Is violet LED light-based photodynamic therapy efficient to treat superficial lesions such as a low-grade cervical intraepithelial neoplasia?

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Abstract. Photosensitizers utilized in photodynamic therapy (PDT) can be exited with light of any wavelengths matching their absorption bands. Thus, superficial lesions, such as those involved in cervical intraepithelial neoplasias (CINs) might be treated with a light of shorter wavelength than red despite the smaller penetration depth in tissue. We review basic aspects of PDT and some investigations related to the use of light-emitting diodes (LEDs) of different wavelength mainly applied to superficial lesions, and highlight the benefits of the use of violet light and cellular spheroids as a PDT dosage model. Our previous results from spheroids of cervix carcinoma cells, suggest that the violet light from a LED source is appropriate for performing PDT of neoplastic lesions involving about 300 μm in depth. In this work we present a high fluence light source emitting at 420 nm and apply it to perform PDT treatment of a HeLa cell tumor model implanted in BALB/c nude mice. The photodynamic reaction is inferred from the fluorescence evolution measured at the tumor and the temperature evolution of the treated surface. Violet LED light could be a promising alternative for treating CINs involving superficial dysplasia, with reduced side effects.

1. Introduction

1.1. *A brief description of photodynamic therapy (PDT) and diagnosis*

In PDT, a photoactive drug (photosensitizer, PS) is administered and accumulated in the affected tissue. In the presence of oxygen, light of appropriate wavelength activate the PS molecules generating highly reactive species that destroy cells with PS concentration above a critical value [1, 2]. The process depends on several interrelated factors, namely the nature of the PS, the characteristics of the illuminated tissue and the fluence rate of deposited light, among others. Each one of the three elements involved in PDT, i.e. PS, oxygen and light, is nontoxic by itself. Most of the approved PSs employed in PDT exhibit natural toxicity at high concentrations in cultures, but these concentrations are not reached in any organ or tissue during clinical procedures. Moreover, the need to combine these three elements to perform PDT may result in a highly selective method for treating malignant affections. A scheme of the basics of PDT is depicted in Fig. 1.

Different mechanisms contribute to the tumor destruction: (a) direct damage of tumor cells; (b) tumor irrigation vasculature injured; (c) the activation of the immune system partially due to the inflammatory response at the tumor microenvironment, a process that may assist the development of systemic immunity. The conditions determined by the PDT application protocols, i.e. the photosensitizer nature and concentration, the amount, rate and wavelength of light deposited on the affected region, and the availability of oxygen to produce reactive species, determine the contribution of each mechanism [1].

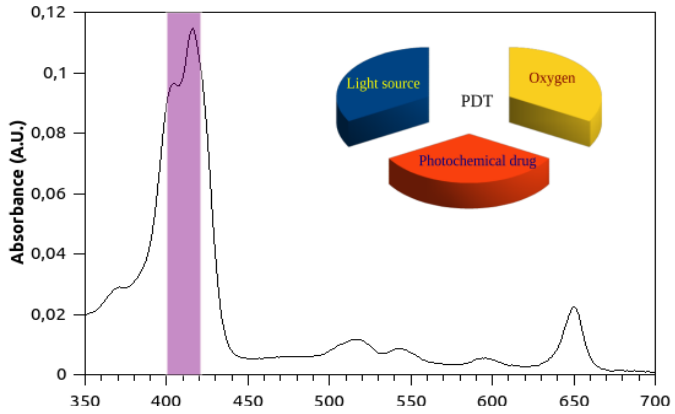

Figure 1. The three principal components in PDT: oxygen, light (represented by the violet band overlapping the photosensitizer absorption spectrum) and the photosensitizer. Absorption spectrum of 2 mg/ml of photosensitizer meta-tetrahydroxyphenyl chlorin (m-THPC) diluted in methanol.

