Effects of Photodynamic Therapy on Rapidly Growing Nontuberculous Mycobacteria Keratitis

Min-Hsiu Shih and Fu-Chin Huang

Purpose. The authors investigated the antimicrobial effect of methylene blue (MB)-mediated photodynamic therapy (PDT) on *Mycobacterium fortuitum* keratitis.

Methods. In the in vitro study, the mycobacterial suspension and colonies were treated with the following: no MB, no light (normal control); MB and no light (dye control); light and no MB (light control); MB and light (PDT). Morphologic characteristics were examined by transmission electron microscopy. The bactericidal effects of combined PDT and antibiotic therapy (ciprofloxacin, moxifloxacin, and amikacin) were determined using the broth microdilution technique. Twenty-one rabbits with *Mycobacterium* keratitis were randomly divided into three groups (no treatment, topical amikacin treatment, and PDT combined with amikacin treatment). The clinical features of keratitis were scored and graded before treatment and before euthanatization. The diseased corneas were trephined for quantitative bacteriologic analysis to determine the antibacterial efficacy of the treatment.

Results. In the in vitro tests, the bacterial count had a 2-log reduction immediately after PDT treatment at 100 J/cm^2 with 10^{-3}% MB. After PDT at 100 J/cm^2 with 10^{-2}% MB, almost no viable bacteria were detected. PDT had a synergistic antimicrobial effect in combination with antibiotics. The phototoxicity occurred in the cytoplasm first and then disrupted the mycobacterial cell walls by lysis. In the rabbit keratitis model, combined PDT resulted in significantly less bacterial burden ($P < 0.01$) than in the amikacin group.

Conclusions. This study demonstrated the effectiveness of MB-mediated PDT against *Mycobacterium fortuitum.* PDT could be a potential alternative treatment for nontuberculous mycobacterial corneal infections. (*Invest Ophthalmol Vis Sci.* 2011; 52:223–229) DOI:10.1167/iovs.10-5593

Nontuberculous mycobacteria (NTM) are rapidly growing acid-fast bacilli that are ubiquitous in the environment and may induce indolent, but intractable, infection of corneal tissue after foreign body trauma or the use of contaminated surgical instruments such as in laser-assisted in situ keratomileusis in ophthalmic surgery.[1–3] Among them, the main pathogens responsible for human infections are *Mycobacterium abscessus*, *Mycobacterium chelonae*, and *Mycobacterium fortuitum*. NTM are usually resistant to the conventional antituberculous agents, including isoniazid, rifampin, streptomycin, ethionamide, and ethambutol, and they exhibit varied susceptibility to other available antibiotics such as amikacin, ciprofloxacin, gatifloxacin, and clarithromycin.[4,5] Medical treatment of NTM keratitis always requires months of therapy even when it is based on the drug sensitivity test results. Surgical debulking of the infection focus is helpful in facilitating the penetration of antibiotics.[1]

Photodynamic therapy (PDT), discovered more than 100 years ago, is known for its killing effect on microorganisms and for decreasing the activity of bacterial virulence factors. Additionally, phenothiazinium dyes, such as methylene blue (MB), used in conjunction with PDT produce antimicrobial activity by effectively photo-inactivating a variety of microorganisms including *Candida albicans* and endospore-forming *Bacillus* spp.[6–18] Previous studies showed that PDT was successful in reducing the bacterial burden of *Mycobacterium bovis* BCG-induced granuloma and *Mycobacterium marinum* infection.[6,7] However, there are little data on the potential efficacy of PDT for the treatment of NTM ophthalmic infections.

In clinical practice, treatment of NTM keratitis remains a great challenge to ophthalmologists.[1] To elucidate the effect of PDT on NTM keratitis, we investigated the microbial killing effect of MB-assisted photosensitization of *M. fortuitum* in vitro and in vivo. The effect of conventional antibiotic amikacin combined with PDT was also determined. We hope this study provides an in-depth understanding of the potential clinical application of PDT for treatment of NTM keratitis in the future.

Materials and Methods

In Vitro Studies

Bacterial Isolates. One clinical isolate of *M. fortuitum* from a patient with infectious keratitis at National Cheng Kung University Hospital was selected as the test organism and was stored at −80°C until use. The isolate was grown in Mueller Hinton broth at 37°C for 72 to 96 hours. The bacterial suspension was then centrifuged and prepared in phosphate-buffered solution (PBS) with 0.02% Tween 80 (J. T. Baker, Phillipsburg, NJ) and was mixed vigorously using a vortex mixer to a final concentration approximating a 0.5 McFarland standard. The stock suspension was diluted 1:100 for minimal inhibitory concentration (MIC) testing and 1:10 for PDT.

