Optical method for monitoring of photodynamic inactivation of bacteria

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Abstract Photodynamic inactivation is a new promising approach to treat bacterial infections. Usually, the evaluation of the efficacy of this method is done through time-consuming and labor-intensive microbiological test methods. This paper describes the development and implementation of an optical method to evaluate the photodynamic inactivation of bacteria based on non-invasive diffuse reflectance measurements. Five *Staphylococcus aureus* cultures and 15 mice have been used in this study. A skin lesion was created on the back of all animals, and it was contaminated with *S. aureus* (5.16 ± 0.013 log CFU/ml). Toluidine Blue O ($c = 8.67 \times 10^{-3}$ M) has been used as a photosensitiser agent. The bacterial cultures and animals were exposed to laser radiation ($\lambda = 635$ nm, $P = 15$ mW, $DE = 8.654$ J/cm²) for 20 min. The photodynamic inactivation of bacteria was monitored by acquiring the wounds' reflection spectra at different time points and by microbiological exams on the bioptical material. The good correlation between the diffuse reflectance and colony-forming units demonstrates the value of this optical method based on diffuse reflectance measurements as a rapid technique to monitor photodynamic bacterial inactivation.

Keywords Inactivation · Photodynamic · Reflectance · Spectroscopy · *Staphylococcus*

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1 Introduction

The more and more frequent use of antibiotics in the treatment of infections has resulted in an increasing number of bacterial strains resistant to a large number of antibiotics. This result has inspired a search for new antibiotics able to inactivate new resistant microorganisms to the existent antibiotics. Among these microorganisms, the pathological bacteria *Staphylococcus aureus* is one of the most resistant to antibiotics with resistance to methicillin (methicillin-resistant *S. aureus*). In this context, the present microbiology research and related studies from biophysics, chemistry, medicine and physics have been oriented towards the development of some alternative methods of antimicrobial therapy, faster and more efficient, non-invasive and non-toxic and which, when repeatedly applied, do not lead to the growth of the resistance of the bacteria [1].

Such non-invasive methods, based on the application of light sources, are currently involved in the research programs of some institutes and universities from around the world, with the aim of developing a new treatment for infections, based on the inactivation of the bacteria through photodynamic effects.

Photodynamic inactivation is based on the administration of a photosensitiser that is located in the bacterial cell followed by exposure to optical radiation with wavelength corresponding with the maximum value of absorption of the photosensitiser. Although it is still in an experimental phase, there are remarkable results reported in the past years on the destruction through photodynamic inactivation of some bacteria able to generate different types of infections. Thus, the in vitro studies concerning the photodynamic inactivation of Gram-positive and Gram-negative bacteria by using different photosensitive compounds and optical radiations with the wavelengths $\lambda \in (350\text{--}800)$ nm have demonstrated a decrease of over 90% of viability of these germs [2–9].

In vivo studies regarding photodynamic inactivation of the contaminated burn wounds with *S. aureus* by using PTMPP (meso-mono-phenyl-tri-*N*-methyl-4-pyridyl) porphyrin as photosensitizing agent and coherent light with $\lambda = 675$ nm demonstrated a decrease of more than 98% of bacterial viability and the clinical exam showed the total healing of burn wounds [10].

In all these studies, microbiological test methods have been used for the evaluation of the photodynamic inactivation of the bacteria (viability tests or CFU counts), methods which are time-consuming and labor-intensive. To determine in vivo the viability or CFU counts, microbiological test methods require biopsy sampling or wiping the surface. Recently, some optical methods have been used as non-invasive characterisation and measurement of optical parameters of tissue, both in photodynamic therapy of malignant tumours (to determine the concentration of photosensitiser in the tumour) and for treatment monitoring [11–18].

One of these optical methods is diffuse reflectance spectrometry. This method can provide, in real time, temporal information about photodynamic action of light radiation on bacteria.

The principle of in vivo diffuse reflectance spectrometry consists in sending optical radiation from a source through an optical fibre onto the sample and collecting the reflected radiation with another optical fibre by a spectrometer for data acquisition.

When the optical radiation is incident on a biological sample, a small fraction of the incident radiation is reflected back due to the jump of the refractive index between the external environment and the sample (specular reflection) and the remaining fraction is transmitted to the sample where it undergoes absorption and scattering events. The

absorption process is due to the presence of the numerous chemical species (chromophores) in biological samples. Water plays the fundamental role, being the main absorber in the infrared spectral region [19]. The scattering events take place in the visible and near infrared spectral regions, because the cellular diameter is comparable to the radiation wavelength [20]. After multiple scattering events on the microscopic structures in the biological sample, the light rapidly loses its intensity. In this way, the changes of specific structures within the sample can be determined, by measuring the changes in the intensity of radiation over a spectral range.

