PHOTODYNAMIC THERAPY OF SUBCUTANEOUS MURINE MAMMARY ADENOCARCINOMA

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ABSTRACT
Photodynamic therapy (PDT) of cancer is a treatment based on the accumulation of a porphyrin-related photosensitizer in tumor cells, and their subsequent destruction based on exposure to light source of specific wave length. Hematoporphyrin derivative has been shown a selective localization in malignant tissues and causing their destruction by generated singlet oxygen when it's activated by appropriate wavelength (λ) of irradiation. Singlet oxygen species are produced and then caused membranes' and organelles' damage leading to cell death and tumor ablation. In this study, Female mice transplanted with AM3 (mouse mammary adenocarcinoma transplantable tumor line) randomly divided into four groups of 10 mice each, the mice of first group were intratumorally injected with 30 mg HPD/kg of body weight and exposed to 10 minutes of irradiation from 20mW He-Ne laser (λ = 632.8 nm). The mice of second group were intratumorally injected with 30 mg HPD/kg of body weight without irradiation. Third group mice were received 10 minutes exposure time of He-Ne laser irradiation without HPD injection, while the fourth group was leaved as a control. Photodynamic therapy, which includes HPD and irradiation, has had the most powerful effect on the tumor growth, while the other groups have not showed any significant response. With more investigations PDT can be promising anti-breast cancer arsenal.

Key words: Photodynamic therapy; Mammary Adenocarcinoma; Hematoporphyrin
العلاج الحركي الضوئي بالحقن داخل ورم سرطان الغدد

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الخلاصة

العلاج الديناميكي الضوئي للسرطان هو علاج يعتمد على تراكم المحسات الضوئية ذات الصلة بالبورفينات في خلايا الورم. وتميزها لاحقاً على أساس التعرض لعنق من الأشعة ذات مواصفات معينة. وقد ظهر أن مشتقات الهيماتوبورفين ينضب بصورة إنتقالية في خلايا الأنسجة الخبيثة مسبباً تحطمها بواسطة توليد جذور الأوكسجين الأحادية وذلك عند تشخيصها بواسطة طول موجي مناسب من الأشعة. إن جذور الأوكسجين الأحادية المنتجة هي التي تنسب في تحطيم الأغذية والعضيات، مؤدية إلى الموت الخلوي وبالتالي استئصال الورم. في هذه الدراسة، تم غرس أنثى الفئران المختبرة بخلايا الخلايا الخبيثة ورم 3 المستزرع من سرطان الغدد اللبية للفئران، وقسمت هذه الفئران عشوائياً إلى أربع مجموعات، كل مجموعة تتألف من 10 فئران، فئران المجموعة الأولى حققت داخل الورم بالمحسض الضوئي المشتق من الهيماتوبورفين بتركيز 30 ملي واط (الطول الموجي 632 نانومتر) مع تمتعت مقدار 10 دقائق، فئة المجموعة الثانية حققت داخل الورم بنفس التركيز من المحسض الضوئي، وتم تثبيت في النظام لمدة 24 ساعة، ولم يتم تمتعت مقدار 10 دقائق، فئة المجموعة الثالثة فئة تمتعت لم تتم تثبيت في النظام لمدة 24 ساعة، ولم يتم تمتعت مقدار 10 دقائق. اظهرت النتائج بأن العلاج الديناميكي الضوئي الذي يتضمن تشتيت الليزر مقتتراً بالمحسض الضوئي (مشتقة الهيماتوبورفين) كان الأكثر قوة تثبيطية لنمو الورم، بينما بقية المجموعات لا تظهر فيها أي فروقات معنوية. ومع المزيد من التحريات المستقبلية ستضاف هذه التقنية الجديدة إلى ترسانة العلاجات المضادة للسرطان خصوصاً سرطان الثدي.
INTRODUCTION

Photodynamic Therapy (PDT) is an approved anticancer therapy that kills cancer cells by the photochemical generation of reactive oxygen species following absorption of appropriate wavelength of light by a photosensitizer, which selectively accumulates in tumors (1,2). The process of PDT can essentially be divided into two stages. Photosensitizers are initially administered, usually systemically (I.V., I.P. or intratumoral), and given time to localize into target tissue. Then using a monochromatic light source or laser, a specific wavelength of non-thermal, visible light (in the red or infrared region of the electromagnetic spectrum) is subsequently delivered to excite the sensitizer. The sensitizer in turn undergoes a sequence of photooxidation reactions that culminate in the generation of highly cytotoxic free radical ions, oxygen-derived species most notably, singlet oxygen (3,4,5).

