Effect of Hydroxyl Radical from Blue Light and Hydrogen Peroxide on *Porphyromonas gingivalis* in Biofilms

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ABSTRACT

Periodontal diseases are inflammatory diseases caused by groups of selected bacteria such as Porphyromonas gingivalis. These bacteria contain black pigment which is sensitive to blue light (BL). The previous studies have shown that hydroxyl radical ($^{\cdot}OH$) produced from BL and hydrogen peroxide (H_2O_2) can cause cellular damage and inhibited this bacterium. Therefore, this study evaluates the use of BL and H_2O_2 in single treatment and combination on the survival of P. gingivalis in biofilms. The $^{\cdot}OH$ produced from BL and H_2O_2 were also studied by using the fluorescence probe technique. P. gingivalis biofilms were prepared from a subject with chronic periodontitis. BL source laser with the wavelength of 405 nm and power density of 50 mW/cm^2 was used as BL treatment. 1% H_2O_2 (v/v) was used in the H_2O_2 treatment. The survival fractions of P. gingivalis were determined at 0, 3 and 6 h post-treatment. This study found that treatment with H_2O_2 alone showed the greatest inhibitory effect against P. gingivalis with an immediate effect while BL alone and BL+H_2O_2 took 6 hour post-treatment to inhibit P. gingivalis.

Keywords: *Blue light/ Hydrogen peroxide/ Porphyromonas gingivalis*

Introduction

Periodontal diseases are inflammatory diseases exhibiting periodontal tissue destruction induced by specific bacteria such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Tannerella forsythia and Prevotella intermedia*.[1-3]

The use of chemical photosensitizer agents, such as methylene blue and toluidine blue in combination with red light in photodynamic therapy (PDT) has shown specificity and selectiveness for periodontal pathogens.[4-5] However, photosensitizing agents have some disadvantages as they can cause temporary discoloration of tissues surrounding the targeted area and the limited availability of suitable photosensitizers for clinical use is concerned.

Hydrogen peroxide (H_2O_2) has been used in the oral cavity to debride infected tissues and kill offending microorganisms. The oxidizing property of H_2O_2 can cause cellular damage through the degradation of DNA and oxidation of proteins and lipids.[6] Leke et al. indicated that H_2O_2 was deleterious for both hemagglutination and Arg-gingipain activity of *P. gingivalis*.[7] Baldeck et al. found that H_2O_2 at low concentration (3 mM) could damage DNA and proteins, while at higher concentration (30 mM), the damage of bacterial membranes without inducing mutation was observed.[8]

The bactericidal effects of visible blue light (wavelengths of 400-500 nm) on various periodontal pathogens have been recently reported. Feuerstein et al. showed that broadband blue light exerted a phototoxic

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effect on *P. gingivalis* and *F. nucleatum.*[9] Oral black-pigmented bacteria, such as *P. gingivalis*, *P. intermedia* and *P. nigrescens* in pure culture were killed with broadband light (380-520 nm) without an exogenous photosensitizer.[10] Fukui et al. found that the growth of *P. gingivalis* was suppressed by 405 nm light irradiation.[11] In addition, Chui et al. reported that blue light LED (425-470 nm) was able to inhibit the growth of *P. gingivalis* suspension.[12]

Feuerstein et al.[13] found that the combination of blue light and 0.3 mM H_2O_2 decreased the growth of *S. mutans*, the important cariogenic bacteria, more efficiently than the application of blue light and H_2O_2 separately. However, the data on antibacterial effect of this combined treatment against periodontal pathogens are currently limited.

The aim of this study is to investigate the effect of blue light and H_2O_2 in separated and combined treatment on *P. gingivalis* biofilms and compared the hydroxyl radicals ($^{\cdot}OH$) produced from H_2O_2 with and without blue light.

