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Antimicrobial effect of photodynamic therapy using high-power blue light-emitting diode and red-dye agent on *Porphyromonas gingivalis*

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Background and Objective: Antimicrobial photodynamic therapy (a-PDT) using a combination of red-colored laser/light-emitting diode (LED) and blue dye has been employed for periodontal therapy and the antimicrobial effect seems promising. Blue light, which has favorable wavelength properties, would be more effective as a light source for a-PDT because blue light itself possesses an antimicrobial effect. This study aimed to investigate the effect of a-PDT using a novel combination of high-power blue LED and red-dye agent on *Porphyromonas gingivalis in vitro*.

Material and Methods: Porphyromonas gingivalis ATCC 33277 suspension was irradiated with blue LED (BL) (425–470 nm) or red LED (RL) (625–635 nm) at 30–90 J/cm^2, or was mixed with erythrosine (ER), phloxine B (PB) or rose bengal (RB) with or without BL irradiation (30 J/cm^2). RL (30 J/cm^2) in combination with toluidine blue was employed as positive control. All the suspensions of *P. gingivalis* were serially diluted, plated and incubated anaerobically, and the numbers of colony-forming units (CFUs) were counted on day 7.

Results: BL irradiation at 60 and 90 J/cm^2 demonstrated a significant reduction in the numbers of CFUs. ER, PB and RB solutions at 160 μg/mL showed almost no or only a minimal reduction in the numbers of CFUs. BL at 30 J/cm^2 combined with ER, PB or RB at 160 μg/mL resulted in a log reduction of 0.9, 1.0 and 7.1, respectively, in the numbers of CFUs; 30 J/cm^2 BL with RB at 1.6, 16 and 160 μg/mL demonstrated a log reduction of 6.3, 8.0 and 5.5, respectively; and a log reduction of 5.2 was obtained after 30 J/cm^2 RL with 16 μg/mL TB.

Conclusion: Within the limits of this study, BL was found to have an antimicrobial/growth-inhibiting effect on *P. gingivalis*, and a-PDT using a combination of BL and RB shows promise as a new technical modality for bacterial elimination in periodontal therapy.

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Periodontal diseases derive from inflammation of the tooth-supporting periodontal tissues in response to chronic infections caused by periodontopathic bacteria. The aim of periodontal treatment is to arrest infection and inflammation of periodontal tissues. However, it has been reported that conventional treatment approaches, such as mechanical therapy (1) and chemotherapy (2), do not necessarily eradicate bacteria completely. Chemotherapy using antibiotics has many disadvantages: it may cause side effects such as allergy; it is difficult to maintain the therapeutic concentration in periodontal pockets; and there is a high possibility that microorganisms develop resistance with repeated usage (3–6). *Porphyromonas gingivalis* is a black-pigmented, gram-negative anaerobic bacterium, which is thought to be an important etiological agent in adult periodontal disease (7,8). It possesses many virulence factors (such as fimbriae, protease, hemagglutinins and capsular polysaccharide) that allow it to cause disease (7). This bacterium is known to mediate the co-aggregation of various oral gram-positive and gram-negative bacteria. In the periodontal pocket, *P. gingivalis* is found predominantly as a component of complex biofilms containing multiple species that colonize specific surfaces and result in interactive nutritional chains (9).

Recently, light energy, including various lasers, has been applied in the field of periodontal therapy. Laser is an artificial light that has certain unique properties, namely high monochromaticity, coherence and directionality, compared with ordinary sources of light, although both are electromagnetic radiations. High-power lasers, which are strongly bactericidal, have been successfully employed as an adjunct tool for the past two decades (10–12). However, they may also cause excessive thermal denaturation, in the form of carbonization, coagulation and melting, on the tooth structures and on periodontal and pulpal tissues during irradiation (10–13).

Quite recently, a new treatment approach using light energy – antimicrobial photodynamic therapy (a-PDT) – based on a photochemical reaction was introduced for effective elimination of periodontal infection. a-PDT is a noninvasive treatment procedure that uses low-level light energy to activate a photosensitizing agent in the presence of oxygen, leading to the production of reactive oxygen species that cause irreparable biological damage to bacterial or target cells (14–17). a-PDT has three components – visible harmless light, a nontoxic photosensitizer and oxygen – and does not produce any resistance in microorganisms (18).

In the field of periodontal therapy, the antimicrobial effect of PDT has been studied and clinically applied using the combination of a red-colored diode laser light and a blue dye such as toluidine blue (TB) or methylene blue (17). On the other hand, considering the wavelength property, blue light would be more promising as the light source in a-PDT than the red light because the blue light itself exhibits an antimicrobial effect (9,19–22). Also, compared with the diode laser, the light-emitting diode (LED) would be more advantageous as a light source in clinical application in terms of the lower device cost.

The aim of this *in-vitro* study was to investigate the antimicrobial effect of a high-power blue-colored LED and that of red-dye agents, separately and in combination, as a novel a-PDT therapy on one of the major periodontopathic bacteria, *P. gingivalis* ATCC 33277.

