SPECTRAL ANALYSIS OF METHYL-AMINOLEVULINATE BASED PHOTODYNAMIC THERAPY USING FRACTIONATED LIGHT

Cristina CÎRTOAJE[1], Emil PETRESCU[2], Mihaela Antonina CĂLIN[3], Gina IZVORANU[4]

Fractionated light effects on malign-cell culture previously treated with methyl-aminolevulinate were studied. Fluorescence spectra were recorded, after a single laser irradiation and compared to the spectra obtained when the beam was divided in two or three parts (fractionated light irradiation). The spectral curves of the culture medium were compared with the ones obtained from samples containing tumor cell cultures before and after each fractionated light irradiation session. The results showed that using multiple fractionated light allows the medium to produce more protoporphyrin IX, increasing the therapy efficiency.

Keywords: photodynamic therapy, protoporphyrin IX, fractionated light

1. Introduction

The first preclinical study on the use of fractionated irradiation schemes in 5-aminolevulinic acid-mediated photodynamic therapy (ALA-PDT) has been reported by Van der Veen et al. in 1994. Relatively recently, in a comparative clinical study on the fractionated ALA-PDT versus non-fractionated ALA-PDT in the treatment of superficial basal cell carcinoma (sBCC), Haas et al. [1] showed that after 12 months follow-up, complete response rate (CR) of the lesions treated with double-fractionated ALA-PDT was significant higher (97 %) than in those treated with single irradiation scheme (89 %). A similar clinical study was performed by Puizina-Ivić et all in 2008 [2] to compare the efficiency of double-fractionated irradiation scheme versus single irradiation scheme in ALA-PDT of the actinic keratosis (AK) and Bowen's disease. The results of this study revealed that double-fractionated ALA-PDT significantly improves the therapeutic outcome in tumor eradication. Many other studies have been conducted until present, aimed mainly to establish the irradiation parameters of the first and the second fractions

[1] Lectureur, Physics Department, University POLITEHNICA of Bucharest, ROMANIA
[2] Professor, Physics Department, University POLITEHNICA of Bucharest, ROMANIA
[3] Senior Researcher, National Institute of Research & Development for Optoelectronics INOE 2000, 409 Atomiştilor Street, 077125 Măgurele, Ilfov ROMANIA
[4] Researcher, Victor Babeş National Institute of Research & Development in Pathology and Biomedical Science, Splaiul Independenţei 99-100, 050096, Bucharest, ROMANIA

of irradiation that are critical for getting a higher response rate [3, 4, 5, 6, 7]. Other research publications [8] have shown the importance of the dark periods between irradiation sessions as the time when the quantity of protoporphyrin IX (PpIX) regenerates. Based on these studies, we were trying to develop a therapy method that combines the advantages of a superior photosensitizer with the ones of the light fractionated irradiation by finding an equilibrium between the number of irradiation sessions and the dark periods between them so we recorded the fluorescence spectra for the simple, double and triple fractionated light irradiation schemes and compared the results.

The photodynamic therapy (PDT) seems to be more efficient in tumor treatment than regular chemical method. PDT is based on combined application of visible light and photoactive compounds - photosensitizers [9].

A crucial step in PDT is the production of singlet oxygen, commonly written as 1O2, or other reactive oxygen species (ROS). The photosensitizer acts as a mediator between the incoming light and the potential ROS. Singlet oxygen seems to be accepted as the most important reactive oxygen species to produce photodynamic injury in biological systems [10, 11], but the other ROS are also capable of inducing cell death.

Administration of methyl-aminolevulinate (MAL) solution into the cell cultures, leads to the production of PpIX inside the tumor cells. PpIX is a member of a class of molecules called macrocycles, a family containing many porphyrins. PpIX's fluorescence spectra can be used to evaluate its intracellular localization or concentration. So the aim of this paper was the evaluation of MAL-PDT efficiency from the fluorescence spectra of MAL-PpIX.

