Bactericidal Effect of Antimicrobial Photodynamic Therapy Using Visible Light-responsive Titanium Dioxide -the First Report-

Chiaki Komine,¹ Chitaka Takahashi,² Hiroko Omori,¹ Yuki Ogura,¹ Yoshimi Konishi,¹ Hidenori Suzuki,¹ Otsuka Issei,³ Mana Fuchigami,¹ Osamu Tsuzukibashi,¹ Akira Fukatsu,¹ Yasuhisa Tsujimoto,⁴ and Masahiko Fukumoto¹

¹Division of Laboratory Medicine for Dentistry, Department of Oral Health Science, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271–8587, Japan

Departments of ²Endodontics, ³Operative Dentistry and ⁴Advanced Dental Treatment & Microscopic Dentistry, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271–8587, Japan

Article History

Received 15 July 2019

Accepted 10 September 2019

Abstract

Background and aim : The aims of the present study were to investigate the relationship between ¹O₂ generated via rutile-TiO₂ and its bactericidal effect on oropathogenic microorganisms, and to evaluate whether antimicrobial photodynamic therapy (a-PDT) via rutile-TiO₂ is applicable to dental treatment.

Materials and methods : The experimental groups were defined as follows: only physiological saline without light-emitting diode(LED)irradiation; control group, physiological saline with LED irradiation; LED group, physiological saline including TiO₂(4 mg/mL)with LED irradiation; and the photocatalyst(PC)group. The LED irradiation time periods were set to 1, 2 and 3 min. A control group was placed without irradiation at room temperature. Reactive oxygen species and the amount of ¹O₂ generated were measured. A representative bacterial species selected from each oral infectious disease was used for bactericidal testing, including *Streptococcus mutans*(*S. mutans*), *Enterococcus faecalis*(*E. faecalis*), *Porphyromonas gingivalis*(*P. gingivalis*), *Aggregatibacter actinomycetemcomitans*(*A. actinomycetemcomitans*), and *Tannerella forsythia*(*T. forsythia*). The colony-forming units per milliliter(CFU/mL)present after incubation was examined by experimental group, and the relative relationships between the amount of ¹O₂ generated and the various sterilization rates were examined.

Results : Whereas *S. mutans* and *E. faecalis* were hardly sterilized in all groups, there were remarkable bactericidal effects in the LED group and PC group for *A. actinomycetemcomitans*, *T. forsythia*, and *P. gingivalis*.

Conclusion : It appears that, by controlling the amount of ¹O₂, one can select the bactericidal effect, and theses findings contribute to the development of an evidence-based medicine approach to a-PDT.

Keywords :

antimicrobial photodynamic therapy, singlet oxygen, rutile type titanium dioxide, 405 nm LED, electron spin resonance

Introduction

Since the discovery of penicillin, the use of antibiotics has increased dramatically in the medical and dental fields. In the dental field, many studies have reported that antibiotics are very effective for marginal(1)or apical periodontitis(2), abscesses(3), and osteomyelitis(4). However, the inappropriate use of antibiotics may result in

the development of resistant bacteria(5). Multiple drug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus*(MRSA)and vancomycin-resistant *Staphylococcus aureus*(VRSA)have already become a problem due to the abuse of antibiotics. In addition, the increased number of elderly people with systemic diseases may also limit their use. Therefore, it is necessary to use antibiotics carefully, and developing a sterilization method that causes no damage to the body and possesses selective bactericidal properties is highly desired.

Correspondence to :

Chiaki Komine

E-mail : komine.chiaki@nihon-u.ac.jp

Fig. 1. Difference in band gap energy due to photoreaction and crystal structure of TiO_2

Recently, antimicrobial photodynamic therapy (a-PDT) using singlet oxygen ($^1\text{O}_2$) has gained significant attention (6, 7). $^1\text{O}_2$ is one of the very interesting reactive oxygen species (ROS), because exogenous $^1\text{O}_2$ is minimally toxic to eukaryotic cells, but toxic to prokaryotic cells (8, 9). Moreover, $^1\text{O}_2$ can be sterilized without producing resistant bacteria. $^1\text{O}_2$ is not normally present, but it is generated by irradiating photosensitizers or photocatalysts such as methylene blue (10), toluidine blue (11), or titanium dioxide (TiO_2) (12) with an excitation wavelength.