Antimicrobial Susceptibility Testing. Ciprofloxacin hydrochloride and moxifloxacin hydrochloride were purchased from Bayer Schering Pharma AG (Berlin, Germany). Amikacin sulfate was purchased from MP Biomedical, Inc. Antimicrobial agent powders were dissolved in sterile water and filtered through 0.22-μm pore size membrane filters to produce stock solutions. MIC drug sensitivities were determined using the Clinical and Laboratory Standards Institute (CLSI)-approved broth microdilution technique.[19] Serial double dilutions of antimicrobial agents were prepared according to CLSI recommendations, ranging from 0.03 to 64 μg/mL. The trays were incubated at 37°C in ambient air and read after 72 hours. The MIC was taken as the lowest concentration of the drug at which no growth was visible. *Escherichia coli* ATCC 25922 and *M. chelonae* ATCC 35752 was used for quality control tests.

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Photosensitization and Irradiation. MB was purchased from Merck KGaA (Darmstadt, Germany), dissolved in sterile water, and filtered through 0.22-μm pore size membrane filters, which served as a 1% stock solution (wt/vol) (31 mM). Then it was diluted and stored before use in brown microcentrifuge tubes. The PDT light source was a metal halogen lamp (PDT-1200; Waldmann, Villingen-Schwenningen, Germany), emitting light at wavelengths of 560 to 780 nm. The light fluence at the irradiated surface was set at 100 mW/cm^2.

Photodynamic Treatment of Bacterial Suspensions. One hundred microliters of the bacterial suspension was added to each well of the 24-well tray and was mixed with 50 μL of each concentration of MB and 350 μL of PBS. Triplicate samples were prepared for each MB concentration. Negative controls contained 100 μL bacterial suspension and 400 μL PBS in a final volume of 0.5 mL After covering with aluminum foil, the plates were incubated and shaken at 240 rpm at 37°C for 10 minutes. For PDT, the foil covering of the plates was removed, followed by light exposure to the indicated power setting. The dark control plates with their foil covering were put side by side with the plates to be exposed to PDT. After treatment, serial 10-fold dilutions were prepared with sterile PBS in a 96-well plate. Ten microliters from each dilution was placed on Mueller Hinton agar and incubated at 37°C for 72 hours. Quantitative analysis of viable mycobacterial colonies was conducted 4 days after inoculation. At least four tests were performed on each of the isolates. Colony counts representing the surviving bacteria were expressed as colony-forming units (CFU)/mL. Data were transformed to logarithmic values. The log value of 2 was the lowest limit of detection in this study.

Combined Antibiotic and PDT. Based on the MIC and PDT results, sublethal concentrations of MB (50 μL) and one of the antibiotics (50 μL) were placed in wells that contained 100 μL of 10-fold dilutions of the 0.5 McFarland stock bacterial suspension and 300 μL Mueller Hinton broth. After exposure to PDT at 100 J/cm^2, the 24-well plates, which contained MB and antibiotic in the medium, were incubated at 37°C for 72 hours in the dark according to the same method used for MIC testing. Next, 500 μL PBS and 200 μL Tween 80 (0.12%) were added to each well ,and the plates were gently mixed to suspend the mycobacteria; serial dilutions were made from all samples and plated on Mueller Hinton agar to count and record the numbers of viable colonies 3 days later.

Transmission Electron Microscopy. With appropriate dilution of the *M. fortuitum* suspension, approximately 10 to 20 colonies grew and became visible on Mueller Hinton agar after inoculation of 10 μL of the suspension and incubation for 72 hours at 37°C. After 15 μL of different concentrations of MB (0.5%, 0.1%, and 0.01%) were applied to cover the colonies for 5 minutes, the plates were exposed to PDT at 100 J/cm^2, 150 J/cm^2, and 200 J/cm^2, respectively. Control colonies included normal controls (no treatment), light only, dye only, and those receiving light exposure before dye application. Immediately after treatment, three colonies of each group were fixed overnight in 4% glutaraldehyde/0.1 M cacodylate buffer consisting of 16 mL glutaraldehyde 25%, 42 mL of 0.2 M cacodylate buffer (sodium cacodylate 4.28 g in 100 mL distilled water), and 42 mL distilled water, and were prepared for ultrathin sectioning and transmission electron microscopy (TEM) examination. The other colonies were kept under observation and photographed on day 4 and day 8.

In Vivo Studies

In Vivo Corneal Infection. New Zealand White rabbits, weighing 3.0 to 4.0 kg, were used as animal models. Institutional guidelines regarding animal experimentation were followed and all animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Anesthesia was induced with intramuscular injection of 30 mg/kg ketamine hydrochloride (Ketalar; Pfizer Inc, Taipei, Taiwan) and 5 mg/kg xylazine hydrochloride (Rompun solution 2%; Bayer Health Care AG, Leverkusen, Germany). Topical anesthesia was achieved with the administration of 1 drop of 0.5% proparacaine hydrochloride to the rabbit eyes. A disposable contact lens (1 Day Visco; Visco, Hsinchu, Taiwan) was laden with a suspension of *M. fortuitum* containing 10^6 organisms/mL for 20 minutes and then was placed over the abraded cornea of one eye. Immediately after contact lens wearing, 20 mg triamcinolone acetonide (Larkcort; Lita Co., Taichung, Taiwan) was injected subconjunctivally, and tarsorrhaphy was performed with two stitches of a 6–0 nonabsorbable suture (Dafilon; Aesculap Inc., Center Valley, PA). The sutures and contact lenses were removed 7 days after inoculation, and the corneas were observed under the surgical microscope.

