation has been confirmed to be mainly dependent on collapsed shock waves and the acoustic streaming of fluid. This would produce a large shear stress on the root canal wall, which could remove debris and the smear layer, and push NaOCl into deep dentine layers (Levy *et al.*, 1996; Matsumoto *et al.*, 2011).

Among the laser systems used in endodontic therapy, the Nd:YAG laser (1064 nm) has been the most widely investigated; it could present an effective bactericidal effect up to 1 mm into the dentine and reduce *E. faecalis* by more than



**Fig. 4.** Root canals after 4-week bacterial incubation. Very few *E. faecalis* could invade the dentinal tubules beyond 500 µm. Root canals sectioned longitudinally. White arrows indicate the bacterial cells.



**Fig. 5.** Bacterial count ( $\log_{10}R$ , mean  $\pm$  SD) before treatment, at different depths ( $\mu\text{m}$ ) inside the dentinal tubules. The value of  $\log_{10}R$  decreased significantly as the depth inside the dentinal tubules increased: it was around 6.50 on root canal walls and only about 1.61 at 500  $\mu\text{m}$  inside the dentinal tubules.  $R$ , c.f.u.; depth 0 shows the count on root canal walls.

99 % in a number of inoculated root canals (Klinke *et al.*, 1997; Moritz *et al.*, 1999). However, it was also reported that Nd:YAG irradiation is not as effective against non-pigmented bacteria or bacterial biofilms because its irradiation is absorbed well only by melanin and dark pigmented tissues (Hellingwerf *et al.*, 1996). Therefore, higher energy densities are required, which might induce a lethal thermal effect (Camargo *et al.*, 2005). It was reported that Nd:YAG

irradiation may cause thermal damage such as structural changes, carbonization, and cracks to dentine when used at higher power (3 W and above) settings (Spencer *et al.*, 1996).

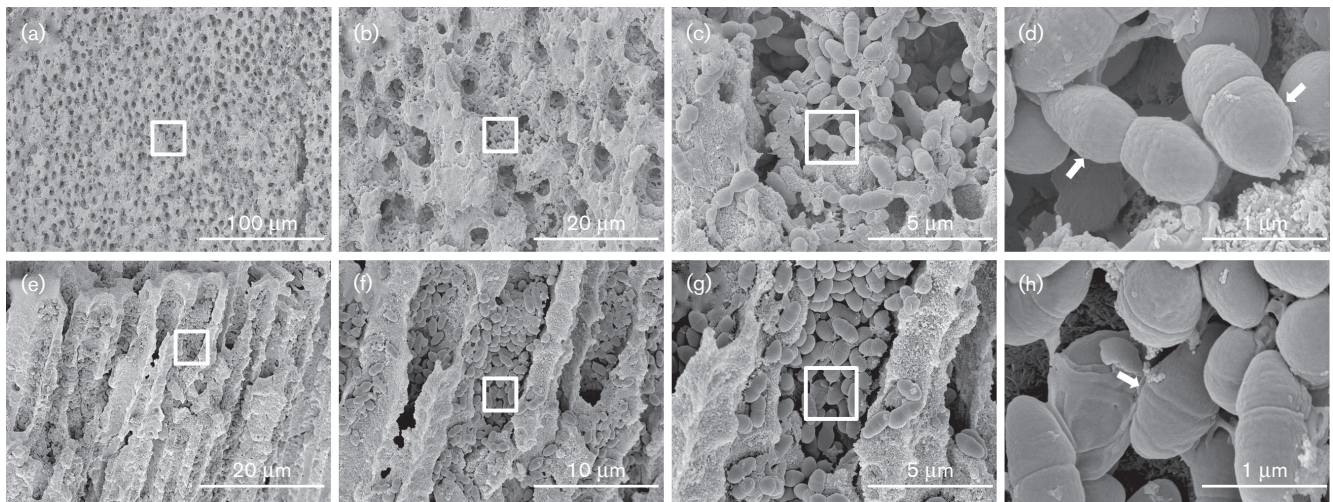
Thermal injury to periodontal tissues is a major concern when laser irradiation is applied during endodontic treatment. Until now, however, no studies have determined the temperature at which the periodontal ligament necrosis or degeneration would occur. An *in vitro* study found that no thermal damage to periodontal tissues occurred when the temperature increased by less than 6 °C in the apical area and less than 3 °C in the central area during Er:YAG laser irradiation for 60 s (Kimura *et al.*, 2002). Another study showed that there was a maximum temperature rise of only 4 °C in a simulated dental pulp irradiated by an Er:YAG laser in combination with water-spray cooling (Attrill *et al.*, 2004). It was also reported that the maximum temperature rose by only 4.58 °C when an Er:YAG laser was used at 1.3 W in endodontically prepared root canals (Schoop *et al.*, 2002). The recommended power setting in Schoop's study was no more than 1 W. In the present study, the emission power of the Er:YAG laser was set at 0.3, 0.5 and 1.0 W. At each emitting power level, the Er:YAG laser was activated for 20 and 30 s. The working mode of super-short pulse would probably reduce the chance of thermal injury to the surrounding tissue. In addition, application of NaOCl would suppress the temperature increase caused by the Er:YAG laser irradiation, similarly to the water spray, and as a result could potentially reduce the chance of thermal damage to periodontal tissues (Attrill *et al.*, 2004). In addition, normal saline and distilled water were used following NaOCl to deactivate the residual NaOCl, which would avoid any damage to the periapical tissues. Therefore, all