TiO_2 has also been studied for tooth bleaching (13, 14) and application to denture base surfaces for antimicrobial purposes (15, 16). Also besides, recent studies have reported the promotion of osseointegration by applying the ROS generated in a photofunctionalization reaction to remove carbon accumulated in TiO_2 formed on the surface of the implant body by ultraviolet (UV) irradiation (17, 18).

TiO_2 has two crystal structures, the anatase and rutile. In general, anatase type TiO_2 (anatase- TiO_2) is used as a photocatalyst, but its excitation wavelength is in the UV region (< 387 nm, band gap energy: 3.2 eV). Thus, there is concern about the use of a-PDT via anatase- TiO_2 due to the potential adverse effects of UV light on the human

body. On the other hand, the excitation wavelength of rutile type TiO_2 (rutile- TiO_2) is in the visible light region (< 413 nm, band gap energy: 3.0 eV) (Fig. 1). Therefore, our previous study reported the safe conditions that optimally generate $^1\text{O}_2$ from TiO_2 by a-PDT. That is, the irradiation of rutile- TiO_2 using a 405-nm light-emitting diode (LED) can be the best a-PDT condition, because visible light is more desirable than UV light from the perspective of patient safety. Moreover, since no hydroxyl radical, which is the most oxidative ROS, was generated directly from irradiated rutile- TiO_2 , it was also confirmed that a-PDT via rutile- TiO_2 could be safely applied to dental treatment (19).

The aims of the present study were to investigate the relationship between $^1\text{O}_2$ generated via rutile- TiO_2 and its bactericidal effect on oropathogenic microorganisms, and to evaluate whether a-PDT via rutile- TiO_2 is applicable to dental treatment.

Materials and Methods

Materials and light source

TiO_2 (rutile type, particle size of 5 μm) and 2,2,6,6-tetramethyl-4-piperidone (4-oxo-TMP) hydrochloride were

Fig. 2. $^1\text{O}_2$ -specific oxidation from 4-oxo-TMP to 4-oxo-TEMPO
 4-oxo-TEMPO was detected by ESR.
 The production of 4-oxo-TEMPO represents the $^1\text{O}_2$ generated by a-PDT.

purchased from Wako Pure Chem. Ind. Ltd. (Osaka, Japan), and 2,2,6,6-tetramethyl-4-piperidone-N-oxyl (4-oxo-TEMPO) was purchased from Sigma Aldrich (St. Louis, MO). All other reagents were of analytical grade. The LED light source (405 nm, 8.69 mw/cm²) was obtained from J. Morita Mfg. Corp. (Kyoto, Japan).

Detection of ROS

In this study, the experimental groups were defined as follows: only physiological saline without LED irradiation; control group, only physiological saline with LED irradiation; LED group, physiological saline including TiO₂ (4 mg/mL) with LED irradiation; and the photocatalyst (PC) group. The LED irradiation time periods were set to 1, 2 and 3 min. A control group was placed without irradiation at room temperature.

The measurement of ROS was performed according to our previous report (20). In brief, a suspension of the above groups (100 μL) was mixed with 4-oxo-TMP (100 μL , 40 mM), and the mixtures were immediately irradiated for 1, 2, and 3 min by LED in the 96-well plate (Fig. 2). All solutions were freshly prepared, mixed, and immediately transferred into a flat quartz cell and then measured using an Electron Spin Resonance spectrometer (ESR, JES JFA-200, JEOL, Tokyo, Japan). The ESR measurements were conducted under the following conditions: magnetic field, 330 $\pm$ 5 mT; modulation width, 0.05 mT; time constant, 0.03 sec; microwave frequency, 9.420 GHz; microwave power, 4 mW; sweep width, 5 mT; sweep

time, 2 min; and amplitude, 500. Signal intensities were normalized to a MnO marker, and the concentrations of stable radical products were determined using an external standard based on the signal height (21).

Finally, the amount of $^1\text{O}_2$ generated was evaluated according to the $^1\text{O}_2$ -specific oxidation of 4-oxo-TMP to 4-oxo-TEMPO, which is detectable with ESR (Fig. 2).

