Journal of Photochemistry & Photobiology, B: Biology 160 (2016) 271–277

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Photodynamic therapy of HeLa cell cultures by using LED or laser sources

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ARTICLE INFO

Article history:
Received 3 September 2015
Received in revised form 6 April 2016
Accepted 11 April 2016
Available online 16 April 2016

Keywords:
Photodynamic therapy
HeLa cultures
LED vs LASER
m-THPC

ABSTRACT

The photodynamic therapy (PDT) on HeLa cell cultures was performed utilizing a 637 nm LED lamp with 1.06 W power and m-tetrahydroxyphenyl chlorin (m-THPC) as photosensitizer and compared to a laser source emitting at 654 nm with the same power. Intracellular placement of the photosensitizer and the effect of its concentration (C_P), its absorption time (T_A) and the illumination time (T_I) were evaluated. It was observed that for $C_P > 40$ μg/ml and $T_A > 24$ h, m-THPC had toxicity on cells in culture, even in the absence of illumination. For the other tested concentrations, the cells remained viable if not subjected to illumination doses. No effect on cells was observed for $C_P < 0.05$ μg/ml, $T_A = 48$ h and $T_I = 10$ min and they continued proliferating. For drug concentrations higher than 0.05 μg ml^{-1}, further deterioration is observed with increasing T_A and T_I. We evaluated the viability of the cells, before and after the treatment, and by supravital dyes, and phase contrast and fluorescence microscopies, evidence of different types of cell death was obtained. Tetrazolium dye assays after PDT during different times yielded similar results for the 637 nm LED lamp with an illuminance three times greater than that of the 654 nm laser source. Results demonstrate the feasibility of using a LED lamp as alternative to laser source. Here the main characteristic is not the light coherence but achieving a certain light fluence of the appropriate wavelength on cell cultures. We conclude that the efficacy was achieved satisfactorily and is essential for convenience, accessibility and safety.

1. Introduction

Photodynamic Therapy (PDT) centers on the photochemical interaction of three principal components: light, photosensitizer and oxygen [1]. This treatment modality uses light of an appropriate wavelength in the presence of oxygen to activate a photosensitizing drug, which then causes localized cell death or tissue necrosis. Singlet oxygen ($^1O_2$) is generally believed to be the major cytotoxic agent during PDT. In 1978, Thomas J. Dougherty conducted the first trials of PDT with Hematoporphyrin Derivative (HpD) in humans using 633 nm laser radiation. PDT has been approved by the US Food and Drug Administration for the treatment of microinvasive lung cancer, obstructing lung cancer and obstructing esophageal cancer, as well as premalignant affections such as actinic keratosis and macular degeneration associated with aging. Studies have shown efficiency in the treatment of head and neck cancer [2,3], lung cancer [4,5,6], mesothelioma [7], Barrett's esophagus [8,9], prostate [10,11,12] and brain [9,13,14,15] tumors. Unlike radiation therapy, PDT uses non-ionizing radiation, which can be administered repeatedly without generating cumulative effects because the target does not seem to be the DNA. The PDT involves a dynamic process, i.e., the distribution of light is determined by the source luminous characteristics and tissue optics, which, in turn, are influenced by the concentration of the photosensitizer and oxygen in the tumor. The distribution of oxygen is altered by the photodynamic process that consumes oxygen and, finally, the distribution of the photosensitizer can change as a result of photobleaching. All this, leads to the following difficulties in the practical application of PDT [16]: 1) the exact determination of the amount of photoactive drug in the tumor and its internal distribution; and 2) precise determination of the amount of light energy in the affected volume delivered from sources. In this respect, considerable progress has been made in understanding the basic of the processes and in developing devices to measure the relevant magnitudes to optimize the PDT treatment [17,18].

In general, any source that emits in the adsorption spectrum range of the photosensitizer, which is able to penetrate enough in tissues and with an appropriate light power can be used in PDT. However, there is in vitro evidence that flash wave (FW) xenon lamps, especially at low frequency of irradiation, has a photodynamic efficiency greater than continuous wave (CW) lights, a fact that could be explained by the greater generation of $^1O_2$ in the cells [19]. Although, the use of different types of light sources has been reported, a further quantitative

