IN VIVO STUDY OF MAL-PDT USING FLUORESCENCE SPECTRA

Emil PETRESCU[1], Mihaela Antonina CĂLIN[2], Bogdan MARINESCU[3], Cristina CÎRTOAJE[4]

The fluorescence spectra of tumor tissues have been studied in vivo using simple, double and triple irradiation schemes. From the analysis of these spectra we could estimate the variations of protoporphyrine IX amount from living tissues. The results have proved an increased efficiency of multiple light fractionated schemes when compared to simple one. We also noticed that the best results were obtained when using triple fractionated light irradiation scheme.

Keywords: photodynamic therapy, fractionated irradiation scheme, fluorescence, mice

1. Introduction

In order to destroy the malign and benign tumor cells, a new treatment method called photodynamic therapy (PDT), combining the effects of a photosensitive drug with optical irradiation was proposed [1,2]. The drugs commonly used for this type of therapy are not toxic in the absence of light both for tumor cells or for the healthy ones. They are systematically applied a few hours, or sometimes days before the irradiation with visible for near infrared light on the affected region. The photosensitive drug must display an absorption peak within the wavelength spectra of the light used for the treatment. The activation of the photosensitizer (methyl aminolevulinate - MAL) determines the excitation of the transition product protoporphyrine IX (PpIX). When PpIX relaxes it induces reactive oxygen species (ROS), i.e. toxic products for cells and mainly for tumor cells which consume a great amount of oxygen.

Most of the photosensitive drugs display such behavior. Some species, known as type II photosensitizers are acting by transforming the stable oxygen species into ROS inside the affected tissue. The singlet oxygen species are strongly reactive and have an average life-time of 0.01-0.04 μs. The triplet oxygen species are stable and no self-transition from the triplet to singlet state is possible. This is the reason why a photosensitizer has to be used to induce the triplet-singlet oxygen transition.

[1] Professor, Physics Department, University POLITEHNICA of Bucharest, ROMANIA
[2] Senior Researcher, National Institute of Research and Development for Optoelectronics INOE 2000, 409 Atomiştilor street, PO BOX MG5, Magurele, Ilfov, 077125, ROMANIA
[3] Researcher, Victor Babes National Institute for Research and Development in Pathology and Biomedical Sciences, 99 – 101 Independentei Street, Bucharest, 050096, ROMANIA
[4] Lecturer, Physics Department, University POLITEHNICA of Bucharest, ROMANIA

Both therapists and physicists have to face two major challenges in this therapy: the amount of drug needed to be used and the irradiation procedure. In order to generate ROS *in vivo*, three things are necessary: a proper drug to stimulate the photosensitizer, a light beam having wavelength in the vicinity of the maximum absorption peaks of the photosensitizer and a proper irradiation scheme needed to generate the greatest amount of ROS possible.

The quantitative evaluation of *in vivo* fluorescence is more difficult than that occurring *in vitro* because any fluorescence measurement is influenced by scattering and absorption phenomena. This is the reason why these effects must to be minimized and explains why different procedures such as photodynamic diagnosis (PD) [2,3] and fluorescence spectra recording methods [4-8] were proposed.

2. Materials and methods

2.1 Animals

The experiments were performed by using the C57BI/6 line female mice (6-8 months old) from the Victor Babes Biobase Institute (Bucharest) in which cancer tumors were induced by transplantation. In order to do that, a donor mouse with carcinoma has been sacrificed and tumor samples were taken. The samples were mixed with physiological serum obtaining a homogenous tumor suspension. From this suspension an amount of 0,4 ml was inoculated to each mouse. After 7 up to 9 days from the transplant the tumors were visible. When the tumors were about 1cm to 3cm diameter (27 days after the transplant) the photodynamic therapy was initiated. The specific microclimate of the mice habitat had an average temperature of 22±2 ^{o}C, relative humidity of 55±10 %, 12 hours light, 12 hours dark and controlled ventilation.

For the experiment, the 3R principle (Replacement, Refinement and Reduction) proposed by Russel and Burch has been applied. The animals were separated in 7 groups depending on the irradiation scheme and MAL incubation time. (Table 1)

Table 1

The animal groups established for testing the efficiency of the fractionated irradiation scheme.