The aim of this paper is to develop an optical method based on diffuse reflectance measurements as a new and useful technique for in vivo monitoring of photodynamic inactivation of *S. aureus* cultures, using Toluidine Blue O as photosensitiser. The relationship between the diffuse reflectance versus colony-forming units (CFU/ml) counts is discussed as well.

2 Materials and methods

2.1 Bacterial culture

The cultures used in this study were *S. aureus* strains (ATCC 25923) as catalogued in the American Type Culture Collection isolated from burn wounds, cultured in brain–heart infusion medium for 18 h at 37°C. The number of colonies formed after 18–24 h incubation at 37°C was 2.31×10^{10} cells/ml.

In order to establish the relationship between the diffuse reflectance and bacterial colony-forming units (CFU/ml), another five cultures were prepared in triplicate through dilution with sterile PBS, the concentration of these samples ranging from 7.22×10^3 to 1.15×10^9 CFU/ml.

2.2 Mice

In this study, we used 15 clinically healthy adult mice from the Swiss strain, with a weight $\cong 25$ g. The animals had been fed and kept in conditions stipulated by the Law 305/2006 referring to the European Agreement for Protection of Animals used in Experiments and Other Scientific Purposes (1986) and by the Convention Protocol Amendment (Strasbourg, 1998); Romania adopted this Law on the 15th of February 2006. The back of these animals was shaved. The shaved area has been disinfected with iodine alcohol, and, under local anaesthesia, a sterile syringe needle was used to scarify the superficial skin layers to bleeding. After haemostasis, mice with single wounds on the middle of the back with an area of 100 mm^2 ($8 \times 12.5 \text{ mm}^2$) received inocula of mid-log-phase of *S. aureus* suspended to appropriate concentration in $50 \text{ }\mu\text{l}$ of PBS. After 24 h, the bacterial concentration was $5.16 \pm 0.013 \text{ log CFU/ml}$. At that moment, $50 \text{ }\mu\text{l}$ of Toluidine Blue O (TBO; $8.67 \times 10^{-3} \text{ M}$) was added to the wounds of animals from study group. Mice in the study group were then illuminated with laser radiation, while the mice from control group were kept in the dark. Mice were covered with a non-light-penetrating plastic sheet with a square opening ($20 \times 20 \text{ mm}^2$) aligned with the wounds during irradiation to avoid whole-body irradiation. After photodynamic treatment, the mice were kept under subdued lighting for 72 h. This duration was selected

because mice that survived for 72 h after bacterial inoculation could totally recover from the infections.

2.3 Photosensitisers and light source

TBO (Fluka), an analytical grade reagent, was used without further purification. Bidistilled water was used to prepare the solution.

For TBO dyes, only monomer forms are present in solution. It is well known from the literature that this dye is active in photodynamic therapy [21].

The solution used for photodynamic inactivation had a concentration of 8.67×10^{-3} M and pH = 7.4.

A portable laser system SCL (INOE 2000, Bucharest, Romania) for the irradiation of the bacterial cultures and infected wounds was used in this study. The equipment consisted of two principal modules: laser probe and control unit. The laser probe is composed of five diode lasers emitting continuously at 635 nm and their drivers being fixed in a special device for an easily maneuverability in different positions.

The main characteristics of the diode laser are: power 3 mW, wavelength 635 ± 10 nm, display current 250 μ A, work current 55 mA (at 5 mW), work tension 1,75 V (at 5 mW). The time of irradiation could be set up to a maximum of 3,600 s, with a step of 1 s.

2.4 Photodynamic inactivation

In vitro studies Five-millilitre aliquots of all *S. aureus* cultures were distributed in Petri dishes, with a diameter of 10 cm, and incubated in the dark with 5 ml TBO ($c_{\text{TBO}} = 8.67 \times 10^{-3}$ M) for 15 min at room temperature. Cultures were then exposed to laser radiation (635 nm, 15 mW, 8.654 J/cm^2) for 20 min at a distance of 1.5 cm. After laser irradiation, the bacterial colony-forming units (CFU/ml) were determined through the method of decimal dilutions and by direct counting on Petri dishes. The photodynamic inactivation of bacteria was monitored by diffuse reflectance measurements at different time points: immediately after administration of TBO, and before and after laser irradiation.