Briefly, there are two main types of photochemical reactions (type I and type II) associated with PDT. The photosensitizer is activated to reach an excited singlet state upon the absorption of photons at the appropriate wavelength, it may subsequently decay from its singlet state back to ground state and emit fluorescence or alternatively undergo intersystem crossing to populate stable, triplet state orbital (6,7). In a type I reaction, once excited to a triplet state energy, the photosensitizer can either undergo intermolecular transfer of electrons with nearby cellular membranes or amino acids and/or nucleic acids. More commonly, the photosensitizer transfers energy to ground-state molecular oxygen in a type II reaction. Either reaction can result in significant intracellular damage of membranes and organelles although, the relative contribution of each will depend largely on the type of sensitizer being used as well as the intracellular environment; i.e. the availability of molecular oxygen (8, 9). The involvement of a type II reaction-associated generation of singlet oxygen ($^1O_2$) in the majority of PDT-induced cytotoxic responses (10,11).

One of the most promising substances for PDT is Hematoporphyrin derivative (HPD) which has been shown to selectively localize in malignant tissues. Porphyrins are a powerful photosensitizing agent that can cause destruction and death of malignant tissues in which they have localized by the generation of singlet oxygen when activated by light of the appropriate wavelength (12,13,14). Tumor necrosis can thus be achieved by irradiation of the neoplastic area with light of 632nm, which corresponds to the longest wavelength absorption band of porphyrins. For the elimination of the tumor the red light emitted from Helium–Neon laser (He–Ne laser, $\lambda = 632.3$ nm) is used in photodynamic treatment because of its deeper penetration into the tissue. Typically, the effective penetration depth is about 2-3mm at 632nm and may be increased to 5mm (15,16).

In a previous study we have shown that the HpD-mediated PDT leads to inhibition of AMN3 (mouse mammary adenocarcinoma tumor cell line) using He–Ne laser in vitro (17). The success HpD photosensitizer combined with He–Ne laser to stimulate the photodamage of AMN3 cell line has open the door wide to apply this technique in vivo.

In recent studies, the PDT has also been shown to induce apoptosis in vivo and in vitro; however, the apoptotic response to PDT seems to depend on both the photosensitizer and the cell line, the combination with He-Ne laser irradiation have been further enhanced the apoptosis signals transduction and finally leading to the apoptosis of neoplastic cells which is one of the mechanisms of the anti-tumor activities of HPD -mediated PDT.
Singlet oxygen is thought to facilitate opening of mitochondrial transition pores which allow for the triggered release of cytochrome C from the mitochondria into the cytosol. The electron transport chain is subsequently disrupted upon the loss of mitochondrial cytochrome C while the newly appropriated cytosolic cytochrome C activates certain cytoplasmic proteins e.g. Apoptosis-activating factor 1 (APAF-1). APAF-1 in turn activates a cascade of caspases which result in apoptotic cell death (18,19,20).

The integral role of mitochondria in PDT-associated apoptotic cell death and the fact that cancer therapy prognosis often correlates with the propensity for malignant cells to undergo apoptosis, implicates mitochondria as an important subcellular target for PDT. Drug resistance or suboptimal tumor response may relate to inappropriate targeting of the sensitizer to PDT-insensitive sites within the cell with subsequent insusceptibility of target cells to apoptosis (20). Thus, besides genetic differences defining a cell’s susceptibility to a given therapy such as PDT, it is also imperative to consider optimizing subcellular targeting of PDT agents in order to achieve maximum tumor response.

The aim of this study is to investigate the ability of PDT to induce anti-tumor effect for mammary adenocarcinoma which is a model for human breast cancer.

