Phenothiazinium dyes in association with diode red laser against B16F10 melanoma cells: in vitro study.

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ABSTRACT

In Brazil solar incidence is high and continuous throughout the year. Body exposure to sunlight may be a key point in the rates of individuals affected by melanoma and other types of skin cancer in many countries. Brazil already occupies the 15th place in the ranking of melanoma cases and the limitations presented by drugs used in the therapy of this cancer, new approaches are being used in an attempt to decrease the mortality of this malignancy. The aim of this study was to evaluate the effects of phenothiazinium dyes (PD) associated with laser light on murine melanoma (B16F10) in vitro by measuring cell growth using colorimetric assay before and after photodynamic therapy. We used a diode laser (λ660nm, 2.4 J/cm², 40 mW, 60 s, CW) associated with PD at 12.5 μg/mL, time pre-irradiation of 30 minutes). The following groups were tested: control (LF-), PD (L-F+), Laser (L+F-), Laser + PD (L+F+). The results showed a significant reduction in cell growth in the group treated by the photodynamic therapy compared to the control at 24 and 48 h ($p < 0.001$). Were showing at 30 min PD has a dose-dependent response on B16F10 cells, but at 24 h did not demonstrated this response.

Keywords: Light, PDT, Melanoma, Laser.

1. INTRODUCTION

Phenothiazinium dyes (PD), like methylene blue and toluidine blue O, are photosensitizer cationic molecules. When this photosensitizer is irradiated, process named Photodynamic Therapy (PDT)[1], they release Reactive Oxygen Species (ROS), *e.g.* oxygen singlet (O^-), promoting cells death via different pathways[2].this technique was show to be effective upon microbes including bacteria[3] fungi[4], parasitic [5], as well as, pathologies such as oral disease [6] and cancer [7]. The clinic its administration may be topical, oral, infusion into the bladder, intravenous, intratumoral injection [8]. Some research link the effect of PDT using other photosensitizer, as porphyrin or 5-aminolevulinic acid in diseases including cancer [9, 10].

Melanoma is the most aggressive skin cancer with low overall survival, but if detected early and removed by surgery there is possibility to cure [11]]. Clark *et al.* [12] describe five stages to prognostic where stage I is most superficial and curable, and stage V is most invasive and metastatic with bad prognostics. The treatment of this malignant neoplasm is inefficient and the drugs used display remarkable adverse effects. Therefore, new approaches to improve chemotherapy efficacy are required [13].

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Mechanisms for Low-Light Therapy IX, edited by Michael R. Hamblin, James D. Carroll, Praveen Arany,
Proc. of SPIE Vol. 8932, 893212 · © 2014 SPIE · CCC code: 1605-7422/14/$18 · doi: 10.1117/12.2038466

In this study we used the PD in association with a diode red light, to evaluate if this PDT has a inhibitory action in melanoma growth cell culture, and also the toxicity of PD before and after irradiation.

2. METHODOLOGY

Cell Culture

Melanoma B16F10 cells were obtained from Institute of Biophysics, Federal University of Rio de Janeiro. The cells were maintained in RPMI 1640 (Gibco), supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen). For experiments, the cells were removed by addition Trypsin/EDTA (Gibco) and centrifuged in 380 g for 10 minutes. They were incubated at 37°C and 5% CO_2.

Photodynamic Therapy (PDT)

The B16F10 after the trypsinization were incubated in 24 well plate in a $2x10^5$ cell/well, for 30 minutes in the dark, with 12.5μg/mL of PD, RPMI 1640 and FBS (10%). Them they were irradiated with diode red laser (Twin Flex®, MMOptics, São Carlos, SP, Brazil), emitting light at 660nm wavelength. We used energy density of 2.4 J/cm^2 and potency of 40 mW for 60 seconds and they were washed with RPMI to remove de PD excesses and incubated at 37ºC and 5% CO_2 for 24 or 48h.

Toxicity of PD in B16F10 cells

The PD (methylene blue and toluidine O blue in a 1:1 proportion) was obtained by Formula, (Salvador, Brazil). To evaluate the growth of B16F10 cells, PD was add on 24 well plate cultures at different concentrations for 24 h at 37 °C and 5% CO_2. The treatment of B16F10 cells with PD were also made for 30 minutes, followed by a wash with RPMI 1640 and incubation at same conditions.