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http://dx.doi.org/10.1016/j.jphotobiol.2016.04.013
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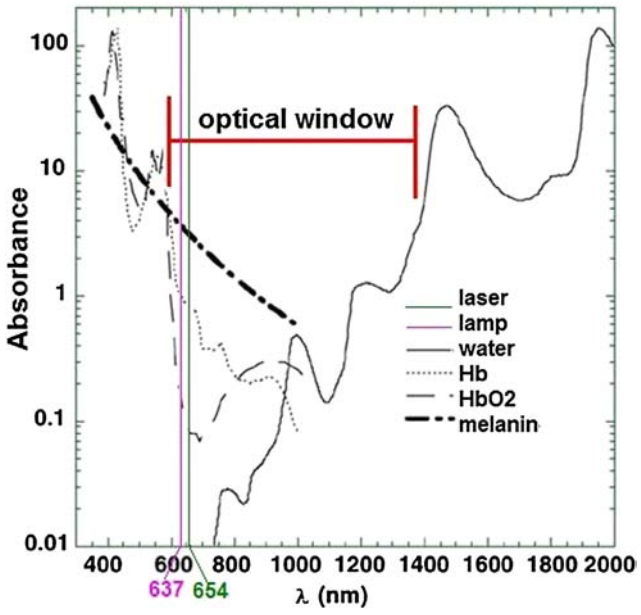

Fig. 1. Optic window and the emission wavelengths of the sources employed in the experiments. This figure was adapted from its original in reference [22] to show the spectral location of the laser and the LED emissions used in this work. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

comparison of their efficiency is needed [20]. Several experiments that take into account the fluence of light and the concentration of the photosensitizer have revealed the existence of a photodynamic threshold. This is defined as the minimum number of photons to be absorbed by the photosensitizer per unit volume of tissue to produce necrosis [21].

The differences between the incident irradiance and in situ fluence rate should be considered due to the contribution of scattered light. However, in practice the dose of light on the surface is specified using the fluence (J/cm^2) regardless of scattered photons. The light fluence is calculated from the output of the light source multiplied by the exposure time. Some protocols give the light dose prescribed as a measure of in situ fluence. Besides, fluorescence measurements provide additional information on the concentration and distribution of the photosensitizer, while the diameter of the incident beam affects the luminous flow rate at all depths. In the present study, we determined the photodynamic efficacy for HeLa cells cultivated in vitro with m-tetrahydroxyphenyl chlorin (m-THPC) using a LED lamp emitting at 637 nm with a power of 1.06 W. These results were compared with those obtained with a clinically used laser.

Table 1
Features and conditions for the use of m-THPC [23].

Properties	Photosensitizer: m-THPC
Absorption maximum (nm)	652
Absorption coefficient (cm^{-1} mol^{-1} L)	22,400
Drug Dose (mg/kg)	0.1–0.15
Absorption time (h)	96
Fluence (J/cm^2)	10–20
Fluence rate (mW/cm^2)	100

2. Materials and Methods

2.1. Light Sources

Human tissue is a highly absorbing and highly scattering medium in which biological chromophores define an optic window. The wavelength range necessary to activate the photosensitizing drug is typically between 600 nm and 800 nm (Fig. 1).

Two illumination sources were utilized in the experiments performed here:

- Laser of 654 nm ± 2.4 nm and varied emitting power up to 1.1 W (Section 4). The light emitted by the laser was coupled through an optical fiber whose free end has a microlens that makes the beam diverge illuminating in a circular area.
- LED lamp (Fig. 2) of 637 nm ± 18.1 nm and emitting power of 1.06 W.

The illuminance of the light sources at different distances was measured placing the lamp in an optical bench and employing an illuminance meter. The source emission spectrum was obtained utilizing an Ocean Optics HR2000 + ES.2 spectrophotometer.

2.2. Cell Culture

HeLa cells (50–60 passages) were grown in an atmosphere of 5% CO_2, at 37 °C and 97% of humidity in RPMI medium containing 10% fetal bovine serum (FBS) and sodium bicarbonate. HeLa cell cultures were prepared by seeding 50,000–100,000 cells into polystyrene Petri dishes 3.5 cm in diameter (Geiner bio-one). All procedures for maintenance of cells and preparation of the cultures were performed under sterile conditions using a laminar flow.