MATERIALS AND METHODS

Experimental animals

Female BALB/c mice, 12 weeks old, weighing 20-25 g were used. They were provided with food and water. A transplantable mammary adenocarcinoma cell line named AM3 (provided by Dr. Ahmed M. Al-Shamery/Iraqi center for cancer and medical genetic research) was propagated by serial transplantation into female BALB/c mice. Tumor material for inoculation was obtained by sterile aspiration to the flank tumors. A 0.25-mm$^3$ sample of macroscopically viable tumor, which is equal to approximately $2\times10^6$ cells, was injected S.C. under the dorsal flank of each mouse. The take rate of the tumors following transplantation was nearly 80-100%. Under these controlled conditions the implant size did not vary by more than 10%. Animals were treated in accordance with guidelines established by the Animal Care and Use Committee of the Iraqi center for cancer and medical genetic research.

Preparation and administration of HPD

The photosensitizer, Hematoporphyrin derivative-HCl (HPD) was purchased from Sigma-Aldrich Chemical Co. (Germany). The hydrochloric salt of HPD was dissolved in Phosphate Buffer Saline (PBS, pH 7.2) in dark chamber at a concentration of 100 mg/ml (17). After completely dissolved by shaken vigorously with a vortex mixer for 5 min at 37°C; it was used immediately for intratumorally administration.

Measurement of spectral properties

The absorbance was measured in the wavelength range of 380–700 nm using double-beam UV/VIS spectrophotometer (Varian Cary 100UV-Vis Spectrophotometer, Australia). Absorbance spectra analyses of HPD were demonstrated as a plot of absorbance against electromagnetic region (300–750 nm).
Laser and irradiations
A helium neon laser (Model DL30, LG Lasers) ($\lambda=632.8$ nm) had been using, the output power of laser was 20 mW. The light was focused into a 7mm diameter light spot, producing a treatment area of uniform intensity exposure time was for 600sec.

Treatment protocol for HPD-based PDT
Ten days after AM3 tumor implantation, when tumors reached the appropriate size of 0.5-0.7 cm$^3$, they were randomly divided into four groups of 10 mice each.

The mice of the first group were intratumorally injected with HPD 30 mg/kg of body weight and superficially irradiated with low power He-Ne laser just 24 h after HPD administration. Each irradiation areas, which encompassed the tumor and 1–1.5 mm of the surrounding skin, was exposed to 10 minutes of irradiation. The second group mice were intratumorally injected with HPD only without irradiation. Third group received irradiation without HPD administration and the fourth group leaved as control without administration and irradiation.

Assessment of tumor response
In all experiments, the tumor growth was recorded every 2 days by measuring a perpendicular diameters using vernier caliper. Tumor volumes were estimated using the following formula according to (21):

$$\text{Tumor volume (mm}^3\text{)} = \frac{a \cdot b^2}{2}$$

a= length of tumor mass (mm), b= width of tumor mass (mm)

Relative tumor volumes (R.T.V.) and Tumor growth inhibition (GI%) were calculated using the following formulas according to (22):

$$\text{R.T.V.}_{(\text{day } x)} = \frac{\text{tumor volume (day } x\text{)}}{\text{tumor volume (day } 0\text{)}} \times 100$$

$$\text{GI\%} = \frac{\text{tumor volume of untreated group} - \text{tumor volume of treated group}}{\text{tumor volume of untreated group}} \times 100$$

To assess the response to treatment an index was defined: $D_X$ (tumor volume $X$ days after PDT/tumor volume before PDT).

Histological studies
After 20 days of PDT, samples of tumor with its surrounding skin were excised. They were extended, sliced, fixed in 10% buffered formalin, embedded in paraffin, sectioned, stained with haematoxylin and eosin and examined by light microscopy. The presence of tumor tissue, necrosis and cells with morphological features of apoptosis was evaluated. Epidermal and dermal damage, vascularity changes and presence of lymphocytic infiltration were also investigated.

Statistical analysis
The unpaired t-test was used to establish the significance of differences between groups. Differences were considered statistically significant when $P > 0.05$. 

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RESULTES AND DISCUSSION

The present study investigated the effect of laser irradiation on the growth of a subcutaneous implanted AM3 mammary adenocarcinoma tumor after intratumoral application of HPD. According to previous study, photodamage effects were observed after Photodynamic action in vitro, when mammary adenocarcinoma (AMN3) cells were incubated with different concentrations of HpD and then illuminated with different doses of He-Ne laser in vitro (17). So the current in vivo work is based on the good results obtained previously from in vitro experiments.

