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ORIGINAL ARTICLE

Photodynamic inactivation of methicillin-resistant *Staphylococcus aureus* by indocyanine green and near infrared light

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ABSTRACT

Background: Antimicrobial photodynamic therapy (APDT) has become a potential regimen to treat multidrug-resistant bacterial infections. Limited data showed indocyanine green (ICG), a safe and inexpensive contrast medium for eye angiography and hepatic function examination, is an effective photosensitizer in APDT to kill methicillin-resistant *Staphylococcus aureus* (MRSA) after excitation with laser.

Objective: We investigated the potentials of ICG-APDT with an inexpensive, non-coherent commercial near infrared (NIR) lamp against MRSA.

Methods: The inhibition of MRSA was studied after exposing bacteria to NIR with different light doses and concentrations of ICG. The selectivity on MRSA was examined on human fibroblasts. Bacterial virulence including the activities of coagulase and enterotoxin was investigated. The effects of singlet oxygen scavengers (tryptophan and ascorbic acid) and H_2O_2 on cell survival were evaluated. The morphology of bacteria after PDT was observed by transmission electron microscopy.

Results: ICG-PDT inhibited the growth of bacteria by 5 log (99.999% inhibition) with 200 J/cm^2 at 65.5 mW/cm^2 in the presence of 100 μg/mL ICG. Adding 0.1% H_2O_2 at a lower PDT dose (25 μg/mL ICG and 100 J/cm^2) increased its efficacy by 5 log. This PDT dose was not toxic to human fibroblasts. PDT significantly reduced the level of bacterial virulence factors. The inhibition effects were decreased by tryptophan and ascorbic acid suggested singlet oxygen involved in the process. TEM showed severe non-selective cell destruction immediately after irradiation.

Conclusion: The study reveals ICG-PDT has the potential to treat MRSA by using a clinical accessible NIR lamp and photosensitizer.

Introduction

Staphylococcus aureus (*S. aureus*), a normal flora on the human skin and mucous membranes, is the most common cause of cutaneous infection.[1] Methicillin is a narrow spectrum beta-lactam antibiotic of the penicillin class. It is widely used in clinic to treat penicillin-resistant *S. aureus* infection. However, with the widespread use of methicillin, the number of methicillin-resistant *S. aureus* (MRSA) isolates have increased globally.[2] In the United States, the mortality rate from MRSA bacteremia is approximately 28% and causes approximately 18,000 deaths per year.[3] Vancomycin has been considered as a drug of last resort; however, vancomycin-resistant

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S. aureus is also becoming quite common.[4] It is therefore important to find alternative ways to overcome MRSA infections.[5,6]

Photodynamic therapy (PDT) has been widely used in oncology to treat head and neck, lung, breast, and skin cancer.[7] The components of PDT include a photosensitizer, light, and oxygen. It has the advantage of dual selectivity, in that the photosensitizer can be directed to its target tissue or cell, and the illumination can be directed to the lesion. PDT has also shown great potential for treating benign dermatoses[8] and bacterial infections.[9] The lethal effect on cell targets are mainly attributed to reactive oxygen species (ROS) and singlet oxygen generated during irradiation via Type I and Type II mechanisms in mammalian cells and microbes.[9–12] PDT causes damages to different organelles in an organism by combining different photosensitizers or various incubation times and photodynamic resistant bacterial strains have so far not been observed.[13]

The use of PDT to kill microorganisms dated back to the last century and began to bloom in the 1990s.[9] It is well documented that PDT is more effective in treating Gram-positive species than Gram-negative species because of the fundamental different cell wall structures of the bacteria. The cytoplasmic membrane of Gram-positive bacteria is surrounded by a relatively porous layer of peptidoglycan and lipoteichoic acid that allows photosensitizer to crossover more readily. Compared with Gram-positive bacteria, Gram-negative bacteria have a relatively impermeable cell wall that prevents effective penetration of photosensitizer. The uses of PDT to treat drug resistant bacteria have shown promising results.[13] For example, aminolevulinic acid-mediated PDT has the potential to eliminate the biofilm of antibiotic-resistant *S. aureus* and *Staphylococcus epidermidis* effectively.[14] However, the absorption peak of this photosensitizer lies within visible light spectrum that prevents a deeper tissue penetration of light.