Twenty-one adult female rabbits with keratitis then underwent one of the treatment regimens. Before further management, deepithelialization and cultures of the corneal scrapings were made for all eyes to confirm the infection. The infected eyes were randomly divided into three equally numbered groups (each group had seven eyes): control group without any treatment, amikacin treatment group, and PDT combined with amikacin treatment group.

PDT Treatment. MB powder was dissolved in sterile water and was filtered through 0.22-μm pore size membrane filters and served as the 1% stock solution (wt/vol), which was diluted to a concentration of 0.5% and stored in brown microcentrifuge tubes. Before light exposure, the corneas were instilled with 0.5% MB every 5 minutes for 6 times and then were washed with 10 mL normal saline.

The halogen lamp (PDT-1200; Waldmann), used for the in vitro studies, was not used for the in vivo studies because of the high heat it generated and the diffused light exposure that resulted in severe corneal dryness. Infected corneas were illuminated using a 125-mW AlGaInP visible laser (Wicked Lasers, Model P125; Energy Technology Development Limited, Shanghai, China), which emitted light at a wavelength of 650 nm, giving a circular spot 4 mm in diameter on the cornea. A donut-shaped piece of cardboard was used as a light shield to protect the rest of the cornea. The total illumination time was 13 minute, and the total light exposure was 97.5 J/cm^2. Lubrication (I-Visc, 1% sodium hyaluronate; I-MED Pharma Inc, Montreal, Canada) was used to prevent corneal dryness.

PDT was performed once before topical amikacin treatment in the eyes of the combined treatment group. Anesthesia was induced with intramuscular injection of 30 mg/kg ketamine hydrochloride and 5 mg/kg of xylazine hydrochloride. Topical anesthesia was achieved with administration of 1 drop of 0.5% proparacaine hydrochloride to the PDT treatment eyes.

Antibiotic Treatment. Topical fortified amikacin (20 mg/mL; Bristol-Myers Squibb, Taipei, Taiwan) was prepared from a parenteral formulation according to routine clinical procedures. Eyes of the amikacin-treated group and the combined treatment group received 20 mg/mL amikacin applied topically four times a day for 7 days.

Clinical Evaluation. Clinical features of NTM keratitis were scored and graded with the aid of a surgical microscope on day 11 (before treatment), day 14 (during treatment), and day 17 after infection (before euthanatization). A grade of 0 to 4 was assigned to each, based on these three criteria: area of corneal opacity, density of opacity, and surface regularity.[20] A normal untreated cornea was given a score of 0 in each category and, thus, had a total score of 0. The score for each eye could yield a possible total score ranging from 0 to 12.

Quantitative Mycobacteriological Analysis. One hour after the final instillation of the antibiotic drops, all animals were killed with an overdose of barbiturate (Beuthanasia D-Special; Schering-Plough Animal Health Corp, Union, NJ), and uniform corneal buttons were excised with a sterile 8.5-mm trephine (Saber Medical Inc., Westchester, PA). Corneal buttons were ground in 1-mL sterile normal saline solution using a disposable tissue homogenizer (The Kendall Co., Mansfield, MA). Then serial dilutions of the corneal suspensions were prepared in normal saline solution. Twenty microliters of each dilution were plated on agar plates and incubated at 37°C in an atmosphere of 5% carbon dioxide. After 4 days of incubation, the numbers of viable *M. fortuitum* colonies were counted and recorded.

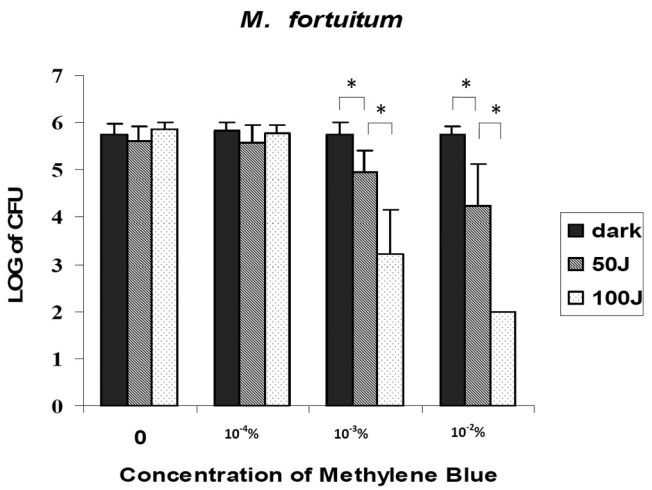

FIGURE 1. Antimicrobial effect of PDT with MB (10^{-4}%, 10^{-3}%, and 10^{-2}%) at two different fluences (50 J/cm^2 and 100 J/cm^2, respectively) for suspensions of *M. fortuitum*. Significant reductions in CFUs occurred for suspensions exposed to 10^{-3}% or 10^{-2}% MB and PDT at 100 J/cm^2 (compared with the dark control; $^*P < 0.05$). No significant bactericidal effect was found after exposure to 10^{-4}% MB and PDT at 50 J/cm^2 or 100 J/cm^2. The log value of 2 was the lowest limit of detection.