Typical fluorescence spectra of cancerous cell with MAL-PpIX were obtained in vitro before and after irradiation with 635-nm laser light and an increase of the 620 nm and 700 nm peaks was noted for MAL-PpIX during irradiation. The fractionated light treatment seems to be more effective due to the "resting" time allowing the protoporphyrin IX regeneration. Thus, the original beam was divided in two parts then in three parts and all the results have been compared. The fluorescence spectra before and after each irradiation session were recorded and compared to the "witness" sample containing only the culture medium and the same MAL quantity as the cancer-cell samples.

2. Theoretical background

Photosensitizers are used to produce the PpIX in order to generate ROS responsible for the cancerous cell death. The decrease of drug concentration into the sample leads to an increased amount of PpIX. According to [12], the photosensitizer concentration decreases in time:

$$x(t) = \frac{x_0}{1+Qt} \tag{1}$$

where x_0 is the initial concentration (for $t = 0$) and Q is a constant depending on the relaxation and excitation parameters for photosensitizer and oxygen.

This decrease of the drug concentration in time appears either because of chemical disintegration or because of PpIX generation (ROS activation) so we may express the amount of drug that produces PpIX as:

$$x_{PpIX} = 1 - x_d(t) - x(t) \tag{2}$$

where $x_d(t)$ is the chemical disintegrated photosensitizer and can be written as a function of the disintegration velocity (v) as $x_d(t) = -vt$.

We can now express the concentration of MAL that produces PpIX as a function of time:

$$x_{PpIX} = \frac{Qvt^2 + (v+Q)t + 1 - x_0}{1+Qt} \tag{3}$$

This means that the amount of PpIX contained into the cancerous cell is also increasing in time by the same law. A laser beam irradiation excites the PpIX to an unstable state. The return to the ground state through a reaction with molecular oxygen generates the ROS responsible for killing the cancerous cells by "suffocation". This process can be observed in a fluorescence spectrum. The answer can be recorded if the target receives enough energy from the laser beam i.e. enough photon-PpIX interactions are produced. These interactions are independent to each other so they obey the Poisson statistic theory and the probability to observe n interactions is:

$$P(n) = \frac{(\xi D)^n}{n!} e^{-\xi D} \tag{4}$$

where ξ is the detector sensitivity and D is the laser radiation dose.

The intensity of the florescence peak is proportional both to the PpIX concentration and the radiation dose:

$$I \simeq \frac{(\xi D)^n}{n!} \cdot \frac{Qvt^2 + (v+Q)t + 1 - x_0}{1+Qt} \cdot e^{-\xi D} \tag{5}$$

The light intensity recorded by the spectrometer is proportional to the PpIX accumulation into the cells and, by consequence, to the destroyed cancerous cell number.

3. Materials and methods

3.1 Cell culture

An established standard line DOK (ECACC No.94122104) has been used. The cell line is a dysplastic oral keratinocyte prior established from a squamous-cell carcinoma and standardized by the European Collection. The cell line is adherent and upon cultivation can prove in confluent cultures stratification properties and contains a keratin profile similar to the original dysplasia. It has an epithelial morphology and has, in our system, a duplication period at 48 h.

The cells were cultured in standard culture medium DMEM enriched with 2 mM Glutamine, 5 μg/ml Hydrocortisone and supplemented with 10% Foetal Bovine Serum. The cells were seeded at $4\times10^4/cm^2$ and when reaching sub-confluent cultures (70-80%) they were detached with 0.25% trypsin/EDTA and further cultivated at the above mentioned concentration in 5% CO_2 at 37 ^oC.