Materials and methods

Experimental Design

In this study, 3 treatments were used *viz.* blue light (BL), hydrogen peroxide (H_2O_2), and BL with H_2O_2. Biofilms which were neither subjected to blue light, hydrogen peroxide nor the combined treatments were used as the control. The post-treatment times were 0, 3, and 6 h. The experiment was conducted in triplicate.

Microorganisms

Samples of dental plaque were taken from one subject who agreed to participate in this study and signed an informed consent. This study followed the guidelines and obtained approval from the Ethics Committee of Khon Kaen University, Thailand (HE562312). The subject had periodontal pockets ≥ 5 mm. with bleeding upon pocket probing and had no history of antibiotic use in the past 3 months. The deepest pockets in each quadrant were chosen and samples of dental plaque were collected by sterile paper points. Plaque samples were placed immediately into a tube containing 4.5 ml of thioglycolate broth (Fluka, Switzerland).

Samples were vortexed for 2 minutes and 100 µl of the sample was used for culture whereas another 100 µl was used for PCR to confirm the expression of *P. gingivalis* in the sample by procedures as previously described.[14] 100 µl of the diluted plaque sample in thioglycolate broth was incubated in 80% N_2-10% H_2-10% CO_2 at 37°C for 7 to 14 days. *P. gingivalis* was identified on the basis of Gram staining and anaerobic growth. The total number of CFU/ml of *P. gingivalis* in positive samples was determined by growing the bacteria for 7 days in trypticase soy agar supplemented with blood (5% by volume), hemin (5 mg/liter) (Sigma, USA), vitamin K (500 µl/liter) (Sigma, USA) and kanamycin (400 µl/liter). The black colonies were selected to create glycerol stocks for *P. gingivalis* and were kept at -80°C until experiment.

Upon experiment, frozen bacterial cells were thawed at room temperature for reactivation before culture in blood agar. The black colonies were selected after culture for 7 days and were used throughout the experiments. The cultivation of *P. gingivalis* was conducted using thioglycolate broth supplemented with blood (5% (v/v)), hemin (5 g/liter), vitamin K (1 g/liter), and kanamycin (100 g/liter) for four days.

Biofilm preparation

Twelve ml of saliva obtained from the subject was mixed with Ringer's solution to obtain a 1:10 dilution. The mixture was then centrifuged at 2,000 rpm for 15 min. The obtained supernatant was filtered by 0.2 µm syringe filter and was kept at 4°C prior to use. The biofilms were prepared by suspending the supernatant of saliva over cover glasses which were placed in 6-well plates (Corning Incorporated, USA). The saliva in the

wells was discarded after incubation for two days. 4 ml of trypticase soy broth (Pronadisa, Spain) containing 10 mg/L hemin was then added to each well on top of cover glasses followed by 100 µl of *P. gingivalis* suspension. The plates were subsequently incubated in anaerobic chamber at 37°C for 4 days. Every 48 h, the medium in the wells were discarded and replaced by fresh trypticase soy broth. Biofilm coated cover glasses were used throughout the study.

Light source

For blue light inhibition test, the blue light laser (Century light (HK) Int'l group limited, Hong Kong) with a wavelength of 405 nm and 500 mW was used as a light source. The system was coupled with lens with a focus of 25 mm and a diameter of 20 mm which formed a uniform circular spot of 1 cm in diameter. The power density of incident radiation was measured using a power meter. The distance between lens and illuminated plates was 80 cm with a fixed power density of 50 mW/cm^2 (or 3 J/cm^2).

Effect of blue light, hydrogen peroxide and combined treatment on *P. gingivalis* biofilm

For blue light exposure, the biofilm coated cover glasses were exposed to blue light for one minute. At 0, 3, 6 h after the exposure, the bacterial cell concentrations in the wells were determined by suspending bacterial cells from the biofilm in Ringer's solution. Cell suspension were dropped on blood agar plates (10 µl/drop) and incubated under anaerobic condition at 37°C for 7 days. To determine survival fractions, colonies on the plates were counted and calculated to obtain colony-forming units/ml.

