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The Role of Some Photosensitizers in Photodynamic Control of Lumpy Skin Disease

[1]Kawther S. Zaher, [1]Sohier M. Syame and [2]Noeman B. Aref

[1]Microbiology and Immunology Department,
National Research Center (NRC), Dokki, Giza, Egypt
[2]Pests and Plant protection Department, NRC, Dokki, Giza, Egypt

Abstract: Photodynamic (PD) activities of Methylene blue, Rose Bengal and tetramethylrosamine were tested on Lumpy skin disease virus (LSDv), the most important secondary bacterial infection accompany it and the vector incriminated in its mode of transmission as a method of controlling the disease. Virucidal assay included estimation of cytotoxic concentration (CC_{50}) using different PD substances concentration (0.5, 0.25, 0.05 and 0.025mM/ ml). A concentration below CC_{50} (0.02mM/ml) of each PD substance at various light exposure time was added to the free virus where the results showed 98, 97 and 94% MDBK cell reviving in case of Methylene blue, Rose Bengal and tetramethylrosamine at 40, 50 and 50 min of light dose of 550 nm; respectively. Moreover, results on virus replicating in MDBK cells for the same periods of illumination were 97, 94 and 92% cell reviving in case of Methylene blue, Rose Bengal and tetramethylrosamine at 50, 40 and 60 min of light with the same intensity; respectively. A bacteriological assay on *Staphylococcus aureus* (*S. aureus*) was performed where 10^6 Colony Forming Unit (CFU)/ml was inoculated on nutrient agar with PD substances (0.05mM/ml). After irradiation to light dose of 550 nm, for the same periods of time, the percentage of *S. aureus* inhibition was calculated. Results showed that Methylene blue, Rose Bengal and tetramethylrosamine induced 99, 91, 93% inhibition of CFU/ml at 50, 50 and 60 min of light exposure; respectively. On the other hand the pesticidal assay was focusing on testing the photodynamic activity against the 3rd instar larvae of *Culex mirificens* and *Aedes natrionus*. Lethal concentration (LC_{50}) was calculated. A concentration of 0.02mM/ml of each PD substance was added on the 3rd instar larvae of each mosquito. After then they were exposed to light dose of 550 nm for the same periods of time. The number of dead larvae was calculated. Results showed100% deaths of larvae were at 40, 50, 60min of light exposure after addition of Methylene blue, Rose Bengal and tetramethylrosamine; respectively on *Culex mirificens* and 50, 60, 40min of exposure after addition of methylene blue, Rose Bengal and tetramethylrosamine; respectively on *Aedes natrionus*.

Key words: Photosensitizers · Photodynamic Activity · Lumpy Skin Disease · Lumpy Skin Disease Control

INTRODUCTION

Photodynamic (PD) sensitizers are molecules that, when activated by light of a specific wavelength, generate reactive oxygen. In-vivo, these substances trigger a cascade of biochemical responses that result in cell death [1]. Virucidal action of illuminated photosensitizers was recognized in the early 1930s [2, 3]. Some observations established that a diverse group of viruses are susceptible to photosensitizers' photo-inactivation where these substances appear to interact with viral nucleic acid [4]. Other studies relay their virucidal activity to denaturation of the viral capsid [5]. Photodynamic therapy (PDT) can become a promising method because of several reasons. PDT has been used in inactivation of some microorganisms, viruses, bacteria, fungi as well as insect. Moreover, the reaction of photosensitizers can be turned on or off since no reaction occurs in the absence of light, the time of its action can be controlled also it can be directed to target locations to be treated [4].

Corresponding Author: Kawther S. Zaher, Department of Microbiology and Immunology,Veterinary Research Division, National Research Centre, Dokki, Giza, Egypt. Potal cod: 12622.

Lumpy skin disease (LSD) is a disease caused by a virus of the family *Poxviridae*, genus *Capripoxvirus*. It mainly affects cattle and zebus and African buffalo [6]. LSD was first seen in Zambia in 1929 and since then has affected cattle throughout Africa, including the countries South Africa, Egypt, Sudan and Israel [7]. Lumpy skin disease virus (LSDv) is spread by biting insects, *Culex mirificens* and *Aedes natrionus*. Morbidity of LSDv can be very high but mortality is low [6].