The advance in PDT and photodynamic diagnosis has demanded the development of appropriate illumination sources [3-6]. Research works have shown a rather large evidence of the PDT successfully treatment of neoplastic diseases using LED light wavelengths different from red, either for *in vitro* and *in vivo* studies [7-12]. In this way, PDT treatment with blue light has been demonstrated to be more effective than with red light in the range 630 – 660 nm, and employing aminolevulinic acid or its derivatives [6, 8], photofrin [10], or even natural photosensitizers [9]. This fact is explained because the Soret absorption band is 10-30-fold larger than that in the red region, for the photosensitizer used by these authors, particularly when dissolved in phosphate buffer. In the case of amelanotic melanomas, it has been reported that a double PDT application, first with light of 420 nm and then with red light, improved the efficiency of the treatment [13]. Other examples are related to dermatologic diseases such as actinic keratosis, Bowen´s disease, and basal cell carcinoma, in which PDT with aminolevulinic acid has been shown to be significantly more efficient when performed with violet light in comparison with red light [14]. Another application of PDT with violet light is the illumination with 405-nm LEDs for the daily disinfection of clinical spaces and also it has been proposed as potential treatments for contaminated wounds [15].

Optical diagnosis is based on the changes in the optical properties of tissues due to a disorder caused by a disease or a pathologic state. PDT procedures can easily be coupled with diagnosis strategies that employ light to exit either endogenous or exogenous chromophores. Nowadays, diagnosis in general relies upon imaging employing endoscopies, computed tomography, magnetic resonance, biochemical techniques employing specific markers, and conventional biopsy followed by histo-pathological confirmation. Although, the latter is considered as the “Golden standard” to confirm a neoplastic disease, it is stressful and may involve additional complications. Thus, optical diagnosis should be considered, either alone or combined with a disease treatment technique. Recently, optical diagnosis has been reviewed concerning *in vivo* medical applications [16]. The use of optical fiber with improved design allows the technique to become localized and with very low risks. In our group, it has been shown that fluorescence spectra from human papilloma virus 6 positive (HPV6+) lesions at the hard palate and genitals exhibit similar characteristics well distinguished from those from adjacent normal zones [17].

On the other hand, enhanced emission from pathologic tissue areas is obtained by the administration of an exogenous chromophore, as is the case of photodynamic diagnosis (PDD). The PS employed for PDT, such as aminolevulinic acid (ALA) or their derivatives, accumulates preferentially in the affected tissue and acts

as a precursor of protoporphyrin IX (PpIX) and it can be used for performing either PDT or PDD, according to the PS concentration and illumination protocol. In general, a short illumination is convenient for fluorescence spectroscopy or enhanced imaging, and a relatively long illumination is convenient for PDT. A review on PDT and PDD for gynecological diseases has been recently reported [18]. PDT with red light from different laser sources has been applied for cervical diseases, and UV-violet light for ovarian, fallopian tube and primary peritoneal cancers. In these three cases, PDD has assisted to further detect malignant regions and to obtain clearer definitions of the pathologic tissue margins than without an administered PS [18]. Furthermore, the fluorescence of ALA or hexaminolevulinae has proved to be efficient to detect neoplastic areas upon illumination of the bladder surface with light in the blue-violet region [19]. PDD detects more tumors than the standard observation with white light, a fact that can help to diminish recurrence. Although sensitivity, particularly for more aggressive tumors, is larger than standard methods, specificity is inferior [19]. PDD is expected to be a valuable diagnostic tool for early detection and better staging of cancers [20].

1.2. *Human papillomavirus (HPV) infection and PDT as a treating tool*

In this subsection, we shall briefly comment on HPV positive infections lesions, as this is the main concern of the present work. HPV infection is the main cause of cervical cancer. It is also a risk factor of other cancers not only in genital areas [21]. Biomarkers correlated with the stage of HPV-diseases, being the most significant, the DNA testing [22, 23, 24]. Cervical intraepithelial neoplasias (CINs) are superficial lesions classified according to the degree of the progression and the extent of the neoplasia along the epithelium, from basal cells. The classification ranges from CIN I to CIN III or carcinoma in situ [25].