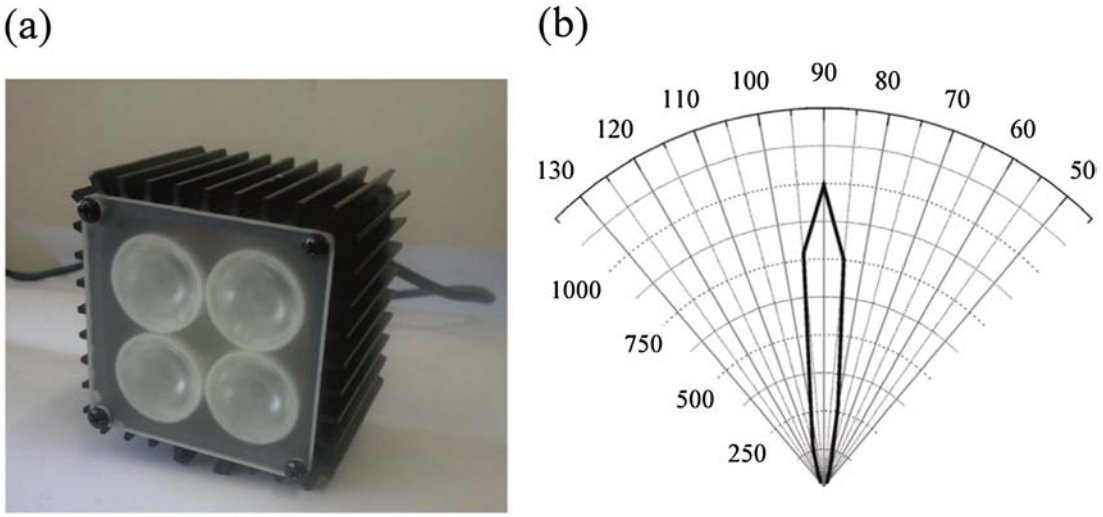

Fig. 2. LED lamp with its characteristic photometric curve.

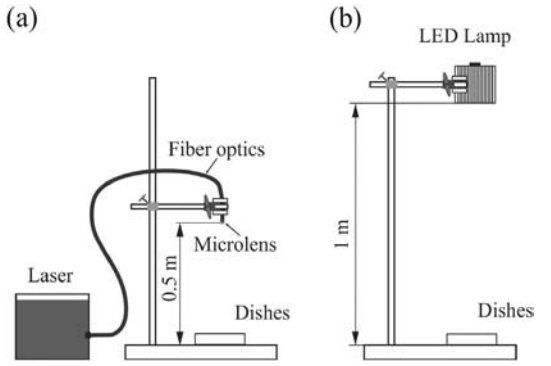

Fig. 3. Schemes of the experimental arrangements for the sources employed. A) laser at 0.5 m from the target; B) lamp located at a variable distance.

2.3. Chemicals

The photosensitizer m-THCP used in the experiments, is a reduced porfirin [23] that has a strong absorption peak in the spectral red zone as indicated in Fig. 1 and Table 1. After seeding, the cultures were incubated with drug in RPMI maintenance medium containing 2% FBS to maintain cells in a living but relatively low metabolic state. The m-THCP concentration (C_P) in the different experiments was varied in the range 0.05 μg/ml ≤ C_P ≤ 80 μg/ml.

Trypan blue staining was used to distinguish viable cells after PDT treatment and for aiding cellular counting. Also, propidium iodide and Hoechst stains were employed to distinguish apoptosis from necrosis by using an Olympus BX51 fluorescence microscope with WIBA filters (excitation: 460 nm–495 nm, emission: 510 nm) and DAPI filters (excitation: 330 nm–385 nm, emission: 420 nm). Cell viability was also evaluated employing thiazolyl blue tetrazolium bromide (MTT) from Life, and dimethyl sulfoxide (DMSO) for solubilizing formazan products was purchased from Sigma-Aldrich.

2.4. Photodynamic Treatment

After culture growth during 24 h, it was incubated in RPMI medium containing m-THPC during a dark interval (T_A) and finally the cells were exposed, by a laser or a non-laser source, a certain time for illumination (T_I). The experimental arrangement is shown in Fig. 3. Then it was decided if to apply some dyes to distinguish cell death or let them proliferate following the evolution of the system.

Cellular counting consisted of taking a representative field of 6 images per dishes. We expressed cell death as the percentage of the number of dead cells against total cells per dish. MTT experiments were performed 12–24 h after PDT treatment. The culture medium was replaced by a new one containing MTT solution (0.5 mg/ml in RPMI growth medium). Cells were incubated in the presence of the MTT solution for 3 h at 37 °C. Then, the culture medium was completely removed, and the formazan products were solubilized by adding 1 ml of DMSO to each capsule. The absorbance spectra were measured at 570 nm by a plate reader (Genios Pro) and the results were expressed as % of nonviability referred to a culture without illumination and without photosensitizer. Measurements were repeated three times, and the mean value and its standard error were reported for each condition.

Table 2
Characteristics of the light emitting sources. The emission wave length (λ), the Full Width at Half Maximum (FWHM) and the power of the sources are included.

Source	λ (nm)	FWHM	Power (W)
Lamp	637	18.1 ± 0.2	1.06
Laser	654	2.82 ± 0.03	0.6
Laser	654	2.38 ± 0.03	1.1