Statistical Analysis

For quantitative analyses, data were transformed to logarithmic values and were expressed as means and standard deviations of at least four separate experiments. The difference between two means was compared using the two-tailed paired Student's *t*-test, assuming equal variances. $P < 0.05$ was considered statistically significant.

RESULTS

In Vitro Studies

Immediate Bactericidal Effect of PDT. Figure 1 shows the viable counts of suspensions of mycobacteria immediately after PDT exposure. Under dark conditions, short-term exposure (<1 hour) to 10^{-2}% (100 μg/mL), 10^{-3}% (10 μg/mL), and 10^{-4}% (1 μg/mL) MB had no significant effect on log CFU for growth and dye controls. No significant effect was found for log CFU between dark controls and those exposed to 100 J/cm^2 of light only, without MB. No bactericidal effect was found for PDT at a concentration of 10^{-4}% MB and exposure to 100 J/cm^2 of light. The antimicrobial effect was proportional to the increase in the dye concentration and exposure to light energy. PDT with 10^{-3}% MB at 100 J/cm^2 resulted in a decrease of 2 or more log CFUs, and no viable mycobacterium was detected on the agar plates from the sample of the suspension after exposure to 10^{-2}% MB and PDT at 100 J/cm^2 (presented as 2 in log value of CFU in Fig. 1). Because the detected limit of CFU was 2 in log value, those suspensions without mycobacterial colony grown on the agar plates were shown as 2 logs despite that mycobacterial burden was <100/mL.

Antimicrobial Susceptibility Testing. The MICs for amikacin, ciprofloxacin, and moxifloxacin were 0.5 μg/mL, 0.06 μg/mL, and 0.06 μg/mL for *M. fortuitum*, respectively. Based on these results, amikacin 0.125 μg/mL/0.25 μg/mL/0.5 μg/mL, ciprofloxacin 0.015 μg/mL/0.03 μg/mL/0.06 μg/mL, and moxifloxacin 0.015 μg/mL/0.03 μg/mL/0.06 μg/mL were used for the combined therapy experiments.

PDT of *M. fortuitum* Suspension Cocultured with Antibiotics. Results of the combined therapy are shown in Figures 2A to 2C.

Antibiotic Controls. The antimicrobial effect of amikacin on *M. fortuitum* did not differ significantly between dark controls and light-exposed suspensions (data not shown). Similar results were found in the ciprofloxacin and moxifloxacin groups. MB at a concentration of 5×10^{-3}% afforded no additional antimicrobial effect on *M. fortuitum* suspensions for the three tested antibiotics after 72-hour incubation without light.

PDT Controls. In the preliminary study, no significant reduction of CFUs was found after 72 hours of culture after PDT with 10^{-3}% MB at 100 J/cm^2. Thus, 5×10^{-3}% MB was used in the combined therapy experiments. After PDT with 5×10^{-3}% MB at 100 J/cm^2 , the bacterial suspension had a 2 to 3 log CFU reduction in viable colony count compared with the dark controls after 72 hours of culture in the absence of antibiotics (Figs. 2A–C, controls).

Effect of Combined PDT and Antibiotic Treatment. Compared with antibiotic controls and MB dark controls, significantly fewer CFUs were noted in the combined treatment groups, except for the controls with the highest concentrations of antibiotics (amikacin 0.5 μg/mL, ciprofloxacin 0.06 μg/mL, and moxifloxacin 0.06 μg/mL).

Effect of PDT on NTM Colonies Grown on Agar. *M. fortuitum* colonies growing on agar plates were treated with PDT of different luminous intensities and concentrations of MB. On day 4, there was no antimicrobial effect in the four control groups (no treatment, light only, dye only, light exposure before dye application). As shown in Figure 3A, no inhibition of growth was found in the colonies exposed to light at 150 J/cm^2 before dye application. For PDT at 100 J/cm^2, proliferation of mycobacteria around the colonies was noted 3

