Effect of antimicrobial photodynamic therapy using rose bengal and blue light-emitting diode on *Porphyromonas gingivalis* in vitro: Influence of oxygen during treatment

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Aims: A combination of rose bengal (RB) and blue LED (BL) has emerged as a new technical modality for antimicrobial photodynamic therapy (a-PDT). The purpose of this study was to clarify the influence of oxygen on the antimicrobial effect of RB + BL treatment on *Porphyromonas gingivalis* in vitro.

Materials and Methods: *P. gingivalis* cells were treated with RB, BL (450–470 nm; 1 W/cm², 5 s), or RB + BL under anaerobic/aerobic conditions. Cells were incubated anaerobically, and the cell density (OD₆₀₀ nm) was measured after 6–48 h. Additionally, cells were cultured anaerobically on blood agar plates for 9 days, and the resulting colonies were observed. Bacterial growth within 1 h of aerobic RB + BL treatment was examined, and RNA degradation due to anaerobic/aerobic RB + BL treatment was measured after 3 h of culture.

Results: Under anaerobic conditions, RB + BL significantly suppressed bacterial growth after 18 h; however, the growth after 48 h and the number of colonies after 9 days were similar to those of the untreated control. RNA degradation in the anaerobic-treatment group was not significantly different from that in the control. Under aerobic conditions, RB + BL immediately affected bacterial growth and completely inhibited growth for up to 48 h. Few colonies were detected even after 9 days of culture, and RNA was completely degraded.

Conclusions: Unlike the bacteriostatic effect of anaerobic treatment, aerobic RB + BL treatment may have a bactericidal action via a-PDT effect, resulting in the destruction of RNA and bacterial cells within a short period.

Keywords: photodynamic therapy • LED • rose bengal • periodontal disease • *Porphyromonas gingivalis*

Introduction

Periodontal diseases arise from inflammatory condi-
need to be developed to address these issues in the aging population.

Conventionally, light energy, including lasers, has been applied as a treatment strategy to destroy infectious pathogenic microorganisms, and antimicrobial photodynamic therapy (a-PDT) based on a photochemical reaction was introduced in dental treatments. a-PDT is a non-invasive treatment procedure that uses low-level light energy to activate a photosensitizing agent; generally, the combination of a red-colored light and a blue dye has been applied in the field of periodontal therapy. On the other hand, considering the wavelength of light, blue light itself exhibits an antimicrobial effect, which may be advantageous in a-PDT.

Recently, the combination of a red dye and blue light emerged as a new technical modality for a-PDT, in vitro, and that a-PDT using RB and BL shows promise as a new technical modality for bacterial elimination in periodontal therapy. a-PDT has three components: visible harmless light, a nontoxic photosensitizer, and oxygen; generally, the combination of a red-colored light and a blue dye has been applied in the field of periodontal therapy. On the other hand, considering the wavelength of light, blue light itself exhibits an antimicrobial effect, which may be advantageous in a-PDT.

Preparation of the photosensitizer

RB (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), often used as a dental plaque tester in the clinical setting, was employed as a photosensitizer. RB solution was freshly prepared in deaerated sterile physiological saline before use. First, the effect of RB concentration on P. gingivalis growth was investigated. P. gingivalis was grown to the mid-log phase. The bacterial suspension (216 µL) and RB (24 µL) at 0.01%, 0.005%, or 0.001% (wt/wt) were added into the wells of a 96-well titer plate (total volume: 240 µL/well; final RB concentration: 0.001%, 0.0005%, or 0.0001% [wt/wt]). Bacterial suspension mixed with saline (24 µL) was used as a control. After treatment, all the suspensions were incubated at 37 °C in an anaerobic chamber, and the cell density (OD600 nm) was measured every 1 h for up to 12 h.

Light apparatus

A high-power blue-colored LED (BL; a device modified from Flashmax 2™, CMS Dental, Denmark; wavelength 425–500 nm, power density 4 W/cm²) was used as the non-laser light source in this study. This light source is also used for composite resin polymerization in dental caries treatment. The apparatuses were fixed onto a stand within an anaerobic chamber, so that the light-emitting end (6.8 mm in diameter) of the LED apparatus was positioned to match the top entrance of a well (6.8 mm in diameter) in a 96-well titer plate during irradiation.

BL 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 bacterial suspension and the light-emitting end was 4.5 mm, and the depth of the mixed bacterial suspension was 7.5 mm. The actual energy level on the surface of the mixed solution was approximately 480 mW (power meter: NOVA II, Optical Metrology Ltd., Jerusalem, Israel; detection head: PD 300, 3 W), and the power density was calculated to be approximately 1.5 W/cm².

