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Antimicrobial Blue Light Inactivation of *Neisseria gonorrhoeae*: Roles of Wavelength, Endogenous Photosensitizer, Oxygen, and Reactive Oxygen Species

Ying Wang^{1,2}, Raquel Ferrer-Espada^{1,3}, Yan Baglo¹, Ying Gu², Tianhong Dai^{1,3,*}

¹Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, 55 Fruit Street, Boston, Massachusetts, 02114

²Department of Laser Medicine, Chinese PLA General Hospital, Medical School of Chinese PLA, 28 Fuxing Road, 100853, Beijing, China

³Vaccine and Immunotherapy Center, Massachusetts General Hospital, Harvard Medical School, 149 13th St, Charlestown, Massachusetts, 02129

Abstract

Background and Objectives: The aim of this study was to investigate the efficacy, safety, and mechanism of action of antimicrobial blue light (aBL) for the inactivation of *Neisseria gonorrhoeae*, the etiological agent of gonorrhea.

Study Design/Materials and Methods: The susceptibilities of *N. gonorrhoeae* (ATCC 700825) in planktonic suspensions to aBL at 405- and 470-nm wavelengths were compared. The roles of oxygen in the anti-gonococcal activity of aBL were studied by examining the effects of hypoxic condition (blowing N₂) on the anti-gonococcal efficiency of 405-nm aBL. The presence, identification, and quantification of endogenous photosensitizers in *N. gonorrhoeae* cells and human vaginal epithelial cells (VK2/E6E7 cells) were determined using fluorescence spectroscopy and ultra-performance liquid chromatography (UPLC). Finally, the selectivity of aBL inactivation of *N. gonorrhoeae* over the host cells were investigated by irradiating the co-cultures of *N. gonorrhoeae* and human vaginal epithelial cells using 405-nm aBL.

Results: About 3.12-log₁₀ reduction of bacterial colony forming units (CFU) was achieved by 27 J/cm² exposure at 405 nm, while about 3.70-log₁₀ reduction of bacterial CFU was achieved by 234 J/cm² exposure at 470 nm. The anti-gonococcal efficacy of 405-nm aBL was significantly suppressed under hypoxic condition. Spectroscopic and UPLC analyses revealed the presence of endogenous porphyrins and flavins in *N. gonorrhoeae*. The concentrations of endogenous photosensitizers in *N. gonorrhoeae* (ATCC 700825) cells were more than 10 times higher than those in the VK2/E6E7 cells. In the co-cultures of *N. gonorrhoeae* and VK2/E6E7 cells, 405-nm aBL at 108 J/cm² preferentially inactivated *N. gonorrhoeae* cells while sparing the vaginal epithelial cells.

*Correspondence to: Tianhong Dai, PhD, Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, BAR 404B, 55 Fruit Street, Boston, MA 02114. tdai@mgh.harvard.edu.

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Conclusions: aBL at 405-nm wavelength is more effective than 470-nm wavelength in inactivating *N. gonorrhoeae* while sparing the vaginal epithelial cells. Reactive oxygen species generated from the photochemical reactions between aBL and endogenous photosensitizers play a vital role in the anti-gonococcal activity of 405-nm aBL. *Lasers Surg. Med.*

Keywords

antibiotic resistance; antimicrobial blue light; *Neisseria gonorrhoeae*; endogenous photosensitizers; porphyrins; flavins; reactive oxygen species; singlet oxygen

INTRODUCTION

Gonorrhea is the second most prevalent sexually transmitted infection globally [1]. Despite the public health efforts to control gonorrhea for 70 years, these infections remain a significant public health concern. In 2015, a total of 395,216 cases of gonorrhea were reported in the United States. Worldwide, 106.1 million people are affected by gonococcal infections annually [2]. If gonococcal infections are not appropriately treated, they can result in severe complications and sequelae such as salpingitis and pelvic inflammatory disease, which may lead to sterility and/or ectopic pregnancy. In addition, epidemiologic and biologic studies have provided evidence that the failure to curb the transmission of gonorrhea facilitates the transmission of HIV infection [3]. Repeated infections are common and no state of protective immunity appears to develop as a consequence of infection. Since there is no gonococcal vaccine, treatment of gonorrhea relies especially on antibiotics. However, *Neisseria gonorrhoeae*, the etiological agent of gonorrhea, is evolving into a superbug and may become untreatable due to its resistance to almost all the antibiotics previously and currently widely used (e.g., sulfonamides, penicillins, earlier cephalosporins, tetracyclines, macrolides, and fluoroquinolones) [4]. As such, the Centers for Disease Control and Prevention (CDC) has declared multidrug-resistant *N. gonorrhoeae* as an urgent threat to public health [5,6]. There is consequently a critical need for the development of alternative therapeutics to combat gonococcal infections in high-risk communities [7].

