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# Photodynamic Action of low power He-Ne laser on photosensitized human Hep-2 and AMN3 cell lines with Hematoporphyrin derivative in vitro

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## Abstract:

Photodynamic Action (PDA) by using appropriate wavelength of irradiation conjugated with porphyrin derivatives is a powerful mechanism of tumor destruction. Hematoporphyrin derivative has been shown to selectively localize in neoplastic cells and then cause destruction of them by generation of singlet oxygen when activated by low power He-Ne laser. Light which used in this study has been emitting from this laser has a wavelength equal to 632.8 nm (red light). Doses of laser had been varied from 3.6 J/cm$^2$ to 14.4 J/cm$^2$. The beam of laser adjusted with a modified tissue culture plate. Cell lines had exposed to Hematoporphyrin D (HpD) for 24 hours before Laser exposure, their concentrations were varied from 5 μg/ml to 80 μg/ml. Results clearly proved a Photodynamic Action of laser conjugated with photosensitizer. No significant difference in cell viability was detected using neither the laser doses alone nor the photosensitizer (HpD) alone. Therefore, we believe that the low power He-Ne laser conjugated with hematoporphyrin derivatives' as a photosensitizer will open the door wide for photodynamic therapy of tumors.

## Introdcution:

Photodynamic Therapy (PDT) is an approved anticancer therapy that kills cancer cells by the photochemical reactions following absorption of visible light by a photosensitizer, which selectively accumulates in tumors (1). It involves systematic administration of a photosensitizer that preferentially accumulates in malignant cells followed by an illumination of the tumor with a monochromatic and collimated beam of laser light which kills cancer cells by the photochemical generation of reactive oxygen species following irradiation with light of a particular wavelength (2), thereby initiating tumor necrosis presumably through formation of singlet oxygen ($^1O_2$). Singlet oxygen ($^1O_2$) is believed to be the major cytotoxic agent involved in photodynamic action (PDA) (3). Thus PDT involves the administration of a photosensitizing drug (photosensitizer), which had affinity to accumulate in malignant cells of tumors and subsequent light activation. PDT is a binary therapy, and a potential advantage of PDT is its inherent dual selectivity. First, selectivity is achieved by an increased concentration of the photosensitizer in target tissue, and second, the irradiation can be limited to a specified volume. Provided that the photosensitizer is nontoxic, only the irradiated areas will be affected, even if the photosensitizer does bind to normal tissues. Selectivity can be further enhanced by binding photosensitizers to molecular delivery systems that have high affinity for target cells (1, 4). Optimal tissue penetration by light apparently occurs in red or infrared region of the electromagnetic spectrum between 600–850 nm (usually at about 630 nm for dihematoporphyrin) (5). The most widely investigated photosensitisers in PDT are hematoporphyrin derivatives (HpD), red light emitted from Low-power He-Ne laser (has a wavelength of 632.8 nm) causes a well-defined and energy dependent cell destruction of in vitro cultured cell lines sensitized by HpD. During the photosensitization process, the red He-Ne laser light, during a one-photon energy activation causes excitation of hematoporphyrin molecules to their triplet state. The excitation of HpD molecules results

in a multi-step, free-radical generating effect (6). Numerous photosensitisers have been developed and many are currently under investigation. The purified active component of HpD is enriched in dihaematoporphyrin ether (DHE). It has become available under the commercial name of Photofrin which was the first substance to receive regulatory approval for PDT applications. Photofrin has recently been approved for clinical treatment (7, 8).

## Material and Methods:

***Laser and irradiations***

The source of laser which used in this study was Helium-Neon atomic gas laser (Model DL30, LG Lasers). The wavelength of light that emitted from this laser was 632.8 nm (red light) and output power equal to 20mW (mill watt) as a continuous wave. Doses of laser had varied from 3.6 J/cm2 to 14.4 J/cm2.