In vivo studies Fifteen animals were grouped into two experimental groups: study group (ten mice) and control group (five mice). After 24 h, the TBO solution ($c_{\text{TBO}} = 8.67 \times 10^{-3}$ M) was administered to the whole area of the wound with sterile gauze for the study group. Every mouse was kept 15 min in the dark after photosensitiser administration and then exposed to laser radiation for 20 min. The animals from the control groups were not incubated with photosensitiser and were not irradiated.

Reflection spectra were recorded before photosensitiser administration, before and after laser irradiation, for every animal. The photodynamic inactivation of bacteria has been corroborated with the wounds' reflection spectra at different times and microbiological exams on the bioptical material, over the course of 24 h.

2.5 Diffuse reflectance measurements

The optical reflectance spectra were obtained with an AvaSpec spectrometer (Avantes, The Netherlands) in wavelength range (500–1100) nm. This portable spectrophotometer is

equipped with a tungsten halogen lamp, CCD detector array (2,048 pixels) and a reflection probe tip FCR-71R200-2 with one illuminating fibre in centre surrounded by six fibres which collect the light reflected from the sample.

2.6 Observation of microbial growth (colony-forming units)

Biological samples in all cases were taken from the central part of the scarified zone before and after laser irradiation. The samples contained in the test tubes were diluted in the proportions 1:10 and 1:100 in Mueller Hinton liquid medium and seeded using the drainage technique on MacConkey agar and on azide blood agar, to observe the bacteria in accordance with the findings from slides stained via the Gram technique. The plates were incubated at 37°C for 24 h and then the colony-forming units were counted using a digital colony counter. The tubes containing the diluted samples were also incubated under the same conditions as for the plates and, after 24 h, they were seeded in new culturing media for confirmation of the results.

3 Statistical analysis

Data are presented as mean and standard deviation. The mean value and its standard deviation were calculated using Microsoft Excel (Microsoft Corp., Redmond, WA), and the differences between data from the control group and the irradiated groups have been tested for statistical significance using the Student's *t* test. Statistical significance was assumed if $p < 0.05$.

4 Results

4.1 In vitro studies

Representative reflection spectra of *S. aureus* culture are shown in Fig. 1. The main spectral characteristics of *S. aureus* culture are two reflection maxima, at $\lambda_{\text{Max-1}} = 613.87$ nm ($R_{\text{Max-1}} = 23.79\%$) and $\lambda_{\text{Max-2}} = 682.69$ nm ($R_{\text{Max-2}} = 27.33\%$), and a minimum at $\lambda_{\text{Sa-min}} = 630.74$ nm ($R_{\text{Sa-min}} = 21.15\%$).

When the *S. aureus* cultures are incubated with TBO solution for 15 min in the dark, the reflection spectra show some changes (Fig. 2). Diffuse reflectance spectrum of *S. aureus* culture with TBO as photosensitiser shows decreased reflectance values and a shift of the reflectance maxima from $\lambda_{\text{Max-1}} = 613.87$ nm to $\lambda'_{\text{Max-1}} = 612.76$ nm ($R_{\text{Max-1}} = 7.80\%$) and from $\lambda_{\text{Max-2}} = 682.69$ nm to $\lambda'_{\text{Max-2}} = 680.79$ nm ($R_{\text{Max-2}} = 8.91\%$; Fig. 2). This shift is caused interactions between the photosensitiser and the biological targets inside the bacteria. The reflection minimum is present at $\lambda_{\text{Sa-min}} = 630.74$ nm. At this wavelength, the bacterial cultures show the greatest absorption and the photodynamic effect on the bacteria can be induced by laser radiation at a corresponding wavelength.

At $\lambda = 635$ nm, the diffuse reflectance of cultures is linearly proportional to bacterial colony-forming units (as determined by method of decimal dilutions and by direct counting on Petri dishes; Fig. 3). Based on this relationship, the effectiveness of the bacterial photodynamic inactivation could be evaluated.

Fig. 1 The reflection spectrum of *S. aureus* culture (culture 1)

Fig. 2 The reflection spectrum of *S. aureus* culture (culture 1) incubated with TBO solution

Fig. 3 The dependence of diffuse reflectance of *S. aureus* culture treated with TBO on colony counts (log CFU/ml)

Fig. 4 Reflection spectra of *S. aureus* culture treated with TBO before (1) and after (2) exposure to laser radiation

Table 1 The correlation between calculated and determined values of bacterial counts (log CFU/ml) during photodynamic inactivation process

Sample number	Before laser irradiation	After laser irradiation	
	log CFU _{det} /ml	log CFU _{det} /ml	log CFU _{calc} /ml
1	3.86 ± 0.016	1.54 ± 0.011	1.49 ± 0.009
2	5.16 ± 0.021	1.98 ± 0.016	1.95 ± 0.008
3	6.46 ± 0.029	3.21 ± 0.020	3.30 ± 0.010
4	7.76 ± 0.037	4.13 ± 0.014	4.15 ± 0.011
5	9.06 ± 0.029	5.76 ± 0.017	5.72 ± 0.019

After laser irradiation ($t_{\text{exp}} = 20$ min), the diffuse reflectance of *S. aureus* cultures decreased (Fig. 4).