Antimicrobial blue light (aBL) in the spectrum of 400–470 nm is an innovative non-antibiotic approach that has demonstrated prominent antimicrobial effects without the involvement of exogenous photosensitizers [8]. Though the mechanism of action of aBL is still not fully understood, a common hypothesis is that aBL excites the endogenous photosensitizing chromophores (e.g., iron-free porphyrins or/and flavins) in microbial cells and, subsequently, leads to the production of cytotoxic reactive oxygen species (ROS) [8–15]. It is envisioned that microbes are less able to develop resistance to aBL than to traditional antibiotics because of the multi-target characteristics of aBL [8]. In addition, it is well accepted that aBL is much less detrimental to host cells than germicidal ultraviolet C irradiation [16,17]. The present study aimed to investigate the efficacy, safety, and mechanism of action of aBL for the inactivation of *N. gonorrhoeae*.

MATERIALS AND METHODS

Culture Conditions of Bacteria and Human Vaginal Epithelial Cells

N. gonorrhoeae (ATCC 700825) cells were grown on gonococcal medium base GC (Remel, Lenexa, KS) agar plates containing GCHI enrichment (Remel) at 37°C and 5% CO₂. VK2/E6E7 cells (ATCC® CRL-2616) were cultured in Keratinocyte-Serum Free Medium (Gibco, Grand Island, NY) supplemented with 5 ng/ml recombinant epidermal growth factor, 50 µg/ml bovine pituitary extract (Invitrogen Corporation, Grand Island, NY) and 100 units/ml each of penicillin and streptomycin (Life Technologies, Grand Island, NY) at 37°C and 5% CO₂ in a humidified incubator.

Light Sources

A light-emitting diode with peak emission at 405 nm (M405L2; Thorlabs, Newton, NJ) and a light-emitting diode with peak emission at 470 nm (M470L2; Thorlabs) were used for irradiation. The full-widths at half maximum of both the LEDs were 10 nm. Irradiance of 60 mW/cm² was used. The irradiance was measured using a PM100D power/energy meter (Thorlabs).

Inactivation of *N. gonorrhoeae* in Suspensions Using 405- and 470-nm aBL

Overnight bacterial cultures were collected from GC agar plates, washed with phosphate-buffered saline (PBS), and re-suspended in PBS to OD_{600nm} = 0.3 (approximately 10⁸ colony forming units [CFU]/ml). Three milliliters of the bacterial suspension was added into a 35-mm petri dish (Transwell®; Costar, New York, NY) prior to aBL exposure. After exposure to aBL at 60 mW/cm² for various radiant exposures (9, 18, 27, 36, 45, and 54 J/cm² for 405 nm and 18, 54, 90, 126, and 234 J/cm² for 470 nm), aliquots of 30 µl were taken and the viability of *N. gonorrhoeae* was measured using serial 10-fold dilution. The experiment was performed in three independent replicates for each condition.

aBL Inactivation of *N. gonorrhoeae* Under Hypoxic Condition

To investigate the role of oxygen in the anti-gonococcal activity of aBL, the inactivation efficiency of 405-nm aBL was evaluated under hypoxic conditions. In brief, the bacterial suspension in a sealed 32-Q-10 cylindrical cell (Starna Cells, Atascadero, CA) was deoxygenated by blowing N₂ for 30 minutes before aBL exposure. Then the deoxygenated suspension was exposed to aBL irradiation through the upper wall of the cylindrical cell with the nitrogen gas purged during the entire course of irradiation. Aliquots of 30 µl of the suspensions were withdrawn with an injection syringe at 0, 5, 10, and 15 minutes, respectively, when 0, 18, 36, and 54 J/cm² aBL had been delivered. The viability of *N. gonorrhoeae* was determined using serial 10-fold dilution. Another bacterial suspension in another sealed 32-Q-10 cylindrical cell without purging nitrogen gas prior to aBL was set as control. The experiment was performed in three independent replicates for each condition.

aBL Illumination of the Co-Cultures of *N. gonorrhoeae* and VK2/E6E7 Cells

To determine the selectivity of 405-nm aBL inactivation of *N. gonorrhoeae* over the host cells, *N. gonorrhoeae* cells were co-cultured with VK2/E6E7 cells and then exposed to aBL.