The traditional therapeutic methods for the treatment of CIN are invasive, like the excision of the pathological tissue. The most common adverse effects are hemorrhage, trauma to underlying tissue, local stenosis, change in the anatomical structure of the cervix, reduction in cervical secretion and increase in the level of perinatal mortality. With the idea of maintaining the functional integrity of the target organ, the PDT appears to be less damaging to normal tissue than surgery, radiation or chemotherapy, and it preserves the fertility [26]. It has been reported that 90% of CIN 2/3 patients have been cured after one and two years of follow-up, placing a 630 nm LED light for a uniform illumination of the entire cervix, with a total dose of 100 J/cm^2 and 1 h of methyl aminolevulinate (MAL) application [27]. Sometimes, in addition to HPV infection, other conditions like bacterial vaginosis coexist. In this case, it has been reported the elimination in 83% of the patients at 3 months post-PDT, administered 4 h of 6 % 5-ALA gel applied with a dose of 200 J/cm^2 [28]. Because the efficiency of PDT therapy differs with photosensitizers and exposure time, future evaluations are needed for it to become a common clinical practice [29].

1.3. *In vitro model of several layers and PDT with violet light*

Three-dimensional cell aggregate spheroids with different preparation procedures have been useful as models to improve PDT dosage as well as for bringing new insight on PDT basics [30-35]. Cell spheroids represent realist models as they mimic some aspect of the tumor microstructure and optical properties. They exhibit a central core of necrotic cells that depend on growth time, and model the decrease of oxygen in going from the external layers to the core. Furthermore, pH, nutrient and PS concentration gradients are established. Spheroids resulted to be more resistant to PDT than cell monolayers, and the impairing of the photodynamic reaction increases with the spheroid size. It has been observed that for spheroids about 500 μm in diameter, the response to PDT is significantly affected by the fluence rate of the light deposited during PDT sessions [36]. This fact has been explained by the relatively reduced oxygen concentration at inner regions of the spheroids, a concept that has been later supported by preclinical trials of solid tumors [36, 37].

From the results of our group, the comparison of PDT treatment efficiency using red and violet light with HeLa cell spheroids allowed us to indicate that violet light is more efficient, at least for a spheroid of about 300 μm in diameter [38] and with m-THPC as the PS. The m-THPC bears a high quantum yield for singlet oxygen production and an absorption band twelve times larger than that at 650 nm (Figure 1). A smaller light penetration for the violet light than for the red one has been reported [39], but it is compensated by the larger PS absorption in the violet region, and with the advantage of increasing selectivity. For early stages of uterine cervical carcinoma, the light should come through a certain number of layers associated with the thickness of the cervix, i.e. a distance around 250 μm [40]. This dimension is similar to that of HeLa spheroids [38], thus the latter would be an appropriate experimental model. In this case, the photosensitizer mainly accumulates in the outer layers but the concentration inside the spheroid, measured by fluorescence

microscopy, exhibits considerable signal [38]. The observed distribution of m-THPC at the spheroid was in agreement with observations reported by other authors [41]. Results from HeLa cell spheroids treated with 395 nm light, indicated that the PDT effect, quantified by fluorescence images of propidium iodide-stained cells, reached the basal layers of the spheroids. Furthermore, an augmented fraction of no-viable cells was observed in comparison with red light-based PDT. This fact is due to the larger absorption coefficient of m-THCP, particularly at planes near the apex where the attenuation is smaller [38]. Findings suggested that PDT applied to spheroids give useful data to evaluate the dosage of PDT in relation, mainly to the spatial distribution of photosensitizer concentration and light fluence rate, at different sections of the spheroid.

In the following section we briefly describe some new results from our group in relation to the use of violet light in PDT.