***Preparation of photosensitizers***

The Photosensitive compounds, Hematoporphyrins derivatives (HpD) (Sigma Aldrich, Germany) were prepared in dark room as stock solution in 5ml of phosphate buffered saline (PBS) and shaken vigorously by a vortex mixer for 5 min at 37 ◦C.

***Spectral analysis of HpD***

UV-Vis absorption spectra for HpD were analyzed by a Double beam spectrophotometer (Varian Cary 100UV-Vis Spectrophotometer, Australia). Absorption spectra of photosensitizing compound were demonstrated as a plot of absorbance against wavelength (300–750 nm).

***Cell line and culture medium***

The human Hep-2 (larynx carcinoma) and AMN3 (murine mammary adenocarcinoma) were obtained from the Iraqi center for cancer and medical genetic research (ICCMGR-Iraq-Baghdad) and maintained in RPMI 1640 (Sigma-Aldrich-Germany) supplemented with 5% calf bovine serum (ICCMGR), 100 units/ml penicillin, and 100 µg/ml streptomycin.

***Photosensitizing agents Cytotoxicity assay***

To determine the cell killing effect of photosensitizing agent alone without light treatment (dark Cytotoxicity), crystal violet assay was conducted as on 96-well plates (Falcon), Hep-2 and AMN3 cells were seeded at 3-4x$10^4$ cells/well after 24 hr or confluent monolayer is achieved. Cells were treated with each photosensitizing agent at 160 µg/ml in two fold serial dilutions reaching to 5 µg/ml. The procedure of adding these therapeutic agents was by adding the agents for 48 hrs at 37°C for all cell lines in dark environment. After the dedicated time, cells were washed with PBS. Cell viability was measured after removing the medium, adding 100 µl of solution of 5mg crystal violet (BDH-England) + 200ml methanol + 50ml formaldehyde 37% and incubating for 20 min at 37°C. After removing the crystal violet solution, cells were washed with water three times. The absorbency was determined on a microplate reader (organon Teknika Reader 230S, Austria) at 492 nm (test wavelength); the assay was performed in triplicate (9). Endpoint parameters that are calculated for each individual cell line include cell proliferation rate, which is the percentage of control absorbance (10, 11, 12 and 13). The inhibiting rate of cell growth (the percentage of Cytotoxicity) was calculated as (A-B)/Ax100, Where A is the mean optical density of untreated wells and B is the optical density of treated wells. $LC_{50}$ is the lowest concentration that kills 50% of cells (14 and 15).

***Phototoxicity assay***

The beam of laser was adjusted with a modified tissue culture plate (3). After cell confluence in 96well tissue culture plate, cells were exposed to Hematoporphyrin in two fold serial dilutions, and after 48hrs; cells were washed and covered with fresh medium prior to irradiation. Each well was exposed to different doses of energy of laser ranging between (3.6 J/cm2 to 14.4 J/cm2) according to matrix showing in table 1. Following irradiation at room temperature (21°C), the cells were incubated in growth medium for 24hrs and growth inhibition was estimated as described in Cytotoxicity assay.

| (D,C) $D_n$ / $C_n$ | $D_0$ | $D_1$ | $D_2$ | $D_3$ | |
|---|---|---|---|---|---|
| $C_0$ | $L^-P^-$ 0,0 | 3.6,0 | 7.2,0 | 14.4,0 | $L^+P^-$ Control $^+$ |
| $C_1$ | 0,5 | 3.6,5 | 7.2,5 | 14.4,5 | $L^+P^+$ |
| $C_2$ | 0,10 | 3.6,10 | 7.2,10 | 14.4,10 | |
| $C_3$ | 0,20 | 3.6,20 | 7.2,20 | 14.4,20 | |
| $C_4$ | 0,40 | 3.6,40 | 7.2,40 | 14.4,40 | |
| $C_5$ | 0,80 | 3.6,80 | 7.2,80 | 14.4,80 | |
| | $L^-P^+$ Control$^-$ | $L^+P^+$ | | | |