Colorimetric Assay

To evaluate if the PDT is able to decease the development of B16F10, we used the colorimetric assay [14] to indicate indirectly the growth of cells. The cells were washed with sterile PBS to remove debris and unviable cells them they were fixed with methanol 100% (0.5 mL/well) for 10 minutes, and washed with borate buffer (pH 8.7) them we added methylene blue (0.1% in borate buffer)(0.5 mL/well) for 10 minutes and supernatants were plated in 96-well plate and read at 655 nm.

Statistical Analysis

All experiments were performed in triplicate and repeated independently. The statistical analysis was performed employing ANOVA and Tukey post-test using GraphPad Prism 5.0 software, with * to $p<0.05$, ** to $p<0.001$ and *** to $p< 0.0001$ as significance level.

3. RESULTS

The aim of this study was to evaluate the effect of PD upon B16F10 cells growth, using PD before and after its association with LASER light.

The effects of PD in association with LASER do not influence in effects of PD without irradiation in B16F10 cells growth.

We used LASER light irradiation to stimulate PD and evaluate the effects on B16F10 growth by colorimetric assay. The conditions were described as Control (L-P-), LASER (L+P-) PD (L-P+) and PDT (L+P+). The cells in L+P- were only irradiated in absence PD to survey if the light has any effect on B16F10. Melanoma cells in L-P+ sample were incubated in presence of PD with no irradiation. All cells in L-P+ and L+P+ sample were incubated for 30 minutes with PD (12.5 μg/mL) and L+P+ after that were irradiated with LASER, and all conditions were incubated for 24 and 48 h. After incubation of 24h (Figure 1-A) was possible evaluate what L+P- as compared to L-P- show a growth, but did not show significance. The L+P+ (PDT) showed statistically significant inhibition as compared to L-P- and L+P-, but do not as compared to L-P+, demonstrating the cytotoxicity of PD. When incubated for 48h (Figure 1-B) the inhibition was similar, L+F- had no statistical significance as compared with L-P. L-P+, L+P+ significantly inhibited B16F10 cells growth, but did not show significant difference.

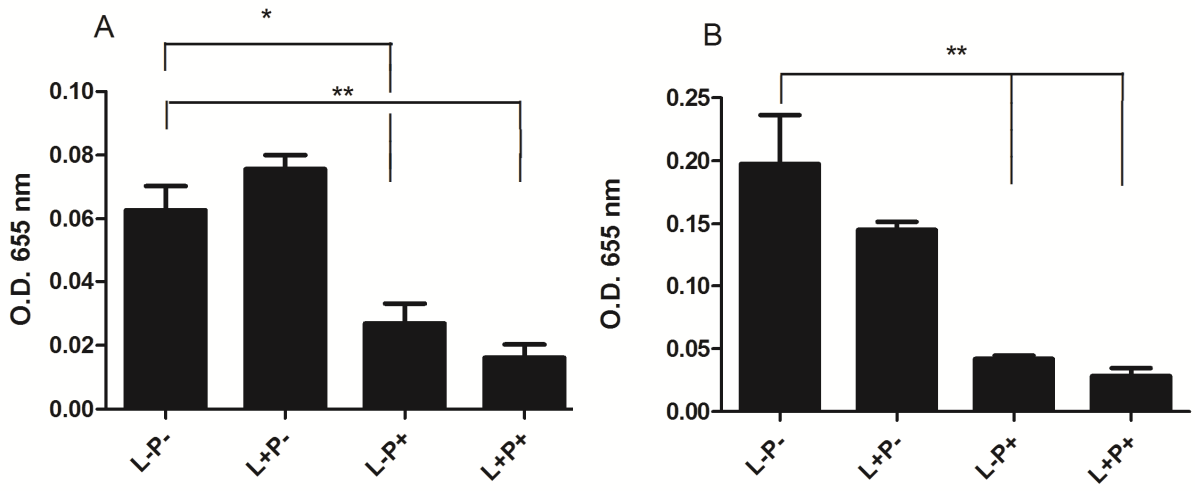

Figure 1. Effect of PDT on B16F10 in 24 and 48h. (A) 24h after PDT. We observed inhibited B16F10 growth on L-P+ and L+P+, as compared to dark control group (L-P- *vs* L+P-), and L+P- did not demonstrate inhibition. Similarly between L-P+ and L+P+ there was no statistical significance. (B) After 48 h PDT we observed that inhibition in growth of melanoma cells was significant only in L-P+ and L+P+ as compared to L-P-. We used ANOVA and Tukey post-test for statistical analysis with *($p<0.05$) and ** ($p<0.001$).