LED irradiation

P. gingivalis was grown to the late-log phase. The bacterial suspension (OD600 nm = 0.1, 216 µL) and 0.001% RB (24 µL) or saline (24 µL) were added in the wells of a 96-well titer plate (total volume: 240 µL/well; final RB concentration: 0.0001% [wt/wt]) and irradiated with BL.
for 5 s or left as is (non-irradiated) under aerobic/anaerobic conditions. The treated samples were incubated at 37 °C for 6, 12, 18, 36, and 48 h in an anaerobic chamber that was impenetrable to light, and the cell density (OD600 nm) was measured.

In addition, after 18 h, each bacterial suspension was diluted 1,000 times, and 100 µL of each diluted suspension was inoculated on tripticase soy agar plates (supplemented with 50 mL/L horse blood, 5 mg/L hemin, and 50 µg/L vitamin K1) and anaerobically incubated at 37 °C for 9 days. Furthermore, in order to examine the effect of aerobic RB + BL treatment on bacterial growth over a short period, a high concentration of the bacterial suspension (OD600 nm = 0.8) was used at the start of the experiment, and the cell density (OD600 nm) was measured every 10 min for up to 60 min after treatment.

Preparation of total RNA
Stabilization of bacterial RNA, bacterial destruction, and RNA purification were carried out as described previously 7). After treatment with BL + RB under anaerobic/aerobic conditions, *P. gingivalis* was incubated for 3 h anaerobically. The cell suspensions were mixed with RNAprotect™ Bacteria Reagent (Qiagen, Tokyo, Japan) to stabilize the bacterial RNAs. Total RNAs were prepared using TRIzol™ reagent (Invitrogen, Carlsbad, CA) and the PureLink™ RNA Micro Kit (Invitrogen) according to the manufacturer’s instructions. Bacterial disruption was performed using a FastPrep FP120 Instrument (Qbiogene, Carlsbad, CA). The RNA quality was examined, and the RNA integrity number (RIN: 1–10 values) was tested for RNA degradation by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) with an RNA 6000 Nano LabChip Kit (Agilent).

Statistical analysis
Data are presented as the mean ± standard deviation (SD). One-way analysis of variance followed with the Tukey-Kramar HSD procedure as a post-hoc test was applied for comparing cell densities of test and control groups. In addition, unpaired t-test with Bonferroni correction was performed to determine whether the cell densities in the anaerobic and aerobic conditions differed. Values of $p < 0.05$ were considered significant.

Results

Effect of RB on bacterial growth
The OD of *P. gingivalis* suspension containing 0.01% RB solution (final concentration: 0.001%) reduced gradually after treatment, suggesting the occurrence of growth inhibition, and the increase of OD was significantly suppressed upon the addition of 0.005% RB (final concentration: 0.0005%), compared with the control. The OD of the suspension containing 0.001% RB (final concentration: 0.0001%) was not significantly different from that of the control, although a very slight effect on growth was observed compared with the control (Fig. 1).

Effect of a-PDT on bacterial growth
Although the OD of *P. gingivalis* suspension treated anaerobically with 0.001% RB + BL was elevated for up to 18 h post-treatment, it was still clearly suppressed in comparison with the non-treated control, while the OD of the group treated aerobically with RB + BL remained low and unchanged during the observation period, indicating that bacterial growth was completely...
inhibited (Fig. 2). Eighteen-hours after treatment, the ODs of the groups treated with either RB or BL alone were lower than that of the control group. Furthermore, significantly lower ODs were observed in the group treated with RB + BL under anaerobic/aerobic conditions ($p < 0.0001$), and the decreasing trend was more noticeable in the groups treated under aerobic conditions than in groups treated under anaerobic conditions ($p < 0.001$; Fig. 3).

Next, we investigated whether the growth inhibition effect of these treatments was bacteriostatic or bactericidal in action. To clarify this aspect, culture of <i>P. gingivalis</i> suspension was continued until 48 h after treatment. In addition, the treated bacterial suspension

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**Fig. 2:** <i>P. gingivalis</i> growth curves after RB + blue LED (BL) treatment under anaerobic/aerobic conditions. <i>P. gingivalis</i> suspension (OD$_{600}$ nm = 0.1) was treated with RB + BL anaerobically (square, solid line) or aerobically (square, dashed line) and then incubated in an anaerobic chamber. Non-treated control (circle, solid line) and control with aerobic exposure for the same period as that of the RB + BL treatment group (circle, dashed line) were also incubated anaerobically. The cell density (OD$_{600}$ nm) was measured as an indication of the bacterial growth at 6, 12, and 18 h after treatment.