Bactericidal test

A representative bacterial species selected from each oral infectious disease was used for bactericidal testing. The stock culture strains of five bacterial species were obtained from the American Type Culture Collection (Manassas, VA) and the Japan Collection of Microorganisms Riken Bioresource Center (Wako, Japan). That is, *Streptococcus mutans* (*S. mutans*, JCM 5705) was used as a caries-causing bacteria, *Enterococcus faecalis* (*E. faecalis*, JCM 5803) was used as a refractory apical periodontitis-related bacteria, and *Porphyromonas gingivalis* (*P. gingivalis*, ATCC 33277), *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*, ATCC 33384), and *Tannerella forsythia* (*T. forsythia*, JCM 10827) were used as periodontitis-related bacteria in this study.

S. mutans, *E. faecalis*, and *A. actinomycetemcomitans* were maintained by cultivating them on BactTM Brain Heart Infusion (BHI, Becton, Dickinson and Co., Sparks, MD, USA) and 1.5% agar (BHI agar). These organisms were cultured at 37°C overnight in an atmosphere of 5% CO₂ in a CO₂ incubator (NAPCO[®] Model 5400; Precision

Scientific, Chicago, IL, USA).

P. gingivalis, and *T. forsythia* were maintained by cultivating them on Anaerobic Blood Agar (CDC), which has a Tryptic soy agar (Becton, Dickinson and Co., Sparks, MD, USA) base supplemented with vitamin K₁ (10 µg/ml), hemin (5 µg/ml), L-cysteine (800 µg/ml), 0.5% yeast extract, and 5% sheep blood. Moreover, N acetyl muramic acid (Sigma-Aldrich Co. LLC., Tokyo, Japan) disk was put in the center of the CDC plate for good growth of *T. forsythia*. These organisms were cultured at 37°C for 72 h in an anaerobic condition using a gas pack system (Anaero-Pack; Mistubishi Gas Chemical Company, Inc., Tokyo, Japan). Each cultured bacterium was collected by centrifugation (10,000 rpm, 15 mins) and washed twice with physiological saline. The suspension was then adjusted to 2×10^8 cells/mL. As well as detection of ROS, the experimental groups were the control, LED, and PC groups, but each bacterial suspension was used instead of 4-oxo-TMP. That is, a suspension of the above groups (100 µL) and a suspension of each bacterium (100 µL) were mixed in a 96-well plate. Immediately after mixing in the 96-well plate, the mixtures were irradiated for 3 min with a 405-nm LED. A control group was placed without irradiation at room temperature. After the irradiation was complete, 10-fold serial dilutions were prepared, and for *S. mutans* and *E. faecalis*, 100-µL aliquots of each dilution were seeded in duplicate onto Mitis-salivarius agar (Difco, Detroit, MI) plates and incubated for 48 h at 37°C. On the other hand, for *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythia*, 100-µL aliquots of each dilution were seeded in duplicate onto the BHI agar described above. Finally, the colony-forming units per milliliter (CFU/mL) present after incubation was determined. Furthermore, the relative relationships between the amount of ¹O₂ generated and the various sterilization rates were examined.

Results

Detection of ROS

The ESR spectra of the PC group displayed a 1 : 1 : 1 triplet signal characteristic of 4-oxo-TEMPO having a hyperfine splitting constant ($a_N = 1.608$ mT), and it increased depending on the irradiation time. However, the control and LED groups did not show any of the spin adduct (Fig. 3). This means that only the PC group generated ¹O₂ efficiently. Furthermore, according to the equation

of the linear relationship between the amount of 4-oxo-TEMPO and the LED irradiation time, the amount of ¹O₂ generated from the PC group during 1, 2, and 3 min of irradiation was 5.5, 11.7, and 14.9 µmol, respectively (Fig. 4).

Bactericidal testing

The bactericidal effects on *S. mutans* and *E. faecalis* were minimal in all groups. On the other hand, *A. actinomycetemcomitans* and *T. forsythia* showed bactericidal effects in the LED group and the PC group. That is, *A. actinomycetemcomitans* showed bactericidal effects of >99.8% in the LED group and >99.999% in the PC group, with *T. forsythia* >99.999% in the LED group and 100% in the PC group. Moreover, *P. gingivalis* was dramatically 100% sterilized in both the LED group and the PC group (Fig. 5).