*Table 1:*
*$D_{n(0-4)}$: Dose of irradiation (J/cm2), n: number of treatment.*

*$C_{n(0-4)}$: Concentration of HpD (µg/ml), n: number of treatment.*

*$L^-P^-$: Cell line without Photosesitizer HpD and without Laser irradiation.*

*$L^-P^+$: Cell line Photosensitized with HpD and without Laser irradiation (negative control).*

*$L^+P^-$: Cell line without Photosesitizer HpD and irradiated with Laser (positive control).*

*$L^+P^-$: Cell line Photosensitized with HpD and irradiated with Laser (test).*

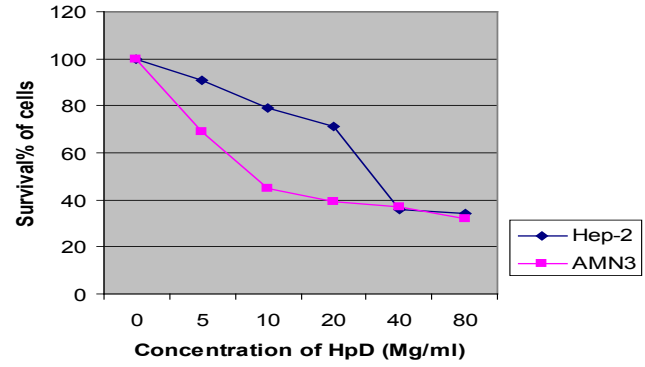
***Statistical analysis:***

The dependent variables were photosensitizer (HpD) and irradiation source (He-Ne laser). First, the data were evaluated to check the equality of variances and scientific errors. To determine the significance of the irradiation alone, the presence of photosensitizer alone and the combination of photosensitizer and laser, the data were analysed by a variance analysis Nova test. The Nova test was chosen for evaluating the significance of all pair wise comparisons with a significance limit of 1%.

## Resultes:

***Hematoporphyrin derivatives Cytotoxicity:***

On long term incubation (72hrs) with Hep-2 and AMN3 cells, Hematoporphyrin D was relatively non-toxic and the proliferation rate of cells was up to 50% up to the concentration of 40µg/ml (Figure 1). On the basis of these data we considered this concentration as nontoxic and used them for photodynamic study.




**Figure(1) Dark cytotoxicity of Hep-2 & AMN3 by HpD**

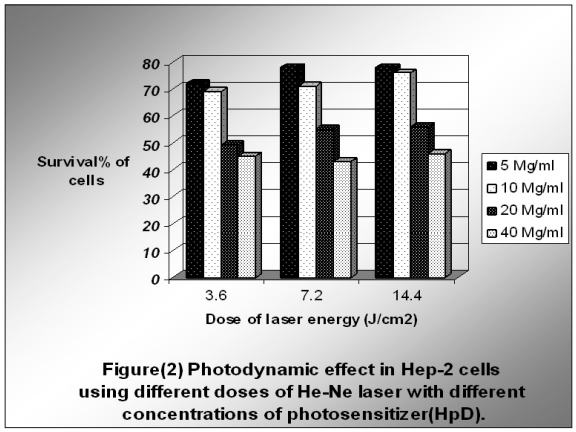
***Photodynamic effect:***

The Hep-2 & AMN3 cells were sensitive to photodamage dependent on the energy of laser applied. Proliferation rate was initially inhibited and later the cells were partially destroyed. The most significant destruction effect ($p > 0.001$) was observed after 24hrs for wells treated with HpD and exposed to (3.6 and 7.2) J/cm$^2$ of laser light (Fig2 & Fig3).

