Photodynamic Therapy with Pyoktanin Blue and Diode Laser for Elimination of *Enterococcus faecalis*

YOSHIKO MASUDA^{1,2}, HIROSHI SAKAGAMI², MASASHI HORIIKE¹, HIROSHI KADOKURA¹,
TAKAHIDE YAMASAKI¹, PERRY R. KLOKKEVOLD³, HENRY H. TAKEI⁴ and SATOSHI YOKOSE¹

¹Division of Endodontics and Operative Dentistry,

Department of Restorative and Biomaterials Sciences, School of Dentistry, Meikai University and

²Meikai University Research Institute of Odontology (M-RIO), Saitama, Japan;

³Section of Postgraduate Periodontics and Implant Surgery,

School of Dentistry, University California, Los Angeles, CA, U.S.A.;

⁴Section of Periodontics, School of Dentistry, University California, Los Angeles, CA, U.S.A.

Abstract. *Background/Aim:* *Enterococcus faecalis* is responsible for most cases of endodontic treatment failure. Despite various conventional disinfection methods, root canals are not completely free of microorganisms. Photodynamic therapy (PDT) is a new antimicrobial strategy that involves the use of a non-toxic photosensitizer (PS) and a light source. The aim of this study was to evaluate the antimicrobial effect of PDT using diode laser and pyoktanin blue (PB) and confirm the nontoxicity of PB as a PS. *Materials and Methods:* Laser irradiation with an output power of 3 W was performed with PB as the PS to a bacterial solution containing *E. faecalis*. Then, the number of colony-forming units was counted. PB cytotoxicity was also assessed by the MTT assay. *Results:* *E. faecalis* counts were reduced after laser irradiation, laser irradiation with PB, or the combination thereof compared to the control, non-irradiation or water. The 50% cytotoxic concentration value for adult human dermal fibroblasts incubated with PB for 1 min was 108 µg/ml. *Conclusion:* Diode laser irradiation in combination with PB as the PS is efficacious for the elimination of *E. faecalis* without toxic effects to human dermal fibroblasts. This strategy might be useful for root canal irrigants.

A high prevalence of *Enterococcus faecalis* is frequently observed in filled root canals associated with persistent apical periodontitis (1). Enterococci are Gram-positive cocci that can resist intracanal procedures and systemic antibiotics because of their morphological and genetic characteristics, even in ecologic conditions of stress (2). Therefore, *E. faecalis* is responsible for most cases of endodontic treatment failure (3). The complex anatomy of the root canal system (*i.e.*, isthmuses, accessory canals, and dentinal tubules) enables the survival of bacteria after conventional cleaning (4). *E. faecalis* is capable of surviving in adequate nutritional conditions and can stay viable as a single microorganism (5). It can also penetrate into the dentinal tubules and form biofilms (6). Bacteria can penetrate into the dentinal tubules up to a depth of 1,250 µm (7). Despite the availability of various conventional mechanical root canal cleaning methods and chemical irrigants, root canals are not completely free of microorganisms (4). The most frequently used irrigant, sodium hypochlorite, can only penetrate into the dentinal tubules up to a depth of 130 µm (8). Therefore, more effective irrigant delivery and agitation systems are needed. Lasers have been proposed as an effective approach to cleaning and disinfecting. Vatkar *et al.* reported that lasers proved to be a valuable adjunct in the elimination of bacteria and could help reduce the incidence of postendodontic treatment failures (9). Various types of lasers have been investigated in an attempt to develop improved treatment methods (10). Recently, high-power lasers have been introduced for endodontic treatment (11). A study showed that an 810-nm diode laser obstructed the dentinal tubules and decreased *Escherichia coli* and *E. faecalis* bacterial counts (12).

Photodynamic therapy (PDT) is an adjunct method for the inactivation of bacteria (8). This antimicrobial strategy involves the use of a non-toxic photosensitizer (PS) and a light source (13). The PS reacts with molecular oxygen to

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Correspondence to: Yoshiko Murakami Masuda, DDS, Ph.D., Division of Endodontics and Operative Dentistry, Department of Restorative and Biomaterials Sciences, School of Dentistry, Meikai University, 1-1 Keyakidai, Sakado, Saitama 350-0283, Japan. Tel: +81 492792787, Fax: +81 492855171, e-mail: yoshik@dent.meikai.ac.jp

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produce highly reactive oxygen species, which injure and kill microorganisms (8, 14). For endodontic treatment, PDT with a diode laser (810 nm) and indocyanine green was shown to reduce bacteria in root canals (15-17).

