

Assessment of Photodynamic Therapy for Staphylococcus Aureus Infected Wounds using Diffuse Reflectance Spectrometry

M.A. Calin, D. Voicu, M.R. Calin, D. Savastru and D. Manea

Abstract—In this paper we evaluated the efficacy of photodynamic treatment of infected wounds on pig animal model by diffuse reflectance spectrometry. The study was conducted on fifteen wounds contaminated with Staphylococcus aureus bacteria that were incubated for 30 min with methylene blue solution ($c = 3.3 \times 10^{-3}$ M) and exposed to laser radiations ($\lambda = 670$ nm, $P = 15$ mW) for 15 min. The efficiency of photodynamic inactivation of bacteria was evaluated by microbiological exams and diffuse reflectance spectrometry. The results of the microbiological exams showed that the bacterial concentration has decreased from 6.93 ± 0.138 logCFU/ml to 3.12 ± 0.108 logCFU/ml. The spectral examination showed that the diffuse reflectance of wounds contaminated with Staphylococcus aureus has decreased from 5.06 ± 0.036 % to 3.36 ± 0.025 %. In conclusion, photodynamic therapy is an effective method for the treatment of infected wounds and there is a correlation between the CFU count and diffuse reflectance.

Keywords—photodynamic inactivation, bacteria, pigs, wounds

I. INTRODUCTION

DETRIMENTAL effect of microbial infection on healing wounds is unknown for decades. With the use of antibiotics began a new era in the evolution of infected wounds, but excessive or wrong use of them led to the selection of microorganisms that have developed mutations that confer resistance to antibiotics. The resistance of bacteria to anti-infectious drugs has been and remains an important problem in the therapeutics currently. Selection of bacterial strains polyresistance to chemotherapy and increased frequency of infected wounds has led to the development of new alternative treatment methods such as photodynamic therapy.

The photodynamic inactivation of the bacteria is based on the concept that a photosensitizer is localized preferentially in

M.A.Calin is with the National Institute of Research and Development for Optoelectronics INOE 2000, Magurele, 077125, Romania (e-mail: micalin@inoe.inoe.ro; mantonina_calin@yahoo.com)

D. Voicu is with the National Institute for Research and Development for Biology and Animal Nutrition, Bucuresti, 077015, Romania, (e-mail: dorica.voicu@yahoo.com)

M.R.Calin is with the "Horia Hulubei" National Institute of Physics and Nuclear Engineering – IFIN-HH, Magurele, 077125, Romania (e-mail: rcalin@nipne.ro)

D. Manea is with the National Institute of Research and Development for Optoelectronics INOE 2000, Magurele, 077125, Romania (phone/fax: (+4021) 457 4522, e-mail: manea_dragos@ymail.com) *corresponding author*

the bacteria and subsequently activated by light to generate reactive oxygen species which produce cell damages and inactivate microorganisms [1].

Although only experimental stages are known up to present, there are remarkable results in killing by photodynamic inactivation of germs which generate several types of infections. For example, in vitro studies of photodynamic inactivation of Staphylococcus aureus and Escherichia coli using different photosensitizers (polylysine-ce6-conjugates, octacationic Zn (II) phthalocyanine, methylene blue, toluidine blue O, deuteroporphyrin, hematoporphyrin derivative) and light radiation with wavelength of 650 nm, 660 nm, 675 nm or 632.8 nm have shown a 90% reduction in bacterial viability [2-8]. Some studies showed that the photodynamic treatment has induced not only lethal effect but also decrease in virulence of bacteria [9,10]. The in vivo studies on photodynamic therapy of infected wounds [11-14] and burns [15-17] showed a faster healing of wounds.

All these results demonstrate that photodynamic therapy is an effective treatment for infected wounds, but it is difficult to be optimized without an appropriate animal model.

The aim of this paper was to evaluate the efficacy of photodynamic treatment of infected wounds on pig animal model using methylene blue as photosensitizing agent. We have chosen a pig model because the pig skin is very similar to human skin. The diffuse reflectance spectrometry has been used as non-invasive method for the monitoring of the treatment.

II. MATERIALS AND METHODS

A. Photosensitizer

Methylene blue (Aldrich), as analytical grade reagent, was used for photodynamic inactivation. Water redistilled from alkaline permanganate was used to prepare the methylene blue solution (MB) with a concentration of 3.3×10^{-3} M and pH = 7.4.

B. Light Sources

The illumination of contaminated wounds was carried out with the laser system SCL (INOE 2000, Bucharest, Romania) with power 15 mW and emitting at wavelength $\lambda = 670$ nm.

C. Bacterial Culture

The cultures used in this study were *Staphylococcus aureus* strains (ATCC 25923), as cataloged in the American Type Culture Collection isolated from burn wounds, cultured in brain-heart infusion medium for 18 h at 37°C.

