light system of a specific wavelength (635 nm) to activate the aqueous solution. The solution selectively targets and tags bacteria when introduced into the root canals. The photosensitizer used in endodontic therapy is a pharmaceutical grade chemical substance. It releases nascent oxygen when exposed to low power light at its peak absorption. The nascent oxygen can cause oxidative injury to the bacterial cell wall and thus kill the microorganism. However, neither the photosensitizer nor the light has any significant antibacterial action when used alone.[10] The advantage of PAD is that it selectively eliminates bacteria. Furthermore, it does not affect any other normal tissue and causes no damage to the surrounding tissues.[11] There is no staining on the gingiva or restorations. It also does not encourage the development of any resistant species. PAD therefore seems a promising method to eradicate bacteria, even the resistant strain such as EF, in the root canal systems. In the previous study, there is limited knowledge of the bactericidal effect of various irradiation energy doses of PAD on EF and the relationship between the PAD power used and the irradiation time. The aims of this study were to examine the bactericidal effect of PAD on EF in a glass tube model to investigate the relationship between the PAD power used, the irradiation time and the energy toward its bactericidal effect, and an infected root canal model, to investigate the bactericidal effect of PAD against EF in the root canal system.

Materials and Methods

Laser devices

The laser irradiation was delivered by a small diode laser designed for clinical use (Denfotex, Denfotex Light Systems Ltd, Inverkeithing, Fife, U.K.). It produced red light at 635 nm with the output power ranging from 50 to 100 mW. The Denfotex handpiece had a 15 mm long endodontic emitter, which was equivalent to a size 40 endodontic file for root canal disinfection. Approximately 70% of the light was emitted from the full 15 mm tapered fiber, and 30% of the light was given out from the tip. This optical fiber was able to distribute uniform illumination of 360 degrees within the entire root canals.

Experiment 1: glass tube experiment

Preparation of bacteria. EF (American Type Culture Collection [ATCC] 29212) was cultured for 48 h at 37 C in brain heart infusion (BHI) broth (Oxoid CM225). A volume of 3,600 μL of bacterial broth were centrifugated with 800 rotations for 2 min, and the supernatant was discarded. Bacterial deposition was introduced into 3,300 μL of photosensitizer (12.7 μg/mL tolonium chloride), and vortexed. The cell suspension was adjusted spectrophotometrically to ensure that the amount of bacteria was $>10^{12}$ colony forming units (CFU)/mL.

Preparation of specimens. A total of 132 glass tubes with an internal diameter of 1.80 0.10 mm were cut to 16 mm length to simulate root canals. One end of each tube was sealed, and the other end was kept patent for the insertion of the laser fiber. All the glass tubes were sterilized by steam autoclaving, and 25 μL of prepared bacterial solution was introduced into each tube.

Test groups. The prepared glass tubes were divided into 16 groups. Each group consisted of 8 specimens, and they were subjected to PAD laser irradiation. The two experimental settings were 50 and 100 mW, with the irradiation time varied from 5 to 55 sec so that the groups received a radiation energy dose ranging from 0.5 to 5.5 J (Table 1). Four specimens received no laser irradiation and were used as controls.

Bacteriological evalsuation. A 5 μL sample was removed from each irradiated specimen and serial 10-fold dilutions were made. Three 5 μL drops of each dilution were deposited onto BHI agar (Oxoid CM225) plates, which were then incubated at 37 C for 24 h under anaerobic conditions. CFU

Table 1. Bactericidal Effect (Log Reduction) of PAD According to the Irradiation Time, Power Output, and Energy Dose