Material and methods

Light apparatus

The following devices were used in this study as nonlaser light sources: a high-power blue LED (BL) (a device modified from Flashmax2™; CMS Dental, Copenhagen, Denmark) (wavelength 425–500 nm, approximate power density 4 W/cm^2) and a high-power red LED (RL) (Fotosan™; CMS Dental) (wavelength 625–635 nm, power density 2 W/cm^2, a light source used in the commercially available Fotosan kit for a-PDT). The apparatus was fixed onto a stand so that the light-emitting end (6.8 mm in diameter) of the LED apparatus was positioned to correspond with the opening of a well (6.8 mm in diameter) in a 96-well plate during irradiation.

Preparation of bacterial suspension

P. gingivalis ATCC 33277 was maintained on brucella agar plates (supplemented with 50 mL/L of horse blood, 5 mg/L of hemin and 50 μg/L of vitamin K_1) at 37°C under anaerobic conditions. A loopful of the bacterium was inoculated into 9 mL of brain–heart infusion medium (supplemented with 5 mg/L of hemin and 50 μg/L of vitamin K_1) and cultured anaerobically for 24 h at 37°C. Immediately before the experiment, the bacterial suspension was diluted and the concentration was adjusted to 1×10^8 cells/mL using a counting chamber.

Preparation of dye and other solutions

Three different red-dye agents – erythrosine (ER), phloxine B (PB) and rose bengal (RB) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) – were employed. Solutions of all three dyes were freshly prepared before each experiment using sterile physiologic saline. Also, two different commercially available dental plaque-disclosing agents were used: Red Dye A [Dent liquid plaque tester® (previous version, not on sale at present); Lion Dental Products Co., Ltd., Tokyo, Japan] and Red Dye B (Butler GUM Red-Cote®, Dental disclosing solution; Sunstar Americas, Inc., Chicago, IL, USA). Both agents contained the same red-colored dye, PB, at undisclosed concentrations. Twenty per cent chlorhexidine gluconate (CHG) solution (Wako Pure Chemical Industries, Osaka, Japan) was

diluted with sterile distilled water to obtain solutions of 0.72% and 1.2% so that the final concentration after mixing with the bacterial suspension was 0.12% and 0.2%, respectively (which are the standard concentrations used in mouthwash). TB powder (Sigma-Aldrich, St Louis, MO, USA) was dissolved in saline to obtain a concentration of 0.1 mg/mL, which is equivalent to the concentration of TB solution commercially available in the Fotosan a-PDT kit.

Experiment 1: antimicrobial effect of high-power BL and RL on *P. gingivalis*

Eight groups were employed in this experiment: one nonirradiated control group; three BL irradiation groups and two RL irradiation groups with different time-periods of irradiation; one positive control group of conventional a-PDT using TB + RL; and a control group of TB only. For each group, a 50-μL aliquot of the bacterial suspension was placed in a well of a sterile 96-well flat-bottom plate and mixed with either 10 μL of reduced dilution buffer (23) for the nonirradiated control group and the BL and RL irradiation groups or 10 μL of TB solution (0.1 mg/mL, the final concentration in the mixed solution 16 μg/mL) for TB + RL and TB only groups. In the TB + RL and TB only groups, the mixed solution was allowed to stand for 1 min to permit the uptake of dye by bacteria before irradiation or culture. In the LED irradiation groups, the mixed solution was irradiated with BL for 30, 60 and 90 s (total power density approximately 30, 60 and 90 J/cm^2, respectively) or with RL for 60 and 120 s (total power density approximately 30 and 60 J/cm^2, respectively). The TB + RL group was exposed to 60 s of RL irradiation (30 J/cm^2). Each bacterial suspension was individually exposed to LED irradiation after preparation of the suspension in each well. The LED irradiation was performed with the light-emitting end in direct contact with the entrance of the well. The distance between the top surface of the mixed bacterial suspension and the light-emitting end was 7.5 mm and the depth of the mixed bacterial suspension was 2.5 mm. The actual energy level at the surface of the mixed solution was approximately 365 mW and 185 mW for BL and RL, respectively (power meter: NOVA II; Optical Metrology Ltd., Jerusalem, Israel; detection head: PD 300, 3W) and the power density was calculated to be approximately 1.0 and 0.5 W/cm^2, respectively.

After treatment, 20 μL of sample from each group was serially diluted by 10^3–to 10^5– fold using dilution buffer and then 10 μL of diluted samples were plated, in triplicate, on brucella agar plates. The experiment was performed under aerobic conditions and all procedures were completed within 30 min. During the experiment, a tube containing the original bacterial suspension was kept in a glass container with crushed ice. After anaerobic incubation for 7 d at 37°C, the numbers of colony-forming units (CFUs) were determined. The experiment was repeated five times, independently.

