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Synergistic antimicrobial activity based on the combined use of a gemini-quaternary ammonium compound and ultraviolet-A light

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ABSTRACT

This study examined the utility of synergistic disinfection employing a gemini-quaternary ammonium salt (a gemini-QUAT, namely 3,3′-(2,7-dioxaoctane)bis(1-decylpyridinium bromide)), as an organic biocide in combination with irradiation by an ultraviolet-A (UV-A) light-emitting diode (LED) with a peak wavelength of 365 nm. The combined system represents a novel disinfection method utilizing facilitated *in situ* oxidation depending on overproduction of reactive oxygen species (ROSs) triggered by the initial action of the gemini-QUAT on the bacterial membrane. We demonstrate that this combination decreased the viability of pathogenic bacteria in a significant and rapid manner, and depended on doses of the gemini-QUAT and the fluence: the viability of *Escherichia coli* was reduced by greater than 5.0-logs by the combination procedure, but the decrease in viability was only 2.3-logs for exposure to UV at the same fluence dose in the absence of the gemini-QUAT. Adding catalase as a radical scavenger decreased bacterial inactivation by the combined disinfection procedure. Flow cytometric analysis indicated superoxide and hydrogen peroxide overproduction within cells treated with the combined disinfection procedure. The excessive superoxide, detected only in the combined system, appeared to be generated by the action of the gemini-QUAT at the bacterial membrane, leading to excessive and rapid generation of ROS in the system. Our data strongly suggested that this ROS promoted bacterial membrane peroxidation during initial treatment by the combination method, resulting in increased oxidative modification of DNA. These oxidative reactions may play an important role in the efficacy of this disinfection procedure.

1. Introduction

Irradiation with ultraviolet-C (UV-C) light traditionally has been used as an effective disinfection procedure. Applications of UV-C irradiation have been employed for disinfection of water, air, and surfaces [1]. Generally, the germicidal potency of UV occurs at a primary wavelength of 254 nm. UV-C at 254 nm facilitates the formation of cyclobutane pyrimidine dimers from adjacent thymidines in DNA. On the other hand, it has been reported that irradiation with ultraviolet-A (UV-A) light at wavelengths of 315–400 nm (particularly light with wavelength of 365 nm) has lethal effects on bacteria, including pathogenic microbes [2–5]. The high bactericidal efficacy of 365-nm UV-A irradiation results from the generation of reactive oxygen species (ROS) such as hydrogen peroxide and hydroxyl radical [3]. It is believed that the ROS generation initiates critical bactericidal events, including the oxidation of lipid membranes [6], DNA [3] and intracellular proteins [7]. However, disinfection by UV-A light alone requires light sources that emit 365-nm light at high fluence rate to inactivate bacteria effectively. With lower fluence rate sources, bacteria exhibit tolerance to UV-A irradiation. For example, *Pseudomonas aeruginosa* induced a *relA*-dependent adaptive response following low fluence rate (2.8 mW/cm^2 at 365 nm) UV-A irradiation, rendering this bacterium resistant to further irradiation [8]. Thus, in order to inactivate bacteria rapidly and consistently regardless of the fluence rate of the UV-A light source, some combined disinfectant systems have been constructed. Similar combined applications are used with antibiotics; for instance, co-administration of two antibiotics provides synergistic antimicrobial activity *in vitro* against clinical isolates of fungi [9]. In an analogous fashion, the bactericidal activity of UV-A light is enhanced in combination with titanium dioxide particles [10,11], with particles of titanium dioxide and silver [12], or with hydrogen peroxide [13]. Increase of the bactericidal activity in the combined systems was attributed to the enhanced oxidative reactions caused by extra-cellular ROS generated by the photocatalysis/photolysis generated by the combined materials. The combination of titanium dioxide and low fluence rate (0.8 mW/cm^2 at 365 nm) UV-A light provided synergistic elevation of bactericidal activity compared to either

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treatment alone, leading to a 96% loss of viability following 30 min of treatment [11]. However, in order to obtain higher bactericidal potency and to permit larger-volume treatment, time-consuming and difficult post-treatment procedures (e.g., separation of insoluble titanium dioxide after the disinfectant treatment) are required. High bactericidal activities were achieved by disinfectant systems combining UV with soluble materials such as hydrogen peroxide, but bacterial suspensions containing hydrogen peroxide had to be irradiated with high fluence rate (110 mW/cm^2 at 400 ± 20 nm) light to provide decomposition of hydrogen peroxide to higher oxidation states by photoreaction [13]. The disadvantages apparent in these examples imply the need to develop more effective and convenient combined disinfection procedures. Advances in these methods would facilitate easier sterile manufacturing; reductions in the required total fluence doses would counteract limitations such as the high cost of light source devices and the low potency of light-permeation resulting from the presence of particulates and contaminants in treated water. For example, 0.1% riboflavin (a photosensitive water-soluble organic substance that is "generally regarded as safe") has been used in combination with UV-A irradiation with a low fluence rate (3 mW/cm^2 at 365 nm) to treat *Acanthamoeba* [14], suggesting a promising photochemical therapy for *Acanthamoeba* keratitis.