Staphylococcus aureus (*S. aureus*) is a facultative anaerobic Gram-positive coccal bacterium and has large, round, golden-yellow colonies. It causes hemolysis when grown on blood agar plates. *S. aureus* is catalase-positive (Meaning it can produce the enzyme catalase). Catalase converts hydrogen peroxide (H_2O_2) to water and oxygen [8]. *S. aureus* is one the main microorganism responsible for secondary skin infection associated with LSDv infection [7].

Mosquitoes differ from the other biting Diptera in having a long slender body, long legs and long needle-shaped mouthparts. The wings sometimes have discernible patterns of scales. The adult insects measure between 2 and 12.5 mm in length [9]. *Culex mirificens* and *Aedes natrionus* were incriminated in transmitting LSDv [7].

The current investigation was designed to monitor the photodynamic activity of Methylene blue, Rose Bengal and tetramethylrosamine on LSDv. Moreover, it was directed on main bacteria involved with the disease complications as well as the vector transporting it. Controlling these microorganisms is consequently a method of controlling this disease. Therefore the result of this study will conclude to possible recommending for the future use of these PD substances in-vivo study.

MATERIALS AND METHODS

The present investigations were carried out at the National Research Centre (NRC) during the period from December 2012-November 2013.

Photosensitizes: Methylene blue, Rose Bengal and tetramethylrosamine were used as models of PD substance. These PD substances were obtained from Pests and Plant protection Department, NRC. They were studied for antiviral, antibacterial and insecticidal effect. These substances were used with light source which is a small device transilluminator emitting UV light (PDT-1200, 560–780 nm, Ultra-Lum, Claremont, CA, USA) also supplied by Noeman B. Aref, Pests and Plant protection Department, NRC.

Viral Assay

Virus Model: LSDv was obtained from Microbiology and Immunology Department, NRC [10]. The virus was isolated from serum and dried scabs then identified and stored in -70 deep freezing.

Cells: MDBK (Madin-Darby Bovine Kidney) cells were obtained from Vaccine and Sera Institute (*VACSERA*), Agoza, Cairo, Egypt. The cells were supplemented with Dulbecco's modified Eagle's medium (MEM) (Gibco, *USA*) containing 10% fetal bovine serum (*Gibco, USA*) and gentamicin (50µg/ml).

Virus Propagation and Purification: LSDv was inoculated and cultivated on confluent MDBK cell monolayer for 3 to 4 days. Infected cells were subjected to freezing and thawing three times to release the viruses, mixed with an equal volume of chloroform and then purified according to Richman *et al.* [11].

Virus Plaque Assay: Plaque assay was performed according to Burleson *et al.* [12]. The plaques on plates containing 5 to 50 plaques were counted and the virus titer was expressed in PFU per milliliter.

Cytotoxicity Examination: MDBK cells were treated with various concentrations (0.5, 0.25, 0.05 and 0.025 mM) of Methylene blue, Rose Bengal and tetramethylrosamine. The toxicity of these substances was tested either by direct count where the cells were counted by a hemocytometer, Morphological changes were daily observed by optical inverted microscope and by MTT assay which was performed as described by Shi [13]. The CC_{50} was determined according to Reed and Muench method [14].

Virus Inactivation

Inactivation of Free Virus: 50 µl aliquots of freshly purified virus suspensions 10^{10} PFU/ml were incubated with 450 µl of each PD substance (methylene blue, Rose Bengal and tetramethylrosamine) (0.02mM). Each well of a 24-well plate was exposed to light dose of 550 nm for up to 60 min (1, 5, 10, 15, 20, 30, 40, 50 and 60 min). After exposure residual infectivity was thereafter titrated as described by Redfield *et al*. [15].

Effect on Replicating Virus: LSDv was inoculated in MDBK cell cultures (seeded 24 h previously with 70, 000 cells per well) using a virus dilution known to cause at least 50% cell death within 72 h. Each photosensitizer was added to the cultures at a concentration of 0.02mM 24 h later. In order to avoid light absorption by the culture medium, which contains phenol red as a pH indicator, the cells were maintained during the illumination period in phosphate-buffered saline (PBS) containing Ca 2+, Mg 2χ and 0.1% glucose. The medium is decanted and the photosensitizers were added in PBS for 3 h. The illumination periods were the same as on free virus. Following this treatment, the cultures were incubated in medium and antibiotics at 37°C for 72 h. and the virus titer was estimated as mentioned above

Antibacterial Assay

Bacteria: The bacterium used in this study was *Staphylococcus aureus* (*S. aureus*). *S. aureus* was propagated on nutrient agar (Difco®, Detroit, USA) and incubated at 37°C.