**Table 2.** Bacterial reduction (mean  $\pm$  SD) in each group at different depths inside the dentinal tubules

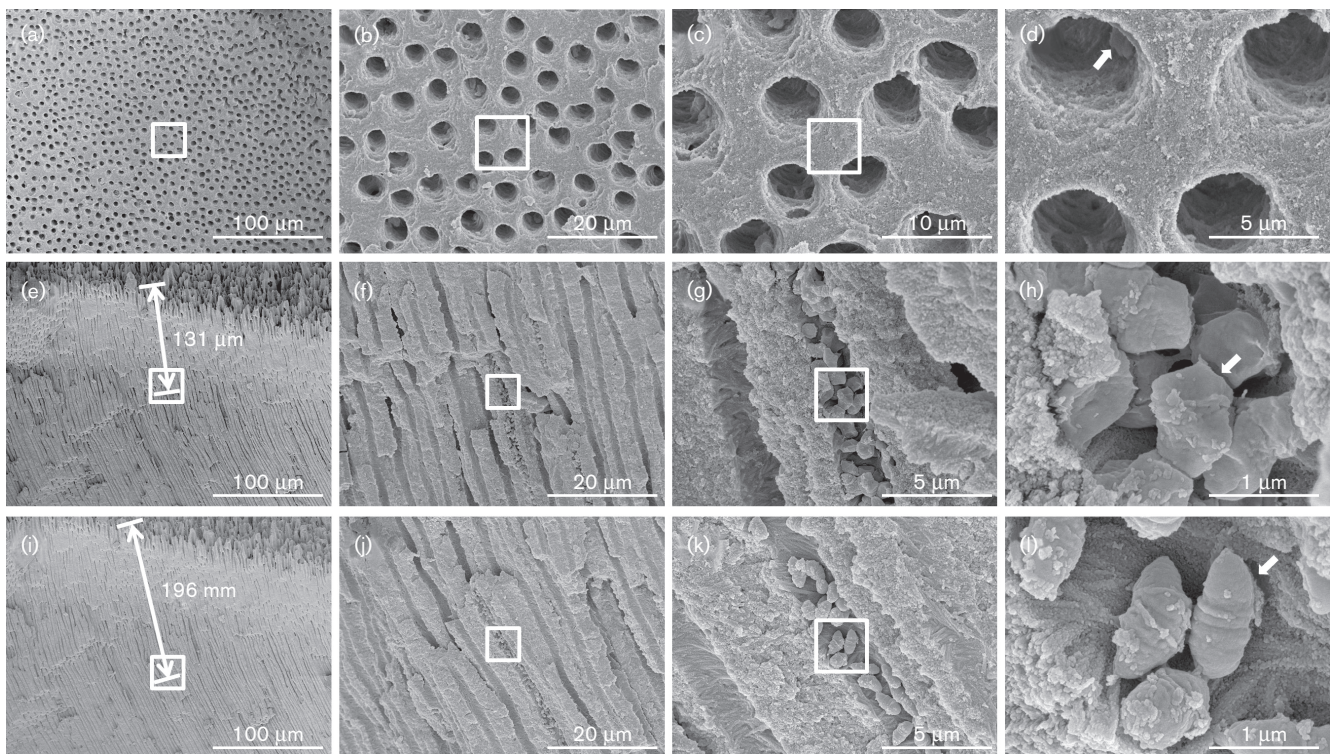
After treatment, bacterial reduction in the experimental groups was greater than that in both the negative control group at each level tested and the positive control group at 300, 400 and 500  $\mu\text{m}$  inside the dentinal tubules; it reached 100 % both on the surface of the root canal walls and at 100 and 200  $\mu\text{m}$  inside the dentinal tubules in all experimental groups. However, only the groups treated at 0.5 and 1.0 W irradiation for 30 s showed no bacterial growth at 300, 400 and 500  $\mu\text{m}$  inside the dentinal tubules. Depth 0, on root canal walls; NS, normal saline; NaOCl, 52.5 g l<sup>-1</sup> NaOCl. <sup>a-d</sup>Comparisons of different labelled groups were statistically significant at the same tested depth ( $P < 0.05$ ).