For hydrogen peroxide treatment, 50 µl of 1% (v/v) hydrogen peroxide was applied to each biofilm coated cover glass in the 6-well plate. The hydrogen peroxide was allowed to soak biofilm coated cover glasses for 0, 3 and 6 h and was analyzed for survival cell concentration as described above. The plate was covered with aluminum foil prior to analysis.

For the combined treatment of blue light and hydrogen peroxide, the biofilm coated cover glasses treated with hydrogen peroxide were subsequently subjected to one-minute blue light before analysis of the survival cell concentration after 0, 3 and 6-h post-treatment.

Controls were biofilm coated cover glasses which were neither subjected to blue light, hydrogen peroxide nor the combined treatments.

All experiments were conducted in triplicates in each treatment and each post-treatment time.

Investigation of hydroxyl radicals ($^{\cdot}$OH) produced from H_2O_2 with blue light and without blue light

The measurement of free hydroxyl radicals produced by H_2O_2 with or without light at various time points was examined by the fluorescence probe technique.[15] Briefly, fluorescein free acid was purchased from Sigma Aldrich (Singapore 117528). Fifty micromoles of fluorescein solution diluted in 1 mg/ml of NaOH was prepared and dropped in 1% hydrogen peroxide (v/v) with 1 min treatment with blue light. The Thermo Scientific ™ Varioskan ™ Flash Multimode Reader, of which excitation wavelength and emission wavelength were set at 490 nm and 512 nm, respectively, was used to analyze hydroxyl radical in H_2O_2 determined by fluorescence intensity.

Statistical analysis

The results were analyzed for their significant differences by *Duncan's Post Hoc test at 95% confident interval using a software SPSS Version 19 (IBM, USA).*

Results

There was no difference in the CFU counts of *P. gingivalis* of the control group at different post-treatment time. Treatment with blue light (BL) alone, *P. gingivalis concentration was significantly decreased* at 6 hours post-treatment as compared with those observed at 0 and 3 hours.

Hydrogen peroxide (H_2O_2) treatments show the lowest *P. gingivalis* concentration at all post-treatment time in compared to the control and other treatments (BL treatment and BL+ H_2O_2 treatment). Moreover, there was no significant difference in the CFU counts at each post-treatment time.

A combined treatment (BL+ H_2O_2) showed a similar trend with BL treatment; however, the combined treatment showed significantly lower bacterial counts than the BL treatment at 6 hours post-treatment time ($P < 0.05$). The results of this study are shown in Figure 1. In addition, Figure 2 shows that the fluorescence intensity of H_2O_2 and BL treatment was lower than H_2O_2 alone which indicated that the combined treatment had a higher hydroxyl radicals.[15]

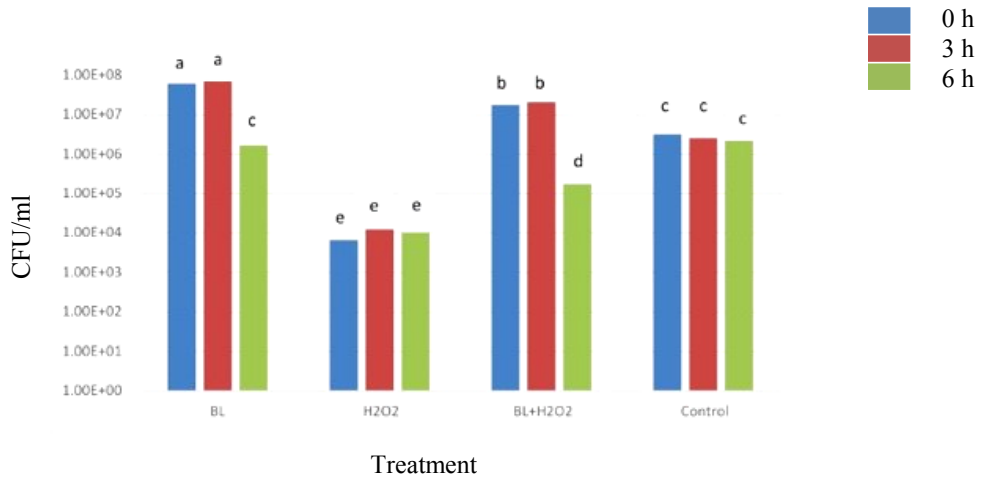

Figure 1 CFU/ml of *P. gingivalis* biofilm after different treatments collected at different time points. Different lower case letters indicate significantly different means at $P<0.05$.