Pyoktanin blue (PB) was used to stain a cyst during cystic brain tumor resection (18, 19).

In this study, we used a new 808-nm diode laser (OPELASER Filio; Yoshida Trade Dental Distribution Co., Ltd., Tokyo, Japan) with PB as the PS for PDT. The aim of this *in vitro* study was to evaluate the antimicrobial effect of PDT using the new diode laser 6uFilio and PB and confirm the nontoxicity of PB as a PS. The hypothesis was that PDT with PB would be efficacious for the elimination of *E. faecalis* without toxicity to human dermal fibroblasts.

Materials and Methods

Bacterial inoculation. *E. faecalis* standard strain (American Type Culture Collection BAA-2128™) was cultured in 5 ml brain heart infusion (BHI) broth (33 g/l; Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C for 24 h. The turbidity of the prepared media was adjusted to 0.2 McFarland standard using a filter colorimeter. Then, 40 µl cultured medium containing approximately 2×10^8 *E. faecalis* and 200 µl 1% pyoktanin blue solution (PB; Wako Pure Chemical Industries, Ltd., Osaka, Japan) were added to a 1.5-ml microtube (Watson Co., Ltd., Tokyo, Japan). As a control, 200 µl sterilized distilled water was added instead of PB to the 1.5-ml microtube containing *E. faecalis*.

Laser irradiation. A diode laser (OPELASER Filio; Yoshida Trade Dental Distribution Co., Ltd., Tokyo, Japan) was used. This laser emits light at a wavelength of 808 nm and has a flexible fiber delivery system ($\Phi=0.2$ mm). Laser irradiation was performed with an output power of 3 W with continuous waves at a distance of 6 mm from the bottom of the 1.5-ml microtube for 10, 20, 40, 50, and 60 sec. Controls were performed without laser irradiation and with laser irradiation for 60 sec without PB. Figure 1 shows the experimental setup including the fiber tip of the diode laser and 1.5-ml microtube containing the PB solution.

Microbiological procedures. Bacterial counts were determined after laser irradiation. First, 5 µl bacterial sample was diluted with 1 ml sterilized distilled water. Then, 10 µl diluted bacterial solution was diluted with 1 ml sterilized distilled water. Finally, 40 µl bacterial solution was spread onto BHI agar plates (52 g/l distilled water) and incubated at 37°C for 24 h. The number of colony-forming units (CFUs) was then counted.

Cytotoxicity assay of PB. Adult human dermal fibroblasts (6 PDL) (HDFa; Gibco, Invitrogen, Grand Island, NY, USA) were plated in 96-well plates (2.42×10^3 cells/well, 0.1 ml/well) containing alpha-minimum essential medium (Gibco, Invitrogen) with 10% heat-inactivated fetal calf serum and incubated for 15 min to allow cell attachment. After 96 h, the medium was replaced with 0.1 ml fresh medium containing different concentrations of PB. Cells were further incubated for 1 min. The medium was removed and cells were washed twice with fresh culture medium and then further incubated in fresh culture medium (Dulbecco's modified Eagle's

Figure 1. Schematic of the experimental setup including the fiber tip of the diode laser. Laser irradiation was performed at a distance of 6 mm from the bottom of the 1.5-ml microtube, which contained the pyoktanin blue solution.

medium + 10% FBS) for 48 h to determine the viable cell number by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (20). Because 1% dimethyl sulfoxide (DMSO) reduced the viability to 86% of the control, cytotoxicity derived from DMSO was subtracted from all data points. The relative viable cell number was determined by reading the absorbance of the cell lysate at 562 nm using a microplate reader (Infinite F50R; TECAN, Kanagawa, Japan). Control cells were treated with the same amounts of DMSO and cell damage induced by DMSO was subtracted from that induced by PB. The concentration of PB that reduced the viable cell number by 50% (CC_{50}) was determined from the dose-response curve. The mean CC_{50} value was calculated from triplicate assays.