***In vitro* Photosensitization Assays:** It was carried out, according to the methods described by Souza *et al.* [16], Shan *et al.* [17] and Khyade and Vaikos [18]. To each well of a sterile 96-well flat-bottomed microtitulation plate, 50µL of nutrient broth (Difco®, Detroit, USA), 50 µL of the photosensitizer (At 0.05mM) or control solution and 5 µL of *S. aureus* suspension (10^6 CFU)/ml) were added. Then, the plate containing the samples was agitated and incubated in the dark for 5 min at room temperature to obtain homogeneous reaction medium and dissolved oxygen content. After this period, the contents of each well were irradiated. The irradiation of the samples was performed under aseptic conditions in a laminar air flow chamber. for positive controls, each photosensitizer (0.05mM) was diluted in sterile saline, was used. For negative controls, distilled water with the inoculum of *S. aureus* incubated in sterile physiological solution and the culture medium alone were used. The experiments were performed in duplicate and one plate was not irradiated (control plate, wrapped in aluminum foil and not exposed to any light). After irradiation to light dose of 550 nm for up to 60 min (1, 5, 10, 15, 20, 30, 40, 50 and 60 min), the plates were incubated at 37°C for 24 or 48 h. After the incubation period, serial dilutions of 10^{-2} and 10^{-3} were obtained from each sample in sterile physiological solution and aliquots of 10 µL were added in duplicate to nutrient broth. After incubation at 37°C for 24 or 48 h, the number of CFU/ml was determined for each photodynamic substance and data were subjected to statistical analysis. The percent reduction of microbial growth for *S. aureus* was calculated for the studied samples. Throughout the experiment, the samples were manipulated in the dark, under aseptic conditions.

Pesticidal Assay

Tested Insects: The tested flies used in this study are *Culex mirificens* and *Aedes natrionus*, which are the vectors transmitting LSDv [7]. The next generation was reared with cabbage and lettuce seedlings in an environmental chamber with the temperature held at 26 ±2°C under a photoperiod of 14:l0 h light: dark.

Lethal Concentration$_{50}$ (LC50): The photodynamic activity was tested against the 3rd instar larvae of *Culex mirificens* and *Aedes natrionus*. The compounds were individually dissolved and serially diluted with acetone. Each resultant solution (0.4 ml) was added to a beaker containing 20 ml of de-chlorinated water and then 30 larvae were transferred into the beaker. Concentration of photosensitizers was 0.5, 0.25, 0.05 and 0.025 mM. The experiments were performed for each test sample, one of which was for ultraviolet-treated trials and another was in the dark. After incubation for 3 h in a dark room, the ultraviolet-treated groups were irradiated for 4 h, receiving irradiation intensity of 3800 pw cm^{-2} at 550 nm and again were incubated in the dark for 24 h. Then some sour dough powder was added to the solution in order to feed the mosquitoes after irradiation. The bioassay was repeated three times and the control treatments were incubated in the dark. Toxicity and media lethal concentration (LC_{50}) were calculated [19].

Insecticidal activities of Methylene blue, Rose Bengal and tetramethylrosamine against the 3rd instar larvae of *Culex mirificens* and *Aedes natrionus:* The compounds were individually dissolved to a concentration of 0.025mM/ml with acetone. The same procedure for determination of LC_{50} was performed and the insecticidal effect was evaluated. The number of dead larvae was calculated [19].