Indocyanine green (ICG) is a relatively less expensive photosensitizer compared to other photosensitizers such as aminolevulinic acid, verteporfin,[15] and porfimer sodium[16] and has a longer absorption peak of light in the near infrared (NIR) region. ICG has been approved by the United States Food and Drug Administration to be used as a contrast agent in retinal and choroidal vascular imaging, and liver function test since 1956 because of its low toxicity and low incidence of side effects.[17] ICG-mediated PDT (ICG-PDT) has been applied in oncological research started in 2002.[18] It inhibits tumor growth in many different cancers including melanoma,[18] leukemia,[19] colon cancer,[20] and oral squamous cell carcinoma by apoptosis and necrosis.[21] In antibacterial research, ICG-mediated PDT with laser has resulted in 5.6 log and 2 log growth reduction of *S. aureus* and *Pseudomonas aeruginosa*, respectively.[11] ICG-PDT on resistant strains of *S. aureus* provided a 95% cell killing with a 1.4 W/cm^2 light power laser.[22] However, cell killing by a thermal effect during irradiation with a high power laser cannot be totally excluded as PDT at high fluence rate and with 200 μg/mL ICG, the temperature of the bacterial suspensions increased to 47 °C.[11] Bacterial virulence proteins including coagulase, and enterotoxin are critical factors affecting the pathogenic capacity of microbes. Coagulase is a pivotal virulence factor used to distinguish *Staphylococcus* from other species of bacteria. The bacteria use coagulase to covert fibrinogen into fibrin in the host to protect them from phagocytosis.[23] Staphylococcal enterotoxins can induce toxic shock syndrome and systemic toxicity, and suppress the immune responses of infected targets.[24] One of the advantages of APDT is the reduction of virulence factors after treatments.[25,26] Near infrared (NIR) lamp emits near infrared light with a wavelength of 780–2500 nm. NIR irradiation using lamp, light emitting diode (LED) or laser has been long applied in low level laser/light therapy (LLLT)[27] or photobiomodulation therapy (PBMT),[28] a term reach a consensus in 2015. It has a wide range of therapeutic applications including hair loss,[29] skin aging,[30] brain disorder,[31] exercise performance,[32] and relieve pain.[27,33] The mechanism is believed to the activation of mitochondrial respiratory chain by photons. Another theory is the photons trigger of light-sensitive ion channels which allowing calcium influx into cells and turns on numerous signaling pathways which lead to initiation transcription factors and genes.[34] A laser light device is relatively expensive compared to non-coherent light source. It also requires specific environment and training to use. A NIR lamp costs around US$60. It is much cheaper and smaller than a laser light source. Using NIR lamp together with the inexpensive ICG makes ICG-PDT easily accessible to most clinics.

In this study, we explore whether ICG-PDT is potential to treat MRSA with a commercial non-coherent NIR light source.

Materials and methods

Bacterial isolates

Different MRSA strains isolated from patients were used in this study. MRSA/JD004 was isolated from a patient's leg ulcer. The other 3 strains, MRSA/L1, L2, L4 were isolated from patients' blood at National Cheng Kung University Hospital, Taiwan. The isolates were stored at −80 °C in glycerol before experiments. MRSA were thawed and grown on Luria–Bertani (LB) agar plates (BD biosciences, California, USA) at 37 °C in a 100% humidity incubator. To reach the log growth phase, overnight culture was diluted with LB broth to a total volume of 5 mL, and incubated at 37 °C for 3 h.

Photoinactivation of MRSA

ICG (Diagnogreen, Taiwan Daiichi Sankyo Co., Taipei, Taiwan) was prepared fresh by dissolving the drug in sterile distilled water before use. Equal volume of different concentrations of ICG (50 μL) and bacteria suspension (5×10^5 CFU/50 μL) were pipetted into a 96-well plate. The plate was exposed to NIR light with different light doses (50, 100, and 200 J/cm^2) at 65.5 mW/cm^2 after 10 min incubation at 37 °C. Control groups included absolute control (neither light exposure nor presence of ICG), dark control (incubated with ICG and kept in the dark), light control (exposed to light without ICG). Bacteria counts in CFU/mL were calculated by serial dilution and bacterial plate count methods.[35]

Phototoxicity in human fibroblasts

In order to find an optimal condition that ICG-PDT inhibits MRSA growth but is harmless to normal cells, human fibroblasts (Hs 68, Bioresource Collection and Research Center, Hsinchu, Taiwan) were treated with ICG-PDT. Fibroblasts were culture in DMEM (Invitrogen, California, USA) complete medium. Each well of a 96-well plate were seeded with 2×10^4 cells in 100 μL medium. Culture medium was replaced by different concentrations of 100 μL ICG solution (12.5, 25, 50, and 100 μg/mL) after overnight culture. Each PDT condition contained 6 repeated samples. After 10 min incubation, cells were washed with LB broth and exposed to different light doses of NIR. Cytotoxicity was determined by tetrazolium salt WST-1 assay (Roche, Mannheim, Germany).[36]

Bacterial virulence

Coagulase activity

The detection limits of the coagulase activity assay (Staphylase test, DR0595, Oxoid, Hampshire, UK) was first determined by adding different amount of MRSA from absolute control (5×10^5 to 1×10^7 CFU/mL) onto the test membrane following the

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