	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6	GROUP 7
Irradiation scheme	Witness mouse (no irradiation)	Simple	Simple	Double	Double	Triple	Triple
Incubation time	Without incubation	2 hours	4 hours	2 hours	4 hours	2 hours	4 hours

2.2 Photosensitizer

The photosensitizer was obtained by mixing 100 mg of methyl delta-aminolevulinate hydrochloride powder with a temperature of 2-8 ^{o}C with 0.625 g of Vaseline resulting 0.725 g of unguent. The whole operation has been performed in a dark room using proper protection equipment. The unguent has been stored into a container at 2-8 ^{o}C before being applied on the tumor.

Each mouse from the group was subjected to the same procedure before treatment. First, they were washed and the fur was gently removed taking care not to harm the skin. The residues were removed by tapping the region with a sterilized wet tampon. The unguent has been applied gently by massage for 30 or 60 seconds. Finally, the mice were placed in a dark room for 2 or 4 hours depending on the protocol.

2.3. Multiple fractionated irradiation scheme and photodynamic therapy

After the incubation period, the animals were anaesthetized and subjected to the irradiation schemes as settled.

After MAL administration the animals from the groups 2, 4 and 6 were kept into a dark room for 2 hours then subjected to irradiation sessions. The same procedure was applied on the animals from groups 3, 5 and 7 excepting the fact that the dark period for these groups was doubled (4 hours).

The first group was used as marker. To these animals were not performed any treatment with MAL or laser radiation.

Fig.1:Laser irradiation of the mice tumors

An AlGaInP multiple laser plunge has been used in continuous wave mode for three types of irradiation schemes:

1. Single fractionated light irradiation scheme consisting of a single irradiation session with the following set of parameters: λ = 635 nm, power density released on the exposed aria P = 15 mW/cm^2, exposure time t_{exp1} = 1800 s. The plunger position was parallel to the tumors (Fig.1)
2. Double fractionated light irradiation scheme consisting of two irradiation sessions with the following characteristic set of parameters: λ = 635 nm, power density released on the exposed aria P = 15 mW/cm^2, The exposure times were t_{exp1} = 900 s for the first session and t_{exp2} = 900 s for the second session interrupted by a dark period, t_{dark} = 30 min. (resting time???)
3. Triple fractionated light irradiation scheme consisting of three irradiation session with 30 min pause between them, using the same parameters (λ = 635 nm, P = 15 mW/cm^2) and the exposure times: t_{exp1} = 300 s, t_{exp2} = 300 s and t_{exp3} = 300 s.

2.4. Fluorescence spectrometry

In order to evaluate the efficiency of the multiple irradiation schemes in photodynamic therapy of the malign tumors, the fluorescence spectra were recorded using an AvaSpec 2048-USB2 optic fiber spectrophotometer (Avantes, The Netherlands, Europe).

3. Results

The fluorescence spectra obtained on mice in GROUP 2 and GROUP 3 using the simple fractionated light irradiation scheme are shown in (Fig. 2). One can notice that in both cases the intensity of the fluorescence spectra peaks of PpIX (the four peaks between 648 nm and 750 nm) recorded 24 h hours after the irradiation (line 3) is considerably smaller than the ones obtained before irradiation (line 1) indicating a decrease of the cancer cells amount. From the intensity of the second group of mice recorded spectra (Fig. 2) we noticed a decrease of the fluorescence maxima of PpIX immediately after the irradiation (line 2) and no consistent difference 24 hours after the treatment (line 3) The spectra recorded from the third group of mice presents a strong increase of the fluorescence peaks of PpIX (line 2) due to a massive absorption of MAL in cancerous cells followed by a drastic decrease 24 hours after the treatment (line 3) as a result of tumor cells death or PpIX exhaustion. A synthesis of the results is presented in Fig. 3 representing the values of the PpIX peaks for each group of mice.