In brief, VK2/E6E7 cells were seeded into a 35-mm Petri dish at a cell density of 2×10^5 cells/dish, and incubated in 2 ml keratinocyte-serum-free medium for 48 hours at 37°C. The supernatant was then discarded and 1 ml suspension of *N. gonorrhoeae* was added. A multiplicity of infection (M.O.I.) of 50:1 (bacteria: VK2/E6E7 cells) was used according to the optimized M.O.I. results of a previous study [18]. The co-cultures of VK2/E6E7 cells and *N. gonorrhoeae* were incubated at 37°C and 5% CO₂ for 4 hours. The invasion of *N. gonorrhoeae* cells into VK2/E6E7 cells was examined by a confocal microscope (Olympus, FV 1000-MPE Confocal, Allentown, PA) as described previously [19]. Immediately after 108 J/cm² aBL exposure (60 mW/cm², 30 minutes), fluorescent dyes propidium iodide (PI, excitation wavelength 559 nm, emission wavelength 603 nm; Invitrogen™) and SYTO9 (excitation wavelength 488 nm, emission wavelength 520 nm; Invitrogen™) were used to determine the viability of *N. gonorrhoeae* and the vaginal epithelial cells.

Membrane-permeable SYTO9 could stain all bacteria and VK2/E6E7 cells green. Membrane-impermeable PI could quench the fluorescence of SYTO9 and stain the nonviable cells red. To stain the intracellular nonviable bacteria with PI, the VK2/E6E7 cells were permeabilized with 0.05% saponin for 15 minutes when incubating PI and SYTO9. This concentration of saponin was found to permeabilize cell membrane without decreasing viability [20]. To discriminate the extracellular bacteria and the bacteria adherent to VK2/E6E7 cells, *N. gonorrhoeae*-infected VK2/E6E7 cells were incubated with Alexa Fluor 647-coupled soybean lectin (Invitrogen™, excitation wavelength 635 nm, emission wavelength 668 nm, false-colored purple) for 10 minutes before adding PI and SYTO9. Because lectins are oligomeric proteins with saccharide-binding sites that can recognize and bind particular glycoconjugates on the cell wall of bacteria. Moreover, Alexa Fluor 647-coupled soybean lectin does not access the cytoplasm of unpermeabilized cells. So, extracellular and adherent viable bacteria exhibited purple + green, extracellular and adherent nonviable bacteria exhibited purple + red. Intracellular viable bacteria and nonviable bacteria could be distinguished in permeabilized VK2/E6E7 cells and nonviable VK2/E6E7 cells. Intracellular viable bacteria exhibited green, intracellular nonviable bacteria exhibited red.

Ten confocal images were taken for the 405-nm aBL-treated and untreated control groups, respectively. The average percentage of nonviable *N. gonorrhoeae* cells were calculated by counting the number of red nonviable *N. gonorrhoeae* cells and the total number of *N. gonorrhoeae* cells using image J software.

Spectroscopic and Ultra-performance Liquid Chromatography (UPLC) Analysis of Endogenous Photosensitizers in *N. gonorrhoeae* and Normal Human Vaginal Epithelial Cells

To identify and quantify the endogenous photosensitizers in *N. gonorrhoeae*, the absorption spectra and fluorescence spectra of gonococcal lysates were analyzed. To extract the endogenous photosensitizers in *N. gonorrhoeae*, overnight bacterial cultures were collected and washed using PBS. The pellets of bacteria were collected after centrifugation, re-suspended in 1.0 ml of extraction solvent (ethanol: dimethyl sulfoxide: acetic acid, 80:20:1 [vol/vol/vol]), and then stored at -80°C for 24 hours. The cell walls of bacteria were then disrupted by sonication for 20 minutes. After centrifugation (13,500g for 6 minutes), the

supernatant was collected as the whole-cell lysates. The Chromatographic Marker kit (Frontier Scientific, Logan, UT) was used as porphyrin standards. It is composed of uroporphyrin I, 7-carboxylporphyrin I, 6-carboxylporphyrin I, 5-carboxylporphyrin I, coproporphyrin, mesoporphyrin IX, mesoporphyrin IX dihydrochloride. A mixture of flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and riboflavin was used as flavin standards. FMN, FAD, and riboflavin were bought from Sigma-Aldrich, Inc (St. Louis, MO).