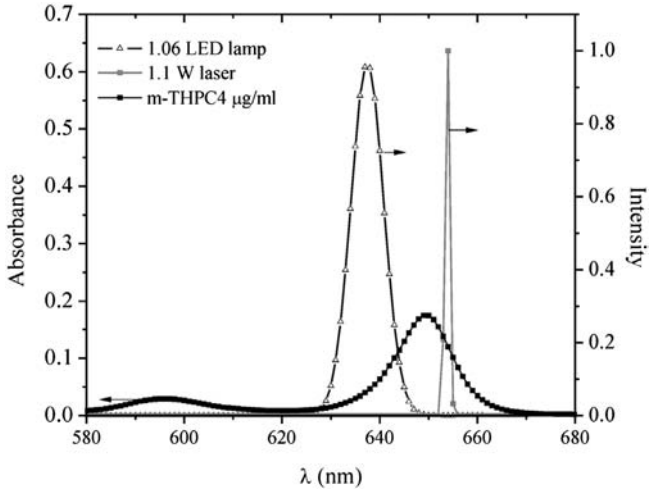

Fig. 4. Comparison between laser and LED lamp with the absorption spectrum of 4 μg/ml m-THPC methanol solution. The intensity scale is arbitrary. The wavelength of the laser at its maximum is closer to the photosensitizer absorption peak.

3. Calculation: Photometry and Radiometry

The luminous flux F emitted by a lamp can be obtained by using an integrating sphere [24], developed by Ulbricht (1920). As the emissions of the LED lamp and the laser used in these experiments are in the visible spectral region, the Ulbricht method is appropriate to measure their luminous fluxes.

Through the spectral sensitivity curve of an universal observer ($V(\lambda)$), the radiant flux or the power spectral density ($\phi(\lambda)$) can be expressed in terms of luminous flux F [25]:

$$F = K \int_{visible} V(\lambda)\phi(\lambda)d\lambda \quad (1)$$

In Eq. (1) K is known as the photopic luminous efficiency constant for photopic vision, whose value is 683 lm/W.

4. Results and Discussions

4.1. Photometric and Radiometric Analysis: Energy Efficiency of the Sources

From the average data obtained from the measurements at the exit aperture of the integrating sphere, the total luminous flux was obtained. Given Eq. (1) and the spectral curve of the Comission Internationale de L'Eclairage (CIE, Publication 18.2, 1983), a computer program was developed to calculate the power of the sources. For the LED lamp the reading in the calibrated instrument at the exit aperture was 47.95; knowing the conversion constant (2.72 lm/reading), the luminous flux of the lamp F = 130.42 lm and the power of 1.06 W were obtained. On the other hand, the values of F obtained for the laser were 53.6 and

Table 3
Fluences of the LED with 1.06 W located at 1 m and 0.5 m from the culture dishes.

Exposures t (s)	Fluence (J/cm^2)		E × t (lm·s/cm^2)	
	0.5 m	1 m	0.5 m	1 m
30	0.11	0.017	13.1	2.12
180	0.64	0.105	79.2	12.77
360	1.29	0.207	158.4	25.55
720	2.58	0.416	316.8	51.09

Table 4
Fluences of the laser source with 0.62 W and 1.1 W located at 0.5 m from the culture dishes.

Power (W)	t (s)	Fluence (J/cm^2)	E × t (lm·s/cm^2)
0.62	30	0.02	1.8
	90	0.06	5.4
	180	0.12	10.8
	360	0.23	21.6
1.1	30	0.03	2.58
	90	0.10	7.74
	180	0.21	15.48
	360	0.41	30.96

83.11 lm, in agreement with the power reported by the manufacturer (0.62 W and 1.1 W, respectively). The nominal wavelength, and the spectral width of the LED lamp emission and those of the laser at two different emission powers were measured. Data are depicted in Table 2.

The Full Width at Half Maximum (FWHM) was a good parameter to examine the overlapping between source emission and the photosensitizer absorption spectrum (Fig. 4). See the next section for more detail.

In the PDT experiments the lamp with 1.06 W of power was located at different distances from the cell culture to attain different illuminances (E) assessed by an illuminancemeter. For instance, at 1 m the LED lamp delivered 709 lux, whereas the laser with a power of 0.62 W and 1.1 W located at 0.5 m from the detector delivered 557 and 860 lux, respectively. The fluences and the amount of light per cm^2 (E x t) that reached cell cultures are depicted in Table 3 and Table 4. Fluences were calculated from illuminances values at different distances from the source employing Eq. (1); they follow the square inverse law of the distance. Furthermore, the illuminances obtained from the lamp were significant larger than those obtained from the laser source at the same distance. But this is only one aspect of photodynamic therapy, the other is the range of wavelength where the m-THCP absorbs light and the source emission wavelength (Figs. 4 and 10).