3.2 Photosensitiser and light source

For photodynamic therapy tests, the solution of MAL (Sigma) (in a final concentration of 1 mM for 2 h in 5% CO_2 at 37 ^oC) has been used.
A portable laser system SCL (INOE 2000, Bucharest, Romania) for the irradiation of the cell cultures was used in this study. The equipment consisted of two principal modules: laser probe and control unit. The laser probe is consisted of five diode lasers emitting continuously at 635 nm, their drivers being fixed in a special device for an easily manevrability in different positions. The main characteristics of the diode laser are: power 3 mW, wavelength 635 ± 10 nm, display current 250 mA, work current 55 mA (at 5 mW), and 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.

3.3 Photodynamic therapy

24h prior the irradiation the cells were cultivated at a density of 3×10^4 cells/well in 24 wells culture plates. Cells were incubated with MAL in a final concentration of 1mM for 2h in 5 % CO_2 atmosphere at 37 ^oC. After 2 hours, the cells were first washed with culture medium for photosensitizer removal and then subjected afterwards to irradiation.

Four groups of cell cultures were considered subjected to irradiation schemes. The group A was exposed to a single fractionated irradiation scheme (SFI) (n = 10, 13.5 J/cm^2, t_{exp} = 900s). The group B was treated using double fractionated irradiation scheme (DFI) (n = 10, 4.5 J/cm^2 +9 J/cm^2 , t_{exp1} = 300 s, t_{exp2} = 600s, dark interval = 30 min); and group C was subjected to triple fractionated irradiation scheme. The last group D serving as witness (n = 10) was not treated with MAL and was not exposed to light irradiation.

4. Fluorescence spectrometry (or measurements)

The evaluation of efficiency of multiple fractionated irradiation schemes in MAL-PDT on human oral keratinocytes was done using the fluorescence spectrometry. For all groups the fluorescence spectra (with wavelength within the range 500 nm - 750 nm) were obtained with AvaSpec 2048-USB2 optic fiber spectrophotometer (Avantes, The Netherlands, Europe) before applying the photosensitizer, before and after each laser irradiation and 24 h after the treatment.

The portable AvaSpec-2048-USB2 spectrophotometer was equipped with an AvaLight-XE Xenon Pulsed Light Source (Spectral Output = 200 nm to 1000 nm; Total Optical Power output = 39 μJ per pulse (average 3.9 mW); Pulse Duration = 5 μs (at 1/3 height), Pulse delay = 6 μs; Pulse rate (max.) = 100 Hz), CCD detector array (2048 pixel) and a special reflection probe type FCR-UV200/600-2-IND with 12 excitation fibers of 200 μm around a 600 μm read fiber, which brings the fluorescence signal back to the spectrometer.

5. Results and discussions

According to the fluorescence spectrum presented in [13], PpIX has two maxima for the wavelength around 620 nm and 700 nm so we may use these maxima as markers for its presence into the cell culture.

The fluorescence spectra for culture medium, MAL and culture medium +MAL were recorded Fig.1 and the presence of these two maxima was observed in MAL containing samples confirming the generation of PpIX.

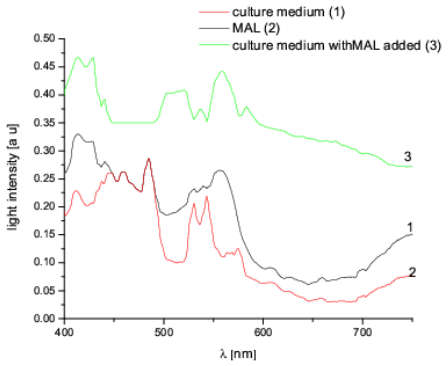

Fig.1 Fluorescence spectra for culture medium, MAL and MAL +culture medium.

Around the 550 nm wavelength, a maximum is observed in the medium and medium+ MAL spectra. This peaks can be used for the study of the medium degradation after the therapy.

When a single fractionated light radiation has been used (Fig. 2), a strong increase (about 40% of the original peak obtained before irradiation) is noticed for the 620 nm and 700 nm peaks so the destruction of the cells (proportional to the

Pp IX) is also significant. Comparing the "before irradiation" spectrum to the one recorded 24 hours after the treatment, a decrease of 24 % of the initial intensity is observed for the two considered MAL peaks.