Discussion

Whereas *S. mutans* and *E. faecalis* were hardly sterilized in all groups, there were remarkable bactericidal effects in the LED group and PC group for *A. actinomycetemcomitans*, *T. forsythia*, and *P. gingivalis*. One of the causes for this is the difference between Gram-positive (G⁺) and Gram-negative (G⁻) bacteria, especially in the cell envelope. The main differences in the cell envelopes between G⁺ and G⁻ bacteria are shown in Fig. 6. Among them, the peptidoglycan layer, which is the basic part of the surface structure, has a large difference in its thickness, 10–100 nm in G⁺ bacteria and 2–15 nm in G⁻ bacteria. We have confirmed the bactericidal mechanism of a-PDT in which ¹O₂ leads to cell death by directly destroying the surface of the cell envelope (22). In other words, this result suggested that the thickness of the cell envelope was very important, and the sterilization rate improved depending on the amount of ¹O₂ generated. Thus, it was suggested that PDT had a more effective bactericidal effect on G⁻ than G⁺ bacteria. In addition, our previous report indicated that 87.2 µmol ¹O₂ was required to achieve >99.99% sterilization of *E. faecalis* (20). Therefore, we considered that G⁺ bacteria could not be sterilized because only 14.9 µmol of ¹O₂ was generated under the conditions of this study. However, not only the PC group but also the LED group showed remarkable bactericidal effects against G⁻ bacteria. The reason for this may be that *P. gingivalis* is an iron-requiring bacterium,

Fig. 3. ESR-Spectra after LED irradiation

The typical ESR-spectra of LED and PC groups after LED irradiation for 1-3 min. The white and black circles indicate the Mn²⁺ marker and the nitroxide radical, respectively.

Fig. 4. Evaluation of the amount of ¹O₂ generated via excited MB at each concentration based on quantitative analysis using ESR. The amount of ¹O₂ generated increases with LED irradiation. A positive correlation is observed between the amount of ¹O₂ generated and irradiation time ($R^2=0.98$).

The equation of the line is $y=5.0514x-4.578$.

The data points indicate the mean values ($n=3$) with a standard deviation bar.

Fig. 5. Bactericidal effect on G^+ bacteria and G^- bacteria by a-PDT
The data points indicate the mean values (n=6) with standard deviation bars.

Fig. 6. Difference in bactericidal mechanism of 1O_2 generated from TiO₂ by G^+ bacteria and G^- bacteria form

and it is known that protoporphyrin IX is retained intracellularly (23). Since protoporphyrin IX has an excitation wavelength around 410 nm, it was considered that protoporphyrin IX as a photosensitizer acted as a-PDT with 405-nm LED irradiation. On the other hand, *A. actinomycetemcomitans* and *T. forsythia* do not have photosensitizers such as porphyrin. That is, it is suggested that only 405-nm LED irradiation affected the sterilization (Fig. 7). Chui et al. reported that the inhibition of growth of *P. gingivalis* by a blue LED (425-500 nm) may be induced

not by a bactericidal effect, but instead due to a bacteriostatic effect mediated by suppression of the genes associated with chromosomal DNA replication and cell division at the transcriptional level (24). Additionally, Uekubo et al. reported that the anaerobic conditions were bacteriostatic, while the aerobic condition became bactericidal via a-PDT (25). In the present experiment, since a-PDT was performed under aerobic conditions, it can be inferred that a bactericidal effect was observed as a result of suppression at the DNA level. However, with a-PDT using

Fig. 7. Difference in bactericidal mechanism with /without protoporphyrin IX in G⁺ bacteria.

rutile-TiO₂, it has been confirmed that ROS generate not only ¹O₂, but also superoxide anion radical(O₂^{•-}), so a bactericidal effect may have occurred by another pathway. O₂^{•-} does not have strong oxidizing power by itself, but neutrophils produce O₂^{•-} with NADH oxidase and use it for bactericidal action. Actually, Chinju et al. reported that a large amount of O₂^{•-} production was frequently observed from polymorphonuclear leucocytes in peripheral blood and gingival crevicular fluid with severe periodontitis, and when the reaction to various anaerobic bacteria was measured, O₂^{•-} production in reaction to *A. actinomycetemcomitans*, *P. gingivalis*, and *Capnocytophaga* was seen(26). However, further detailed studies are necessary to clarify the O₂^{•-} pathway for a bactericidal effect. Based on the findings obtained from this study, selective sterilization, which was difficult with conventional disinfectants, may be possible with a-PDT using rutile-TiO₂. Since the resident oral flora play a role in preventing colonization and growth of foreign pathogenic microbes, it is important to determine how to selectively sterilize the foreign pathogen microbes without destroying the resident flora. In addition, selective sterilization

can also be achieved by changing the irradiation time, power output, or the concentration of rutile-TiO₂. Then, because the wavelength of 405-nm is in the visible region but close to the UV region, it will be necessary to examine the cellular toxicity when irradiated for a long time.