2. Experimental

2.1. *Construction and characterization of a LED source for PDT and PDD*

A LED source emitting at 420 nm was constructed employing four 3 W LEDs, coupled to individual lens and dissipators. They were placed on a metallic platform that allows the adjustment of the illumination direction of each lens-LED system (Figure 2a). The metallic platform is provided with channels in the radial direction and independent springs that allow the free movement of the lens-LEDs systems in order to focus on a convenient point by adjusting the lens holders at the upper part of the lamp (Figure 2a). Each LED is fed by an independent electrical circuit with variable electric power to modulate the light intensity.

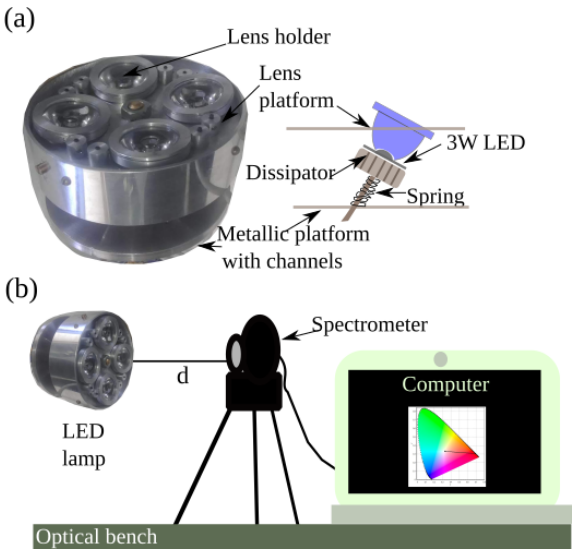

Figure 2. (a) 12W LED source of 420 nm utilized in *in vivo* and *in vitro* assays. It is possible to converge light beams to a region of interest. A scheme of a single lens-LED system is included in the figure; (b) experimental arrangement to carry out the photometric/ radiometric characterization of the lamp. The spectrometer is positioned at a distance d from the sources on an optical bench.

The characterization of the illumination source was performed with a spectrometer AvaSpec-ULS3648-USB2-UA-25 (Figure 2b). The illuminance, the irradiance and the angular output of the intensity of the light beam, and the wavelength spectrum were measured.

2.2. Application and follow-up of PDT on the tumor model

Immunocompromised BALB/c nude mice were inoculated with HeLa cells (from human cervix cancer), subcutaneously into the flank. The tumor growth was monitored with calipers. Tumors with average diameter in the range 0.4 - 0.6 cm were obtained after two weeks approximately. Five mice were used for the experiments, after selections of tumor with similar size.

Before the PDT treatment, the transmission of the violet light through the skin and the ear of the mouse was determined, the experimental procedure is outlined in Figure 3a.The photosensitizer used was m-THPC, Foscan^{R}, Biolitec Pharma Ltd., and was gently provided by Dr. H. Poteca. A strong absorption peak around 420 nm can be appreciated in Figure 1 for m-THPC. For injection into the mice, a PS solution was prepared in a 2:1 molar ratio mix of ethanol-propylene glycol. A 0.75 mg / kg dose of m-THPC was employed and the injection made at the medial portion of the mouse tail.

After 24 h m-THPC injection, mice held in a dark cage to avoid undesired effects due to accumulation of PS in the skin and eyes, were illuminated [39] with a light dose of 20 J/cm^2 and the 12W-LED lamp of 420 nm was placed at a distance of 0.2 m (Figure 3b). During the illumination, the animals were covered with double black cloth with a cut silhouette, which made the illumination of the tumor possible and minimized the effect of light on other parts of the animal body. Temperature and fluorescence follow-up was carried out to monitor the evolution of PDT (Figure 3b and 3c). The fluorescence was measured at 652 nm, the average wavelength of the m-THPC fluorescence maximum, by means of the spectrometer indicated above. The infrared camera utilized for surface temperature measurement was a SDS INFRARED E8NN/S E8210040 with a sensitivity of 8 µm to 14 µm.