Experiment 2: antimicrobial effect of red-dye agents

The antimicrobial effect of red-dye agents on *P. gingivalis* was investigated and the optimal concentration of the dye for a-PDT in combination with BL was estimated. Twelve groups were employed in this experiment: one untreated control group; two groups of commercially available dental plaque-disclosing agents; and nine groups of the experimental red-dye agents ER, PB or RB, at three different concentrations each.

For each group, a 50-μL aliquot of fresh *P. gingivalis* suspension was placed in a well of a sterile 96-well flat-bottom plate and mixed with 10 μL of dilution buffer (the untreated control) and 10 μL of Red Dye A (Dental liquid plaque tester®) or 10 μL of Red Dye B (Butler GUM Red-Cote®) (both contain PB) at the original concentration for plaque-disclosing agent groups, or with 10 μL of the experimental red dyes RB, ER or PB (1, 10 and 50 mg/mL of each dye solution; the final concentration: 0.16, 1.6 and 8.3 mg/mL, respectively). The mixed solutions were allowed to stand for 1 min. Finally, these solutions were separately serially diluted with dilution buffer, plated onto brucella agar plates in triplicate and incubated anaerobically. The numbers of CFUs were determined. The experiment was repeated five times independently.

Experiment 3: antimicrobial effect of BL in combination with ER, PB and RB

The antimicrobial effect of BL in combination with red-dye agents was investigated. ER, PB and RB solutions were prepared at 1 mg/mL in normal saline. Eight groups were employed in this experiment: one untreated control group; six groups of ER, PB or RB with/without BL irradiation; and one group of BL only.

In each group, 50 μL of bacterial suspension was placed in a well of a sterile 96-well flat-bottom plate, mixed with 10 μL of dilution buffer for control and BL-only groups or with 10 μL of each dye solution (1 mg/mL; the final concentration was 0.16 mg/mL) for ER, PB and RB groups, and allowed to stand for 1 min. One of the two wells treated with each dye solution or dilution buffer was irradiated (30 J/cm^2) with BL for 30 s. Finally, each group was serially diluted, plated in triplicate onto brucella agar plates and incubated anaerobically.

Experiment 4: antimicrobial effect of BL in combination with graded concentrations of RB

The antimicrobial effect of PDT with different concentrations of RB solutions was examined. Thirteen groups were employed in this experiment: one untreated control group; eight groups of RB at four different concentrations with/without BL irradiation; one group of BL irradiation; two groups of CHG (positive controls); and another positive control of conventional a-PDT with TB + RL.

In each group, 50 μL of bacterial suspension was placed in a well of a sterile 96-well flat-bottom plate and

then mixed with dilution buffer (control group), with each concentration of RB solutions (0.001, 0.01, 0.1 and 1 mg/mL; the final concentrations were 0.16, 1.6, 16 and 160 μg/mL, respectively) for the RB with/without BL groups, with each concentration of CHG (0.72% and 1.2%, the final concentrations were 0.12% and 0.2%) for the CHG groups or with TB solution (0.1 mg/mL, the final concentration was 16 μg/mL) for the TB + RL group and allowed to stand for 1 min. One of the two wells mixed with RB solution at each concentration was exposed to BL for 30 s (30 J/cm^2). One well mixed with TB was irradiated with RL for 60 s (30 J/cm^2). Finally, each group was serially diluted, plated in triplicate onto brucella agar plates and incubated anaerobically.

Experiment 5: temperature measurement of the bacterial suspension during LED irradiation

A thermocouple (Digital Thermometer IT-2000; Iuchi Seieido, Osaka, Japan) was used to measure the degree of temperature elevation of the bacterial suspension during the BL (30–90 J/cm^2) and RL (30–60 J/cm^2) irradiation and a-PDT using 30 J/cm^2 BL with RB (0.001–1 mg/mL). Before and immediately after irradiation, a thermocouple was inserted into the suspension and the temperature at the vertical and horizontal middle of the suspension was measured. Temperature measurement was performed in five independent experiments.

Statistical analysis

For each experiment, data are presented as mean ± standard deviation of five independent experiments. All statistical analyses were performed using JMP® 9.0.3 (released by SAS Institute Inc., Cary, NC, USA). One-way analysis of variance was used for all the group comparisons except for experiment 2 (Table 1). Post-hoc Dunnett's test was used to compare differences between each group and control, except for experiments 2 (Table 2), 4 (Fig. 4), and 5 (Tables 3 and 4) where Tukey's test was performed to compare the differences among all the groups. In experiment 2 (Table 1), two-way analysis of variance was performed followed by Tamhane to compare the factor of dye, factor of concentration and the interaction between the two factors. A p value of < 0.05 was considered significant.