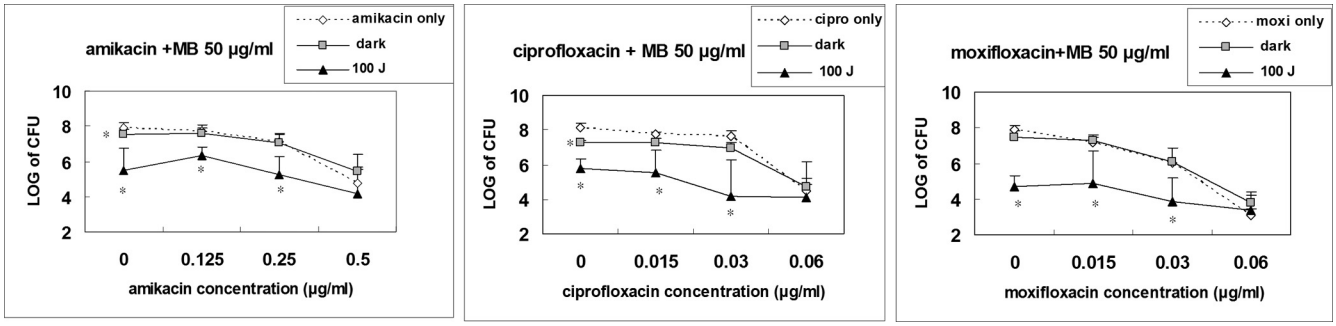

FIGURE 2. Final CFU counts of *M. fortuitum* after treatment of the antibiotic-containing suspension with photodynamic therapy followed by culture for 72 hours with (**A**) amikacin, (**B**) ciprofloxacin, or (**C**) moxifloxacin. Previous treatment with 5×10^{-3}% methylene blue and 100 J/cm^2 resulted in two or more log reductions of CFUs compared with controls for the wells with lower antibiotic concentrations, but this effect became insignificant at higher doses of antibiotics. $^*P < 0.05$, compared with the antibiotic control.

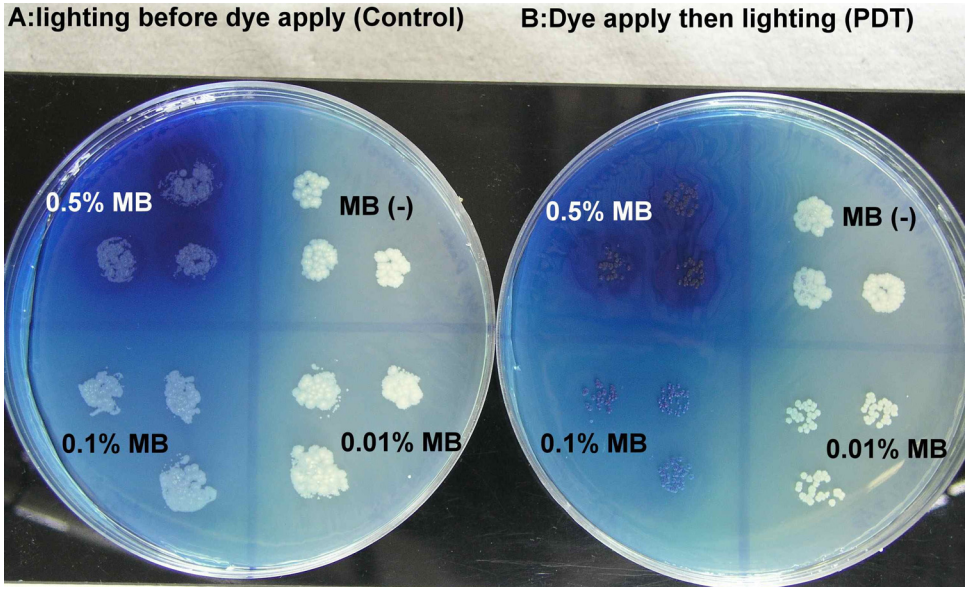

FIGURE 3. *M. fortuitum* colonies were exposed to light at a fluence of 150 J/cm^2 before application of the different concentrations (0.01%, 0.1%, 0.5%) of MB as (**A**) controls or (**B**) after the dye application (PDT). After treatment, the plates were kept in the dark for 3 days and then were photographed as shown. On the PDT plate, moderate inhibition was found for the colonies treated with 0.01% MB. The colonies treated with MB at concentrations of 0.1% or 0.5% discolored deeply and maintained their original size without apparent bacterial proliferation.

days later. The colonies that underwent one PDT treatment with 0.1% or 0.5% MB at 150 J/cm^2 became deeply blue and did not increase in size during the following 3 days (Fig. 3B); however, proliferation of mycobacteria was noted at the edges of the colonies later on day 8 (data not shown). Only colonies covered with 0.5% MB that received PDT at 200 J/cm^2 showed complete cessation of growth (data not shown).

TEM of Bacterial Colonies after PDT. TEM revealed that the characteristics of mycobacterial bacteria treated with PDT at 150 J/cm^2 before application of 0.5% MB (Fig. 4A) did not differ from those of normal controls (data not shown). Disorganized nuclei and heterogeneous cytoplasm were noted after PDT with 0.5% MB at 150 J/cm^2, although the rigid cell wall remained intact (Fig. 4B). After PDT treatment with 0.5% MB at 200 J/cm^2, the presence of many tiny vacuoles in the cytoplasm and cell wall disruption were observed (Fig. 4C).

In Vivo Studies

Mycobacterial keratitis developed within 10 days after inoculation in all 21 rabbits. Certain clinical features mimicked those observed in human infections, such as indolent progression, granular opacity with satellite lesions, feathery margins, and corneal vascularization (Fig. 5A–C). Progression of corneal ulcers was noted in the control group (Figs. 5A, 5D). The lesions remained stationary in the amikacin group. Localized edematous stroma occurred on the light-exposed cornea after PDT treatment the next day and improved during the following 4 days. Figure 6 demonstrates the average clinical scores (mean ± SD) of the separate groups before, during, and after treatment. In the clinical evaluation, despite higher scores (6.1 ± 2.0) in the combined group, no significant difference in scores was noted before treatment among the three groups (control group, 4.7 ± 1.4; amikacin group, 4.7 ± 1.8). One week later, scores of the control group significantly differed from their corresponding values in the previous week (P = 1.98E-05). A significant increase in scores was noted during 1-week follow-up in controls (Fig. 6, control group). The posttreatment scores on day 14 and day 17 were similar to those before treatment in the amikacin group ($P > 0.05$). The scores of the combined group differed statistically before and after treatment (P = 0.00161). The combined treatment improved