Treatment group	Bacterial reduction (%) at:					
	0	100 $\mu\text{m}$	200 $\mu\text{m}$	300 $\mu\text{m}$	400 $\mu\text{m}$	500 $\mu\text{m}$
NS	72.34 $\pm$ 1.57 <sup>a</sup>	72.16 $\pm$ 1.65 <sup>a</sup>	67.26 $\pm$ 1.45 <sup>a</sup>	48.86 $\pm$ 1.13 <sup>a</sup>	20.35 $\pm$ 1.38 <sup>a</sup>	19.43 $\pm$ 0.99 <sup>a</sup>
NaOCl	99.98 $\pm$ 0.03 <sup>b</sup>	99.98 $\pm$ 0.03 <sup>b</sup>	99.96 $\pm$ 0.04 <sup>b</sup>	99.37 $\pm$ 0.29 <sup>b</sup>	96.72 $\pm$ 0.35 <sup>b</sup>	91.99 $\pm$ 2.51 <sup>b</sup>
Er:YAG						
0.3 W, 20 s	100.00 $\pm$ 0.00 <sup>b</sup>	100.00 $\pm$ 0.00 <sup>b</sup>	100.00 $\pm$ 0.00 <sup>b</sup>	99.83 $\pm$ 0.07 <sup>c</sup>	98.81 $\pm$ 0.67 <sup>c</sup>	96.44 $\pm$ 0.85 <sup>c</sup>
0.3 W, 30 s	100.00 $\pm$ 0.00 <sup>b</sup>	100.00 $\pm$ 0.00 <sup>b</sup>	100.00 $\pm$ 0.00 <sup>b</sup>	99.88 $\pm$ 0.08 <sup>c</sup>	99.54 $\pm$ 0.23 <sup>d</sup>	96.82 $\pm$ 1.97 <sup>c</sup>
0.5 W, 20 s	100.00 $\pm$ 0.00 <sup>b</sup>	100.00 $\pm$ 0.00 <sup>b</sup>	100.00 $\pm$ 0.00 <sup>b</sup>	99.96 $\pm$ 0.04 <sup>c</sup>	99.86 $\pm$ 0.20 <sup>d</sup>	99.61 $\pm$ 0.82 <sup>d</sup>
0.5 W, 30 s	100.00 $\pm$ 0.00 <sup>b</sup>	100.00 $\pm$ 0.00 <sup>b</sup>	100.00 $\pm$ 0.00 <sup>b</sup>	100.00 $\pm$ 0.00 <sup>c</sup>	100.00 $\pm$ 0.00 <sup>d</sup>	100.00 $\pm$ 0.00 <sup>d</sup>
1.0 W, 20 s	100.00 $\pm$ 0.00 <sup>b</sup>	100.00 $\pm$ 0.00 <sup>b</sup>	100.00 $\pm$ 0.00 <sup>b</sup>	99.98 $\pm$ 0.03 <sup>c</sup>	99.89 $\pm$ 0.15 <sup>d</sup>	99.75 $\pm$ 0.57 <sup>d</sup>
1.0 W, 30 s	100.00 $\pm$ 0.00 <sup>b</sup>	100.00 $\pm$ 0.00 <sup>b</sup>	100.00 $\pm$ 0.00 <sup>b</sup>	100.00 $\pm$ 0.00 <sup>c</sup>	100.00 $\pm$ 0.00 <sup>d</sup>	100.00 $\pm$ 0.00 <sup>d</sup>