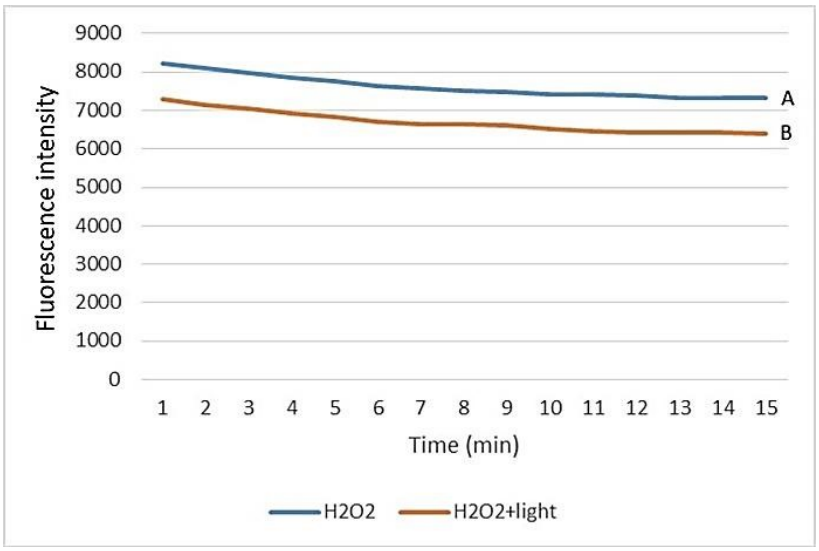

Figure 2 Fluorescence intensity of hydroxyl radicals generated during a reaction of fluorescein in the presence of H_2O_2 with and without light were detected by Varioskan™ Flash Multimode Reader of which excitation wavelength of 490 nm and emission wavelength of 512 nm were set. Hydroxyl radicals were generated in accordance with a linear proportion. H_2O_2 with light group (trace B) showed more hydroxyl radicals intensity than H_2O_2 without light group (trace A).

Discussion

The results of this study were in agreement with the previous *in vitro* study on the bactericidal efficacy of phototoxicity of blue light in combination with H_2O_2 against *P. gingivalis* biofilms.[9]

Feuerstein et al. showed that blue light exerted a phototoxic effect on *P. gingivalis* and *F. nucleatum*. The authors suggested that this was possibly due to high amounts of endogenous photosensitizers such as cytochromes, porphyrins, flavins and NADH within bacterial cells.[9] The results of our study showed that *P. gingivalis* biofilms tended to significantly decrease at 6 h ($P<0.05$) for groups treated by blue light or blue light in combination with H_2O_2.

Hydrogen peroxide (H_2O_2) is a mild oxidizing agent that at low concentrations (≤ 3 mM) can cause degradation of bacterial DNA and oxidation of proteins and lipids. At higher concentrations (≥30 mM), it damages bacterial membrane without inducing mutation.[8] The H_2O_2 concentration used in our study is 30 mM which was relatively high. However, even with a high concentration, there was no significant difference in the reduction of *P. gingivalis* biofilms when the exposure time was increased ($P > 0.05$). The possible explanation could be that the H_2O_2 is unstable at room temperature. Furthermore, *P. gingivalis* has the ability to survive by adapting to and protecting itself from the deleterious effects of oxidative stress by heam acquisition on its cell surface.[8, 16-17]