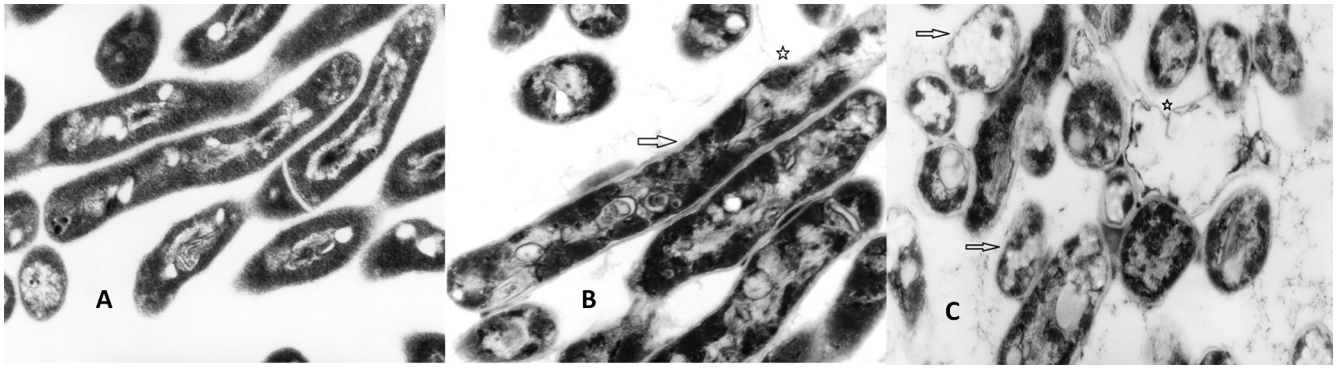

FIGURE 4. (**A**) TEM shows the characteristic ultrastructure of *M. fortuitum* after exposure to PDT (at 150 J/cm^2) before 0.5% MB application. No significant difference in morphology was found compared with the nontreated control (not shown). (**B**) After PDT at 150 J/cm^2 with 0.5% MB, the cytoplasm became electron-dense and discontinuous (*star*), and the nuclei became disorganized (*arrow*); the rigid cell walls became more distinct but maintained their integrity. (**C**) For colonies exposed to PDT at 200 J/cm^2 with 0.5% MB, TEM shows vacuole formation in the cytoplasm of the mycobacteria, with total loss of chromatin material (*arrow*). The rigid cell walls were disrupted, with leakage of intracellular contents (magnification, 30,000×; *star*).

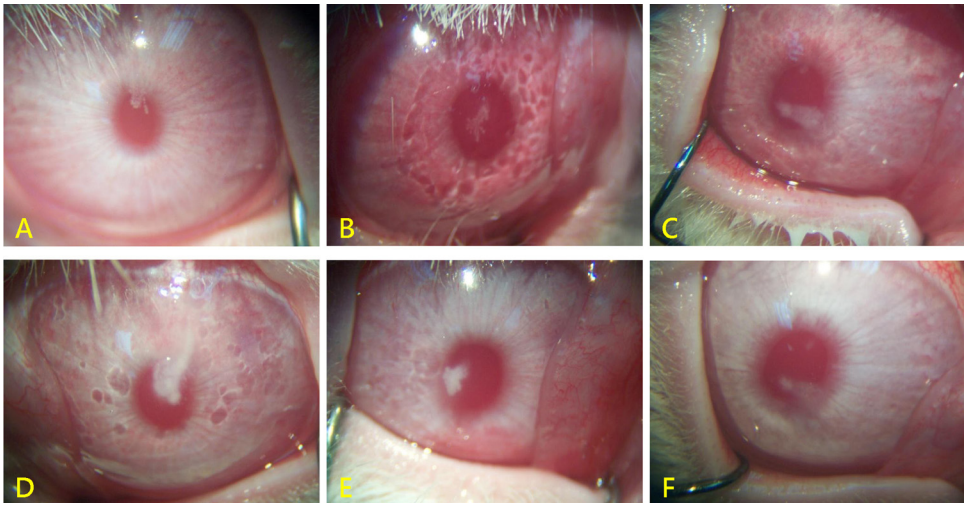

FIGURE 5. Clinical manifestations of nontuberculous mycobacterial keratitis of all three groups: (**A**) control without treatment, (**B**) amikacin group, (**C**) combined amikacin and PDT treatment group, on day 11 after infection (before treatment). (**D–F**) The three groups on day 17 after inoculation (before euthanatization).

the clinical picture significantly, especially from day 14 (3 days after PDT, $P = 8.77E\text{-}05$, day 14 V.S., day 17).