**Fig. 6.** Root canals treated with normal saline. Bacterial content decreased a little on root canal walls (a–d). The cells remaining both on the surface of the root canal walls and located inside the dentinal tubules had a smooth, uniform and bright surface (d, h). (a–d) Root canal wall; (e–h), root canals sectioned longitudinally. White arrows indicate the bacterial cells.



**Fig. 7.** Root canals treated with NaOCl. (a–d) Few bacteria, with shrunken surfaces, on the root canal walls. (e–h) Rough, shrunken and fractured bacterial cells inside the dentinal tubules. (i–l) Intact bacterial cells inside the dentinal tubules around 200 μm. (a–d) Surface of root canal wall; (e–l), root canals sectioned longitudinally. White arrows indicate the bacterial cells.



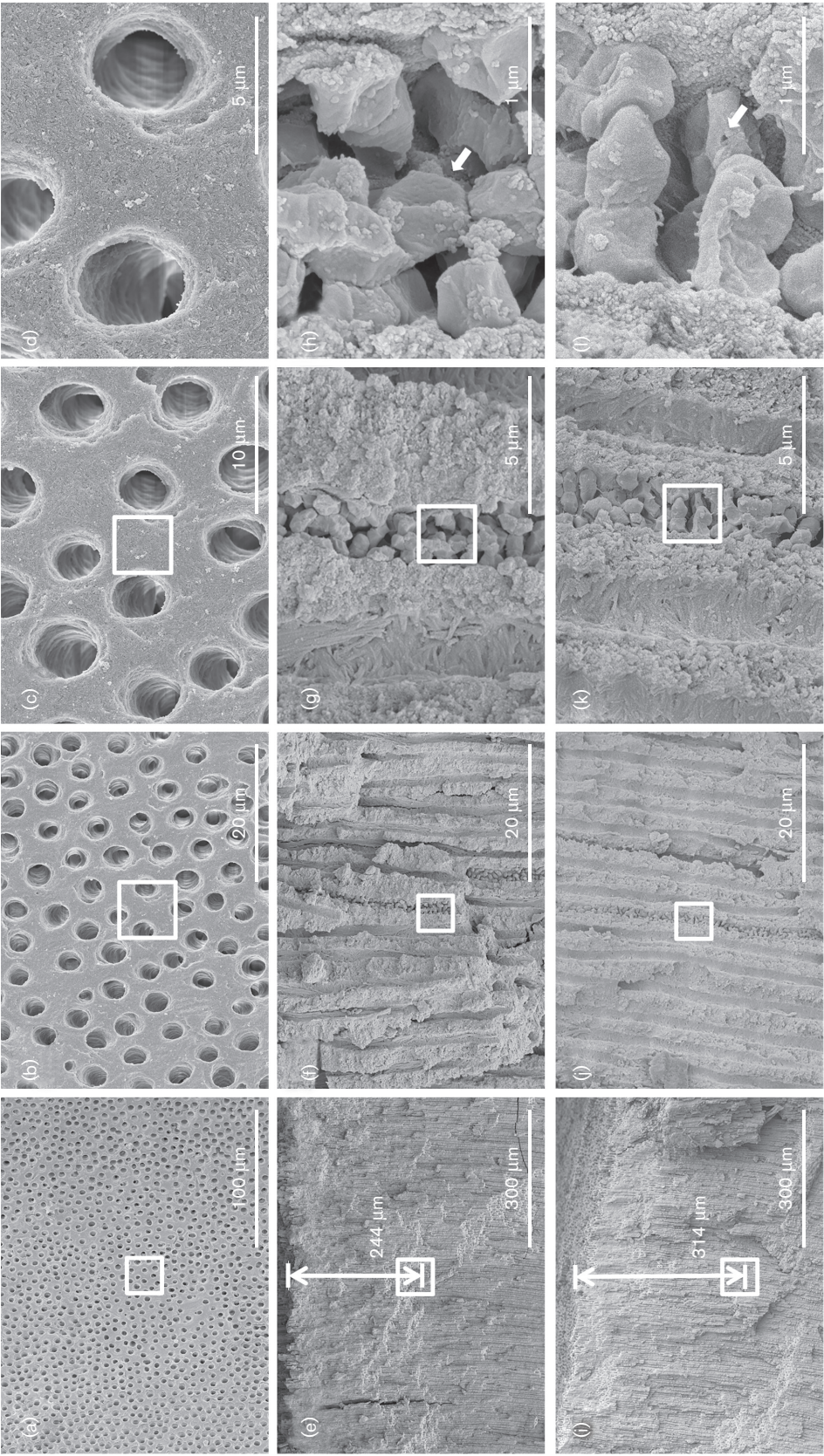
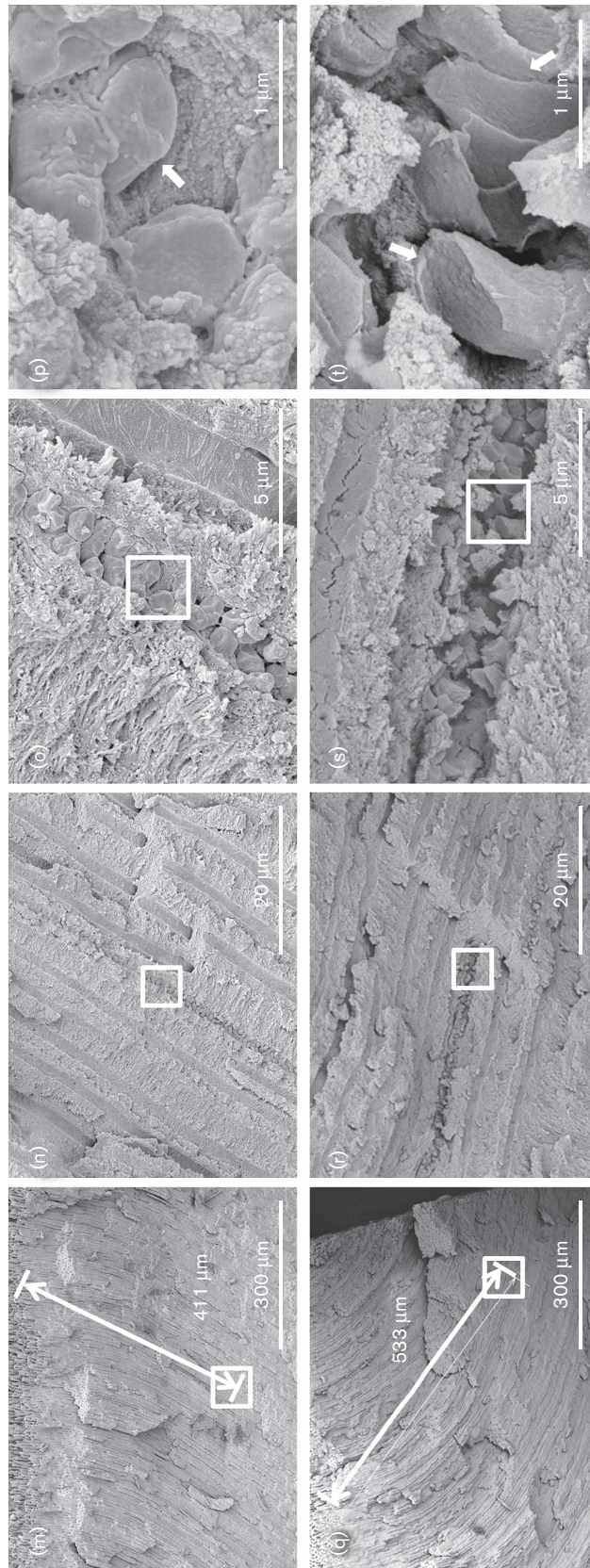