RESULTS AND DISCUSSION

Virological Assay: Virus Propagation LSDv was propagated on MDBK cells. In this experiment MDBK cells were chosen to be used in virus propagation because they are susceptible to LSDv infection,

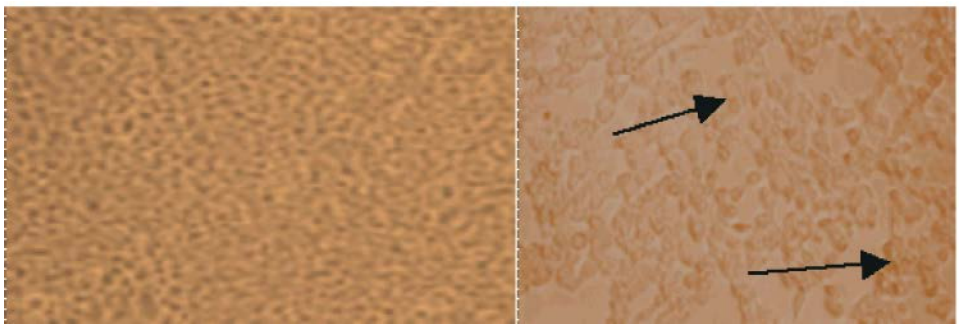

Fig. 1: Normal MDBK cells are in photo A. The cells showed pyknosis and karyorrhexis of the nucleus 7 days post LSDv inoculation in photo B.

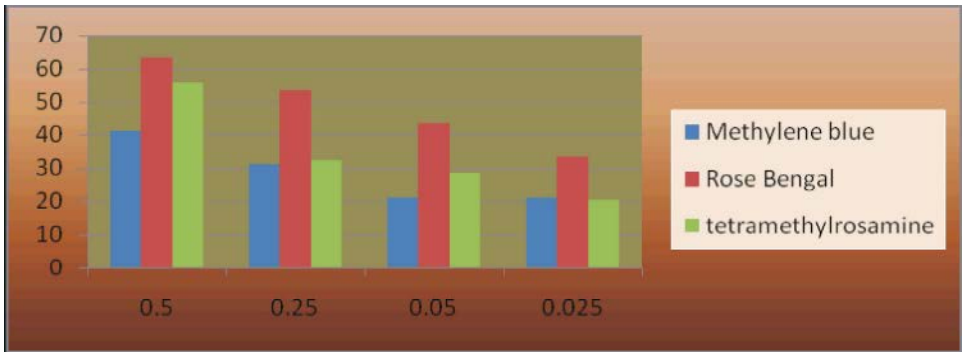

Fig. 2: Chart shows cytotoxicity examination for Methylene blue, Rose Bengal and tetramethylrosamine.

Table 1: Cytopathic effect expressed in percentage of cell reviving in MDBK cells

	PD substances	1	5	10	15	20	30	40	50	60	χ^2value
On free virus	Methylene blue	50	60	70	80	90	95	98	98	98	1.17**
	Rose Bengal	40	55	65	73	86	91	95	97	97	8.46
	Tetramethylrosamine	35	50	55	67	79	90	92	94	94	2.38*
On replicating virus	Methylene blue	30	40	45	60	80	92	95	97	97	19.98
	Rose Bengal	29	38	49	62	75	91	94	93	94	8.8
	Tetramethylrosamine	22	28	37	55	62	78	82	91	92	1.4**

which showed typical Cytopathic Effect (CPE) and cell line live long enough to ensure the propagation of the virus. In the present study the appearance of CPE took sometimes only 3 days to develop but it usually became apparent between the 5th to the 6th day (Fig. 1). The titer of LSDv was determined by a plaque assay and was estimated to be approximately 10^{11} PFU/ml.

Cytotoxicity Examination: Different concentrations of each PD substance (0.5, 0.25, 0.05 and 0.025 mM) were added to MDBK cell monolayers for three days and the cytotoxicity of these photosensitizers was evaluated by different assays as described above. Low cytotoxicity was observed at concentrations below 0.025mM/ml in all examined PD substances and the concentrations found to cause 50% toxicity (CC_{50}) were approximately 0.5, 0.25 and 0.5 for Methylene blue, Rose Bengal and tetramethylrosamine, respectively (Fig. 2).

Virucidal Results

On Free Virus: Different times of illumination were used (1, 5, 10, 15, 20, 30, 40, 50 and 60min) at constant concentration of PD substances. These substances were added to the free virus. Results showed that the action of PD substance need short time to perform its virucidal action (98, 97 and 94% in case of Methylene blue, Rose Bengal and tetramethylrosamine at 40, 50 and 50min; respectively) (Table 1).