In the present study, we constructed a new disinfection system that combines a soluble organic biocide (a gemini-quaternary ammonium compound (gemini-QUAT; 3,3′-(2,7-dioxaoctane)bis(1-decylpyridinium bromide)), also referred to as 3DOBP-4,10) with UV-A irradiation. Gemini-QUATs are composed of two similar QUAT moieties linked by hydrocarbon chains with or without heteroatoms. Such gemini-QUATs have greater surface activities [15], wider antimicrobial spectra, and more potent antimicrobial activities than conventional QUATs, such as benzalkonium chloride [16–18]. Moreover, 3DOBP-4,10 has two advantages: 3DOBP-4,10 has lower toxicity to human cells than conventional QUATs such as benzalkonium chloride, and the compound has high water-solubility (589 g/100 g H_2O) [17]. We hypothesized that the powerful antimicrobial activity of 3DOBP-4,10 permits increased entry of oxygen into cells following the interaction of the gemini-QUAT with the cell wall and membrane, resulting in the generation of ROS in the cells [19]. 3DOBP-4,10 appears to synergize with the bactericidal activity of the ROS generated by UV-A light. Unlike some combined systems described previously, the combination of gemini-QUAT + UV-A is expected to generate ROS as an intracellular (not extracellular) species. Fluorescence probes that respond to intracellular ROS demonstrated that an ordinary QUAT, cetyltrimethylammonium bromide (CTAB), produced ROS in cells treated with inhibitory concentrations of CTAB [20].

The purpose of the present study was to demonstrate the synergistic bactericidal activity of the combination of low concentration 3DOBP-4,10 and low fluence rate UV-A irradiation. The high bactericidal activity associated with the combined treatment was also investigated mechanistically by focusing on the associated generation and action of ROS.

2. Materials and methods

2.1. Reagents

A gemini-QUAT, 3DOBP-4,10, was synthesized in our laboratory [17]. 3DOBP-4,10 shows no absorption of UV-A light in the wavelength range used in the present study (Fig. 1).

2.2. Light source and irradiation

A light-emitting diode (LED) device manufactured by SAN Electronics Ltd. (Aizumi, Japan) was used as a light source. The device is

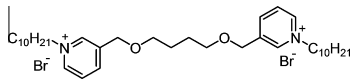

Fig. 1. Chemical structure of 3DOBP-4,10, a gemini-QUAT used in the combination disinfection process described here.

constructed with 27 LED elements (NCSU033A; Nichia Corp., Anan, Japan) arranged in a three-column-by-nine-row array on a basal plate of 100 mm × 300 mm. The fluence rate of the light (wavelength range: 350–385 nm; peak wavelength: 365 nm) emitted by this device was 4.77 mW/cm^2 (Fig. 2). The spectrum and light fluence rate were measured at 65 mm from the device, using a cumulative UV meter (MCPD-3700A; Otsuka Electronics Co., Ltd., Hirakata, Japan) that was tested while shielded by the cover of a plastic Petri dish, consistent with the LED's use in all bactericidal tests. The fluence rate was expressed in mW/cm^2 and the total fluence dose, which was calculated based on the fluence rate and exposure time, was expressed in J/cm^2. The distance (65 mm) was applied as the irradiation distance between the UV-A–LED device and the surface of bacterial suspensions through all bactericidal assays. In the assays, UV-A irradiation was performed in a dark room, using bacterial suspensions in plastic Petri dishes or six-well culture plates that were held at 30 or 37 °C in a water bath.

2.3. In vitro assay for determination of bactericidal level

Bactericidal level was determined by counting of the number of colony-forming units (CFUs).

The bacteria used were *Escherichia coli* NITE Biological Resource Center (NBRC) 12713, *Salmonella enterica* NBRC 13245, *Serratia marcescens* NBRC 12648, *Staphylococcus aureus* NBRC 12732, *S. epidermidis* NBRC 12993, *P. aeruginosa* American Type Culture Collection (ATCC) 10145, and *S. aureus* ATCC 700699 (MRSA). All bacteria were purchased from ATCC and NBRC.

After cultivation (15 h, 37 °C) in Luria-Bertani medium (LB broth, Lennox; Nacalai Tesque Inc., Kyoto, Japan), bacterial cells were harvested by centrifugation (6570*g*, 3 min, 4 °C) and washed twice with the phosphate-buffered saline (D-PBS(−); Nacalai Tesque Inc.). Bacterial suspensions (2.0 × 10^6 cells/ml) were prepared in sterile ion-exchanged water, and transferred to a plastic Petri dish (90 mm × 20 mm; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), at 20 ml/plate. A stock solution of 3DOBP-4,10 formulated in sterile milliQ water was added to the bacterial suspension by diluting by a minimum of 1000-fold or more to the prescribed concentration before UV-A irradiation. Following irradiation, aliquots (0.2 ml) of each suspension were subjected to 10-fold serial dilutions with 0.8% (w/v) physiological saline containing 0.7% (w/w) Tween 80 (Kanto Chemical Co., Inc., Tokyo, Japan). In this context, the Tween 80 serves as an inactivating agent that quenches the

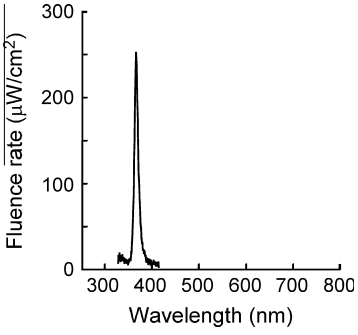

Fig. 2. Emission spectrum of UV-A–LED used here. This UV-A–LED provides a spectral maximum at 365 nm. The fluence rate reaches 4.77 mW/cm^2 at a 65-mm distance between the illumination source and UV meter.