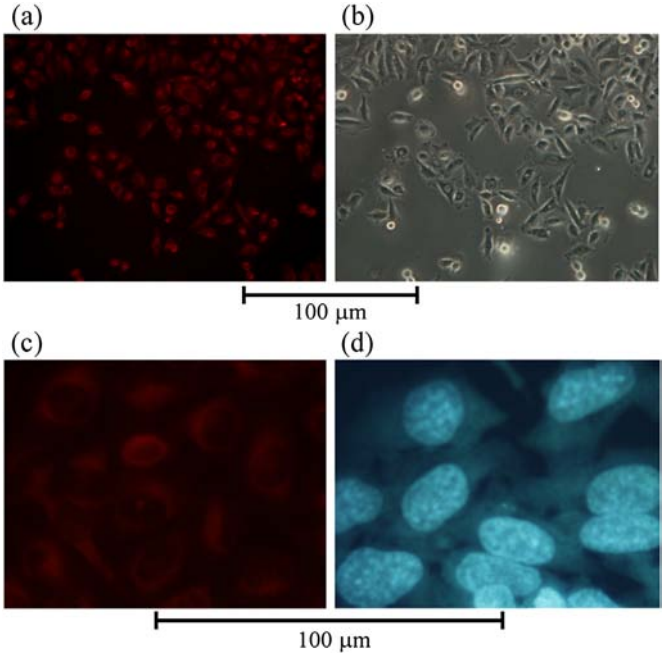

Fig. 5. HeLa cell images containing m-THPC. (a) Fluorescence microscopy; (b) phase contrast microscopy; (c) higher magnification of (a) and Hoescht nuclei fluorescence (d). The photosensitizer is located surrounding the nucleus. These sites correspond to the endoplasmatic reticulum and the Golgi apparatus.

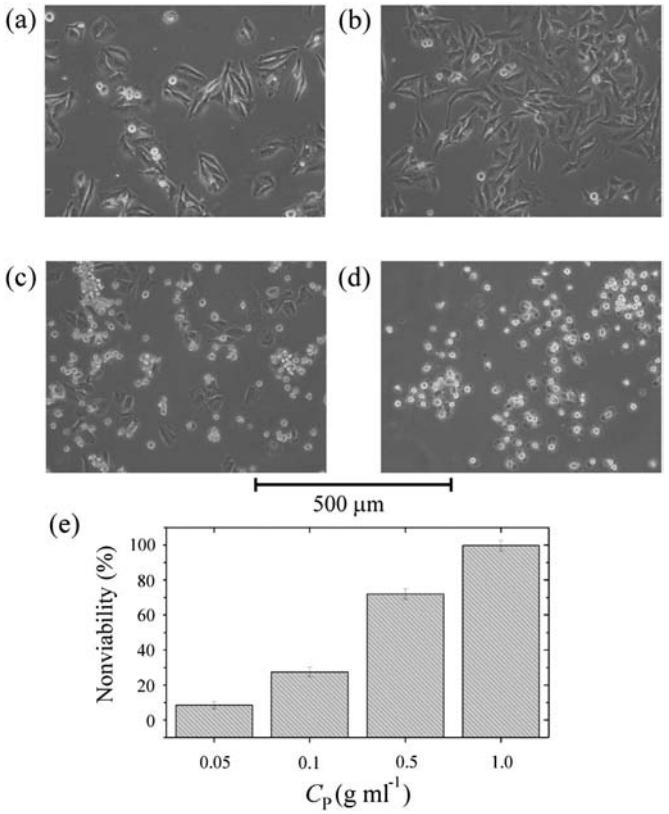

Fig. 6. HeLa cell culture microimages after PDT employing the LED lamp at 1 m. (a) C_P = 0 μg/ml; (b) C_P = 0.05 μg/ml; (c) C_P = 0.5 μg/ml and (d) C_P = 1 μg/ml. Cells that are still alive present a large spreading area, whereas dead cells are rounded in shape and rather rough. (e) MTT viability test was performed for each treatment condition. Data are referred to a control culture without m-THPC and illumination. The standard error is included.

4.2. Photodynamic Therapy

4.2.1. Photosensitizer Location in Cells

HeLa cultures were incubated in the dark for 90 min in maintenance medium containing 20 μg/ml of m-THPC. The culture was fixed employing a solution of one part of Glacial acetic and one part of ethanol. Hoechst stain was also used as to better identify the position of the nucleus (Fig. 5). Thus, the fluorescence of m-THPC was observed in all cells surrounding their nuclei in accordance of previous observations [17,22,26].

4.2.2. Range of Toxicity of m-THPC on Cell Cultures

To asses this issue, 50,000 cells/ml were cultivated, varying the photosensitizer concentration in the range 10 μg/ml < C_P < 80 μg/ml for a constant T_A = 3 h 15 min. When cells were illuminated with light of 637 nm, cellular injuries appeared during the PDT treatment. For C_P > 40 μg/ml and T_A > 24 h, the drug provided natural toxicity to cells in culture, even in the absence of illumination.

In addition, cells were incubated for 24 h alternatively in maintenance medium containing 1, 0.5 and 0.05 μg/ml m-THCP, and subsequently illuminated with the lamp during T_I = 20 min. Then, microscopic images of the cultures (Fig. 6a–d) were taken 18 h post illumination. Additionally, the MTT viability test was performed for each treatment condition to quantify the photodynamic effect (Fig. 6e). It was noted that for higher C_P the proportion of dead cells increased. For the lowest C_P cell culture went on growing similarly to the control.