D. Animals

In this study, we used three young male pigs, uniform in weight (11 kg), Large White breed as experimental animals. The animals have been fed and housed in a specially designed space in conditions stipulated by the Law 305/2006 referring to European Agreement for protection of animals used in experiments and other scientific purpose (1986) and by the Convention Protocol Amend (Strasbourg 1998); Romania adopted this Law in 15th of February 2006.

Five wounds have been made, under local anesthesia, on the back of each animal using a sterile syringe needle to scarify the superficial skin layers to bleeding, after those areas were previously shaved and disinfected with iodine alcohol. This experimental protocol was approved by the Ethics Commission.

Each wound has received inocula of mid-log-phase of *Staphylococcus aureus* suspended to appropriate concentration in 50 μ l of PBS, immediately after the injuries have been made.

To ensure the infectious process, after completion of primary infection on each wound a sterile gauze soaked in the cultures of bacteria was applied and then fixed with adhesive tape a separate buffer. After 24 h, the mean bacterial concentration was 6.9309 ± 0.138 logCFU/ml.

The data are presented as means \pm SD. The mean value and its standard deviation were calculated using Microsoft Excel.

E. Photodynamic Inactivation

For each animal, three of the five wounds made on the back were considered as study group and the other two wounds were the control group.

After 24 h, 50 μ l of methylene blue solution was administered topically on the wounds from study group. These wounds were covered with sterile dressing for 30 min and then were exposed to laser radiation (670 nm, 15 mW, 9.854 J/cm²) for 15 min. The wounds from the control group were not incubated with methylene blue solution and were not exposed to laser radiation.

F. Diffuse Reflectance Spectrometry

Diffuse reflectance spectrometry was used as a method of monitoring the process of photodynamic inactivation of bacteria [18].

Optical reflectance spectra in the wavelength range (500 - 1100) nm corresponding to each wound were obtained with AvaSpec optic fiber spectrophotometer (Avantes, The Netherlands, Europe) before applying the photosensitizer, before and after laser irradiation and 48 h after the treatment.

This portable spectrophotometer is equipped with a tungsten halogen lamp, CCD detector array (2048 pixel) and a reflection probe type FCR-7IR200-2 with one illuminating fiber in center surrounded by six fibers which collect the light reflected from the sample.

G. Statistical Methods

The data are presented as means \pm SD. The mean value and its standard deviation were calculated using Microsoft Excel.

III. RESULTS

To assess the biological response to treatment, we acquired diffuse reflectance spectra of the contaminated wound before applying photosensitizer, before and after laser irradiation together with clinical and bacteriologic monitoring.

Diffuse reflectance spectra acquired before applying the photosensitizer show a different behavior of the wounds contaminated with bacteria both from each other and from healthy skin (Fig 1). This is due to wound characteristics (size, depth) and their degree of contamination with *Staphylococcus* bacteria.

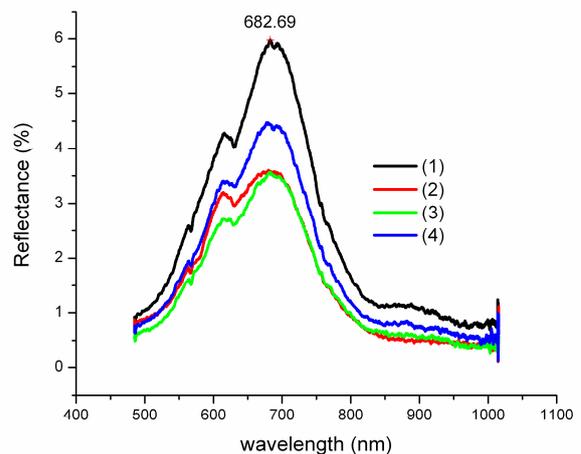


Fig. 1 Diffuse reflectance spectra of wounds contaminated with bacteria (pig no. 1); (1), (3) and (4) - contaminated wounds, (2) - normal

Normal skin shows a main maximum reflection $R_2 = 3.51\%$ ($\lambda = 682.69$ nm) while the contaminated wounds show a high reflection, diffuse reflectance values ranging from 4% to 6%.

Diffuse reflectance spectra of the wounds from the study group, acquired 30 min after the administration of methylene blue solution and before exposure to laser radiation show a reflection peak shift to higher wavelengths (from $\lambda_1 = 682.69$ nm to $\lambda_2 = 712.87$ nm) and a decrease of reflectance (Fig. 2).

The mean variation of diffuse reflectance was $\Delta R_{med} = 0.73 \pm 0.018\%$ (Table 1). This decrease of diffuse reflectance is due to the presence of methylene blue, which absorbs strongly at $\lambda = 698$ nm and provides information on the degree of accumulation of photosensitizer in the bacterial cell.