On Replicating Virus: At low concentrations of the PD substances (0.02mM/ml) and for rather long illumination periods (20-40 min) the PD substances exerted a marked protective effect on the cells against the cytopathic action of the virus. This "intracellular photoinactivation" is dependent on the illumination period for a given concentration (97, 94 and 92% cell

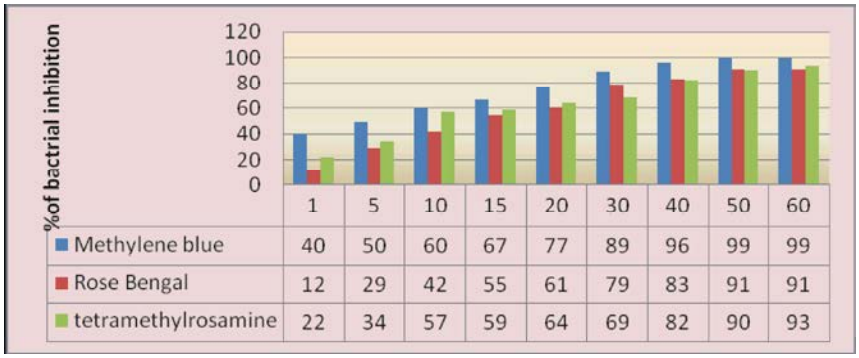

Fig. 3: Chart showing percentage of bacterial colonies inhibition with different times of light exposure with each PD substance

Table 2: Photoactivated insecticidal activities of Methylene blue, Rose Bengal and tetramethylrosamine against Culex mirificens and Aedes natrionus

Mosquitoes	PD substance	1	5	10	15	20	30	40	50	60	χ^2value*Culexmirificens*
	Methylene blue	53	66	70	77	88	99	100	100	100	55.87
	Rose Bengal	50	62	68	75	82	91	98	100	100	9.64
	Tetramethylrosamine	46	55	65	71	80	90	97	99	100	6.44
Aedes natrionus	Methylene blue	51	65	69	75	85	98	99	100	100	9.4
	Rose Bengal	49	58	66	71	80	96	97	99	100	2.39*
	Tetramethylrosamine	49	59	69	75	88	99	100	100	100	2*

mortality in case of Methylene blue, Rose Bengal and tetramethylrosamine at 50, 40 and 60min; respectively) (Table 1). These results match with Redfield *et al.* [15].

Antibacterial Assay: The results of the three PD substances showed no inhibition of *S. aureus* colonies in plates kept in the dark. Figure 3 shows percentage colonies inhibition for each PD substance at different times of exposure, where best results for Methylene blue, Rose Bengal and tetramethylrosamine were 99, 91, 93% at 50, 50 and 60 min of light exposure; respectively. This result was attributed to the fact that the reactive oxygen released by PD substances when exposed to light denaturated nucleic acid and epitopes on bacterial wall [4].

Pesticidal Assay: The best results for LC_{50} were achieved by the concentration of 0.025mM/ml and that is why it was used for testing the insecticidal activity of the three PD substances. Table 2 shows the results of insecticidal activities of these substances. The results showed that 100% deaths of larvae were at 40, 50, 60min of light exposure after addition of Methylene blue, Rose Bengal and tetramethylrosamine; respectively on *Culex mirificens* and 50, 60, 40min of light exposure after addition of Methylene blue, Rose Bengal and tetramethylrosamine; respectively on *Aedes natrionus*. The results showed high sensitivity of *Culex mirificens* to Methylene blue at exposure time 40 min. On the other hand *Aedes natrionus* was found to be sensitive to tetramethylrosamine at the same time of exposure. Pesticidal effect of these PD substances was attributed to the release of oxygen during exposure to light penetrating the cells and denaturizing its proteins causing death of the 3rd instar larvae of mosquitoes.

In summary, the current *in-vitro* evaluation results indicated that Methylene blue, Rose Bengal and tetramethylrosamine have efficient photodynamic activities on LSDv, *S. aureus* and the 3rd instar larvae of *Culex mirificens* and *Aedes natrionus*. Therefore a highly promising use of photosensitizer for controlling LSDv *in-vitro* is established. Further studies of this system in-vivo are in progress.

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