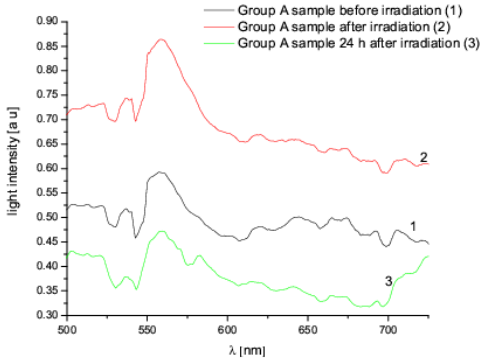

Fig.2: The fluorescence peaks obtained for the single fractionated light irradiation scheme.

Analyzing the 550 nm peak, a massive decrease (about 37 % of the original peak) is noticed suggesting that the culture medium is very much affected by the therapy.

In Fig.3, we present the fluorescence spectra of a cancer cells culture when a double light fractionate irradiation scheme was used with the same amount of laser power. We have noticed an average increase of MAL-Pp IX peaks intensity of 30% but the 24 hours after the irradiation process the total decrease of the PpIX containing cell is only 10%.

The medium peak (corresponding to the 550 nm wavelength) decreases only 25% now, suggesting a serious improvement of the therapy since the medium is less damaged.

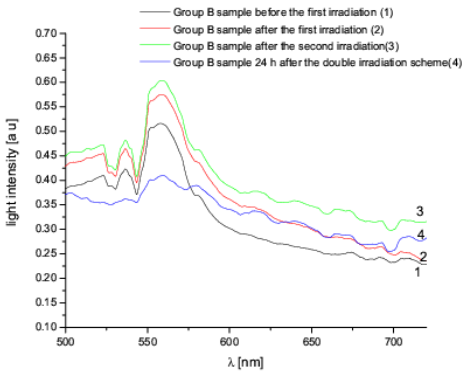

Fig.3: The fluorescence peaks obtained for the double fractionated light irradiation scheme.

When the triple light fractionated irradiation scheme was used (Fig. 4), the MAL-PpIX peaks are increasing with 8% so we may assume that the PpIX is produced at smaller rates. The decrease of 24 hours after irradiation of the 540 nm peak is also smaller (15% of the original peak) as compared to the other types of irradiation schemes and so are the PpIX peaks suggesting that the efficiency of the triple fractionated irradiation scheme is not as high as we expected.

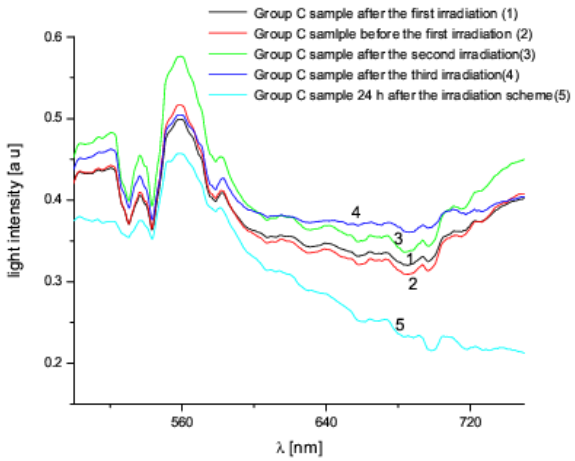

Fig. 4: The fluorescence spectra obtained when the triple light irradiation scheme is used.

Although the Pp IX quantity is not the only indicator of ROS generation and cancerous cell destruction, a low level of MAL-PpIX, indicates a lower cancerous cell death rate so the double light fractionated scheme is more efficient than the triple one. This suggests that the treatment efficiency strongly depends on the power of the radiation, and a small amount of electromagnetic energy cannot generate enough ROS to destroy all the tumor cells.