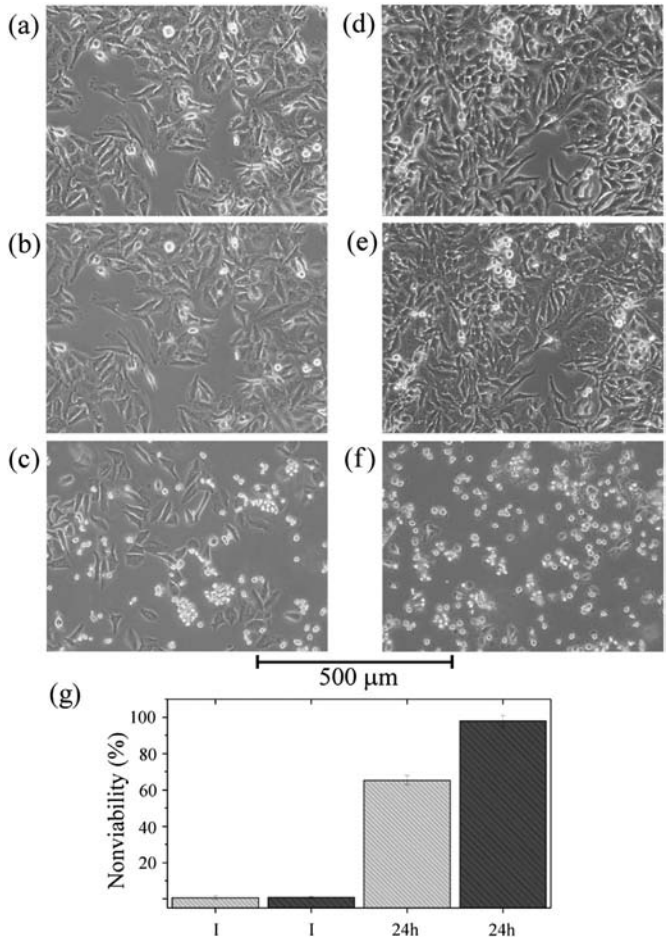

Fig. 7. Images of HeLa cell cultures after PDT for $T_A = 24$ h (a–c) and $T_A = 48$ h (d–f); (a and d) before illuminate, (b and e) immediately after illumination, and (c and f) 24 h after illumination. The LED lamp was located at 1 m from the dishes. (g) MTT viability test was performed for each condition mentioned above: for $T_A = 24$ h (light gray) and $T_A = 48$ h (dark gray). The test was performed immediately after illumination (I) and after 24 h post-illumination (24 h). The standard error is included.

4.2.3. Effect of the Photosensitizer Absorption Time on PDT

HeLa cells were incubated with 0.1 μg/ml of m-THPC in the dark for $T_A = 24$ h (Fig. 7a–c) and $T_A = 48$ h (Fig. 7d–f). Both cultures were illuminated with 637 nm LED light for $T_I = 20$ min at 1 m light source-cell dish distance employing the experimental arrangement shown in Fig. 3.

For the same photosensitizer concentration and the same time of illumination, the larger T_A, the lower the proportion of living cells. Furthermore, HeLa cells incubated with 0.5 μg/ml of m-THPC for $T_A = 24$ h and $T_A = 48$ h were illuminated with LED lamp for 20 min, and imaged after 6 h of the PDT treatment. The same trends described above for $C_P = 0.1$ μg/ml were observed.

4.2.4. Variation in Time of Illumination with LED Lamp on Cell Cultures

The cell cultures were incubated for 24 h with 0.25 μg/ml of m-THPC, and the PDT treatment was performed. Different T_Is were employed and the effect of the PDT treatment was evaluated 12 h post-treatment (Fig. 8a–e) by microimages and the MTT assay (Fig. 8f).

The larger T_I, the greater the proportion of cells detached from the culture dish bottom, i.e., the smaller the cell viability. Cellular counting was performed by the observation of the respective monolayer. Through the dyes used in the experiments, a greater proportion of dead cells by necrosis was observed.