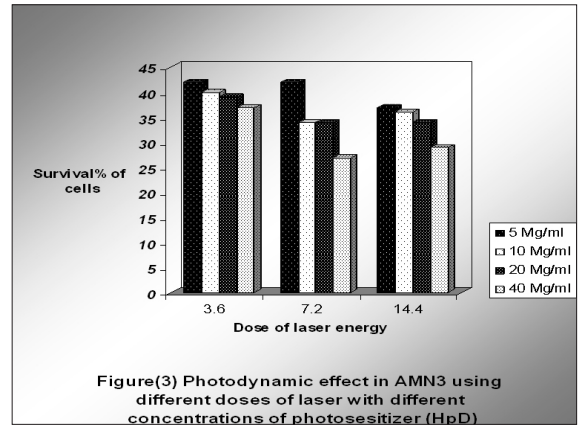
Control experiment showed that irradiation alone did not inhibit cell growth (Fig4). Controls were carried out for all exposure doses and all ages of cell lines. Neither irradiation of the cell lines in the absence of HpD ($L^+P^-$) nor incubation with HpD alone ($L^-P^+$) had a significant effect on the viability of cell lines at any stage.

Significant decreases in the viability of Hep-2 & AMN3 cell lines were only observed when cell line were exposed to both HpD and laser irradiation. There was a significant relationship between the photosensitizer and irradiation doses ($P < 0.001$). The photodynamic action using different energy doses of He-Ne on the viability of cell lines after irradiation with (3.6, 7.2 and 14.4) J/cm$^2$, Cell proliferation rate is shown in Figures 2 and 3. The Hep-2 & AMN3 cell lines were sensitized with HpD for 24 hours before Laser exposure; their concentrations were varied from 5 µg/ml to 80 µg/ml, and then irradiated with a HeNe laser as mentioned above. When Hep-2 cell line was submitted to photodynamic action there

was a considerable reduction in proliferation rate in compare with controle after (3.6, 7.2 and 14.4) J/cm$^2$ of irradiation with HeNe laser, respectively. Similar results of growth inhibition were obtained in AMN3 cell line after (3.6, 7.2 and 14.4) J/cm$^2$ of irradiation with HeNe laser, respectively.




**Figure(2) Photodynamic effect in Hep-2 cells using different doses of He-Ne laser with different concentrations of photosensitizer(HpD).**




**Figure(3) Photodynamic effect in AMN3 using different doses of laser with different concentrations of photosesitizer (HpD)**

***Absorption spectra of HpD***

The emission spectra of HpD were obtained in a Phosphate Buffer Solution (PBS) environment and in (PBS) with Human Serum Albumin (HAS).

Final concentration of HpD was 3µg/ml. It could be seen that the photosensitizer has weak absorbance above 630 nm. However, HpD was undergoing a red shift in its emission maxima around 600 nm when dissolve in PBS in presence HAS. (Fig. 4).

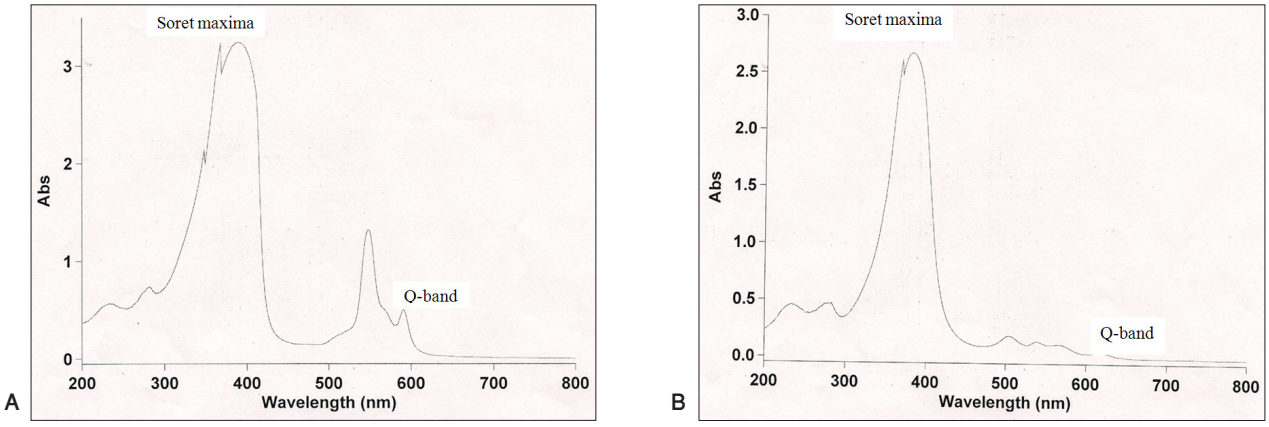



*Figure 4: UV-VIS absorption spectrum of HpD (3 µg/ml): (A) In PBS buffer, and (B) in HAS.*