The phototoxic effect of photodynamic therapy on various periodontal pathogens including *P. gingivalis* has been reported. Feuerstein et al., showed that the mechanism of killing of *P. gingivalis* and *F. nucleatum* is oxygen-dependent, which might result in the formation of hydroxyl radicals.[9] Hope et al. suggested the possibility that lethal photosensitization occurs by the oxygen-independent type I pathway as opposed to oxygen-dependent type II pathway. The mechanism of cytotoxicity on micro-organisms is generally reported to be due to the production of hydroxyl radicals.[18] Soukos et al. reported that the synergic antibacterial effect between blue light and H_2O_2 on *P. gingivalis* was a result of light excitation on their endogenous porphyrins.[10] Ikai et al. have reported that the bactericidal effect on *P. gingivalis* by H_2O_2 in combination with laser light at 405 nm was through the generated hydroxyl radical. The bactericidal effect was dependent on the time of laser irradiation, the concentration of H_2O_2 and the yield of hydroxyl radical.[19]

The result of fluorescence probe showed a decrease of fluorescence intensity with time, suggesting an increase of hydroxyl radicals over time. However, the result of blue light plus H_2O_2 at 6 hours post-treatment showed significant decrease in *P. gingivalis* CFU count compared to those at 0 and 3 hours post-treatment. This may be due to the Fenton reaction which involves some chemicals with ferrous compounds and H_2O_2. The excitation of blue light on endogenous porphyrins and photohydrolysis of H_2O_2 results in the production of hydroxyl radical.[9]

The high peak of bactericidal activity in this study was at 6 hr. for BL, H_2O_2 and BL+ H_2O_2. This can be explained that one of the signaling and changes in cellular metabolism of bacterial cells from the PDT has been shown to raise the levels of free calcium within cells and this has been associated with cell death. The increase of cytoplasmic free calcium in Chinese hamster ovary cells and T24 human bladder transitional carcinoma cells treated with the PS aluminum phthalocyanine and red light reached the peak within 5-15 min after exposure and then returned to basal level. So this study had to investigate the bactericidal activity from 0-6 hr period of time.[20]

There is an evidence to support the bactericidal effect of H_2O_2 and LED light in the dental microenvironment. One of the best properties of H_2O_2 is its covalent and uncharged molecule that readily mixes with water. This allows diffusion and ease of penetration even into mature

biofilm. As a consequence, it allows the hydroxyl radical to disinfect bacteria in the deeper layer of the biofilm. Conventional dye photosensitizers in PDT have low capacity to penetrate the deep layers of the biofilm. They are relatively much larger molecules as compared to H_2O_2. It is now believed that the oxidizing power and reactivity of hydroxyl radical are higher than that of singlet oxygen. This allows H_2O_2 to exert a greater bactericidal effect compared with conventional PDT.[21]

From the literatures given above, it could be implied from our results that the strong inhibition effect of H_2O_2 on *P. gingivalis* biofilm might be due to the oxidation properties of H_2O_2. The toxicity of H_2O_2 to bacteria is by the hydroxyl radical formed through the reaction of oxidant with divalent ions. Protein and lipids were oxidized, damaging the bacterial cell membrane and DNA. Moreover, its covalent and uncharged molecule that can readily mix with water allows diffusion of hydroxyl radical in deeper layer of the biofilm. In the treatment with H_2O_2 alone, *P. gingivalis* was immediately inhibited at 0 hours post-treatment. This was possibly because the highly hydroxyl radicals could damage DNA quickly.

Conclusions

The use of blue light, H_2O_2 and combined treatment resulted in the reduction of bacteria counts of *P. gingivalis* biofilms. H_2O_2 alone is the most effective method for the inhibition of *P. gingivalis* as cleary evidenced by the immediate inhibition effect of bacteria and the level of inhibition is most potent. Blue light and blue light in combination with H_2O_2 had a prolong and lower inhibition effect in comparison to H_2O_2 alone.