In conclusion, irradiation of rutile-TiO₂ for 3 min by a 405-nm LED showed no bactericidal effect on *S. mutans* and *E. faecalis*, but showed that sterilization of *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythia* was possible with generation of only 14.9 μmol ¹O₂.

In other words, the present findings suggest that, by controlling the amount of ¹O₂, one can select the bactericidal effect, and they further contribute to the development of an evidence-based medicine approach to a-PDT.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Number 19K19076.

Conflict of Interest

No potential conflicts of interest were disclosed.

References

1. Saquib SA, AlQahtani NA, Ahmad I, Kader MA, Al Shahrani SS, Asiri EA: Evaluation and Comparison of Antibacterial Efficacy of Herbal Extracts in Combination with Antibiotics on Periodontal pathobionts: An in vitro Microbiological Study. *Antibiotics*, <https://doi.org/10.3390/antibiotics8030089>, 2019.
2. Arruda MEF, Neves MAS, Diogenes A, Mdala I, Guilherme BPS, Siqueira JF Jr, Rôças IN: Infection Control in Teeth with Apical Periodontitis Using a Triple Antibiotic Solution or Calcium Hydroxide with Chlorhexidine: A Randomized Clinical Trial. *J Endod*, 44: 1474–1479, 2018.
3. Mosailova N, Truong J, Dietrich T, Ashurst J: *Streptococcus gordonii*: A Rare Cause of Infective Endocarditis, Case Rep Infect Dis. <https://doi.org/10.1155/2019/7127848>, 2019.
4. Liu D, Zhang J, Li T, Li C, Liu X, Zheng J, Su Z, Wang X: Chronic osteomyelitis with proliferative periostitis of the mandibular body: report of a case and review of the literature. *Ann R Coll Surg Engl*, 101 : 328–332, 2019.
5. Al-Ahmad A, Ameen H, Pelz K, Karygianni L, Wittmer A, Anderson AC, Spitzmüller B, Hellwig E: Antibiotic resistance and capacity for biofilm formation of different bacteria isolated from endodontic infections associated with root-filled teeth. *J Endod*, 40 : 223–230, 2014.
6. Takasaki AA, Aoki A, Mizutani K, Schwarz F, Sculean A, Wang CY, Koshy G, Romanos G, Ishikawa I, Izumi Y: Application of antimicrobial photodynamic therapy in periodontal and peri-implant diseases. *Periodontol* 2000, 51: 109–140, 2009.
7. N.S. Soukos, J.M. Goodson: Photodynamic therapy in the control of oral biofilms. *Periodontol* 2000, 55: 143–166, 2011.
8. Nakano M, Kambayashi Y, Tatsuzawa H, Komiyama T, Fujimori K: Useful $^1\text{O}_2$ (1 delta g) generator, 3-(4'-methyl-1'-naphthyl)-propionic acid, 1',4'-endoperoxide (NEPO), for dioxygenation of squalene (a skin surface lipid) in an organic solvent and bacterial killing in aqueous medium. *FEBS Lett*, 432: 9–12, 1998.
9. Tatsuzawa H, Maruyama T, Misawa N, Fujimori K, Hori K, Sano Y, Kambayashi Y, Nakano M: Inactivation of bacterial respiratory chain enzymes by singlet oxygen. *FEBS Lett*, 439: 329–333, 1998.
10. Petrellis MC, Frigo L, Ribeiro W, Leal-Junior EC, Oliveira FR, Maria DA, Lopes-Martins RÁ: Proinflammatory effects of photoactivated methylene blue on rat model of Walker 256 carcinosarcoma. *Exp Oncol*, 41 : 112–122, 2019.
11. ElZorkany HE, Youssef T, Mohamed MB, Amin RM: Photothermal versus photodynamic treatment for the inactivation of the bacteria *Escherichia coli* and *Bacillus cereus*: an in vitro study. *Photodiagnosis Photodyn Ther*, 27: 317–326, 2019.
12. Yurt F, Ince M, Colak SG, Ocakoglu K, Er O, Soylu HM, Gunduz C, Avci CB, Kurt CC: Investigation of in vitro PDT activities of zinc phthalocyanine immobilised TiO_2 nanoparticles. *Int J Pharm*, 524: 467–474, 2017.
13. Kurzmann C, Verheyen J, Coto M, Kumar RV, Divitini G, Shokoohi-Tabrizi HA, Verheyen P, De Moor RJG, Moritz A, Agis H: In vitro evaluation of experimental light activated gels for tooth bleaching. *Photochem Photobiol Sci*, 18 : 1009–1019, 2019.
14. Saita M, Kobayashi K, Yoshino F, Hase H, Nonami T, Kimoto K, Lee MC: ESR investigation of ROS generated by H_2O_2 bleaching with TiO_2 coated HAp. *Dent Mater J*, 31 , 458–464, 2012.
15. Mori K, Tsuji M, Ueda T, Sakurai K: Color and gloss evaluation of titanium dioxide coating for acrylic resin denture base. *J Prosthodont Res*, 59: 249–253, 2015.
16. Arai T, Ueda T, Sugiyama T, Sakurai K: Inhibiting microbial adhesion to denture base acrylic resin by titanium dioxide coating. *J Oral Rehabil*, 36: 902–908, 2009.
17. Aita H, Att W, Ueno T, Yamada M, Hori N, Iwasa F, Tsukimura N, Ogawa T: Ultraviolet (UV) light-mediated photofunctionalization of titanium to promote human mesenchymal stem cell migration, attachment, proliferation and differentiation. *Acta Biomaterialia*, 5 : 3247–3257, 2009.
18. Su EP, Justin DF, Pratt CR, Sarin VK, Nguyen VS, Oh S, Jin S: Effects of titanium nanotubes on the osseointegration, cell differentiation, mineralisation and antibacterial properties of orthopaedic implant surface. *Bone Joint J*, 100-B: 9–16, 2018.
19. Takahashi C, Tsujimoto Y, Yamamoto Y: The effect of irradiation wavelengths and the crystal structures of titanium dioxide on the formation of singlet oxygen.