Fig. 8. Continued





**Fig. 8.** Root canals treated with Er:YAG laser combined with NaOCl. (a–d) No bacteria were present on the root canal walls. (e–t) The disinfection depth of Er:YAG laser combined with NaOCl increased with the irradiation power and time. In the group irradiated with 0.3 W for 20 s the dentinal tubules were disinfected up to 300 μm in depth [244 μm, for example, in (e)]. In the groups treated with 0.3 W for 30 s and those treated with 0.5 and 1.0 W for 20 s the dentinal tubules were disinfected to approximately 300 [314 μm, for example, in (i)] and 400 μm depth [411 μm, for example, in (m)]. In addition, the affected bacterial cells left inside the dentinal tubules became rough, shrunken and even fractured (h, i, p, t). (a–h) Root canal treated with 0.3 W for 20 s; (i–l) root canal treated with 0.3 W for 30 s; (m–p) root canal treated with 1.0 W for 20 s; (q–t) root canal treated with 1.0 W for 30 s. (a–d) Surface of a root canal wall; (e–t) root canals sectioned longitudinally. White arrows indicate the bacterial cells.



the protocols we tested might be feasible for clinical application. Moreover, treatment with 0.5 and 1.0 W for 30 s completely eliminated *E. faecalis* located at 500 µm inside the dentinal tubules, and might be able to produce an ideal disinfection effect as discussed above.

In the present study, the extracted human root canals were prepared to a constant diameter throughout their full length, so that a standard quantification analysis could be performed to evaluate the number of viable bacteria before and after treatments. To further understand the clinical bactericidal effect of Er:YAG laser irradiation combined with NaOCl, evaluations *in vivo* with standard instrumentation will be explored in future investigations.

In conclusion, all the Er:YAG laser irradiation protocols, combined with NaOCl irrigation, demonstrated effective bactericidal effects at all tested depths inside dentinal tubules. However, 0.5 W irradiation for 30 s was confirmed to be optimal and might be considered as a new alternative to conventional root canal disinfection.