Absorption spectra

Absorption spectra of HpD are characterized by a Soret band in the violet region and four wide Q-bands located in visible region (500nm–630 nm). They were comparable to those achieved in previous study (17). No tendency to aggregate was observed. However, this does not exclude the possibility of aggregation inside cells, which is often found for such compounds. There was a difference was observed in the shape of the UV-VIS spectra of the HpD in two solvents studied. Only a small difference in the peak positions was seen. This difference may indicate that the PDT properties of HpD should not vary considerably from one solvent to another. The high absorbance in the Soret band and significantly lower one for the Q bands indicate suitability of the studied HpD in PDT. The longest-wave absorption band at 632 nm observed for HpD when activated by UV irradiation, it is the most important one regarding PDT, because only red light has sufficient tissue penetration ability.

Effectiveness of HPD-based PDT in delaying tumor growth

The effectiveness of HPD as a photosensitizer for PDT was determined by assessing the extent of tumor growth after one PDT application. Two response indexes were defined, measuring the ratios between tumor volumes before and after treatment. D4, D8, D12, D16 and D20 indexes for intratumoral HPD-PDT are shown in table(1). After a single application all indexes were lower than those of the untreated tumors, indicating that if not a complete reduction, a delay of tumor growth occurs. Irradiation on HPD injected intratumorally appeared to induce a greater reduction in tumor volume earlier. An index of 57 was observed at 4D and 70 at 8D, but differences between times were not statistically significant. In order to compare the response of PDT between groups, tumor growth curves were used figure(1). Animals received one intratumoral HPD-PDT; a single application induced a clear tumor growth delay for intratumoral HPD administration which continued to the end of the experiment at day 20. The current study is about using intratumoral PDT technique in vivo for treatment a subcutaneous murine mammary adenocarcinoma by the combination of HpD with low power He-Ne laser, then killing tumor cells through photo damaging. The results showed very powerful antituomr activity with marked tumor mass regression, this showed that photodynamic actions including photosensitization is the efficient selective technique for killing of target tumor cells which exposed to certain photosensitizers and corresponsing laser light with appropriate wavelenght which confirmed by other researchers (23,24). In this study, the photodynamic action induced by the excitation of HPD photosensitizer after exposing to red light of He-Ne laser at wavelength 632.8 nm.
The laser-activated process necessarily requires the presence of a light-absorbing substance, the photosensitizer HpD, which initiate photochemical processes in a non-absorbing substrate (AM3 Mammary adenocarcinoma tumor cells). Current study has illustrated a significant antitumor effect of PDT with single application. The pathway which involved a photosensitizer triplet state reacted initially with a substrate rather than molecular oxygen, this is termed Type I photochemical reaction. In the alternative Type II photochemical reaction the photosensitizer triplet state reacts first with molecular oxygen producing a singlet oxygen (\( ^1\text{O}_2 \)), it is the main free radical ion which responsible for cell death (6,7,10,11). The most important photosensitizer is HPD which localizes and retains for many days in tumors anywhere in the body after intravenous administration. There is no therapeutic effect until HPD in tumor tissue is exposed to appropriate visible light, which is usually the red region of the electromagnetic spectrum. The irradiation exposure induces necrosis followed by sloughing of the necrotic tissue and re-growth of normal tissues. The putative action mechanism in PDT is \( ^1\text{O}_2 \) generated by energy transferring from the HPD triplet state to tumor oxygen, and then initiated a lipid peroxidation in the endothelial cells of the small blood vessels which supply the tumor cells with blood (6,7). The tumor oxygen supplying is blocked by this process afterward the observed necrosis is induced. Direct cancer cell killing may be involved as well. The selectivity mechanism is depended on the total amount of HPD in tumor tissue which were several times higher than normal tissue, that is due to thier ability to accumulate in malignant tissue (25 and 26), which is intradermally located murine adenocarcinoma. These findings may be explained by either the existence of a saturating amount of HPD-formed porphyrins necessary to produce the photodynamic damage (17) or alternatively by an uneven distribution of porphyrins in different tumour layers which eventually result in a similar or equivalent amount of porphyrins directly exposed to light in either route of HPD administration. According to tumor HPD concentration ratios and total amounts of porphyrins accumulated in tumour tissue, irradiation would be optimal to 31.5 J/cm\(^2\) of He-Ne laser after intratumoral administration of 30 mg HPD/kg of body weight.