**Fig. 3:** <i>P. gingivalis</i> growth at 18 h after various treatments. <i>P. gingivalis</i> was treated with RB, BL, or RB + BL anaerobically or aerobically and then incubated in an anaerobic chamber. Control with aerobic exposure for the same period as that of the RB + BL treatment group was also incubated anaerobically. The cell density (OD$_{600}$ nm) was measured at 18 h after treatment. Data are presented as the mean ± standard deviation (SD; n = 9). **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$ (one-way analysis of variance [ANOVA] followed by Tukey-Kramer HSD procedure as post-hoc). #: $p < 0.001$ (between the aerobically and anaerobically treated RB + BL groups, unpaired t-test with Bonferroni correction).
was inoculated on a blood agar plate and cultured for 9 days. All groups, except for the aerobically treated RB + BL group reached the stationary phase at 36 h post-treatment. The lowest OD value was observed in the group treated aerobically with RB + BL (data not shown), and therefore, it was monitored until 48 h after treatment, and the same results, with no change in the aerobic RB + BL treatment, were confirmed at 48 h post-treatment (Fig. 4). On the blood agar plates, after 9 days, few colonies were observed in the aerobic RB + BL treatment group, compared with the non-treated control and the anaerobic RB + BL treatment group, both of which showed numerous colonies (Fig. 5).

To further verify the bactericidal effect of a-PDT, a-PDT with blue LED + red dye

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**Fig. 4:** *P. gingivalis* growth at 48 h after various treatments. *P. gingivalis* was treated with RB, BL, or RB + BL anaerobically or aerobically and then incubated in an anaerobic chamber. The cell density (OD600 nm) was measured at 48 h after treatment. Data are presented as the mean ± SD (n = 3). *: p < 0.05, ****: p < 0.0001 (one-way analysis of variance [ANOVA] followed by Tukey-Kramer HSD procedure as post-hoc). #: p < 0.001 (between the aerobically and anaerobically treated RB + BL groups, unpaired t-test with Bonferroni correction).

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**Fig. 5:** Colony formation on a blood agar plate after 9 days. *P. gingivalis* was treated with RB + BL anaerobically or aerobically and then incubated for 9 days in an anaerobic chamber. Non-treated control was also incubated anaerobically.
we prepared a high-density *P. gingivalis* suspension (OD600 nm = 0.8) and measured the short-term change of OD values after aerobic RB + BL treatment (Fig. 6). The OD reduced immediately after treatment and reached a plateau with a value of under 0.2 after 40 min, suggesting destruction of cells, in contrast to the increasing tendency of the OD in the non-treated control (where the OD finally reached 1.0).

**Investigation of bacterial viability**

Degradation of total RNAs was examined by determining the RIN using samples prepared after 3 h of incubation post treatment with RB + BL. The RNA fragments were analyzed by microcapillary electrophoresis, and typical peaks for 23S, 16S, and 5S rRNA were confirmed in the control (A) and anaerobic RB + BL treatment (B) groups; however, no peaks of rRNAs were detected in the aerobic RB + BL treatment group (C; Fig. 7).

The RINs are valued from 1 to 10, with a value of 10 indicating a perfect RNA sample without any degradation products, whereas a value of 1 indicates a completely degraded sample. In this study, the RIN values of the anaerobic non-treated control (A) and the anaerobic RB + BL treatment group (B) were 9.3 and 8.1, respectively. The RIN value of the aerobic RB + BL treatment group (C) could not be measured because of excessive degradation.

**Discussion**

*P. gingivalis* is a black-pigmented, Gram-negative anaerobic bacterium believed to be an important etiological agent in adult periodontal disease. Many virulence factors, such as fimbriae, protease, hemagglutinins and capsular polysaccharide, allow this organism to cause disease. This bacterium is known to mediate the coaggregation 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, which colonize specific surfaces and result in interactive nutritional chains. The regulation of this bacterium may thus lead to biofilm regulation, and may thus serve as a strategy for improving or maintaining gingival health.

Lately, a-PDT has been applied in bacterial elimination for the treatment of periodontal pockets in periodontal therapy. Currently, a-PDT in combination with blue dye agents, such as methylene blue and toluidine blue, and red diode laser or LED, is employed. Recently, we focused on the antimicrobial effect of blue lights, and analyzed the suitability of RB as a photosensitizer and their combined effects on a-PDT. RB has been approved as a food additive for red coloration in Japan and is one of the dyes used for dental plaque disclosing. Ishiyama et al. and Kato et al. showed that, among the three red dyes...
employed as plaque disclosing agents (erythrosine, phloxine, and RB), RB exhibited the highest a-PDT activity. BL is usually employed in daily dental practice as a photopolymerization device of resin materials used for dental caries treatment. Our group had previously investigated the effect of BL on *P. gingivalis* in anaerobic condition and demonstrated that BL has a growth-inhibiting effect on *P. gingivalis* owing to a bacteriostatic action mediated by the suppression of genes associated with chromosomal DNA replication and cell division at the transcriptional level.