## Discussion:

The photosensitizing agents used in this study were chemically defined porphyrin. Haematoporphyrin derivative (HPD), its 'purified', and its high molecular weight fraction analogues have been used for many extensive clinical trials as the 'original' sensitizer for the photodynamic therapy of cancer (PDT).(16). Thus, the possible enhancement of PDA effects after Hep-2 & AMN3 cell incubation with HpD was investigated by irradiating cells successively with red light of He-Ne laser (wave length = 632.8 nm).

Haematoporphyrin has several absorbance peaks, such as Soret maxima at 400 nm, and Q-band absorbance in the red spectral region at ~600 nm. Furthermore, its structural formula is porphyrin-derived with additional carboxyl-groups which can be activated using certain biological substances to facilitate attachment to N-terminal amino groups.

Thus for optimal PDA the HpD should have an activate Q-band approaching 600 nm and/or higher.

The interactions with biological substances induce changes in the fluorescence emission spectra of HpD (17). Interaction with HSA causes red shift of HpD emission spectrum. In the emission spectrum of HpD the red shift is even more significant and is followed by the broadening of the second emission band. The emission spectrum of HpD in tumor cells includes both above-mentioned spectral changes (18, 19). Photo bleaching rate of porphyrin molecules localized in protein rich environment would be enhanced due to the interaction of porphyrins with proteins since the proteins accelerate the photo bleaching and photoproduct formation of porphyrin-type sensitizers (19, 8). It is now recognized that an adequate photosensitizing agent should possess specific chemical and biological properties. It should be exhibit minimal dark toxicity, have a substantial absorbance band above 630 nm and an efficient yield of singlet oxygen generation. It should also be a chemically pure substance (20). It looks like, that a detailed understanding of the mechanisms involved in photosensitized cell lines by porphyrin-type photosensitizers is hampered, at least in part, by the heterogeneous chemical composition of them substances (19, 6, 7). It is therefore unlikely that the photosensitizing effects are due to a direct interaction between the porphyrins (e.g. HpD) and irradiation with appropriate laser. Nevertheless of particular interest is the finding that the choice of photosensitizer is of critical importance. It is worldwide accepted that the physico-chemical properties of every photosensitizer are different and determine their accumulation capacity in tumor cells.

The other question arises, what might be explanation of observed phenomenon, that other less purified porphyrin-type photosensitizers exert lower photosensitizing properties. It looks like, that the more purified form of sensitizer was used, the higher photosensitization was reached (for instance HpD) (19, 17, 6). However, this photosensitizing agent exerted their photodynamic effect at non-toxic concentration towards cell lines in vitro. HpD has induced a slight dose-dependent toxicity in AMN3 cells two times greater than that of Hep-2.

In addition, investigation of intracellular concentration of photosensitizers accumulated in Hep-2 & AMN3 cells has indicated that there was a remarkable difference in accumulating potential of HpD. Our data may be support the idea, that the quantity of the porphyrin-type drug accumulated in the cell might determine the efficiency of photosensitization. In any case, the photosensitizing efficiency of HpD observed in Hep-2 & AMN3 cells was evident.