Group (n)	Irradiation time/sec	Output/mW	Energy dose/J	Mean/CFUmL^{-1}	SD	Log reduction
Baseline (4)	-	-	-	5.95E+14	9.47E+13	-
1 (8)	10	50	0.5	2.94E+10	6.25E+09	4.31
2 (8)	5	100	0.5	3.03E+09	7.32E+08	5.3
3 (8)	20	50	1	5.76E+08	7.15E+07	6.01
4 (8)	10	100	1	3.18E+08	7.11E+07	6.28
5 (8)	30	50	1.5	4.25E+06	7.58E+05	8.15
6 (8)	15	100	1.5	2.99E+06	7.40E+05	8.31
7 (8)	40	50	2	7.78E+05	8.00E+04	8.88
8 (8)	20	100	2	6.50E+05	7.82E+04	8.96
9 (8)	50	50	2.5	8.19E+04	9.36E+03	9.86
10 (8)	25	100	2.5	5.56E+04	7.19E+03	10.03
11 (8)	30	100	3	5.04E+04	4.69E+03	10.07
12 (8)	35	100	3.5	3.63E+03	8.38E+02	11.22
13 (8)	40	100	4	4.31E+03	8.94E+02	11.14
14 (8)	45	100	4.5	0	0	14.77
15 (8)	50	100	5	0	0	14.77
16 (8)	55	100	5.5	0	0	14.77

PAD, photoactivated disinfection; CFU, colony forming units.

from every irradiated and control specimen was measured. The mean bacterial concentration of each group was calculated as the primary outcome measured in this study.

Statistical analysis. Statistical analysis was performed using SPSS version 17 software (SPSS Inc., Chicago, IL). All data were assessed for normal distribution using the Shapiro–Wilk test for normality. The differences in the mean EF concentration of the test (irradiated) and control group were assessed by Student's t test. Linear regression was used to study the relationship between the bactericidal effect (log reduction) of PAD and irradiation energy dose. The cutoff level of significance was taken as 5% for all analyses.

Experiment 2: root canal experiment

Preparation of bacteria. EF (American Type Culture Collection [ATCC] 29212) was cultured for 48 h at 37 C in BHI broth (Oxoid CM225).

Preparation of specimens. Sixty single-rooted teeth with straight canals were selected. The crowns and the coronal parts of the roots were removed, and the length of the roots was uniformed as 12 mm. The canals were enlarged to an apical size of 40# using Ni-Ti ProTaper instrumentation and sterilized with 10 mL of 5.25% sodium hypochlorite solution and 10 mL 17% EDTA solution between each endodontic file. The apical foramens and the surface of the roots were sealed with flowable composite resin and all the specimens were sterilized by autoclaving for 15 min 121 C.

Each of the specimens was incubated in a sterile centrifuge tube with 1 mL of the EF ATCC29212 at 37 C under anaerobic conditions for 21 days. The medium in each tube was refreshed every 3 days. After the incubation, the samples were collected by using three sterile paper points per canal, which were immediately placed in sterile centrifuge tubes. The extracted fluid was diluted in log 10 steps, and then 50 μL of each dilution was spread out on BHI agar plates, which were then incubated for 24 h at 37 C under anaerobic conditions. After incubation, the number of the CFU was counted on those plates containing between 20 and 200 colonies.

Test groups. All the specimens were randomly divided into three groups with 20 teeth in each group: (1) 20 root canals were disinfected by PAD, (2) 20 root canals were irrigated with 10 mL 5.25% sodium hypochlorite (NaOCl) solution for 5 min as the positive control, and (3) 20 root canals were irrigated with 10mL 0.9% sterile saline as the negative control.

The laser irradiations were done as the procedures below. Photosensitizer (12.7 micrograms/mL tolonium chloride) was injected into the root canals, then the optical fiber was slowly inserted into the root canals, and irradiated for 150 sec with 100 mW (according to the user's manual of the PAD device), so that this group received a radiation energy dose of 15 J.

Bacteriological evalsuation. After irradiation or irrigation, the surfaces of the roots were disinfected with 5.25% NaOCl. Then 50 μL of physiological saline solution was delivered to each root canal, and the samples were collected by using three sterile paper points per canal, which were immediately placed in sterile centrifuge tubes. Then the residual bacteria were counted by the method described previously.

After the samples were collected by paper points, 10 μL BHI broth was injected into each root canal, which was then incubated for 72 h at 37 C under anaerobic conditions. The surfaces of the roots were disinfected with 5.25% NaOCl, and the samples were collected by using three sterile paper points per canal, which were immediately placed in sterile centrifuge tubes. Then, the 72 h recovery bacteria were counted by the method described previously.