In order to compare the PDT efficiency of the LED lamp and the medical laser, experiments were carried out by changing T_I and light

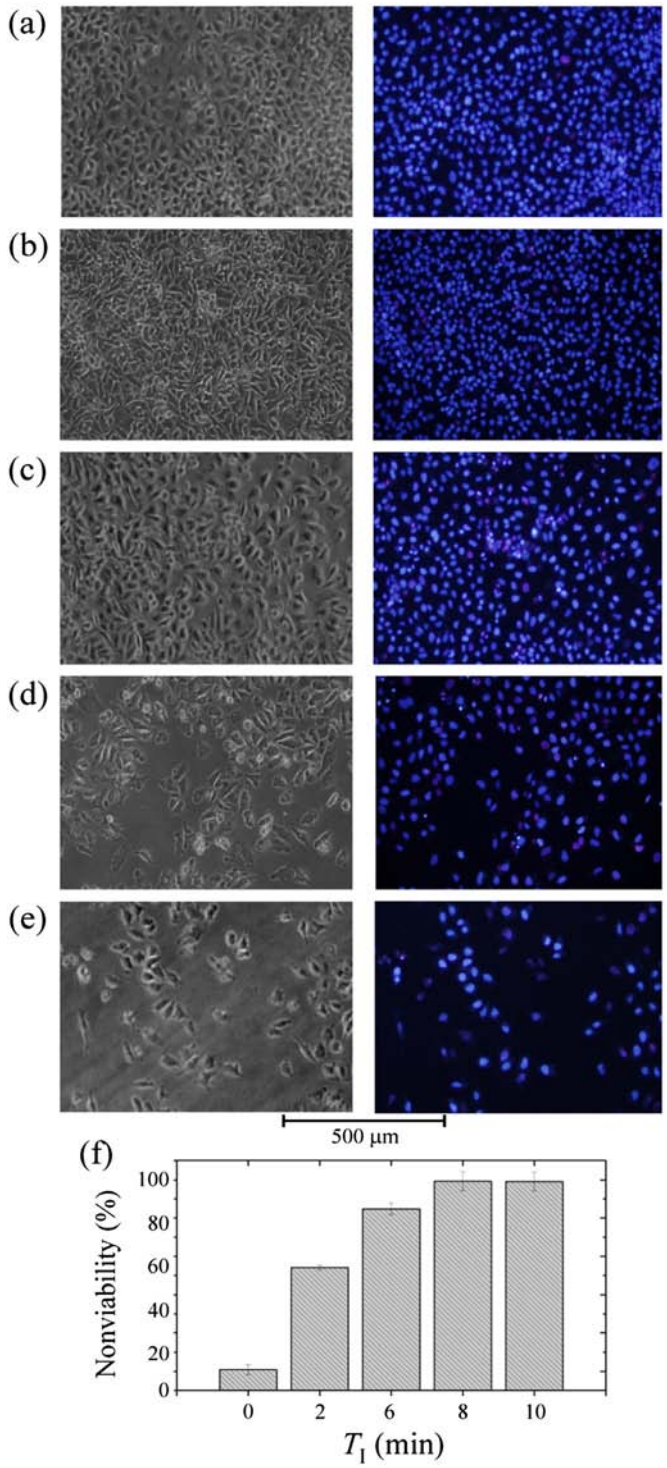

Fig. 8. Phase contrast images (left) and staining with propidium iodide and Hoechst satin (right) of HeLa cell cultures after PDT with the LED lamp at 1 m. (a) $T_I = 0$ min; (b) $T_I = 2$ min; (c) $T_I = 6$ min; (d) $T_I = 8$ min and (e) $T_I = 12$ min. (f) MTT viability test was performed for each condition mentioned above. The standard error is included.

fluences, and evaluating cell viability by the MTT assay. Formazan was measured 24 h after culture illumination employing the LED lamp or the laser source during different T_Is and distances from the culture dishes (Fig. 9).

Both illumination sources at 0.50 m exhibited similar PDT efficiency. The cell viability after $T_I = 0.5$ min was almost null. On the other hand, the LED lamp at 1 m showed the same result for $T_I = 4$ min (inset Fig. 9).

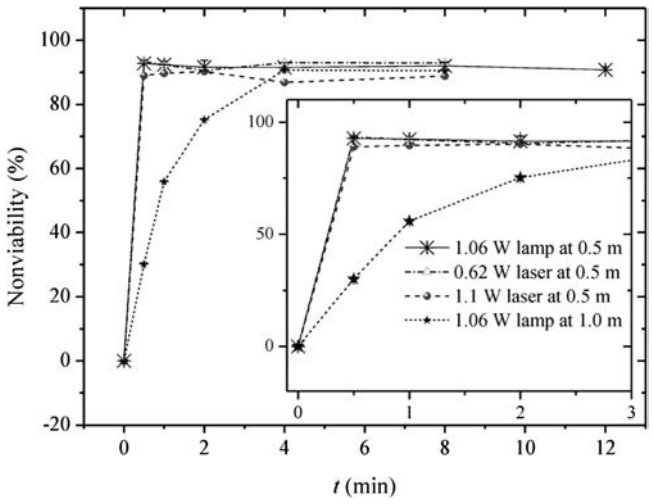

Fig. 9. Results from MTT assay of HeLa cell cultures with $C_P = 0.25$ μg/ml and varying T_I. Cell cultures were illuminated with the LED lamp of 1.06 W at either 0.5 m or 1 m of distance and with the laser (1.1 W or 0.62 W) at 0.5 m of distance, as indicated in the figure. For each T_I at least three experiments were averaged and the standard error is included.