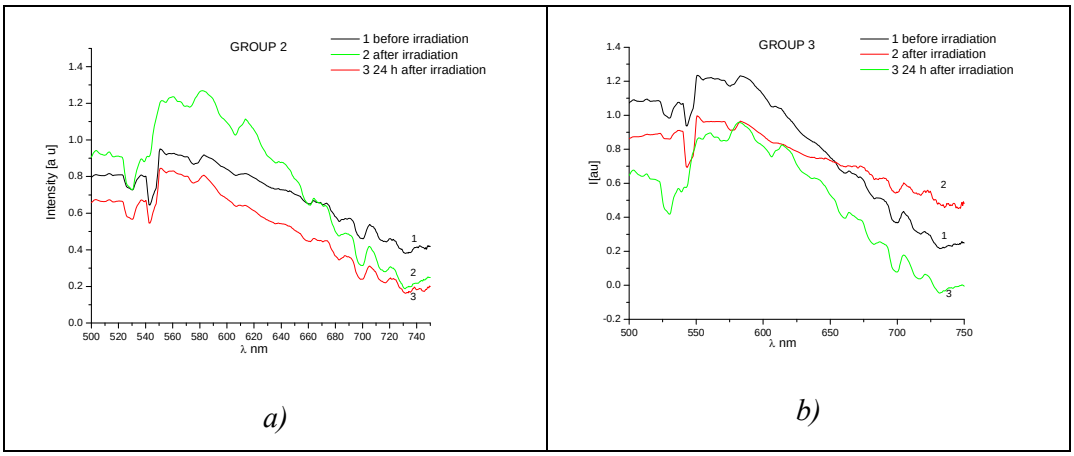

Fig.2 Fluorescence spectra obtained using a single fractionated light irradiation scheme a) GROUP 2 (2 hours of incubation) b) GROUP 3 (4 hours of incubation).

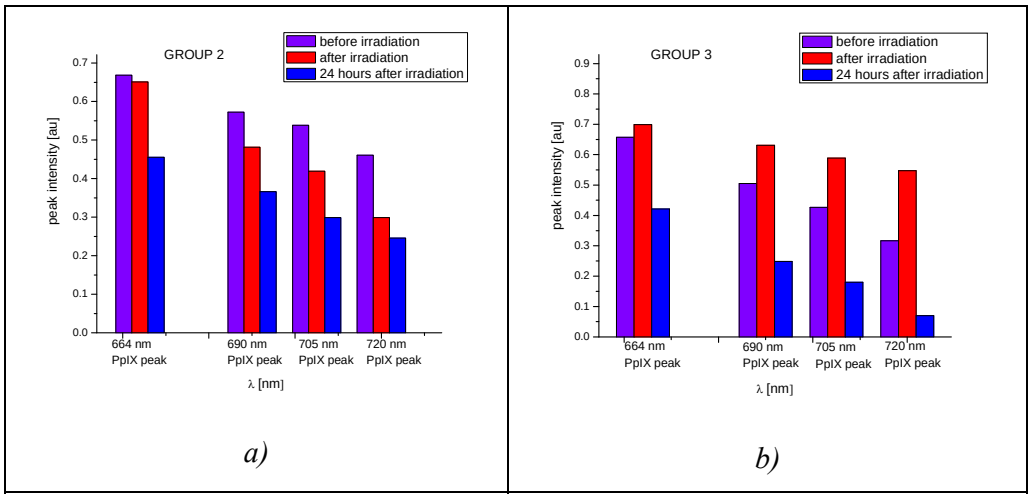

Fig.3 Peak intensity after applying a single fractionated light irradiation scheme a) GROUP 2, (2 hours of incubation) b) GROUP 3 (4 hours of incubation).

When a double fractionated light irradiation scheme has been used on GROUP 4 and GROUP 5 (Fig. 4 and Fig. 5) one can notice a decrease (about 20%) of fluorescence peaks of PpIX after the second irradiation session (line 3) when compared to the ones obtained before the first irradiation (line 1) and an increase 24 hours after the treatment (line 4). It results that this type of irradiation is more efficient than the previous one because the number of the cells containing protoporphyrine PpIX decreases after the first irradiation session and the light fractionation allows the regeneration of PpIX instead of chemical destruction of MAL.