To identify and quantify the endogenous photosensitizers in normal VK2/E6E7 cells (not incubated with *N. gonorrhoeae*), the absorption spectra and fluorescence spectra of VK2/E6E7 cells lysates were also analyzed. The extract of the endogenous photosensitizers in VK2/E6E7 cells were prepared following the same procedure as that for preparing extracts from *N. gonorrhoeae*.

The UPLC quantitation of different flavin species was carried out using a Waters® Acquity UPLC™ System. The excitation was set at 260 nm and emission at 470 nm emission.

Statistical Analysis

Data were presented as the mean $\pm$ standard error of the means. The differences between different conditions were analyzed using one-way analysis of variance. *P* values < 0.05 were considered statistically significant.

RESULTS

Susceptibilities of *N. gonorrhoeae* to aBL Inactivation at 405- and 470-nm Wavelengths

As shown in Figure 1, about 3.12- $\log_{10}$ reduction of bacterial CFU was achieved by 27 J/cm² (60 mW/cm², 7.5 minutes) 405-nm aBL. When the 405-nm aBL exposure was increased to 54 J/cm² (60 mW/cm², 15 minutes), eradication of bacterial CFU was achieved ($>6\text{-}\log_{10}$ CFU reduction). By contrast, less than 1- $\log_{10}$ reduction of bacterial CFU was observed at 54 J/cm² 470-nm aBL. When 470-nm aBL was increased to 234 J/cm² (60 mW/cm², 65 minutes), about 3.70- $\log_{10}$ reduction of bacterial CFU was produced.

Anti-Gonococcal Efficiency of 405-nm aBL Under Hypoxic Condition

The anti-gonococcal effect of 405-nm aBL was significantly inhibited under hypoxic condition by blowing N₂ (Fig. 2). An exposure of 54 J/cm² only induced less than 1- $\log_{10}$ reduction of bacterial CFU in contrast to the complete eradication of bacterial CFU ($>6\text{-}\log_{10}$ CFU reduction) under the same aBL exposure but without blowing N₂ ($P < 0.0001$).

Effects of 405-nm aBL on the Co-Cultures of *N. gonorrhoeae* and the Vaginal Epithelial Cells

The percentage of nonviable *N. gonorrhoeae* cells in co-cultures of *N. gonorrhoeae* and the vaginal epithelial cells was 32.85% $\pm$ 7.70%. This percentage increased to 78.15% $\pm$ 6.52% when exposed to 108 J/cm² 405-nm aBL ($P < 0.0001$) (Figs. 3 and 4). In contrast, no obvious increase in the number of nonviable VK2/E6E7 cells was observed. This

demonstrated that 405-nm selectively inactivated *N. gonorrhoeae* cells while sparing the vaginal epithelial cells.

Confocal microscope imaging under higher magnification ascertained the invasion of VK2/E6E7 cells by *N. gonorrhoeae* (Fig. 5). Viable adherent *N. gonorrhoeae* cells depicted as green “dots” by SYTO9 were observed. Because the sizes of *N. gonorrhoeae* cells are only about 1/50th of those of the vaginal epithelial cells, intracellular viable bacteria could only be identified at certain cross sections of the vaginal epithelial cells (solid white arrows). When VK2/E6E7 cells (aBL–saponon, without being permeabilized) were treated with aBL, increased number of nonviable adherent *N. gonorrhoeae* cells were observed. After permeabilizing (aBL + saponon), nonviable *N. gonorrhoeae* cells (hollow white arrows) were also observed in VK2/E6E7 cells. While, in the untreated VK2/E6E7 cells, nonviable intracellular *N. gonorrhoeae* cells were hardly observed after permeabilizing the membranes (NT-saponin).

Species and Quantity of Endogenous Photosensitizers in *N. gonorrhoeae*

The normalized absorption spectra of *N. gonorrhoeae* lysates, porphyrins standards and flavins are shown in Figure 6. The presence of porphyrins and flavins in *N. gonorrhoeae* (ATCC 700825) is supported by the spectroscopic results. The absorption peak of *N. gonorrhoeae* lysates at approximately 400 nm overlapped with the Soret band of porphyrins. There is only a slight elevation (shoulder) at approximately 460 nm, which corresponds to one of the absorption peaks of flavins. In the normalized fluorescence emission spectra of *N. gonorrhoeae* lysates, the emission peaks of around 520 and 620 nm overlapped with the peaks of the flavins and porphyrins, respectively (Fig. 7).