Additionally, we reported the excellent antimicrobial...
effect of a-PDT using a combination of RB and BL against *P. gingivalis* under aerobic conditions. However, the precise effects of each component, as well as the combined effect under the presence and absence of oxygen, remained unclarified.

Therefore, in the present study, the antimicrobial effects of RB, BL, or RB + BL on *P. gingivalis* under anaerobic and aerobic conditions were investigated, and the influence of oxygen during the a-PDT procedure with a combination of BL and RB was clarified. RB or BL alone inhibited bacterial growth to similar extents, and the growth inhibition was more significant upon combined treatment with RB + BL than with RB or BL individually. Moreover, the effect was more significant under aerobic conditions than under anaerobic conditions at 18 h post-treatment. In the anaerobic condition, although RB + BL application significantly retarded *P. gingivalis* growth for up to 18 h after treatment, the bacterial growth continued and recovered to the same level as that of the non-treated control after 48 h, suggesting that the effect may be temporal bacteriostatic action. On the other hand, under aerobic conditions, RB + BL application did not increase the cell density for up to 48 h, and very few bacterial colonies were formed even after 9 days. Thus, aerobic RB + BL treatment almost completely inhibited bacterial growth, suggesting bactericidal action.

In order to confirm this finding, we performed the following two experiments. First, we investigated the change in bacterial growth after irradiation under aerobic conditions by using a high-density bacterial suspension. Secondly, we extracted RNA from *P. gingivalis* after RB + BL treatments and examined the quality of RNA by determining the RIN values. Immediately after RB + BL application under aerobic conditions, the cell density decreased rapidly and reached a plateau (<2.0) at 40 min post-treatment (the value of 2.0 may be attributable to the bacterial debris remaining after bacterial destruction). This suggested that the destruction of bacterial cells was completed within 40 min after treatment. Furthermore, after aerobic RB + BL application, the primary peaks of rRNA were completely diminished, suggesting that the bacterial cells may have lysed completely. On the other hand, the quality of RNA following anaerobic RB + BL application showed no remarkable change in comparison with the non-treated control. Thus, it may be concluded that the effect of RB + BL treatment under anaerobic conditions was bacteriostatic, similar to the effect of RB or BL treatment individually, and that the effect of the aerobic treatment was bactericidal, probably because the a-PDT effect occurred in the presence of oxygen.

Regarding the current clinical applications of a-PDT in periodontal pocket therapy, Sgolastra et al. reported that a-PDT used adjunctive to conventional scaling and root planning (SRP) provided short-term benefits in terms of probing depth reduction and attachment level. In addition, Sculean et al. reported that, in patients with chronic periodontitis, the combination of SRP and a-PDT may result in substantial short-term clinical improvements, as evidenced by probing depth and bleeding on probing reductions compared with SRP alone. Therefore, the positive effect of a-PDT is still limited to a short term, suggesting that the antimicrobial effect may be clinically weak.

Various reasons may account for the minor clinical effects of a-PDT, such as the power and irradiation time of lights, concentration of dyes, and combination of dyes and lights, etc. Periodontal pockets become more anaerobic with the deepening of the pockets, and thus, the oxygen-deficient conditions could potentially be one of the causes for the lowered effectiveness of a-PDT in the pockets. The original amount of oxygen contained in the dye solution might also be insufficient for effective a-PDT. Therefore, considering the results of the present study, intentionally supplying oxygen into the periodontal pockets by modifying the dye solution during the a-PDT procedure might be an effective strategy for enhancing the antimicrobial effects of PDT in periodontal pockets. On the other hand, in the case of preventive application of a-PDT, such as in dental plaque control, there is no concern about the presence of oxygen, since a-PDT is performed in the oral cavity under aerobic conditions. Thus, further analysis is required to determine the optimal oxygen concentration in the clinical application of a-PDT in the periodontal pockets.

In conclusion, the effect of RB + BL treatment under anaerobic conditions was bacteriostatic while that under aerobic condition became bactericidal by exerting the a-PDT effect. Furthermore, after RB + BL application under aerobic conditions, destruction of RNA as well as bacterial cells occurred within a short period.
References


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