Statistical analysis. Statistical analysis was also performed using SPSS version 17 software (SPSS Inc., Chicago, IL). The differences of the mean EF concentration of each group after contamination were compared by one way ANOVA. The differences in the mean EF concentration before, after irradiation or irrigation, and after 72 h incubation were assessed by Student's t test. The cutoff level of significance was taken as 5% for all analyses.

Results

The mean bacterial concentrations of each group after laser irradiation are presented in Table 1. The initial concentration of EF was 10^{14} CFU/mL. The bactericidal effects of application of PAD for each dose of irradiation were calculated by counting the difference in the CFU. The concentration of EF was notably reduced when the energy dose reached 0.5 J in group 1 (10 sec, 50 mW) and group 2 (5 sec, 100 mW), and no viable EF was detected on the culture plates in group 14 (45 sec, 100 mW), group 15 (50 sec, 100 mW), and group 16 (55 sec, 100 mW). For the same energy dose, the difference between the two output power settings was significant ($p < 0.05$), of which the group with 100 mW output demonstrated a better bactericidal effect than the group with 50 mW output exposed to doubled irradiation time.

A kill ratio for each energy dose of irradiation was calculated by dividing the logarithmically transformed initial EF concentration by the logarithmically transformed killed EF concentration. The PAD energy dose (J) and the bactericidal effect (kill ratio) are shown in Fig. 1. A high kill ratio means high bactericidal effect. As the energy dose increased, less EF survived the disinfected procedure. The log reduction in EF was related to the energy dose (E) by linear regression:

$$\log \text{ reduction} \quad 4{:}31 \quad 2{:}1E \ (R^2 \quad 0{:}95)$$

The bacterial numbers before, after laser irradiation or irrigation, and after 72 h recovery are presented in Table 2. After contamination with EF for 21 days, the bacteria count in all the samples reached to 10^6 or 10^7 CFU/mL, and there was no significant difference among the three groups ($p > 0.05$). After laser irradiation or irrigation, the number of bacterial cells within the root canal in all the three groups reduced clearly. No bacterium was detected after irrigation in NaOCl group, and bacteria were detected in all the specimens in the PAD group and the saline solution group, PAD was significantly more effective than saline solution in reducing the number of bacterial cells within the root canals ($p < 0.05$). After 72 h, the recovery of bacteria was detected in

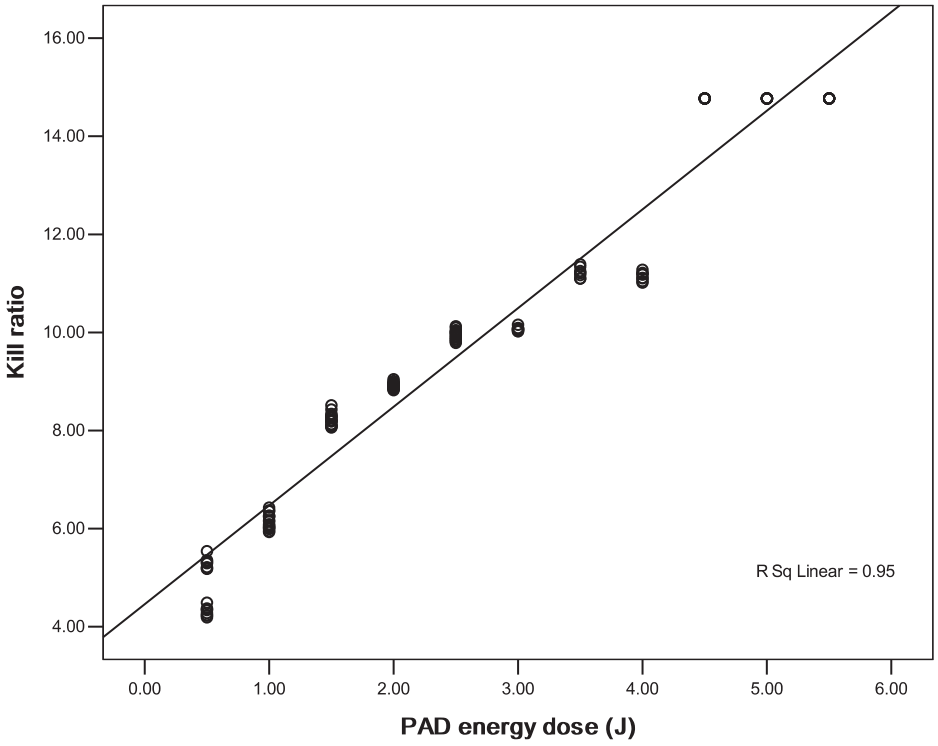

FIG. 1. Photoactivated disinfection (PAD) energy dose (J) and bactericidal effect (kill ratio).