Quantitative UPLC analysis results are listed in Table 1. For *N. gonorrhoeae* (ATCC 700825), the total amount of endogenous flavins was 363.63 nmol/g. FAD and FMN each accounted for about 40% amount of the total flavins.

Species and Quantity of Endogenous Photosensitizers in Normal VK2/E6E7 Cells

The light absorption of normal VK2/E6E7 cells (not incubated with *N. gonorrhoeae*) was very low from 350 to 700 nm. No obvious absorption peaks around 400 nm were observed (Fig. 8). In the normalized fluorescence emission spectra, the emission peaks around 520 nm overlapped with those of the standard flavins (Fig. 9). Only a slight peak around 620 nm was observed. This indicated that there is only little amount of porphyrins in VK2/E6E7 cells. Quantitative analysis results of UPLC showed that the amount of flavins in VK2/E6E7 cells was also significantly lower than that in *N. gonorrhoeae* (Table 1).

DISCUSSION

The results of this study showed that *N. gonorrhoeae* (ATCC 700825) was more susceptible to 405-nm aBL than to 470-nm aBL. Compared with the other bacterial species previously studied by our group, *N. gonorrhoeae* (ATCC 700825) turned out to be the most susceptible bacterium to 405-nm aBL. Over 50 J/cm² aBL exposure are commonly required to achieve a 3-log₁₀ reduction of CFU for *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Escherichia coli* [21]. For all these bacterial species we tested, 470-nm wavelength almost

showed no effect (data not shown). Many studies reported that, in contrast to most other bacterial species, *N. gonorrhoeae* contains no superoxide dismutase, which is the major antioxidant defense system in bacteria [22]. This might explain the high susceptibility of *N. gonorrhoeae* to aBL inactivation.

In this study, 405 and 470 nm were initially chosen as they correspond to the maximum absorption peaks of porphyrins and riboflavin, respectively, which are hypothesized to be the major endogenous photosensitizers in bacteria. However, according to the absorption spectra of flavins (Fig. 6), flavins also absorb light at 405 nm although the maximum absorption peak is around 450 nm. As a result, 405-nm aBL can excite both porphyrins and flavins. The 470-nm aBL can only excite flavins because the light absorption from 450 to 470 nm by porphyrins is close to nil. In this study, spectroscopic and UPLC analysis verified the presence of porphyrins and flavins in *N. gonorrhoeae* (ATCC 700825). The content of FAD and FMN accounted for about 90% of the total amount of flavins in *N. gonorrhoeae* (ATCC 700825). Our previous results showed that the total amount of the endogenous porphyrins was 22.26 nmol/g and coproporphyrin (19.16 nmol/g) accounted for 86.06% [23]. It was reported that the endogenous porphyrins patterns (e.g., porphyrin quantity and porphyrin species) *in vitro* are highly affected by the culturing conditions (e.g., time of culturing, passaging, culture media) [24]. In this study, the culture conditions of *N. gonorrhoeae* were kept consistent before testing the inactivation effects of aBL and the quantity of endogenous photosensitizers. The presence of endogenous photosensitizing chromophores in some aBL-sensitive microbial strains were verified in several recent studies [11–15,25,26]. However, quantitative analysis were only carried in some of these studies. In these studies, different units (nmol/CFU, nmol/bacteria weight) were used to present the amount of endogenous photosensitizers [14,24]. Therefore, the data in different studies are not comparable, and it is hard to correlate the aBL efficacy with the amount of endogenous photosensitive compounds in bacteria from the data in different studies.

The spectroscopic and UPLC analysis results indicate that 405-nm aBL excites both porphyrins and flavins in *N. gonorrhoeae* and subsequently leads to phototoxicity to *N. gonorrhoeae* cells. Therefore, the mechanism of the anti-gonococcal activity of aBL was preliminarily explored based on 405-nm aBL. The present results showed that the anti-gonococcal effects of 405-nm aBL was oxygen dependent. Moreover, in our previous study [23], it was observed that a high concentration of NaN_3 significantly suppressed but didn't completely inhibit the anti-gonococcal effect of 405-nm aBL. This suggests that ROS other than $^1\text{O}_2$, such as superoxide anion, hydrogen peroxide and hydroxyl radical through type I photochemical pathway, were also involved. All these phenomena indicate that beside porphyrins, flavins also play some role in the anti-gonococcal activity of 405-aBL, the sensitized reactions of which include both type I and type II pathways [27].