The above results were in agreement with photometric measurements of the LED lamp. The illuminance showed a reciprocal law with the square of the distance to the detector (Fig. 10). At 50 cm from the detector it resulted in a maximum value circa three times the value of the laser source, although the nominal wavelength of the former was smaller ($\lambda = 637$ nm) than that of the photosensitizer absorption peak ($\lambda = 650$ nm). The larger illuminance of the light emitted by the LED lamp at 50 cm from the detector increased the overlapping of the emission with the photosensitizer absorption peak and consequently the number of photons with appropriate λ to generate reactive species. This fact compensates the larger match between the relatively narrow laser emission and the photosensitizer absorption peaks. Furthermore, similar spectra for the LED light passing through 2 μg/ml and 4 μg/ml m-THPC methanol solutions were obtained. The intensity of the transmitted light for the greater concentration solution of m-THPC, resulted smaller according to the Lambert–Beer law for light absorption. These results explained the similar photodynamic effect obtained for both illuminating sources (Fig. 9).

Thus, LED lamp could replace or even, be more convenient for illuminating large areas, at least with the present lamp design, i.e., without the use of any concentration lens system. Thus, provided that the photosensitizer and oxygen concentration are in excess, the light absorption appears to be the main parameter for PDT efficiency at least for in vitro experiments. Illuminating devices based on light emitting diodes have been characterized and proposed for low cost supplements to conventional light sources for PDT [27,28,29]. For instance, the high PDT efficiency towards A431 squamous carcinoma cell line using a device based on LED sources has been demonstrated [30], and even more, battery-powered LED has been characterized to be used in proven PDT protocols [31].

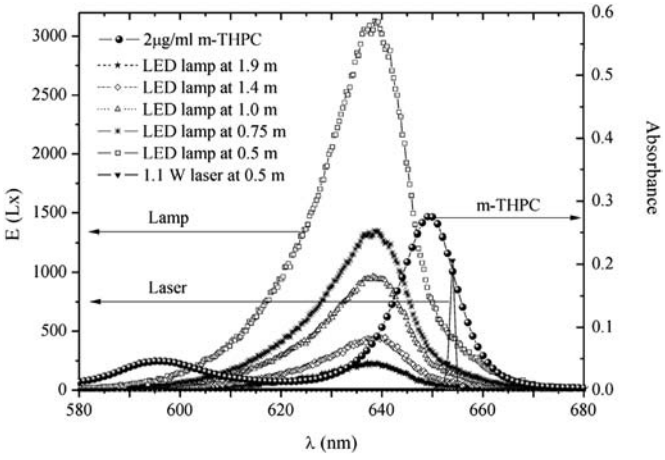

Fig. 10. Luminous flux measurements for the LED and laser sources. The different distances from the LED lamp to the detector are indicated in the figure. The photosensitizer absorption spectrum from 580 to 680 nm is also included.

5. Conclusions

A convenient, low cost and portable LED lamp prototype has been tested to be applied in cells cultivated in vitro for photodynamic studies. The LED lamp was capable of delivering over 1 W of wider bandwidth light than the laser. The overlapping area between the peaks of the sources and the absorption band of m-THPC is related with the efficiency of the photoactivation and therefore with the percentage of dead cells. Effective ranges of m-THPC concentrations were determined and a threshold above which existed natural toxicity ($C_P > 40$ μg/ml and $T_A > 24$ h) was identified. In addition, for $C_P = 0.05$ μg/ml and $T_A = 3$ h 15 min the cells showed morphological growth without apparent damage. Our finding showed that the cell death percentage increased with T_I, while C_P and T_A were constants.

Although the emission peak of the LED source was displaced from the absorption peak of the m-THPC, the photodynamic efficiency was similar to that of the laser source, increasing the illuminance of the lamp when it was placed closer to the sample.

The use of photometric units is associated to the necessity of describing the total photons that arrive at the illuminated area (perception) according to the luminous characteristic of the sources.

Finally, comparably with the laser, the LED device can photoactivate the photosensitizer and be effecive in trials of HeLa cell cultures safely and in a simple to use and maintain way.

Acknowledgments

The authors would like to thank Eng. Anibal Laquidara, Dr. Fausto Bredice, and Eng. Pablo Ixtaina for their electronic, espectrometric and photometric-radiometric contributions, respectively. This work received the financial support from the Universidad Nacional de La Plata grants through Facultad de Ciencias Médicas (11-M/170) and Facultad de Ingeniería (I 170), and the Fundación Innovatec, Arentina.

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