From these reflectance measurements, the number of colonies was calculated by using the calibration curve (log CFU_{calc}/ml) and compared to the method of decimal dilutions and direct counting on Petri dishes (log CFU_{det}/ml). The results are shown in Table 1.

Table 1 shows a good correlation between log CFU_{calc} and log CFU_{det} determined by standard methods, demonstrating that optical methods based on the diffuse reflectance measurements have a value as a non-invasive, rapid and non-toxic method for evaluation of photodynamic inactivation for in vivo and in vitro studies.

4.2 In vivo studies

In vivo studies were performed on mice using the same optical method together with clinical and bacteriological monitoring. The repeated bacteriological exams proved the presence of *S. aureus* in the first 3 to 4 days after photosensitiser incubation followed by laser irradiation. In the control group, *S. aureus* was isolated as well. The spectral exam showed a decreased reflectance (at $\lambda = 635$ nm) for wounds contaminated with *S. aureus* after 20 min of laser exposure. The mean variation of reflectance was $\Delta R_{\text{med}} = 1.21 \pm 0.023\%$, and the colony counts decreased with 3.17 ± 0.015 log CFU_{det}/ml (Table 2). After 24 h, mean reflectance of contaminated wounds was $R_{\text{med}} = 5.57 \pm 0.024\%$, and bacterial counts was 1.06 ± 0.022 log CFU/ml. By comparison, for the control group the reflectance was $R_{\text{med}} = 7.22 \pm 0.025\%$, corresponding to 5.16 ± 0.013 log CFU_{det}/ml. After 24 h, bacterial counts became 5.78 ± 0.019 log CFU_{det}/ml (as determined by standard method) and the reflectance of these cultures $R_{\text{med}} = 7.46 \pm 0.029\%$.

Table 2 Variation of diffuse reflectance and colony counts (log CFU/ml) for contaminated wounds with *Staphylococcus aureus* bacteria as a result of photodynamic inactivation

Time	Study group		Control group	
	log CFU _{det} /ml	R_{med} (%)	log CFU _{det} /ml	R_{med} (%)
Before laser irradiation	5.16 ± 0.013	7.13 ± 0.038	5.16 ± 0.013	7.22 ± 0.025
After laser irradiation	1.98 ± 0.016	5.92 ± 0.027	—	—
24 h Post-treatment	1.06 ± 0.022	5.57 ± 0.024	5.78 ± 0.019	7.46 ± 0.029

5 Discussion

Photodynamic inactivation of bacteria has proved to be an effective method to control infection in many experimental studies [22–24]. The present study shows a significant decrease in CFU counts in vitro for bacterial species. The in vitro experiment was also designed to set a calibration curve relating classical CFU counting to diffuse reflectance. The relationship between the two parameters (determined and estimated CFU count) proved to be linear and it shown a good correlation between actual bacterial counts (log CFU/ml) and the reflectance measurements at the wavelength corresponding to the laser radiation ($\lambda = 635$ nm). The in vitro approach gave the authors a basis for establishing diffuse reflectance spectrometry as a method for monitoring of photodynamic inactivation of bacteria.

The in vitro findings were then tested in vivo. The same decrease in bacterial counts (log CFU/ml) after laser irradiation was observed and the correlation between log CFU/ml and reflectance measurements at $\lambda = 635$ nm proved to be consistent for in vivo conditions.

This non-invasive and rapid method based on the measurement of diffuse reflectance was shown to be useful for evaluating modifications of colony counts, but it should be confirmed in future studies on different species of bacteria (both Gram-positive and Gram-negative).

6 Conclusions

In order to evaluate the photodynamic inactivation of bacteria, the variation of the number of log CFU/ml has been determined through two methods: decimal dilution method (log CFU/ml_{det}) and diffuse reflectance spectrometry (log CFU/ml_{calc}).

A good correlation between log CFU/ml_{calc} and log CFU/ml_{det} has been demonstrated, as a proof that the diffuse reflectance method is a good monitoring method of bacteria photodynamic inactivation. This method is simple, non-invasive and non-toxic and does not necessitate biological sampling for clinical cases.

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