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## REFERENCES

- Aoki, A., Ishikawa, I., Yamada, T., Otsuki, M., Watanabe, H., Tagami, J., Ando, Y. & Yamamoto, H. (1998). Comparison between Er:YAG laser and conventional technique for root caries treatment *in vitro*. *J Dent Res* **77**, 1404–1414.
- Attrill, D. C., Davies, R. M., King, T. A., Dickinson, M. R. & Blinkhorn, A. S. (2004). Thermal effects of the Er:YAG laser on a simulated dental pulp: a quantitative evaluation of the effects of a water spray. *J Dent* **32**, 35–40.
- Berutti, E., Marini, R. & Angeretti, A. (1997). Penetration ability of different irrigants into dentinal tubules. *J Endod* **23**, 725–727.
- Blanken, J., De Moor, R. J., Meire, M. & Verdaasdonk, R. (2009). Laser induced explosive vapor and cavitation resulting in effective irrigation of the root canal. Part 1: a visualization study. *Lasers Surg Med* **41**, 514–519.
- Camargo, S. E., Valera, M. C., Camargo, C. H., Fonseca, M. B. & Menezes, M. M. (2005). Effects of Nd:YAG laser irradiation on root canal dentin wall: a scanning electron microscopic study. *Photomed Laser Surg* **23**, 399–404.
- Chen, X., Li, P., Wang, X., Gu, M., Zhao, C., Sloan, A. J., Lv, H. & Yu, Q. (2013). *Ex vivo* antimicrobial efficacy of strong acid electrolytic water against *Enterococcus faecalis* biofilm. *Int Endod J* **46**, 938–946.
- Cheng, X., Guan, S., Lu, H., Zhao, C., Chen, X., Li, N., Bai, Q., Tian, Y. & Yu, Q. (2012). Evaluation of the bactericidal effect of Nd:YAG: Er:YAG, Er,Cr:YSGG laser radiation, and antimicrobial photodynamic therapy (aPDT) in experimentally infected root canals. *Lasers Surg Med* **44**, 824–831.
- De Moor, R. J., Meire, M., Goharkhay, K., Moritz, A. & Vanobbergen, J. (2010). Efficacy of ultrasonic versus laser-activated irrigation to remove artificially placed dentin debris plugs. *J Endod* **36**, 1580–1583.
- George, R., Meyers, I. A. & Walsh, L. J. (2008). Laser activation of endodontic irrigants with improved conical laser fiber tips for removing smear layer in the apical third of the root canal. *J Endod* **34**, 1524–1527.
- Haapasalo, M. & Orstavik, D. (1987). *In vitro* infection and disinfection of dentinal tubules. *J Dent Res* **66**, 1375–1379.
- Hellingwerf, K. J., Hoff, W. D. & Crielaard, W. (1996). Photobiology of microorganisms: how photosensors catch a photon to initialize signalling. *Mol Microbiol* **21**, 683–693.
- Huang, T. Y., Gulabivala, K. & Ng, Y. L. (2008). A bio-molecular film ex-vivo model to evaluate the influence of canal dimensions and irrigation variables on the efficacy of irrigation. *Int Endod J* **41**, 60–71.
- Kimura, Y., Yonaga, K., Yokoyama, K., Kinoshita, J., Ogata, Y. & Matsumoto, K. (2002). Root surface temperature increase during Er:YAG laser irradiation of root canals. *J Endod* **28**, 76–78.
- Klinke, T., Klimm, W. & Gutknecht, N. (1997). Antibacterial effects of Nd:YAG laser irradiation within root canal dentin. *J Clin Laser Med Surg* **15**, 29–31.
- Levy, G., Rizioi, I., Friedman, S. & Lam, H. (1996). Pressure waves in root canals induced by Nd:YAG laser. *J Endod* **22**, 81–84.
- Macedo, R. G., Wesselink, P. R., Zaccaro, F., Fanali, D. & Van Der Sluis, L. W. (2010). Reaction rate of NaOCl in contact with bovine dentine: effect of activation, exposure time, concentration and pH. *Int Endod J* **43**, 1108–1115.
- Matsumoto, H., Yoshimine, Y. & Akamine, A. (2011). Visualization of irrigant flow and cavitation induced by Er:YAG laser within a root canal model. *J Endod* **37**, 839–843.
- Meire, M. A., Coenye, T., Nelis, H. J. & De Moor, R. J. (2012). Evaluation of Nd:YAG and Er:YAG irradiation, antibacterial photodynamic therapy and sodium hypochlorite treatment on *Enterococcus faecalis* biofilms. *Int Endod J* **45**, 482–491.
- Moritz, A., Schoop, U., Goharkhay, K., Jakolitsch, S., Kluger, W., Wernisch, J. & Sperr, W. (1999). The bactericidal effect of Nd:YAG, Ho:YAG, and Er:YAG laser irradiation in the root canal: an *in vitro* comparison. *J Clin Laser Med Surg* **17**, 161–164.
- Nair, P. N., Sjögren, U., Krey, G., Kahnberg, K. E. & Sundqvist, G. (1990). Intraradicular bacteria and fungi in root-filled, asymptomatic human teeth with therapy-resistant periapical lesions: a long-term light and electron microscopic follow-up study. *J Endod* **16**, 580–588.
- Ordinola-Zapata, R., Bramante, C. M., Apécio, R. M., Handysides, R. & Jaramillo, D. E. (2014). Biofilm removal by 6 % sodium hypochlorite activated by different irrigation techniques. *Int Endod J* **47**, 659–666.
- Peeters, H. H. & Gutknecht, N. (2014). Efficacy of laser-driven irrigation versus ultrasonic in removing an airlock from the apical third of a narrow root canal. *Aust Endod J* **40**, 47–53.
- Peters, L. B., Wesselink, P. R., Buijs, J. F. & van Winkelhoff, A. J. (2001). Viable bacteria in root dentinal tubules of teeth with apical periodontitis. *J Endod* **27**, 76–81.
- Schoop, U., Moritz, A., Kluger, W., Patruta, S., Goharkhay, K., Sperr, W., Wernisch, J., Gattringer, R., Mrass, P. & Georgopoulos, A. (2002). The Er:YAG laser in endodontics: results of an *in vitro* study. *Lasers Surg Med* **30**, 360–364.