PD dose-dependent and time-dependent cytotoxicity on B16F10 cells

In order to evaluate the PD cytotoxicity upon melanoma cells, the B16F10 cultures were exposed to different concentrations (100; 50; 25; 12.5; 6.25; 3.12; 1.6 µg/mL) for 30 minute and 24 hours proliferation was accrued by colorimetric assay. The PD treatment produced a dose-dependent effect in B16F0 growth in 30 minute (Figure 2-A). In the major concentrations, ranging from 100 – 6.25 µg/mL displayed marked 100µg/mL, shows great inhibition compare with control and last concentration with hyper statistical significance (p<0,0001) on cancer cell growth. In 24h (Figure 2-B) all concentrations were highly cytotoxic with no apparent dose-dependently under these conditions.

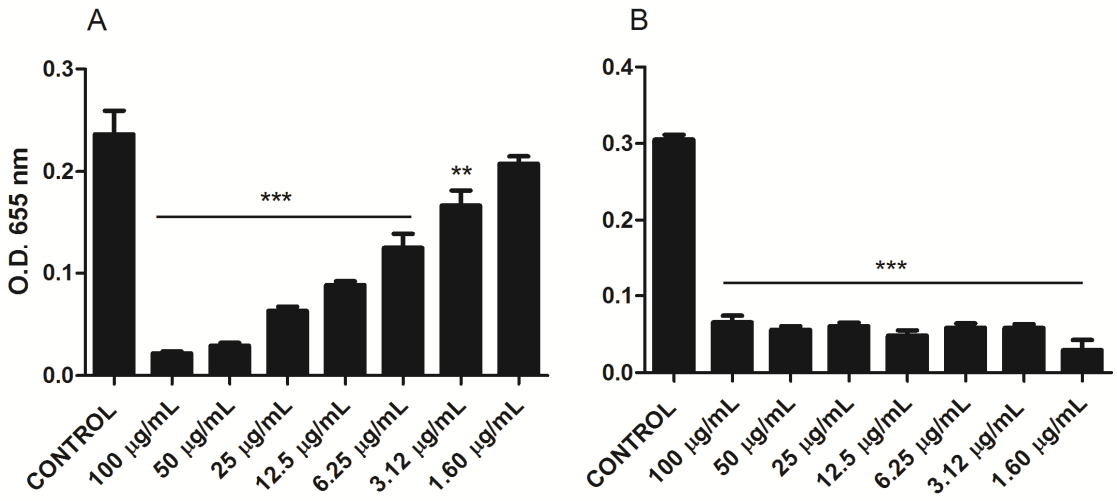

Figure2. Cytotoxicity of PD on B16F10. (A) On 30 min. Dose-dependent response showing in the 3.12–100 µg/mL range PD as highly significant (*** p<0.0001) (B) In 24h all concentration showed high inhibition with no apparent dose-dependently under these conditions Data were analyzed using ANOVA and Tukey post-test with *** (p<0.0001)

4. DISCUSSION

The photosensitizer used on PDT must preferentially be non-toxic before irradiation, quickly absorbed by cells among other features[15]. The methylene blue and toluidine blue O used in this study have been show to be rapidly absorbed by tissues and is actuated at 600-660 nm wavelength [16]. The PDT using PD has demonstrated in some bacterial models to be effective against *Staphylococcus aureus* and multi-drug resistant *Escherichia coli* [17] and *Candida albicans* [18]. As performed in other cancer models like sarcomas PDT show high antitumoral activity [19]. In our work we verified that PDT using PD and LASER, produced remarkable effects on B16F10 proliferation, demonstrating efficacy against B16F10,

We also notice that PD has a high cytotoxicity on B16F10. Cytotoxity was reported in *Staphylococcus aureus* [20], *Leishmania braziliensis* [21] models. Furtherer approaches are under way to determine suitable, safe conditions for PD use both in vitro and in vivo.

5. CONCLUSION

The present study indicates that PDT using PD as photosensitizer is a possible approach against melanoma cells, but further investigation is refused to elucidate optimal conditions and mechanisms of melanoma death envolved.

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