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ผลของไฮดรอกซี แรดิคัลจากแสงสีฟ้าและไฮโดรเจนเปอร์ออกไซด์ต่อเชื้อพอร์ไฟโรโมแนส จิงจิวาลิสในไบโอฟิล์ม

อังคณา แสงปัญญา เบญญาวรรณ เอื้ออรัญโชติ** จุฑามาศ รักษ์ชันแสง*** อุบลวรรณ ทัพสุรีย์**** มนัสวีส์ ธนะภูมิชัย***** อรุณ ทีรฆพงศ์**

บทคัดย่อ

โรคปริทันต์เป็นโรคที่เกิดการอักเสบเนื่องจากกลุ่มของแบคทีเรีย เช่น พอร์ไฟโรโมแนส จิงจิวาลิส ซึ่งเป็นแบคทีเรียที่ประกอบไปด้วยเม็ดสีดำและไวต่อแสงสีฟ้า จากการศึกษาที่ผ่านมาพบว่าไฮดรอกซีลเรดิเคิลที่ผลิตมาจากแสงสีฟ้า และไฮโดรเจนเปอร์ออกไซด์ สามารถทำให้เกิดความเสียหายต่อเซลล์ได้ ดังนั้น ในการศึกษานี้ได้ใช้แสงสีฟ้าและไฮโดรเจนเปอร์ออกไซด์ทั้งที่ใช้แบบเดี่ยว และแบบร่วมกันมาทดลองกับพอร์ไฟโรโมแนส จิงจิวาลิส บนฟิล์มชีวภาพ ใช้เทคนิคฟลูออเรสเซนต์โพรบ ทำการศึกษาหาไฮดรอกซีลเรดิเคิลที่ผลิตจากแสงสีฟ้า และไฮโดรเจนเปอร์ออกไซด์ เตรียมพอร์ไฟโรโมแนส จิงจิวาลิส บนฟิล์มชีวภาพจากตัวแทนที่เป็นโรคปริทันต์อักเสบเรื้อรัง ใช้เลเซอร์ที่มีแหล่งกำเนิดเป็นแสงสีฟ้า ความยาวคลื่น 405 นาโนเมตร และพลังงาน 50 มิลลิวัตต์ต่อตารางเซนติเมตร สำหรับการทดลองศึกษาดูผลของแสงสีฟ้า และใช้ไฮโดรเจนเปอร์ออกไซด์ที่ความเข้มข้น 1 เปอร์เซ็นต์ โดยปริมาตรสำหรับการทดลองศึกษาผลของไฮโดรเจนเปอร์ออกไซด์ เพื่อยับยั้งพอร์ไฟโรโมแนส จิงจิวาลิส ทำการประเมินเศษส่วนความอยู่รอดของ พอร์ไฟโรโมแนส จิงจิวาลิส ช่วงหลังการทดลองทันที หรือ 0 ชั่วโมง หลังการทดลอง 3 ชั่วโมง และ 6 ชั่วโมง จากการศึกษาครั้งนี้พบว่า ผลของไฮโดรเจนเปอร์ออกไซด์ เพียงอย่างเดียวมีฤทธิ์ยับยั้งพอร์ไฟโรโมแนส จิงจิวาลิส ได้ดีที่สุด และยับยั้งได้ทันที หรือที่เวลา 0 ชั่วโมงหลังการแช่ในไฮโดรเจนเปอร์ออกไซด์ ขณะที่การใช้แสงสีฟ้า เพียงอย่างเดียวและ การใช้แสงสีฟ้าร่วมกับไฮโดรเจนเปอร์ออกไซด์ ต้องใช้เวลา 6 ชั่วโมงหลังการทดลอง จึงสามารถยับยั้ง พอร์ไฟโรโมแนส จิงจิวาลิส ได้

คำสำคัญ: *แสงสีฟ้า/ ไฮโดรเจนเปอร์ออกไซด์/ พอร์ไฟโรโมแนส จิงจิวาลิส*

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