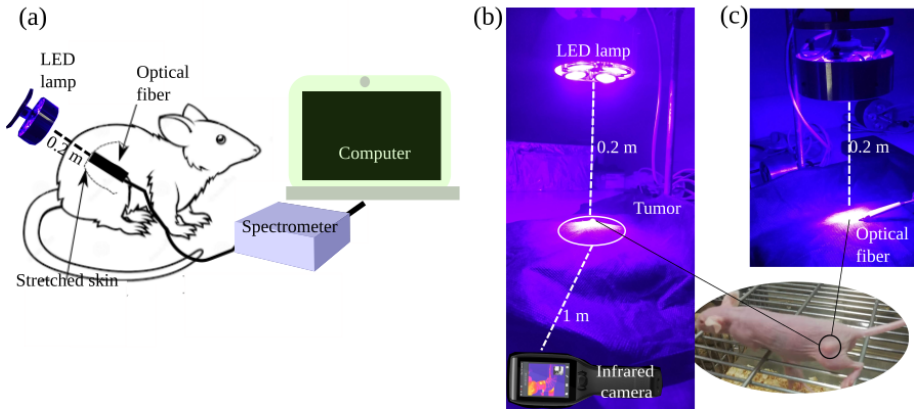

Figure 3. (a) Transmission measurements. The lamp is positioned at a 0.2 m distance from one side of the stretched skin, and on the other side, the optical fiber in contact with the skin records the transmitted light; (b) photograph of PDT treatment with temperature control by infrared camera; (c) fluorescence follow-up during the PDT treatment.

3. Results

3.1. *Characterization of the violet light source*

The LED source was characterized by means of radiometric and photometric parameters, and then utilized in PDT experiments. With the arrangement shown in the scheme of Figure 2b, it was possible to follow up the illuminance and irradiance at different angles from the perpendicular to the source plane.

The light source presented a homogeneous illumination on, at least, an area equivalent to that of the projected tumor surface (Figures 2a and 4a). For the analysis of the LED lamp performance at different angles, the lamp was fixed and the spectrometer was moved from an axis perpendicular to the surface of the lamp to the horizontal axis, recording illuminance values for 0°, 45°, 60°, and 90°. The relative maximum power of the light beam decreased with the source-detector distance following the expected square relationship, i.e., the inverse square law. At 0.2 m of distance the values measured were 280 lx and 4.2 x 10^4 µW/cm^2 for the illuminance and the irradiance, respectively (Figure 4b).