Low-power He-Ne laser irradiation causes a well-defined and energy dependent cell destruction of in vitro cultured cell lines sensitized by HpD. Evidently, the mechanism of this photosensitization was studied by Berki T. et al by measuring with polarization microscopic, scanning electron microscopic and electron-spin-resonance (ESR) spectroscopic parameters(21). The cell damage caused by photosensitization and laser irradiation seems to be a complex process; however the biological membranes seem to be one of the primary targets. The energy of laser light causes rotation and resonance changes of macromolecules and the water molecules, resulting in an increased structural order of the submembraneous components in the living cells, detectable microscopically. During the photosensitization process, the red (632.8 nm) He-Ne laser light, during a one-photon energy activation causes excitation of hematoporphyrin molecules to their triplet state. The excitation of HpD molecules results in a multi-step; free-radical generating effect (21) measured by ESR spectroscopy and studied by the ultrastructural changes of membrane organization and cell shape. Similar effects could be observed on in vitro lipid-water liposome membranes (21).

Light microscope observation for stained cell at the end of the experiments and diagram analyses of cellular damage may be indicated that cell death due to necrotic and apoptotic cell destruction was the predominant biological effect of photodynamic action of HpD in cell lines under study (Fig. 2, 3).
The frequency of cell destruction induced by photodynamic treatment with either photosensitizing agent combain with He-Ne laser irradiation was significantly higher as compared with the effect of

irradiation alone, HpD alone or no treatment at all ($P < 0.01$), these results supported with more recent study of Li et al. (2010) who found that the combination of photosensitizer with He-Ne laser irradiation leads to the effects of PDT was further enhanced; facilitating the apoptosis signals transduction and finally leading to the apoptosis of neoplastic cells may be the mechanism of the anti-tumor activities of photosensitizes mediated PDT (22).

We believe that more attention should be paid to further understanding the Molecular mechanisms of PDA in neoplastic cells. Learning the details of how PDA is activated will be crucial to understanding the effects of PDT in tumors.

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# الفعل الحركي الضوئي لليزر الهليوم-نيون ذي القدرة الواطئة على الخطوط الخلوية ( Hep-2 and AMN3x ) مقترنا بمشتق المحسس الضوئي الهيماتوبورفرين خارج الجسم

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**الخلاصة:**

يعد الفعل الحركي الضوئي باستعمال الطول الموجي المناسب من الاشعة بالاقتران مع مشتقات البورفرين من الاليات ذات القدرة العالية في تحطيم خلايا الاورام السرطانية, حيث ظهر ان مشتق الهيماتوبورفرين يتراكم بصورة انتقائية في هذه الخلايا الخبيثة ومن ثم تحطيمها بواسطة توليد جذور الأوكسجين الأحادية عندما يتم تحفيزانتاجها بواسطة ليزر الهليوم-نيون.

في هذه الدراسة تم استعمال ليزر الهليوم نيون ذو القدرة الواطئة (20 ملي واط) وبطول موجي 632.8 نانوميتر مقترنا مع المحسس الضوئي الهيماتوبورفرين. و الجرع الليزرية تراوحت بين 3,6 – 14,4جول/سنتمتر مربع . وتم ظبط توجيه الحزمة الليزرية بأستخدام صفيحة الزراعة النسيجية المحورة. حيث عرضت الخطوط الخلوية السرطانية لمشتقات الهيماتوبورفايرين لمدة 24 ساعة قبل تطبيق الجرع الليزرية. وكانت تراكيز المحسس الضوئي متدرجة ابتداءاً من 5 ملغم/مل إلى 80 ملغم/مل. أثبتت النتائج بوضوح قدرة الفعل الحركي الضوئي لليزرالهيليوم-نيون مقترنا بالمحسس الضوئي )مشتق الهيماتوبورفرين( في قتل الخلايا السرطانية ، ولم يلاحظ هذا الفعل الحركي الضوئي عند تطبيق الجرع الليزرية او تراكيز المحسس الضوئي كلا على حدة. لذا فإن العلاج باستخدام ليزر الهيليوم – نيون ذو القدرة الواطئة مع مشتق الهيماتوبورفايرين كمحسس ضوئي يفتح الباب واسعا لتطبيق هذه الالية العلاجية ضد الاورام السرطانية على المستوى السريري .