Results

Experiment 1: effect of high-power BL and RL on *P. gingivalis*

BL irradiation at 60 and 90 J/cm^2 showed a similar weak, but statistically significant, antimicrobial effect with a 1.1 and 1.2 log reduction in bacteria compared with the control ($p < 0.05$). There was almost no effect of RL at 30 and 60 J/cm^2. a-PDT with RL (30 J/cm^2) and TB (16 μg/mL) showed a moderate effect, with a 2.9 log reduction in bacteria compared with the control ($p < 0.0001$) (Fig. 1).

Experiment 2: effect of red-dye agents

The antimicrobial effect of red dyes on the viability of the *P. gingivalis* suspension is shown in Tables 1 and 2. Dyes A and B used at the commercially available concentration resulted in 7.0 log ($p < 0.0001$) and 1.7 log ($p < 0.05$) reductions of bacteria, respectively, compared with the control (Table 2). All three experimental red-dye agents showed low to high

Table 1. Effect of red-dye agent

		Bacterial count ($\log_{10}$ CFU/mL)			
		Red-dye agent			Control
Dye concentration (mg/mL)	Final concentration (mg/mL)	ER	PB	RB	Dilution buffer
1	0.16	8.5 ± 0.9	8.7 ± 0.8	7.9 ± 1.4	
10	1.6	8.0 ± 0.8	7.6 ± 0.4	6.0 ± 1.2	8.1 ± 0.3
50	8.3	7.4 ± 0.6	3.8 ± 2.4	1.7 ± 1.7	

Results are given as mean ± standard deviation.
CFU, colony-forming units; ER, erythrosine; PB, phloxine B; RB, rose bengal.
The factor of dye concentration and the interaction between the two factors were statistically significant at $p < 0.0001$.

Table 2. Effect of plaque-disclosing agent

	Bacterial count ($\log_{10}$ CFU/mL)		
	Red-dye agent		Control
Group	Dye A	Dye B	Dilution buffer
	1.1 ± 1.4	6.5 ± 0.3	8.1 ± 0.3
	***	***	
	*		

Results are given as mean ± standard deviation.
Dye A: Dental liquid plaque tester®. Dye B: Butler GUM Red-Cote®.
CFU, colony-forming units
Statistically significant differences among experimental groups ($n = 5$) are shown as *($p < 0.05$) and ***($p < 0.0001$).

Table 3. Temperature elevation after blue and red light-emitting diode (LED) irradiation

	BL			RL	
				TB (−)	TB (+)
Energy density (J/cm^2)	30	60	90	30	30
Temperature rise (ºC)	3.8 ± 0.1	6.4 ± 0.7	8.7 ± 0.9	1.9 ± 0.2	7.2 ± 0.2
	***	***	***	***	

Results are given as mean ± standard deviation.
BL, blue LED; RL, red LED; TB, toluidine blue.
Statistically significant differences among experimental groups ($n = 3$) are shown as ***($p < 0.0001$).

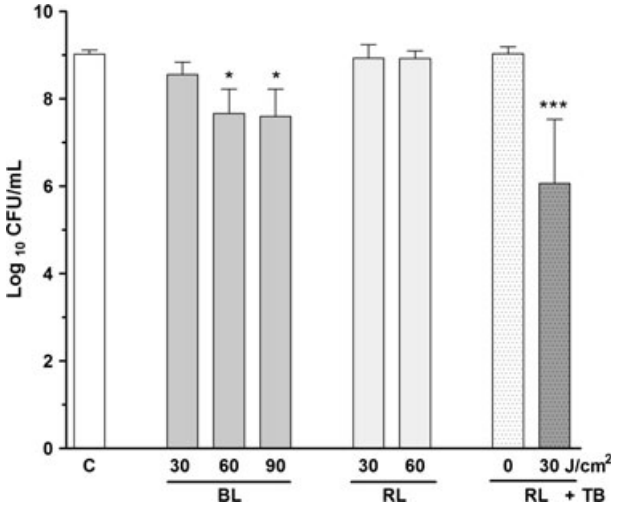

Fig. 1. Effect of blue light-emitting diode (BL) and red light-emitting diode (RL) on *Porphyromonas gingivalis* viability. *P. gingivalis* suspension was irradiated with BL at 30–90 J/cm^2 (1 W/cm^2, 30–90 s, respectively) or with RL at 30–60 J/cm^2 (0.5 W/cm^2, 60–180 s, respectively), or was mixed with 16 μg/mL of toluidine blue (TB), with or without RL irradiation at 30 J/cm^2. Nonirradiated bacterial suspension was employed as the control (C). Statistically significant differences between experimental groups ($n = 5$) and the control group ($n = 5$) are shown as *($p < 0.05$) and *** ($p < 0.0001$). CFU, colony-forming units.

reduction in bacteria in a dye concentration-dependent manner. The antimicrobial effect was highest for RB, followed by PB and then ER. At 160 μg/mL, the three dyes showed almost no or only a very minimal antimicrobial effect. The effect of Dye A was higher than that of PB at 8.3 mg/mL (the original dye concentration was 50 mg/mL) and the effect of Dye B was between those of PB at 1.6 and 8.3 mg/mL (original dye concentrations were 10 and 50 mg/mL). The factor of dye concentration and the interaction between the two factors were statistically significant at $p < 0.0001$ (Table 1).