Figure 7 shows the surviving CFUs for the three groups after 7 days of treatment. Amikacin (20 mg/mL) alone (3.57 ± 0.53) and combination therapy (2.66 ± 0.58) with amikacin and PDT significantly reduced the number of *M. fortuitum* CFUs compared with controls (4.76 ± 0.80) without any therapy (both $P < 0.0001$). Combination antibiotic therapy and MB-mediated PDT demonstrated greater antimycobacterial activity than antibiotic therapy alone ($P < 0.0001$).

DISCUSSION

Previous studies showed that multi–antibiotic-resistant bacteria were as easily killed by PDT as were naive strains, and the bacteria did not readily develop resistance to PDT.[8–10,13,21,22] Because of its characteristic cellular wall and multidrug resistance, the infectious loci of NTM are usually intractable despite the use of traditional antibiotics. PDT could be a potential alternative treatment for NTM corneal infections.

In contrast to porphyrin derivatives, which require long incubation periods for photosensitization, phenothiazinium derivatives such as MB yield results more quickly. Thus, MB was chosen as the photosensitizer for this study. Such phenothiazinium-based photosensitizers have phototoxicity to tumors, bacteria, viruses, and other microbes.[8–17,23,24] MB, toluidine blue, and other phenothiazinium-based photosensitizers attack bacteria at multiple sites, including nuclear DNA and the cell wall, and have different sites of action for different species.[8,10,13] The intrinsic bacterial toxicity of MB was previously demonstrated[17] but was negligible in our study. Short-term exposure to MB concentrations of 10^{-4}% to 10^{-2}% during PDT in the dark did not affect mycobacterial viability. After incubation for 72 hours, 5×10^{-3}% MB did not significantly influence the number of mycobacterial CFUs. Even 0.5% MB without light exposure did not significantly inhibit the growth of the mycobacterial colonies on the agar plates. For corneal instillation in our preliminary study, intact epithelia prevented staining of MB, and there was no complication after instillation of 1% MB four times per day on the denuded cornea for 3 days.

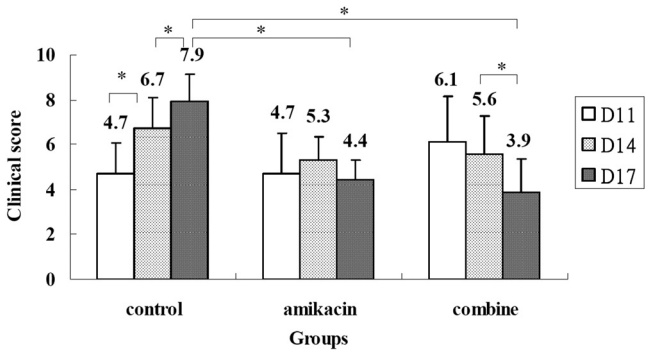

FIGURE 6. Clinical scores of the three groups with nontuberculous mycobacterial keratitis on day 11 (D11) after infection (before treatment), day 14 (D14) after infection (during treatment), and day 17 (D17) after infection (before euthanatization). *$P < 0.05$.

We found that PDT in combination with 10^{-2}% MB and light exposure of 100 J/cm^2 eradicated all the mycobacteria in the *M. fortuitum* suspension. However, 0.1% or 0.5% MB at 150 J/cm^2 or greater exposure was needed for a significant bacteriostatic effect on visible mycobacterial aggregates on the agar plates. After PDT, TEM demonstrated disorganized cytoplasm and nuclei with intact cell walls, which was apparently reversible because the colonies regrew later, as noted on the agar plates. With time, surviving mycobacteria proliferated again, which suggested the necessity of more powerful PDT, repeated PDT, or antibiotic treatment combined with PDT. PDT at 200 J/cm^2 halted the proliferation of mycobacteria when administered with 0.5% or more MB, as shown by the disruption of cell walls and the disorganized and vacuolated cytoplasm on TEM. The major target of phenothiazinium-based PDT is thought to be DNA in both Gram-negative and Gram-positive bacteria.[13,14,25] Although the mechanisms that induce mycobacterial death could not be clarified in this study, the characteristic cell wall appeared to be the stronghold against damage by MB-aided PDT.

We observed a synergistic effect for PDT and antibiotic therapy, suggesting that for treating an intractable, localized infection such as infectious keratitis, PDT could be a valuable adjuvant to antimicrobial therapy. Sublethal concentrations (less than the MIC concentration) of antibiotics, such as ciprofloxacin at concentrations of 0.015 μg/mL or 0.03 μg/mL, plus PDT with a sublethal dosage of 5×10^{-3}% MB at 100 J/cm^2 had a significantly greater bactericidal effect than ciprofloxacin alone (Fig. 2B). With increasing concentrations of antibiotics, combined treatment with PDT did not result in significantly greater bactericidal effects than antibiotics alone (Fig. 2). This absence of synergy for combined therapy at high concentrations of antibiotic could be attributable to the detection limit (CFU 100/mL) at our facility or a lower initial bacterial burden. The photosensitizer MB is the substrate of an efflux pump,