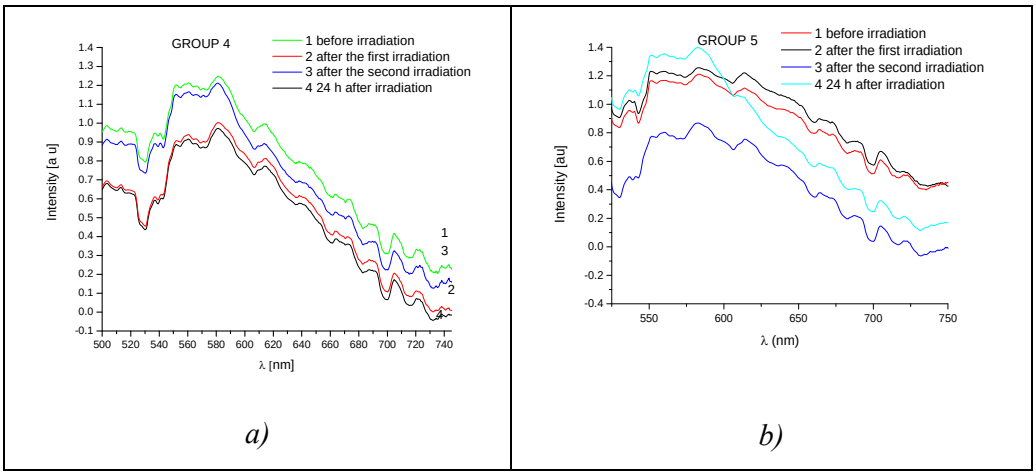

Fig.4 Fluorescence spectra obtained after the double fractionated light irradiation scheme a) GROUP 4 (2 hours of incubation) and b) GROUP 5 (4 hours of incubation).

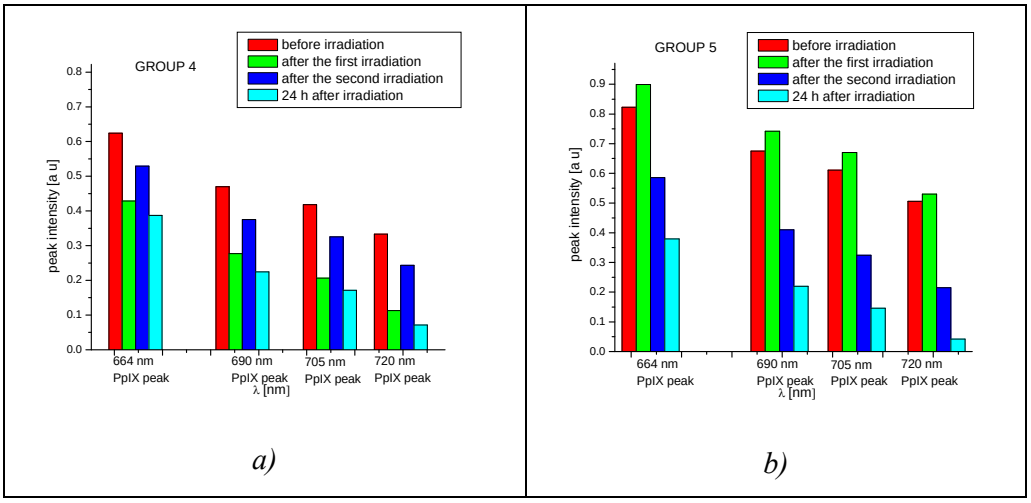

Fig.5 Peak intensity after applying a double fractionated light irradiation scheme. a) GROUP 4 (2 hours of incubation) and b) GROUP 5 (4 hours of incubation).

The triple fractionated light irradiation scheme (Fig. 6, Fig. 7) treatment does not seem to be more efficient than the double fractionated one because changes in the fluorescence peaks before the first irradiation (line 1) and after the third irradiation (line 5) are small when compared to the difference before the first irradiation spectrum (line 1) and after the second irradiation one (line 3). From (Fig. 7) we noticed a decrease of the protoporphyrine IX peaks recorded 24 hours after the treatment suggesting a lower rate of regeneration.