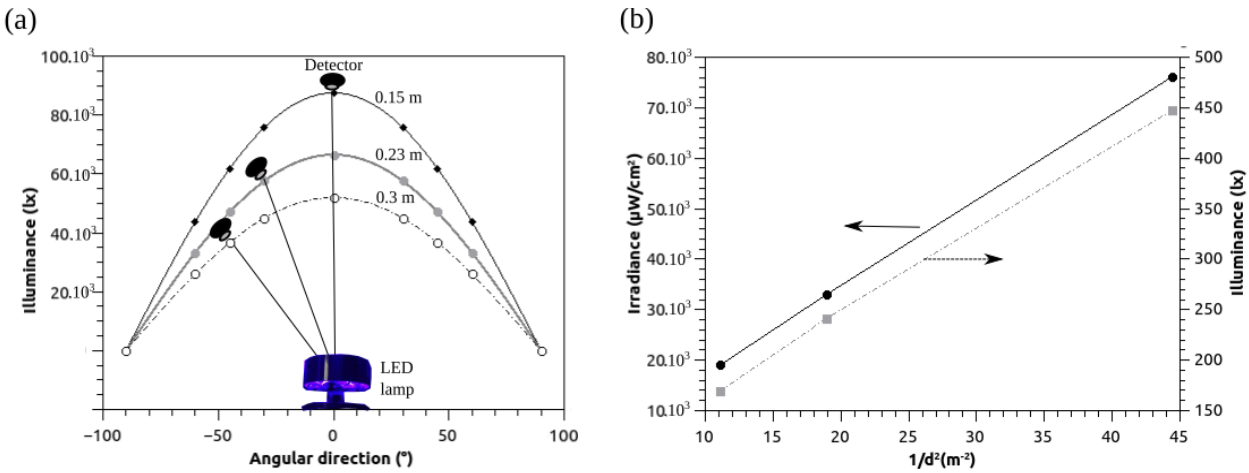

Figure 4. (a) Illuminance versus angular direction of the violet LED light source beam at 0.3, 0.23 and 0.15 m; (b) inverse square law for the violet LED light source and distances indicated in (a).

3.2. *In vivo model and PDT with violet light*

3.2.1. Transmission experiments

We measured the light passing through the skin of the murine tumor model. We compared the violet light with the red one to assess the ability of the violet light to activate the photoactivatable molecules of the photosensitizer at the tumor. Using the experimental setup shown in Figure 3a, both the tumor skin and the mouse ear were illuminated with 420 nm and 660 nm light with the same output power (Figure 5). The relative light transmission for the red light was about 20 times larger than the violet one for the tumor skin. For the ear, the transmission of the red light was 2.5-fold larger than the violet one (Figure 5).

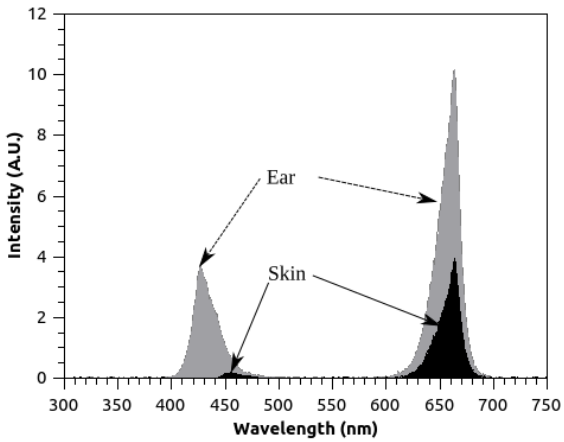

Figure 5. *In vivo* transmission spectrum for light of 420 nm and 660 nm in the tumor skin and ear of BALB/c nude mice, before the injection with m-THPC and PDT illumination.

3.2.2. PDT treatment

After one day of the m-THPC administration, the follow-up of the fluorescence was carried out by the arrangement depicted in Figure 3c. *In vivo* m-THPC fluorescence spectra from tumors were obtained by excitation with the violet light source employed in the PDT treatment, and recorded with the spectrometer coupled to an optical fiber in the 500-800 nm range during the PDT sessions. The emission intensity at 652 nm, after correcting for auto-fluorescence, was associated with m-THPC. The fluorescence intensity decreased as the treatment time elapsed. At the end of the treatment, the fluorescence intensity was also measured in another area, namely, the eye. In a previous work [39], we showed that without illumination the m-THPC concentration remained approximately constant in the range 1 – 4 days.