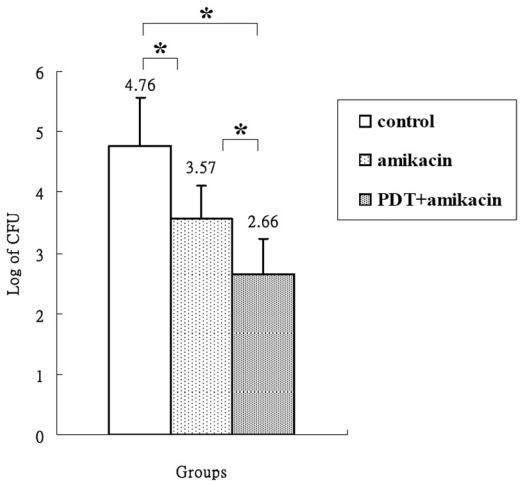

FIGURE 7. Infectious keratitis in rabbit eyes was treated with amikacin or combined amikacin and PDT. After 7 days of treatment, quantitative bacteriologic analysis of viable colonies showed significant synergy resulting in bactericidal efficacy for the combined antibiotic and MB-PDT treatment. $^*P < 0.05$.

which contributes to multidrug resistance.[26] It is possible that there is competition between MB and the antibiotics for efflux pump. Additionally, free radicals produced by PDT might intervene in the efficacy of cocultured antibiotics. More studies will be needed to elucidate the interaction between PDT and antibiotic therapy in the future.

To date, there have been few studies of the effects of PDT on mycobacteria. One patient with a digital skin infection caused by *M. marinum* was cured after PDT.[7] In this report, the authors demonstrated the accumulation of fluorescent 5-aminolevulinic acid in the skin after 3 hours of incubation, without in vivo and in vitro bacterial studies of PDT.[7] O'Riordan et al.[6] demonstrated that PDT—using the benzoporphyrin derivative verteporfin (5 μM) at a fluence of 60 to 100 J/cm^2—decreased *M. bovis* BCG cells by >70% in suspension and by 50% in J774.2 cells. Nonetheless, PDT resulted in only a 0.7-log reduction of burden for 10^5 BCG in a rodent model of *M. bovis* BCG-induced granuloma.[6] The possibility of multiple administrations of PDT could be necessary to achieve a better therapeutic effect.

Because corneal thickness is limited to 540 to 570 μm, corneal ulcers are thought to be good candidates for the investigation of PDT effects, not only because of the availability of photosensitizer instillation but also because of their facility for light exposure. However, there is no literature regarding PDT on infectious keratitis. In this study, there was no PDT-only control in the rabbit model for lack of dramatic clinical effect as our formula. In the in vitro study, one course of PDT with 0.5% MB at 150 J/cm^2 did not eradicate the mycobacteria growing on the agar plate (Fig. 3). In a preliminary study, mycobacterial keratitis progressed even after two PDT treatments per week (data not shown). Therefore, PDT was used as an adjuvant, not an alternative, to antibiotic therapy. For monotherapy of PDT in clinical practice, further research regarding the appropriate photosensitizer and therapeutic formula for infectious keratitis is needed. Furthermore, the side effects of corneal and possible retinal toxicities caused by PDT must also be considered.[27] PDT combined with antibiotic therapy is an appropriate alternative modality to prevent such complications.

The choice for treatment of NTM keratitis is topical amikacin combined with fluoroquinolone or clarithromycin.[28] However, topical amikacin reportedly has poor corneal penetration.[29] In antimicrobial susceptibility testing in our study, *M. fortuitum* was sensitive to amikacin in vitro and seemed a reasonable candidate for our in vivo study. The aim of our study design was to investigate whether PDT had any synergistic antimicrobial effect in combination with low-efficiency antibiotics. In the present study, the efficacy of combination PDT and antibiotic therapy was superior to that of monotherapy with amikacin. In the amikacin group, though *M. fortuitum* was sensitive to amikacin in vitro, fortified amikacin eyedrops did not improve the clinical pictures of the rabbits significantly. Our in vivo study demonstrated the synergy of PDT with certain antibiotics. We assumed the synergy was attributed not only to PDT-induced antimicrobial activity but also to enhanced antibiotic efficacy because of localized corneal edema after PDT rather than to poor corneal penetration of topical amikacin monotherapy. Local edematous stroma surrounding the lesion was noted after PDT and lasted for several days. Localized tissue trauma associated with infection and inflammation can alter the pharmacokinetics and increase the penetration of drugs used to treat ocular infections.

The current experiment was designed to compare the in vivo efficacy of MB-mediated PDT. Thus, experimental keratitis was treated during a short period. Long-term animal studies of MB-mediated PDT treatment for the treatment of NTM keratitis are currently under way to evaluate the safety of MB-PDT on the cornea and the appropriate protocol for PDT treatment. These in vitro and in vivo studies demonstrated MB-mediated lethal photosensitization of NTM. TEM revealed that the phototoxicity of MB occurred in cytoplasmic targets first, whereas the disruption of the mycobacterial cell wall occurred during subsequent bacteriolysis. We suggest that PDT has the potential to provide adjuvant antimicrobial effect when combined with antibiotic treatment for intractable mycobacterial infection.

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