Experiment 3: effect of BL in combination with ER, PB and RB

Figure 2 presents the antimicrobial effect using the combination of 30 J/cm^2 BL and 160 μg/mL of red dye on *P. gingivalis*. Alone, ER, PB, RB and BL resulted in minimal reductions of bacteria, of 0.2, 0.3, 0.6 and 0.5 log, respectively. ER and PB, in combination with BL, also showed similar, minimal, reductions of 0.9 and 1.0 log, respectively. RB + BL demonstrated a significant reduction of 7.1 log compared with the control ($p < 0.0001$) and clearly exhibited an enhanced antimicrobial effect (a-PDT effect).

Experiment 4: effect of BL in combination with graded RB

Figure 3 shows the colors of the RB solution at the original concentrations and after being mixed with the bacterial suspension, and also the conditions before and after irradiation. There were no detectable changes in the condition of the mixed suspensions following irradiation.

In Fig. 4, 30 J/cm^2 BL in combination with 1.6, 16 and 160 μg/mL of RB showed 6.3, 8.0 and 5.5 log reductions, respectively, of bacteria, with a statistically significant difference compared with the control ($p < 0.0001$). RL (30 J/cm^2) combined with 16 μg/mL of TB also demonstrated a significant, 5.2 log, reduction of bacteria ($p < 0.0001$). At 0.12% and 0.2%, CHG showed 2.2 ($p = 0.35$) and a significant 4.3 ($p < 0.001$) log reduction of bacteria, respectively. Among the experimental groups RB + BL and CHG, there was a significant difference between 16 μg/mL of RB + BL and 0.16 μg/mL of RB + BL ($p < 0.001$), between 16 g/mL of RB + BL and 0.12% CHG ($p < 0.0001$) and between 16 μg/mL of RB + BL and 0.2% CHG ($p < 0.05$). A significant difference was also observed between the PDT reactions using 1.6 and 0.16 μg/mL RB ($p < 0.0001$) and between 160 μg/mL of RB + BL and 0.12% CHG ($p < 0.05$). The combination of 16 μg/mL of RB + 30 J/cm^2 BL showed the maximal bacterial reduction among all the groups and a significantly higher reduction than in CHG groups, and there was a tendency for a higher reduction than for the 16 μg/mL TB + 30 J/cm^2 RL group.

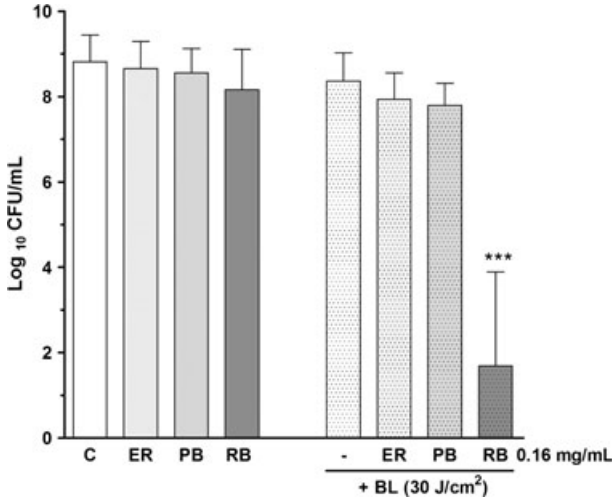

Fig. 2. Effect of blue light-emitting diode (BL) in combination with erythrosine (ER), phloxine B (PB) and rose bengal (RB). *Porphyromonas gingivalis* suspension was mixed with ER, PB or RB (1 mg/mL; the final concentration was 0.16 mg/mL) and irradiated with 30 J/cm^2 BL or not irradiated. Bacterial suspension without dye and irradiation was employed as the control (C). Statistically significant differences between experimental groups ($n = 5$) and the control ($n = 5$) are shown as *** ($p < 0.0001$). CFU, colony-forming units.

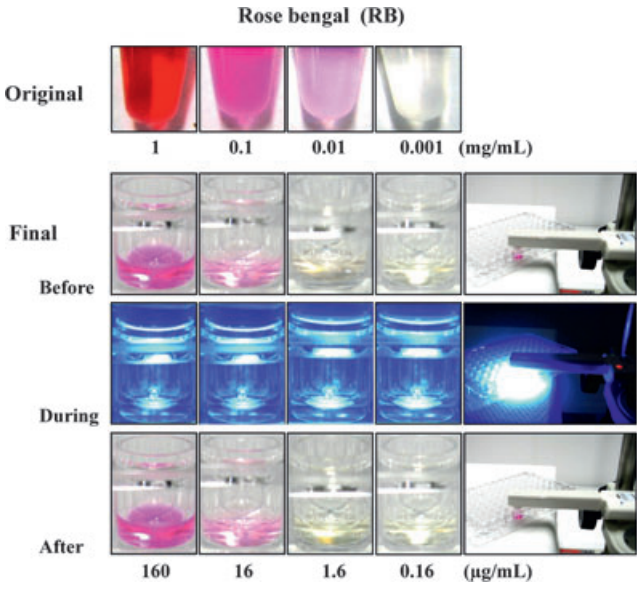

Fig 3. Color of rose bengal at the original concentrations (Original) and at the final concentrations after being mixed with the bacterial suspension (Final); and the conditions of bacterial suspension before, during and after blue light-emitting diode irradiation.