Statistical analysis. Comparisons between groups were statistically analyzed using the Mann-Whitney *U*-test. The percentage of reduction in colony count (%RCC) was calculated using the following equation (15):

$$\frac{\text{CFUs (before treatment)} - \text{CFUs (after treatment)}}{\text{CFUs (before treatment)}} \times 100 = \%RCC.$$

Results

Figure 2 shows *E. faecalis* colonies on BHI agar plates. Greater reductions in *E. faecalis* colony numbers were observed with longer laser irradiation time compared with the control (without laser and PB). At 60 sec, few colonies were detected on the plate (Figure 2). Furthermore, reductions in *E. faecalis* counts were observed after laser irradiation, PB, or the combination thereof compared with the control (without laser and PB) (Figure 3).

Greater reductions were observed in the PB without laser group compared with the laser irradiation for 10 and 20 sec group. Moreover, the reduction in *E. faecalis* count in the PB with laser irradiation at 40 sec group was greater than that in the PB without laser irradiation group. At 50 sec after laser irradiation with PB, RCC was about 99% compared

Figure 2. *E. faecalis* colonies on brain heart infusion agar plates. (A) Control without laser irradiation and pyoktanin blue (PB); (B) laser irradiation without PB; (C) PB without laser irradiation; (D) PB with laser irradiation for 10 sec; (E) PB with laser irradiation for 20 sec; (F) PB with laser irradiation for 40 sec; (G) PB with laser irradiation for 50 sec; (H) PB with laser irradiation for 60 sec.

with the baseline value. At 60 sec after laser irradiation with PB, RCC was about 100% compared with the baseline value (Table I). Without PB, at 60 sec after laser irradiation RCC was 48.45%. Statistically significant differences were observed for all group comparisons (Mann-Whitney *U*-test; Table II).

Figure 4 shows the results of the cytotoxicity assay. HDFa cells were incubated with a range of PB concentrations and cytotoxicity was analyzed using the MTT assay after 1 min of incubation (Figure 4). Viable cell number was affected by both the PB incubation time and concentration. A shorter incubation time resulted in higher viable cell counts. Each value represents mean+S.D. ($n=6$) The CC_{50} values of pyoktanin blue was 108 $\mu\text{g/ml}$.

Discussion

Elimination of *E. faecalis* in the root canal has been explored by many investigators, and different irrigation techniques and devices have been developed to enhance the efficacy and distribution of irrigants (21). Lasers have been proposed as an alternative to conventional cleaning and disinfection approaches (22). PDT involves the interaction between light and a PS (a chemical compound that can be excited by light of a particular wavelength) in the presence of oxygen. The PS accumulates in the target cells (including bacterial and tumor cells) and then light of a specific wavelength is applied to the target cells. This causes oxidative damage to the target cells by inducing the production of reactive oxygen species (23).

Figure 3. *E. faecalis* counts (colony-forming units). (A) Control without laser irradiation and pyoktanin blue (PB); (B) laser irradiation without PB; (C) PB without laser irradiation; (D) 0.83% PB with laser irradiation for 10 sec; (E) PB with laser irradiation for 20 sec; (F) PB with laser irradiation for 40 sec; (G) PB with laser irradiation for 50 sec; (H) PB with laser irradiation for 60 sec.

Figure 4. Cytotoxic activity of pyoktanin blue (PB). Confluent HDFa cells (6 PDL) ($2.42 \times 10^3/\text{cm}^2$) were inoculated into the 96-microwell plate and incubated for 3 days. Cells were exposed for 1 min to the indicated concentrations of pyoktanin blue. After washing twice with culture medium, cells were further incubated for 48 h with fresh culture medium to determine the viable cell number by the MTT method. Data represent the mean ± standard deviation ($n=6$). * $p < 0.01$ CC_{50} of pyoktanin blue was 108 µg/ml.

Siddiqui *et al.* reported in their systematic literature review that PDT effectively reduced *E. faecalis* counts in infected root canals compared with traditional endodontic instrumentation/irrigation treatment protocols (3). However, controversial results have also been reported. Nagayoshi *et*

Table I. *Enterococcus faecalis* colony-forming units.