Two seemingly paradoxical phenomenon were observed in this study. The first one is that although the total amount of flavins in *N. gonorrhoeae* was about 10-fold of that of porphyrins, 470 nm wavelength, which corresponds to the maximum absorption peak of flavins, exhibited less inactivation efficacy. This phenomena can be attributed to the lower singlet oxygen quantum yields of flavins and the lower optical absorbability of *N. gonorrhoeae* (ATCC 700825) at 470 nm. The singlet oxygen yield ratio of 405-nm aBL to

470-nm aBL can be calculated based on the following formula:

$\Phi_{O_2}^1 = \Phi_{\Delta} \cdot I_0 \cdot (1 - 10^{-\epsilon(\lambda) \cdot c})$, where $\Phi_{O_2}^1$ is the yield of singlet oxygen, Φ is the quantum yield of singlet oxygen, and $I_0 (1 - 10^{-\epsilon(\lambda) \cdot c})$ is the number of photon absorbed by photosensitizer. I_0 is the fluence rate of light irradiated on the reaction system, $\epsilon(\lambda)$ is the molar extinction coefficient of photosensitizer, and c is the concentration of photosensitizer. As discussed above, coproporphyrin, FAD, and FMN are the prominent endogenous photosensitizing compounds in *N. gonorrhoeae* (ATCC 700825). If only coproporphyrin is taken in to account for the 1O_2 yield quantum by 405-nm excitation, and only FAD and FMN is taken into account for the 1O_2 quantum yield by 470-nm excitation, the single oxygen yield ratio of 405-nm to 470-nm wavelength in *N. gonorrhoeae* can be calculated

$$\frac{\Phi_{405nm}}{\Phi_{470nm}} = \frac{\Phi_{\Delta\text{Coproporphyrin}} \times I_0 \times \left(1 - 10^{-\epsilon_{\text{Coproporphyrin 405 nm}} \times C_{\text{Coproporphyrin}}}\right)}{\Phi_{\Delta\text{FAD}} \times I_0 \times \left(1 - 10^{-\epsilon_{\text{FAD 470nm}} \times C_{\text{FAD}}}\right) + \Phi_{\Delta\text{FMN}} \times I_0 \times \left(1 - 10^{-\epsilon_{\text{FMN 470nm}} \times C_{\text{FMN}}}\right)}$$

The singlet oxygen quantum yields were determined to be 0.58 for coproporphyrin [28], 0.07 for FAD, and 0.51 for FMN [29]. The molar extinction coefficient of coproporphyrin at 405 nm is $4.78 \sim 5.3 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ [30]. The molar extinction coefficient of FAD and FMN around 470 nm is $1.13 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ [31] and $1.06 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ [32], respectively. By putting all the corresponding values, the coproporphyrin content (19.16 nmol/g), FAD content (168.03 nmol/g), and FMN content (161.48 nmol/g) into the above equation, the singlet oxygen yield ratio of 405-nm aBL to 470-nm aBL is calculated to be 5.24:1. If the singlet oxygen yield induced by the photoexcitation of FAD and FMN at 405 nm is taken into account, the singlet oxygen yield of 405-nm aBL will be higher.

The other phenomena is that the absorption peak at 470 nm of *N. gonorrhoeae* (ATCC 700825) is much lower than that at 405 nm ($A_{405nm}:A_{470nm} \approx 1:0.25$), even though the total amount of flavins in *N. gonorrhoeae* was over 10-fold of that of porphyrins (363.63 vs. 22.26 nmol/g). The reason is that the molar extinction coefficient of FAD and FMN around 470 nm are more than 40 times lower than that of coproporphyrin at 405 nm.