Experiment 5: temperature elevation of the bacterial suspension after LED irradiations

At room temperature (approximately 24°C), BL irradiation increased the temperature significantly in an irradiation time-dependent manner ($p < 0.0001$). There was an average elevation in temperature of 3.8, 6.4 and 8.7°C with 30, 60 and 90 J/cm^2 BL irradiation, respectively. An elevation of 1.9°C was observed with 30 J/cm^2 RL irradiation only and an elevation of 7.2°C was observed with 30 J/cm^2 RL + 16 μg/mL of TB ($p < 0.0001$) compared with 30 J/cm^2 RL irradiation only (Table 3).

In the combination of BL and RB, the temperature of the bacterial suspension was elevated in a dye concentration-dependent manner. Elevations of 3.8, 4.0, 4.3, 4.6 and 7.9°C were observed at 30 J/cm^2 BL with 0, 0.16, 1.6, 16 and 160 μg/mL of RB, respectively. In particular, the temperature showed a marked increase at 160 μg/mL of RB. There was a significant difference in temperature elevations at 1.6, 16 and 160 μg/mL RB compared with BL without RB ($p < 0.05$, $p < 0.001$ and $p < 0.0001$, respectively) (Table 4).

Discussion

The present study demonstrates that BL had a moderate antimicrobial effect on *P. gingivalis* ATCC 33277, whilst RL did not affect the bacteria. The difference in the antimicrobial/growth-inhibiting effect between BL and RL may be a result of the difference in the wavelength property of both lights. On BL irradiation, it has been speculated that endogenous porphyrin produced by bacteria is excited, leading to a photodynamic reaction through singlet oxygen production, resulting in an antimicrobial effect (24). In contrast, some *in-vitro* studies reported that red light or near-infrared light exerted no antibacterial effect (25–27).

So far, many studies have reported an antimicrobial effect with blue light. Five minutes of BL delivery (32 J/cm^2) was sufficient to reduce the viability of *Helicobacter pylori* by 99.99% (28). BL irradiation at 58.8 J/cm^2 (9.8 min) reduced the viability of methicillin-resistant *Staphylococcus aureus* US-300 by 92.1% and irradiation at 50.4 J/cm^2 (8.4 min) reduced the viability of *S. aureus* IS-853 by 93.5% (20). BL at a wavelength of 405 nm (15 J/cm^2) achieved reduction rates of 95.1% and nearly 90% for *Pseudomonas aeruginosa* and *S. aureus*, respectively (29). Regarding periodontal pathogens, BL at 94 J/cm^2 (3 min) reduced the survival rates of *P. gingivalis* and *Fusobacterium nucleatum* by 90% and 40%, respectively (25). Moreover, BL irradiation at 4.2 J/cm^2 (1 min) resulted in complete killing of *Prevotella*