11 samples in the NaOCl group. There was no significant difference between the PAD group and the saline solution group ($p > 0.05$) and the bacteria count in these two groups rebounded to the level before disinfection.

Discussion

PAD is an innovative approach for the disinfection of the root canal system. It involves the use of low-power lasers on photosensitisers to produce reactive oxygen species. The reactive oxygen species are short range free radicals that can disrupt bacterial membrane, which leads to rapid death of the microorganisms.[12] *In vitro*[13,14] and *in vivo*[9,15] studies were performed to explore PAD as an alternative approach to disinfection in endodontic therapy. Seal et al.[16] reported that PAD has the potential to eradicate a wide range of oral bacteria, including EF. However, the clinical parameters remained to be optimized.

NaOCl solution is considered by many to be the preferred irrigant for root canal treatment, because of its proteolytic effect.[17] In the experiment, no bacterium was detected after irrigation in the NaOCl group. It seems that 5.25% NaOCl was more effective in discriminating EF in root canals. However, the recovery of bacteria after 72 h was detected in 11 samples in the NaOCl group, which means that after irrigation by 5.25% NaOCl, there were still some bacteria remaining in the root canals, such as deep dentin tubules and canal irregularities. These results of the infected tooth model experiments show that it is hard to eradicate EF from the root canals. This is because of the complexities of root canal system, the deep invasion of microorganisms into dentinal tubules, and the formation of biofilms on the surface of the root canal walls.[18,19]

Unlike PAD, NaOCl is highly toxic to vital tissues. Heggers et al. suggested that the safe concentration of NaOCl for debridement of wounds should not be > 0.025%.[20] However, such a low concentration has no significant antimicrobial effect for endodontic treatment. At present, there is no consensus on the optimal concentration that is safe and effective for NaOCl use in endodontic therapy. A low concentration of 1% NaOCl and a high concentration of 5.25% NaOCl can all provides tissue dissolution and antimicrobial effects.[21,22]

Table 2. Bacteria Controls in the Three Groups (CFU/mL)

Groups n = 20	Before bacteria counts (Mean, SD)	After		72 h recovery	
		Positive number	Bacteria counts (Mean, SD)	Positive number	Bacteria counts (Mean, SD)
NS	7.14E + 06 (7.39E + 06)	20	3.11E + 05 (1.56E + 05)	20	4.66E + 06 (5.26E + 06)
5.25%NaOCl	1.14E + 07 (1.30E + 07)	0	0	11	5.66E + 04 (8.27E + 03)
PAD	6.07E + 06 (8.83E + 06)	20	1.67E + 04 (1.92E + 04)	20	3.81E + 06 (3.64E + 06)

CFU, colony forming units; PAD, photoactivated disinfection.

However, NaOCl accidents could occur between 1 and 5.25% concentrations of NaOCl; therefore, if a perforation or open apex exists, great care should be exercised to prevent an NaOCl accident, or an alternative irrigation solution should be considered.[23] Unlike NaOCl, the photosensitizers used in PAD are nontoxic to vital tissues.[24] PAD is also harmless to periodontal tissues because the increase in temperature is far below the threshold level to cause periodontal injury.[25] Common photosensitizers in PAD include tolonium chloride and methylene blue. They are organic dyes belonging to the phenothiazine family. Tolonium chloride was chosen as the photosensitizing agent in this experiment because it absorbs light at wavelengths ranging from 620 to 660 nm, and the red light irradiated from the device in this study was 635 nm. In another aspect, tolonium chloride is unchanged by the process, in which activity ceases as irradiation stops.[10]