TABEL I
DIFFUSE REFLECTANCE AND COLONY COUNTS (LOGCFU/ML) OF CONTAMINATED WOUND
AT DIFFERENT MOMENTS OF PHOTODYNAMIC THERAPY

Time	Study group		Control group	
	R_{med} (%) ($\lambda = 712.87$ nm)	logCFU/ml	R_{med} (%) ($\lambda = 712.87$ nm)	logCFU/ml
Before photodynamic therapy	5.06±0.036	6.93±0.138	4.94±0.033	6.93±0.138
Before laser irradiation	4.33±0.028	-	-	-
After laser irradiation	3.54±0.031	-	-	-
48 h Post-treatment	3.36±0.025	3.12±0.108	7.64±0.026	7.37±0.121

The photodynamic effect induced to the bacterial cells (in which the photosensitizer was preferentially localized) by exposing the contaminated wounds for 15 min to laser radiation leads to modifications of the optical properties of the wounds (Fig. 3), the mean diffuse reflectance decreasing from $R_{med} = 4.33 \pm 0.028$ % to $R_{med} = 3.54 \pm 0.031$ %. This low reflectance can be correlated with the large absorption that dead bacteria which contain the photosensitizer present.

After 48 h, the spectral exam showed a decreased reflectance (at $\lambda = 712.87$ nm) for wounds contaminated with *Staphylococcus aureus* after 15 min of exposure to laser radiation and the bacteriological exam proved the presence of *Staphylococcus aureus*. The medium variation of reflectance was $\Delta R_{med} = 0.18 \pm 0.015$ % and the concentration of bacteria decreased with 3.81 ± 0.119 logCFU/ml.

By comparison, for control group initially at the bacterial concentration of 6.93 ± 0.138 logCFU/ml, after 48 h, the medium variation of reflectance was $\Delta R_{med} = 2.70 \pm 0.020$ % and the mean bacterial concentration reached the value of 7.37 ± 0.122 logCFU/ml.

IV.DISCUSSION

Many experimental studies have highlighted the efficiency of photodynamic therapy in infection control [19,20]. In these studies, the mouse has been commonly used as animal model. Since pig skin is very similar to human skin, in this study we used this animal model and we have proved that photodynamic therapy is an effective method to control infected wounds with bacterial specie taken into account. The mean decrease in bacterial counts, 48 h post-photodynamic treatment was 3.81 ± 0.120 logCFU/ml.

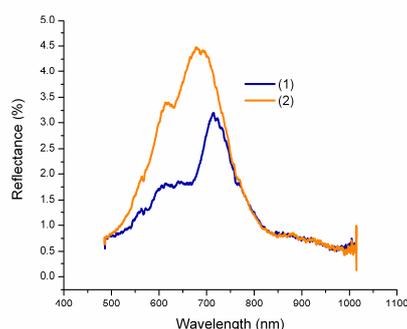


Fig. 2 Diffuse reflectance spectra of contaminated wound incubated with methylene blue solution for 30 min (wound no. 4 / pig no.1); (1) contaminated wound incubated with methylene blue solution; (2) contaminated wound without photosensitizer

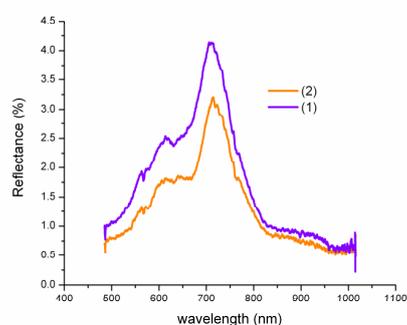


Fig. 3 Diffuse reflectance spectra of contaminated wound after laser irradiation (wound no. 4 / pig no.1); (1) – before laser irradiation; (2) – after laser irradiation

In addition to biological tests conducted at the beginning and end of experiments, we also used the diffuse reflectance spectrometry as a non-invasive method to monitor changes in optical properties (absorption and scattering) of *Staphylococcus aureus* infected wounds during photodynamic therapy.

The results have shown that the decrease in bacterial counts was accompanied by a decrease of diffuse reflectance with 1.70 ± 0.031 % (48 h post-treatment).

These preliminary results demonstrate that there is a correlation between the CFU count and diffuse reflectance and the assessment of changes in CFU count during photodynamic treatment could be done in real time using diffuse reflectance spectroscopy.

The relationship between these two parameters (calibration curve) and optimization of treatment parameters will be the subjects of future studies on different bacteria, photosensitizers and pig as animal model.

V.CONCLUSION

The results obtained on pigs have demonstrated that photodynamic therapy is an effective method of treating wounds infected with *Staphylococcus aureus*. Furthermore, on the basis of the correlation between the CFU count and diffuse reflectance highlighted in this study, a diffuse reflection method for the monitoring of photodynamic inactivation of bacteria can be developed. This method is simple, non-invasive and non-toxic and does not necessitate biological sampling.

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