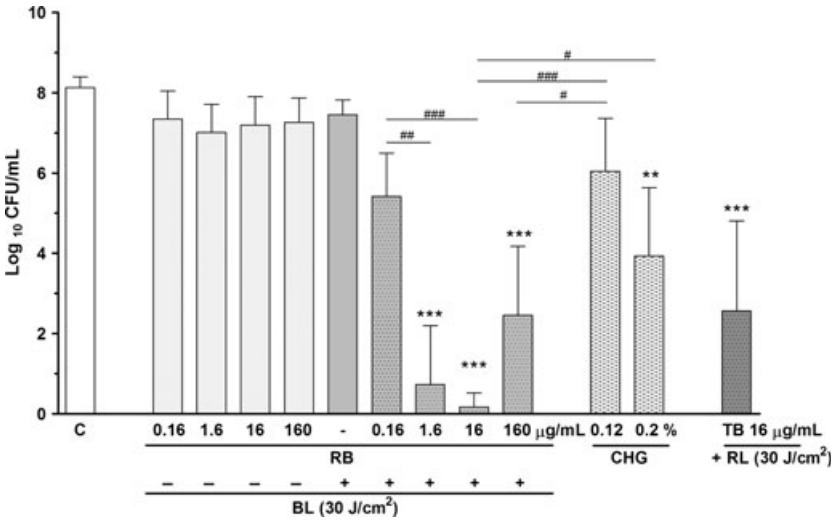

Fig. 4. Effect of blue light-emitting diode (BL) in combination with graded rose bengal (RB). Fresh *Porphyromonas gingivalis* suspension was mixed with RB (0.001, 0.01, 0.1 and 1 mg/mL; the final concentrations were 0.16, 1.6, 16 and 160 μg/mL, respectively) and irradiated with 30 J/cm^2 BL or not irradiated. Bacterial suspension incubated with toluidine blue (TB) (0.1 mg/mL, final concentration 16 μg/mL) followed by red light-emitting diode (RL) irradiation at 30 J/cm^2 was employed as the positive control. Bacterial suspension without dye and irradiation was employed as the control (C). Statistically significant differences between experimental groups and the control are shown as **($p < 0.001$) and ***($p < 0.0001$) on each bar. Statistically significant differences among the experimental groups of RB + BL, chlorhexidine gluconate (CHG) and TB + RL are shown as $^{\#}$($p < 0.05$) and $^{\#\#\#}$($p < 0.0001$). CFU, colony-forming units.

intermedia and *Prevotella nigrescens*, and irradiation at 42 J/cm^2 (10 min) reduced *P. gingivalis* by 98.5% (30). Our recent study demonstrated that BL does not kill, but just inhibits the growth of *P. gingivalis* ATCC 33277 (9).

In the present study, we used a high-power BL, expecting a higher antimicrobial effect as well as a shorter irradiation time, which are important in clinical practice. The antimicrobial effect of high-power BL on *P. gingivalis* was detected after short time periods of irradiation of 60 and 90 s, but there was only a 1 log reduction of CFUs. This level of bacterial reduction following treatment with BL alone is not sufficient for clinical application. Therefore, we further investigated the effect of a-PDT using the combination of BL with red-dye agents. Three xanthene dyes – ER, PB and RB – were selected, based on previous studies, as red-dye agents (31,32). Regarding the antimicrobial effects of the dye itself, previously PB has been reported to inhibit methicillin-resistant *S. aureus* (33). RB has also been shown to reduce the viability of all 12 strains of *Enterobacteriaceae* (32) and of planktonic *Aggregatibacter actinomycetemcomitans* (34). Therefore, in the present study, we examined, first of all, the antimicrobial effect of these dyes in order to investigate the toxicity of the red dyes and thereby determine the concentration with the lowest toxicity suitable for a-PDT. RB showed the highest antimicrobial effect on *P. gingivalis* followed by PB and ER. These results coincided with those of quite recent studies by Kato *et al.* (35) on *S. aureus* and by Ishiyama *et al.* (36) on *Streptococcus mutans*. As for the antimicrobial effect of commercially available dental plaque-disclosing agents on *P. gingivalis*, interestingly both Red Dye A and Red Dye B (both contain PB) demonstrated strong (7 log CFU reduction) and slight (1.7 log CFU reduction) effects, respectively. Therefore, the plaque-disclosing procedure in periodontal therapy may have an additional effect on bacterial elimination in the clinic.

The concentration of red dye employed for a-PDT was 160 μg/mL after being mixed with the bacterial suspension in experiment 3 because, at this concentration, the red dyes showed no or only minimal toxicity. In the analysis of a-PDT with BL, among the three dyes in our study, only RB clearly showed an enhanced antimicrobial effect. Recently, Rossoni *et al.* (32) demonstrated that a-PDT with BL and RB was more effective than a-PDT with BL and ER: a-PDT with 50 μmol/L (0.05 mg/mL) of RB and 180 s (200 mW) of irradiation resulted in the reduction of *Enterobacteriaceae* species by 6–7 log whilst a-PDT with 50 μmol/L (0.041 mg/mL) of ER did not show any reduction in the number of CFUs compared with the control (32). Quite recently, Ishiyama *et al.* (36) and Kato *et al.* (35) also showed that among the red dyes employed, RB in combination with green laser/wide-spectrum halogen light exhibited the highest a-PDT activity against *S. mutans* and *S. aureus*, respectively. The result of the present study, indicating the highest effect of a-PDT with RB on *P. gingivalis*, is in agreement with the results of the abovementioned studies.

In a-PDT, matching of the light and the dye is important. However, in the present study, the wavelength of BL (425–500 nm) did not completely match with the maximum absorption wavelength of the red dyes employed: PB (538 nm), ER (526 nm) and RB (549 nm) (35). This discrepancy between the emission wavelength of BL and the absorption wavelengths of red dyes might have influenced the results of the a-PDT effect. However, in previous studies, Ishiyama *et al.* (36) used 532-nm laser light, which matches well with the absorption wavelengths of the three dyes, and they reported that only RB exerted an a-PDT effect. Also, in the study of Kato *et al.* (35), halogen light, which produces a continuous spectrum of light from near-ultraviolet to deep into the infrared region, was employed and the result also showed

Table 4. Temperature rise after antimicrobial photodynamic therapy (a-PDT)