Table 1: Group one (L+P+) showing impressive tumor growth inhibition and delay in tumor growth in compare with first day of experiment.

<table>
<thead>
<tr>
<th>P injection day</th>
<th>L+P+</th>
<th>L-P+</th>
<th>L+P-</th>
<th>L-P-</th>
</tr>
</thead>
<tbody>
<tr>
<td>laser treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 4</td>
<td>62</td>
<td>84</td>
<td>98.85</td>
<td>67.6</td>
</tr>
<tr>
<td>day 8</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>day 12</td>
<td>57</td>
<td>136.4</td>
<td>164.7</td>
<td>125.1</td>
</tr>
<tr>
<td>day 16</td>
<td>70</td>
<td>211.08</td>
<td>375.5</td>
<td>332.85</td>
</tr>
<tr>
<td>day 20</td>
<td>109</td>
<td>362.79</td>
<td>516.1</td>
<td>445.3</td>
</tr>
</tbody>
</table>

L+P+ = Laser and photosensitizer treatment at the same time
L-P+ = Photosensitizer treatment alone
L+P- = Laser treatment alone
L-P- = control without treatment
Figure(1): Tumor growth curve of treatment groups, group one of photodynamic therapy cause marked growth inhibition in compare to photosensitizer alone or laser alone which show no response or delay in tumor growth, especially laser group which have similar growth pattern to control untreated group.

L+P+ = Laser and photosensitizer treatment at the same time
L-P+ = Photosensitizer treatment alone
L+P- = Laser treatment alone
L-P- = control without treatment

Macroscopic and histological analysis

There was no macroscopic or microscopic changes were observed for tumor or skin exposed to either HPD or laser alone at all times analyzed. After 24 h of HPD-based PDT showed macroscopically necrotic zones, gross tumor volume reduction, ulceration and scar formation were induced. At day 20 preserved tumor tissue with necrosis and cells with apoptotic images up to a depth of 4 mm from the epidermis were seen figure(2). Necrotic cells with vacuolization of the cytoplasm, pycnotic appearance of the nucleus and loss of cellularity were seen. Also there were large areas of necrosis and presence of apoptotic cells in tumor tissue. Congestion was developed and some lymphocytic infiltration was found. Laser alone and HPD alone treatment groups tumor sections showed massive tumor growth without any pathological changes figure(3).
Histopathological findings of our experiment refer to the presence of a large area of necrosis characterized by loss of cellularity due to direct cell injury from the reactive oxygen radicals generated by HPD when exited by laser, moreover we noticed the presence of apoptotic cells in the treated tumor tissue where cell undergoing apoptosis showed presence of vacuolated cytoplasm and condensed nuclear chromatin. Kessel and Luo have proposed that mitochondrial damage may be an important step in PDT-induced apoptosis (27).

Our tumor sections from treated groups showed infiltration of inflammatory cells although anti-tumor immunological responses have been mainly associated with necrosis; apoptosis-associated immune responses have been recently suggested as well. Zhou et al. (28) have investigated anti-tumor immune responses and regulatory mechanisms using apoptotic cells induced by PDT, their results showed that apoptosis can potentially have higher impact in inducing immunological responses, hence clarifying the immunological regulatory mechanisms under cell apoptosis and necrosis induced by PDT treatment. These findings could lead to an optimal PDT treatment based on immunological responses.
Survival study

Results of dark toxicity experiment showed no significant difference in survival rate between HPD treated group and untreated group as shown Kaplan-Meier curve figure(4).

Figure(4): Kaplan-Meier curve showing the influence of PDT treatments on prolong surviving of treated mice with HPD and laser (G1) in compare to only HPD treated group (G2).

In summary, our finding in the current study showed the possibility of using PDT as new antitumor modulation for breast cancer model in mice bearing murine mammary adenocarcinoma were there were interesting antitumor activity which can lead to clinical applications.

REFERENCES