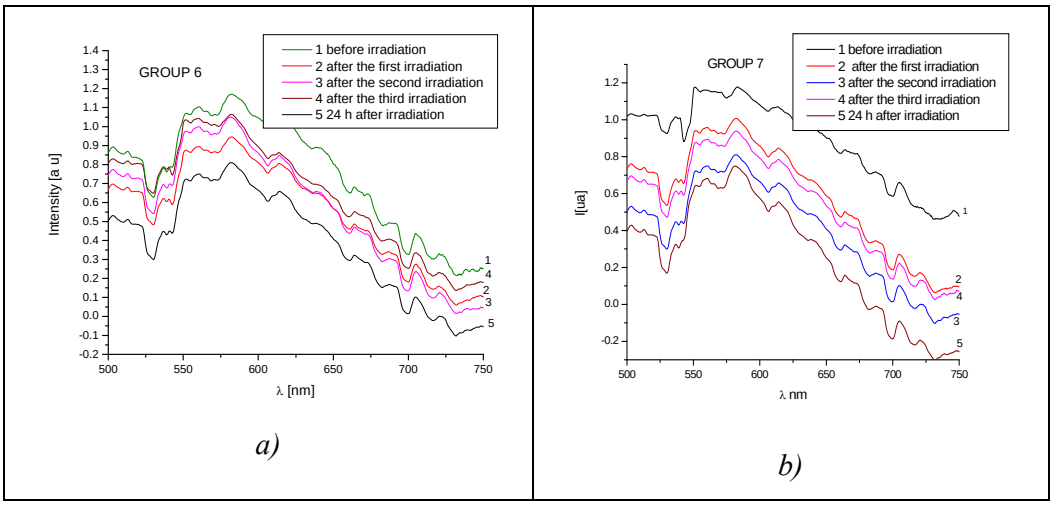

Fig.6: Fluorescence spectra obtained after the triple fractionated light irradiation scheme treatment a) GROUP 6 (2 hours of incubation) and b) GROUP 7 (4 hours of incubation).

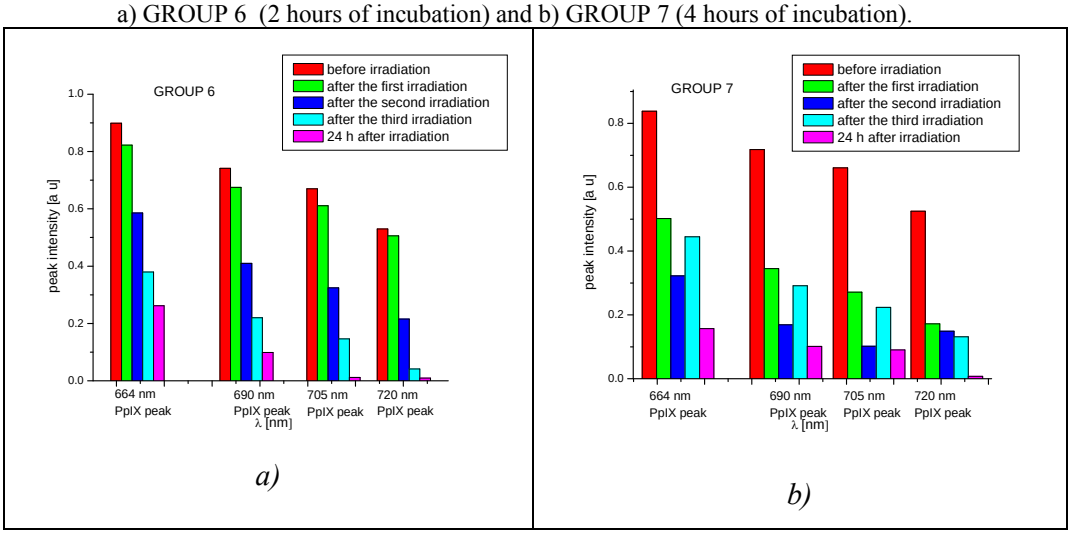

Fig.7 Fluorescence spectra obtained after applying the triple fractionated light. a) GROUP 6 (2 hours of incubation) and b) GROUP 7 (4 hours of incubation).