- Seal, G. J., Ng, Y. L., Spratt, D., Bhatti, M. & Gulabivala, K. (2002).** An *in vitro* comparison of the bactericidal efficacy of lethal photosensitization or sodium hypochlorite irrigation on *Streptococcus intermedius* biofilms in root canals. *Int Endod J* **35**, 268–274.
- Siqueira, J. F., Jr., MacHado, A. G., Silveira, R. M., Lopes, H. P. & de Uzeda, M. (1997).** Evaluation of the effectiveness of sodium hypochlorite used with three irrigation methods in the elimination of *Enterococcus faecalis* from the root canal, *in vitro*. *Int Endod J* **30**, 279–282.
- Spencer, P., Cobb, C. M., McCollum, M. H. & Wieliczka, D. M. (1996).** The effects of CO<sub>2</sub> laser and Nd:YAG with and without water/air surface cooling on tooth root structure: correlation between FTIR spectroscopy and histology. *J Periodontal Res* **31**, 453–462.
- Stabholz, A., Sahar-Helft, S. & Moshonov, J. (2004).** Lasers in endodontics. *Dent Clin North Am* **48**, 809–832.
- Wang, Q. Q., Zhang, C. F. & Yin, X. Z. (2007).** Evaluation of the bactericidal effect of Er,Cr:YSGG, and Nd:YAG lasers in experimentally infected root canals. *J Endod* **33**, 830–832.
- Williams, J. A., Pearson, G. J. & Colles, M. J. (2006).** Antibacterial action of photoactivated disinfection PAD used on endodontic bacteria in planktonic suspension and in artificial and human root canals. *J Dent* **34**, 363–371.
- Yilmaz, H. G., Kurtulmus-Yilmaz, S., Cengiz, E., Bayindir, H. & Aykac, Y. (2011).** Clinical evaluation of Er,Cr:YSGG and GaAlAs laser therapy for treating dentine hypersensitivity: a randomized controlled clinical trial. *J Dent* **39**, 249–254.
- Yonemoto, K., Eguro, T., Maeda, T. & Tanaka, H. (2006).** Application of DIAGNOdent as a guide for removing carious dentin with Er:YAG laser. *J Dent* **34**, 269–276.