Conclusions

As we expected when using MAL as a photosensitiser we obtained a massive destruction of the cancerous cells proving the efficiency of the MAL based drugs.

Photodynamic therapy using multiple fractionated light sessions is obviously more efficient than the single fractionated light irradiation therapy, as it has already been shown by other studies [14], [7]. Increasing the number of irradiation sessions will not only allow the PpIX to be generated and activate the ROS but also reduce the impact of the laser on the healthy cells and the damages on the medium making the method less invasive.

The results of our research showed that there must be an equilibrium between the irradiation period, the resting time (dark periods between irradiation) and the laser power. As it can be noticed from the analyses of the fluorescence spectra, the double fractionated light irradiation scheme is more efficient than the triple one so increasing the irradiation session number is not always the most efficient method.

Acknowledgment

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

R E F E R E N C E S

[1] *E. R. De Haas, B. Kruijt, H. J. Sterenborg, H. A. Martino Neumann, D. J. Robinson,* Journal of Investigative Dermatology **vol. 126,** (2006), 2679.

[2] *N. Puizina-Ivić, H. Zorc, L. Vanjaka-Rogosić, L. Mirić, A. Persin*, Collegium Antropologicum, **vol. 32** Suppl 2, (2008), 67.

[3] *M. R. Mhissen, M. W. De Blois, D. J. Robinson, H. S. De Bruijn, R. P. Dutrieux, W. M. Staret et al*. Journal of Investigative Dermatology, **vol. 118**, (2002), 239.

[4] *D. J. Robinson, H. S. De Bruijn, W. M. Star, H. J. Sterenborg*, Photochemistry and Photobiology, **vol. 77**, (2003), 319.

[5] *W. M. Star, A. J. Van't Veen, D. J. Robinson, K. Munte, E. R. De Haas, H. J. C. M. Sterenborg,* Acta Dermato Venereologica, **vol. 86**, (2006), 412.

[6] *I. Seiichi, K. T. Schomacker, G. Wagnieres, M. Rajadhyaksha, M. Bamberg, T. Momma, T. Hasan*, Cancer Research, **vol. 59**, (1999), 6164.

[7] *E. R.. M. De Haas, H. S. De Bruijn, H. J. C. M. Sterenborg, H. A. M. Neumann, D. J. Robinson*, Acta Dermato Venereologica, **vol. 88**, (2008), 547.

[8] *H. S. De Bruijn, N. Van der Veen, D. J. Robinson, W. M. Star*, Cancer Research, **vol. 59**, (1999), 901.

[9] *M. Dellinger, G. Moreno, C. Salet, H. Tapiero, T. J. Lampidis*, International Journal of Radiation Biology, **vol. 62**, (1992), 735.

[10] *O. A. Gederaas*, Biological mechanisms involved in 5-aminovulinic acid based photodynamic therapy, PhD thesis, NTNU, The Norwegian Radium Hospital 2000.

[11] *K. Berg, P. K. Selbo, A. Weyergang, A. Dietze, L. Prasmickaite, A. Bonsted, B. Ø. Engesaeter, E. Angell-Petersen, T. Warloe, N. Frandsen, A. Høgset*, Journal of Microscopy, **vol. 218**, (2005), 133.

[12] *M. S. AlSalhi, M. Atif, A. A. Alobiadi, A. S. Aldwayyan*, Laser Physics Letters, **vol. 9**, (2012), 611.

[13] *Ingvild Kinn Ekroll* Ph. D thesis Light induced cell death in the cancer cell line AY-27: Modes of cell death after red-light hexylaminolevulinate photodynamic therapy and effects of fractionated light delivery, Norwegian University Science and Technology, 2009

[14] *I. Seiichi, K. T. Schomacker, G. Wagnieres, M. Rajadhyaksha, M. Bamberg, T. Momma, T. Hasan*, Cancer Research, **vol. 59**, (1999), 6164.