4. Discussions

Photodynamic therapy has been applied *in vivo* to a set of tumor tissues on different mice groups. As photosensitive drug we used methyl delta-aminolevulinate hydrochloride (MAL). During the treatment, simple double and triple fractionated light irradiation schemes were applied. The results proved the efficiency of the multiple fractionated light irradiation schemes comparing to the classical single irradiation indicating a strong decrease of the tumor tissue 24 hours after the treatment. Our studies proved once again the treatment's efficiency when fractionated light irradiation schemes are used allowing the PpIX

regeneration inside the cells instead of their chemical destruction. Both the double and triple fractionated light irradiation scheme proved to be more efficient than the single one but, double one is clearly more efficient.

5. Conclusions

After recording the fluorescence spectra of tumor tissues, we found that the best results were obtained when using a double fractionated light irradiation scheme. This is in an indication that by multiplying the fascicle fractions the amount of reactive oxygen species (ROS) is not sufficient for a massive destruction of tumor cells. Similar results were obtained when an in-vitro analysis of MAL based photodynamic therapy using fractionated light was performed [9].

Acknowledgements

This work was supported by The Ministry of Education, Research and Youth by the PN II Program, Grant No. 62074/2008 and the Sectorial Operational Program Human Resource Development 2007-2013 of the Ministry of European Funds through the Financial Agreement POSDRU 159/1.5/S/132397.

R E F E R E N C E S

[1]. C. *Jarod,* Reflectance and Fluorescence Spectroscopies in Photodynamic Therapy PhD Thesys, Department of Physics and Astronomy, The College of Arts and Sciences, University of Rochester, Rochester, New York, 2003

[2]. *N.K. Chaudhury, S. Chandra and T. L. Mathew*, "Oncologic Applications of Biophotonics" in Applied Biochemistry Biotechnology, **vol. 96**, issue 1-3 Oct-Dec 2001, pp. 183–204.

[3]. *S.L. Marcus, R.S. Sobel, A.L. Golub, R.L. Carroll, S. Lundahl, and D. Shulman,* "Photodynamic Therapy (PDT) and Photodiagnosis (PD) Using Endogenous Photosensitization Induced By 5-Aminolevulinic Acid (ALA): Current Clinical and Development Status." in Journal of Clinical Laser Medicine and Surgery, **vol. 14,** issue 2, Apr. 1996, pp. 59–66.

[4]. *R.W. Weersink, M.S. Patterson, K. Diamond, S. Silver, and N. Padgett,* "Noninvasive Measurement of Fluorophore Concentration in Turbid Media with a Simple Fluorescence/Reflectance Ratio Technique." in Applied Optics, **vol. 40**, issue 34, Dec. 2001, pp. 6389–6395.

[5]. *J. Wu, M.S. Feld, and R. Rava,* (1993). "Analytical Model for Extracting Intrinsic Fluorescence in Turbid Media." in Applied Optics, **vol. 32**, issue 19, Jul. 1993, pp. 3585–3595.

[6]. *M. Müller, I. Georgakoudi, Q. Zhang, J. Wu and M. Feld*, "Intrinsic Fluorescence Spectroscopy in Turbid Media: Disentangling Effects of Scattering and Absorption." in Applied Optics, **vol. 40**, issue 25, Sept. 2001, pp. 4633–4646.

[7]. *S.G. Vari, T.G. Papazoglou, V.R. Pergadia, M. Stavridi, W.J. Snyder, T. Papaioannou, J. T. Duffy, A.B. Weiss, R. Thomas, and W.S. Grundfest*, "Blood Perfusion and Ph Monitoring in Organs by Laser Induced Fluorescence Spectroscopy." in Proc. SPIE. 2081, 1993, pp. 117–128.

[8]. *R.E.N. Shehada, V.Z. Marmarelis, H.N. Mansour, and W.S. Grundfest,* "Laser Induced Fluorescence Attenuation Spectroscopy: Detection Of Hypoxia." in IEEE Transaction on Biomedical Engineering, **vol. 47**, issue 3, 2000, pp. 301–312.

[9]. *C. Cîrtoaje, E. Petrescu, M.A. Călin, G. Izvoranu*, "Spectral Analysis of Methyl-Aminolevulinate Based Photodynamic Therapy Using Fractionated Light",U.P.B. Scientific Bulletin, Series A, Appliede Mathematics and Physics, **vol. 76**, issue 4, 2014, pp. 191-198.