	BL (30 J/cm^2)				
RB final concentration (μg/mL)	0	0.16	1.6	16	160
Temperature rise (ºC)	3.8 ± 0.1	4.0 ± 0.1	4.3 ± 0.2	4.6 ± 0.2	7.9 ± 0.4
		*			
			**		
				***	

Results are given as mean ± standard deviation.
BL, blue light-emitting diode; RB, rose bengal.
Statistically significant differences among experimental groups ($n = 3$) are shown as *($p < 0.05$), **($p < 0.001$) and ***($p < 0.0001$).

that only RB enhances the a-PDT effect. Thus, the a-PDT effect is influenced by the photosensitizing ability, which, in turn, is dependent on the chemical structure of the dye agent and not on the complete matching wavelength of dye and light. In the present study, an a-PDT effect was obtained when BL and RB were combined; however, for BL, a more suitable dye with an absorption wavelength that matches well with the emission wavelength of BL might enhance the efficiency of the photochemical reaction.

The mechanism of killing of a-PDT is hypothesized to be as follows. Upon light irradiation, the dye is excited, leading to the production of cytotoxic reactive oxygen species and resulting in cell death (17). Among all the reactive oxygen species, singlet oxygen is highly cytotoxic in PDT (37,38), indicating that the greater the amount of singlet oxygen produced, the more effectively undesired cells, such as cancer cells and bacteria, are killed. However, quite recently, Ishiyama *et al.* (36) and Kato *et al.* (35) demonstrated that the ability of the dye to produce reactive oxygen species, such as singlet oxygen, is not the only factor contributing to the a-PDT effect but that the penetration efficiency of dye into the bacterial cells is also a factor. In their studies on singlet oxygen production among the red dyes, PB produced the highest amount and RB produced the lowest amount. However, RB was incorporated into the bacterial cells more efficiently compared with the other two photosensitizers, which probably led to the highest bactericidal activity observed for RB (35,36).

In the present experimental condition, the a-PDT effect increased in a dye concentration-dependent manner up to 16 μg/mL; however, the a-PDT effect decreased at the highest concentration of dye (160 μg/mL). This reverse phenomenon could be explained as follows: the 160 μg/mL concentration was so dark that the power of BL would have been reduced by being absorbed during light penetration into the red-colored solution, resulting in a decrease in the photochemical reaction.

Heat generation after light irradiation is one of the main drawbacks of light therapy (11). In the present study, in the BL-only group, there was a temperature elevation of 3.8–8.7°C at 30–90 J/cm^2. When using 30 J/cm^2 BL (30 s) in combination with RB up to a concentration of 16 μg/mL, the elevation was within 4.5°C. It is speculated that this minimal temperature elevation for a short time-period would not have any negative effect on the periodontium. In particular, if applied *in vivo*, the degree of temperature elevation would be less compared with that of the present experiment, which was performed at room temperature. Although there was a temperature elevation of 7.8°C when using 160 μg/mL of RB, this concentration is not useful because the antimicrobial effect was lower compared with the a-PDT using 16 μg/mL of RB. This result also suggests that temperature elevation is not directly associated with the death of bacteria.

RB is currently used in the medical field. It has been employed as a diagnostic tool for dry eye (39) and has been under investigation in clinical trials as a candidate for cancer therapy (40,41). Its role in photoactivated tissue bonding, a novel tissue-sealing technology that immediately cross-links proteins between tissues planes, thereby sealing on a molecular scale, has also been studied (42). In Japan, RB is approved as a food additive for red coloration. Regarding the concern of the negative effect of BL on the periodontium, a previous *in-vivo* study showed no tissue alteration following BL irradiation at 144 J/cm^2 (20 min) on rat palatal mucosa. Interestingly, tissue healing induced by LED biostimulation has also been demonstrated (43). However, further detailed studies are necessary to clarify the toxicity of BL or RB, alone, and in combination, on the periodontium in the present experimental condition before clinical application. In addition, in this study all experiments were conducted using one laboratory strain of *P. gingivalis* (ATCC 33277). The experiments should be conducted in clinical isolates and in other bacteria to determine the effects of BL and a-PDT using red dye in the real clinical situation.

Basically, the light and the dye for a-PDT should ideally not exert any toxic effect; however, if both light and dye independently possess some antimicrobial effect, a-PDT could be more effective and advantageous for disinfection in the complex environment of highly advanced deep periodontal pockets with bacterial invasion into the soft tissues. Both BL and RB have shown some antimicrobial effects on *P. gingivalis* ATCC 33277 in our study. Therefore, this novel a-PDT system can be considered as a viable alternative tool for pocket disinfection in the treatment of periodontal and peri-implant diseases. Also, this a-PDT system could effectively complement mechanical plaque control if a toothbrush is combined with BL after applying a red-colored mouth rinse.

Conclusion

Within the limits of this study, BL has an antimicrobial/growth-inhibiting effect on *P. gingivalis*, and a-PDT, using the combination of BL and RB, shows promise as a new treatment modality for bacterial elimination in periodontal therapy.

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