- for bacterial killing. J Clin Biochem Nutr, 51: 128-131, 2012.
20. Komine C, Tsujimoto Y: A small amount of singlet oxygen generated via excited methylene blue by photodynamic therapy induces the sterilization of *Enterococcus faecalis*. J Endod, 39 :411-414, 2013.
21. Nakamura K, Ishiyama K, Ikai H, Kanno T, Sasaki K, Niwano Y, Kohno M: Reevaluation of analytical methods for photogenerated singlet oxygen. J Clin Biochem Nutr, 49: 87-95, 2011.
22. Komine C, Kageyama M, Otsuka I: Elucidation of sterilization mechanism in antibacterial photodynamic therapy. J Jpn. Soc. Laser Dent, 27: 146, 2016.
23. Yasuo T, Akira A, Koichi H, Chui C, Akiko I, Ayana U, Yuichi I: Application of Antimicrobial Photodynamic Therapy in Dental Treatments. JJSLSM, 38: 457-470, 2018.
24. Chui C, Hiratsuka K, Aoki A, Takeuchi Y, Abiko Y, Izumi Y: Blue LED inhibits the growth of *Porphyromonas gingivalis* by suppressing the expression of genes associated with DNA replication and cell division. Lasers Surg Med, 44: 856-864, 2012
25. Uekubo A, Hiratsuka K, Aoki A, Takeuchi Y, Abiko Y, Izumi Y: Effect of antimicrobial photodynamic therapy using rose bengal and blue light-emitting diode on *Porphyromonas gingivalis* in vitro: Influence of oxygen during treatment. Laser Ther, 25: 299-308, 2016.
26. Chinju N, Maeda K, Iwamoto Y, Akamine A, Hara Y, Ohga N, Tanigawa K, Hatakeyama T, Aono M: [Bactericidal effect of human polymorphonuclear leukocytes--superoxide anion production of human polymorphonuclear leukocytes from peripheral blood, or gingival crevicular fluid] . Nihon Shishubyo Gakkai Kaishi, 29: 522-529, 1987.