Potential antimicrobial strategy used in the clinic should selectively inactivate pathogenic microbes while sparing the normal host cells and tissues. As a safety study of 405-nm aBL, the effects of aBL on co-cultured *N. gonorrhoeae* and VK2/E6E7 cells were investigated in this study. This “side-by-side” comparison results indicated that the susceptibility to aBL of *N. gonorrhoeae* cells was much higher than that of the host cells. The selectivity of aBL inactivation of *N. gonorrhoeae* over VK2/E6E7 cells is likely to be attributed to the difference in the content of endogenous photosensitizers between *N. gonorrhoeae* cells and VK2/E6E7 cells. Optical fiber diffusers have been successfully used in the clinic to deliver 630 nm laser in photodynamic therapy for tumor or condyloma acuminata in vagina. So, 405

nm laser (2 W) combined with optical fiber diffuser with certain length (4 cm) could be used to deliver 60 mW/cm² 405 nm light to the vaginal canal in the clinic. Further studies, which are underway, are warranted in assessing the effectiveness and safety of aBL therapy for gonococcal infections in animal models and exploring the potential clinical applications.

To sum up, the results of our present study suggested that 405-nm aBL is a potential strategy to control gonococcal infections.

CONCLUSION

The 405-nm aBL is effective in inactivating *N. gonorrhoeae* while sparing the normal vaginal epithelial cells. Reactive oxygen species generated from the photochemical reactions between aBL and endogenous photosensitizers play a vital role in the anti-gonococcal activity of 405-nm aBL.

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Fig. 1. Inactivation of *Neisseria gonorrhoeae* (ATCC 700825) by 405-nm aBL (60 mW/cm², for 9, 18, 27, 36, 45, and 54 J/cm²) and 470-nm aBL (60 mW/cm², for 18, 54, 90, 126, and 234 J/cm²). Bars denote standard error of the means. aBL, antimicrobial blue light; CFU, colony forming units.

Fig. 2. Effects of hypoxic condition on the inactivation efficiency of 405-nm aBL on *Neisseria gonorrhoeae* (ATCC 700825). Bars denote standard error of the means. aBL, antimicrobial blue light; CFU, colony forming units.

Fig. 3.

Confocal images of vaginal epithelial cells (VK2/E6E7) infected with *Neisseria gonorrhoeae* (ATCC 700825). aBL: treated with 405-nm aBL. Viable VK2/E6E7 cells and *N. gonorrhoeae* were stained green by SYTO9, and nonviable VK2/E6E7 cells and *N. gonorrhoeae* were stained red by PI. A few, spontaneously dead VK2/E6E7 cells and *N. gonorrhoeae* cells were observed in the culture without aBL irradiation. Bar: 5 μ m. aBL, antimicrobial blue light; NT, no treatment; PI, propidium iodide.

Fig. 4. The percentage of nonviable *Neisseria gonorrhoeae* cells in the co-cultures of *N. gonorrhoeae* and VK2/E6E7 cells. aBL: treated with 405-nm aBL with exposure of 108 J/cm² at 60 mW/cm². $P < 0.0001$ (aBL vs. NT). aBL, antimicrobial blue light; NT, no treatment.

Fig. 5.

Confocal images of the co-cultures of *Neisseria gonorrhoeae* and VK2/E6E7 cells. aBL: treated with 405-nm aBL. +Saponin: incubated with saponin. –Saponin: not incubated with saponin. Extracellular viable bacteria exhibit purple + green, extracellular nonviable bacteria exhibit purple + red. Intracellular viable bacteria exhibit green, and intracellular nonviable bacteria exhibit red in the permeabilized VK2/E6E7 cells or nonviable VK2/E6E7 cells. Solid white arrows: viable intracellular *N. gonorrhoeae*. Hollow white arrows: nonviable intracellular *N. gonorrhoeae*. Bar: 5 μm. aBL, antimicrobial blue light; NT, no treatment; PI, propidium iodide.

Fig. 6. Normalized absorption spectra of *Neisseria gonorrhoeae* (ATCC 700825) lysates, porphyrins, and flavins. FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

Fig. 7. Normalized fluorescence emission spectra of *Neisseria gonorrhoeae* (ATCC 700825) lysates, porphyrins, and flavins. Excited by 405 nm. FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

Fig. 8.
Absorption spectrum of VK2/E6E7 cell lysates.

Fig. 9.
Normalized fluorescence emission spectrum of VK2/E6E7 cell lysates, porphyrins, and flavins. Excited by 405 nm. FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

TABLE 1.

Concentrations of Flavins in *Neisseria gonorrhoeae* ATCC 700825 and VK2/E6E7 Cells (nmol/g [protein weight])

Sample	FAD	FMN	Riboflavin	Sum
ATCC 700825	168.03	161.48	34.12	363.63
VK2/E6E7 cells	23.89	1.31	2.92	28.12

FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.