EF is often associated with persistent endodontic infections, and is commonly found in the root canals of failed endodontic therapy patients.[2] EF can survive long periods of time in root canals without nutrient support.[3] In an *in vitro* study, EF maintained its viability in root-lled canals 12 months after root canal treatment.[26] The resistance of EF to endodontic treatment has long been recognized.[27] EF has displayed resistance to various medicaments and mechanical preparation during the treatment.[4] Soukos et al. compared the kill percentage of the different species by PAD and found that all bacterial species were eradicated except for EF (kill percent was only 53%). It indicated that EF was more resistant to the current PAD regimen than other bacteria, thereby supported the ndings that EF is a highly resistant pathogen.[28]

The effectiveness of PAD in killing EF in planktonic solution was measured by calculating the reduction in EF concentration after treatment. The $\log_{10}$ reduction increased linearly with the energy dose up to 4.5 J. Williams et al. reported similar ndings with the energy dose up to 2.4 J.[10] Paulino et al. also found that fewer bacteria survived with increased energy dose when they used PAD to disinfect canal colonized with *Streptococcus mutans*.[29] In our pilot study, bacterial concentrations $< 10^9$ CFU / mL were used, and we found that the kill percentage was 100% with a low energy dose of 0.5 J. Therefore, a higher concentration, which was $> 10^{12}$ CFU / mL, was chosen in this experiment to investigate the relationship between the irradiation energy and its bactericidal effects.

According to the results of our experiment, PAD had a profound bactericidal effect on EF *in vitro*, and the effect was related to the energy dose delivered. For the same energy dose, the group with 100 mW output demonstrated a higher effectiveness in bacterial killing than the group with 50 mW output exposed to longer irradiation time. Our study supports the parameter of increasing the output power of the diode laser with shortened irradiation time. Therefore, this study obtained a good result when bacteria concentration of 10^{14} CFU / mL was exposed to shorter irradiation time compared with prolonged irradiation.

In this experiment, PAD could signi cantly destroy EF in planktonic solution at low energy doses, which can reduce the risk of unwanted side effects. Therefore, the glass tube was modeled to simulate the root canal, but it was different from the complex root canal systems of human teeth. Therefore, another experiment was performed on an infected tooth model. In the experiment, we found that PAD could eliminate 10^{14} CFU / mL bacteria in glass tubes when the irradiation energy dose reached 4.5 J. But in root canals, even when the energy dose reached 15 J (according to the user's manual of the PAD device), 10^6 or 10^7 CFU / mL bacteria in root canals could not be totally eliminated. Therefore, it is noteworthy that PAD could not completely eradicate the root canal infection. One possible reason for the incomplete bacterial elimination could be attributed to the low concentration of available nascent oxygen in the canals, especially in dentinal tubules and canal irregularities, where the photosensitizer agent might not diffuse well into deep dentinal tubules and canal irregularities.[30] And it has been demonstrated that the ability of EF to form a bacterial biofi lm on root canal dentine may be a factor that contributes to their persistence after endodontic treatment.[19]

In experiment 2, all the specimens were sterilized by steam autoclaving. Steam autoclaving is the most efcient and popular method of sterilization used in the dental eld, but it affects enamel microhardness,[31] and alters dentin structure.[32] In this study, teeth served only to represent a more realistic model than the planktonic solution, and the alternation of teeth by steam autoclave was not investigated.

Conclusions

The results of this study demonstrated that PAD with an energy dose 4.5 J was able to completely eradicate viable EF in the bacteria suspension with 10^{14} CFU / mL; however, in the root canal model, PAD with an energy dose at 15 J could not thoroughly eliminate EF with 10^6 or 10^7 CFU / mL. The effectiveness of PAD in killing EF in the planktonic solution is greater than in an infected tooth model, and the bactericidal effect increased linearly with the irradiation energy dose and was superior using higher output power. It is noteworthy that these two simple models did not reproduce the complex situation as found in infected root canals in the patient's mouths. It should not be surprising that a high energy dose may be required for effective disinfection, and this is worth further investigation.

Author Disclosure Statement

No competing nancial interests exist.

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Address correspondence to:
Chengfei Zhang
Department of Endodontics
Faculty of Dentistry
University of Hong Kong
Hong Kong SAR
China

E-mail: zhangcf@hku.hk