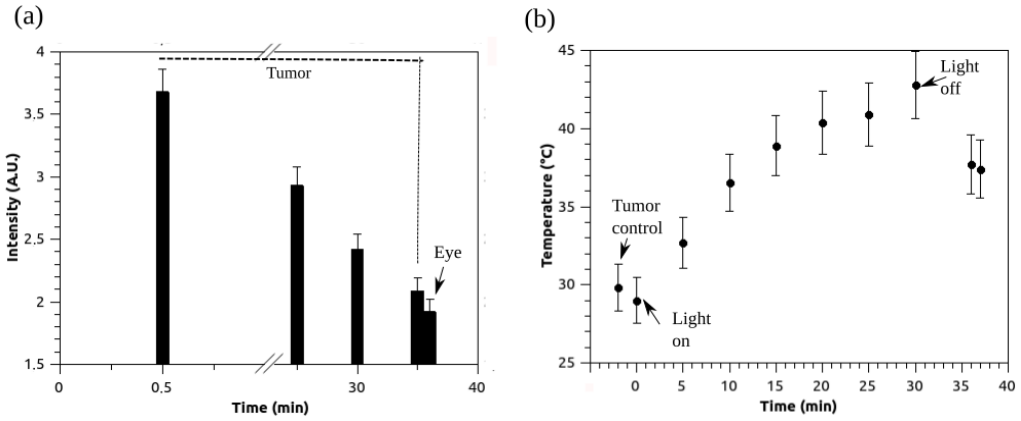

Figure 6. (a) Fluorescence measurement of m-THPC at 652 nm in the cervix tumor model in BALB/c nude mice during the PDT illumination with the setup shown in Figure 3. A decrease in the fluorescence of m-THPC is observed at the indicated wavelength (652 nm), due to the decrease in the concentration of photoactivatable molecules; (b) BALB/c nude mouse thermography throughout PDT by the infrared camera.

In addition, tumor temperature monitoring was carried out throughout the treatment (Figure 6b), employing the infrared camera. During the PDT treatment, the temperature gradually increased in the tumor from 29 to 42.8 °C. In clinical practice temperature control is an important tool since it would avoid reaching the pain threshold, as fractioning of lighting could be conveniently used for treatment.

4. Discussion

In this work, we have briefly reviewed the application of violet light both in PDT and PDD. We have shown some important results from 3D spheroid models used for studying the photodynamic dosage [36, 38], a matter of great interest for improving PDT protocols. Cell spheroids are appealing models that can reduce experiments with animals. Spheroids reproduce many real characteristics of real tumors and have been employed, similarly to cell suspensions, for testing quantitative models of PDT dosage and for predicting treatment outcome in multicellular systems [35, 42-44]. According to the results of our research group, violet light would be effective to treat superficial lesions about 250 – 300 μm in depth.

The use of violet light for treating several diseases has been briefly reviewed in Section 1. This light has been proposed even for sophisticated technologies for the application of PDT, such as a wireless implantable miniaturized photonic device for illuminating selected small regions of the body [45]. This type of approach increases the potential applications of PDT with violet light, minimizing normal tissue damage. In this vein, UV-violet light properly delivered to the target tissue and in the presence of a PS, enhances the detection of tissue regions harboring pathologic conditions.

Although the mouse tumor model does not reflect the multistage process of carcinogenesis and the complexity of the disease, valuable data can be extrapolated to humans. Thus, it can be inferred that violet light is able to induce the photochemical reaction expected to destroy the malignant tissue. The largest fluorescence was obtained by exiting with light of wavelength close to the Soret band indicating that, although tissue absorption is larger than for the red light, the amount of light transmitted is enough for PDT performing [46]. This is reflected by the decrease in the m-THPC fluorescence and the temperature increase measured at the BALB/c nude mice tumor surface with the 420 nm-LED illumination.

As indicated in the Introduction, in clinical trials, patients with a CIN1/2 infection were PDT treated with the topical administration of hexaminolevulinate and red light from lasers [18, 24] or more recently with a LED-based device [27, 28, 47], and it has been concluded that this treatment strategy should be considered as very competitive among other more traditional ons. Violet light for PDT treatment of CINs could be a valuable alternative that can be combined with PDD.

The incorporation of the fluorescence measurement during the PDT treatment is valuable in order to improve the dosage of the treatment. Furthermore, the photobleaching of the photosensitizer, as well as the

quantitative determination of singlet oxygen, which is considered the most general measurement of the PDT dose, have been reported [39]. Temperature monitoring would complement fluorescence determination and make the PDT application comfortable without reaching pain thresholds.

To sum up, the review notes and the results presented here and those from our previous work [34] reveal advantages of employing violet light to expand PDT applications with good outcome as well as to improve early cancer detection by fluorescence –based methods.

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