	PB	Laser	Colony count	%RCC		
				Before treatment		
A	(-)	(-)	3107±735	0		
B	(-)	60 s	1601±123	48.45	0	
C	(+)	(-)	715±110	77		
D	(+)	10 s	1125±70	63.8	29.77	(-) 57.37
E	(+)	20 s	846±14	72.76	47.15	(-) 18.42
F	(+)	40 s	120±4	96.14	92.51	83.21
G	(+)	50 s	9±3	99.7	99.42	98.6
H	(+)	60 s	0±0	100	100	100

2×10^8 *E. faecalis* were mixed with a final concentration of 0.83% pyoktanin blue solution (PB). RCC: The percentage of reduction in colony count.

Table II. Comparisons between groups according to *E faecalis* colony-forming units.

Comparison	p-Value*	Comparison	p-Value*
Group 1 × group 2	<0.001	Group 2 × group 7	<0.001
Group 1 × group 3	<0.001	Group 3 × group 4	<0.001
Group 1 × group 4	<0.001	Group 3 × group 5	<0.005
Group 1 × group 5	<0.001	Group 3 × group 6	<0.001
Group 1 × group 6	<0.001	Group 3 × group 7	<0.001
Group 1 × group 7	<0.001	Group 3 × group 8	<0.001
Group 1 × group 8	<0.001	Group 4 × group 5	<0.001
Group 2 × group 4	<0.001	Group 5 × group 6	<0.001
Group 2 × group 5	<0.001	Group 6 × group 7	<0.001
Group 2 × group 6	<0.001	Group 7 × group 8	<0.001

Group 1, PB (-) Laser (-); group 2, PB (-) Laser 60 sec; group 3, PB (+) Laser (-); group 4, PB (+) Laser 10 sec; group 5, PB (+) Laser 20 sec; group 6, PB (+) Laser 40 sec; group 7, PB (+) Laser 50 sec; group 8, PB (+) Laser 60 sec. *Mann-Whitney *U*-test.

al. evaluated PDT using a diode laser and indocyanine green in a well-established root canal model and concluded that diode laser irradiation in combination with the PS had nearly the same antimicrobial effect as 2.5% sodium hypochlorite (23).

The results of the present study, which evaluated the effects of a diode laser (808 nm) and PB, demonstrated that PDT with PB effectively decreased *E. faecalis* counts. The number of bacteria was increased following treatment with PB and laser irradiation at 10 and 20 sec compared with PB without laser irradiation. We considered that the small exposure of the laser increased the activity of the bacteria and cells. Adequate laser power can increase the activity of

bacteria and cells. However, too much power causes damage to the bacteria and cells (24-26). Biostimulatory effects of laser are governed by the Arndt-Schulz law of biology, *i.e.*, weak stimuli excite physiological activity, whereas strong stimuli retard it (27). Previous investigators mentioned adverse effects of laser irradiation, such as temperature increase on the periapical tissue and enhancement of mammalian cell proliferation (28, 29). Thus, the reduction in *E. faecalis* cell viability was likely from laser irradiation itself, not from the heat produced by irradiation, and the addition of a PS was essential for antimicrobial effects (23). Thus, selection of the appropriate PS should be considered in PDT.

PB has been used to stain a cyst during cystic brain tumor resection (18, 19) and as a mouthwash (Honzou; Honsou Biological Research Institute Co., Ltd., Nagoya, Japan). In this study, incubation with PB for 1 min resulted in a significant loss of viable cells (CC_{50} value=108 μ g/ml). In the clinical setting, it takes less than 1 min to complete root canal irrigation with laser irradiation and PB. Our results suggest about 10 μ g/ml PB concentration (1%) and 1 min irrigation time might be optimal as PB was not cytotoxic to HDFa cells under these conditions.

Conclusion and Summary

Diode laser irradiation in combination with PB as the PS is efficacious for the elimination of *E. faecalis* without toxicity to HDFa cells. This treatment might be useful for root canal irrigants.

Conflicts of Interest

No competing financial interests exist.

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