



Photodynamic Therapy for Leiomyosarcoma: *In vitro* study

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Abstract: Leiomyosarcoma (smooth muscle tumor) is a rare type of a malignant neoplasm of smooth muscle. It is a resistant cancer, generally not very responsive to chemotherapy, radiation and surgery. The aim of this study was to evaluate photodynamic therapy (PDT) as a possible basis for treatment using (650 nm) diode laser with methylene blue (MB) as a photosensitizer on leiomyosarcoma (LMS) cell line (L20B) *in vitro*. The cytotoxic effects of 650nm diode laser 0.052 W/cm² (power density) at different exposure time (10, 20, and 30 min) with methylene blue at different concentrations (0.01% ,0.1 % ,1%) was investigated on LMS cell line (L20 B). The cytotoxic effect of laser was increased with increasing irradiation time for a certain concentration of methylene blue. The exposure time 30 min of diode laser with 1% (MB) has the great inhibitory rate (IR) for LMS cells growth which reaches to 80.46% and reducing the proliferation rate (PR) which reaches to 19.54%.

Introduction

Leiomyosarcoma (LMS) is a relatively rare form of cancer, comprising between 5-10% of soft tissue sarcomas, which are in themselves relatively rare. Leiomyosarcomas can be very unpredictable. They can remain dormant for long periods of time and recur after years. It is a resistant cancer, meaning generally not very responsive to chemotherapy or radiation and surgery. Smooth muscle cells make up the involuntary muscles, which are found in most parts of the body, including the uterus, stomach and intestines, the walls of all blood vessels, and the skin. It is therefore possible for leiomyosarcomas to appear at any site in the body (including the breasts); they are most commonly found in the uterus, stomach, small intestine and retroperitoneum (Arnold et al., 2010, Piovanello, et al. 2007).

The conventional methods of treating cancer generally include surgery, radiation therapy, and chemotherapy (Kirn, 2000). Photodynamic therapy (PDT) is a promising modality for the management of various tumors and non-malignant diseases, based on the combination of a photosensitizer that is selectively localized in the target tissue and irradiation of the lesion with visible light, resulting in photodamage and subsequent cell death (Dougherty 1992, Kalka, Merk, Mukhtar 2000, Brown SB, Brown EA, Walker, 2004). Numerous worldwide clinical trials have shown that PDT represents an effective and safe modality for various malignant conditions.

Photodynamic therapy (PDT) has emerged as a noninvasive regimen for cancer treatment thus representing an attractive alternative to conventional therapies (Kessel 2004, Moan, Peng. 2003, Ackroyd, et al., 2001, Detty, Gibson, Wagner. 2004, Allison, et al.,

2004, Nyman, Hynninen, 2004, Berg, et al., 2005, Sharman, van Lier, Allen, 2004).

A photosensitizer absorbs energy directly from a light source, which it may then transfer to molecular oxygen to create an activated form of oxygen called singlet oxygen (1O_2). 1O_2 is extremely electrophilic and can oxidize directly electron-rich double bonds in biological molecules and macromolecules and it is believed to be the main cytotoxic agent related with PDT (Ochsner 1997). The photosensitizer can also get involved in electron transfer reactions initiating radical-induced damage in biomolecules (Baptista, Indig 1998), methylene blue (Fowler, Rees, and Devonshire 1990) is a potential photosensitizer for PDT.

The objective of this in vitro study was to investigate the photodynamic effects of diode laser (650nm) with methylene blue (MB) photosensitizer on leiomyosarcoma cell line.

Materials and Methods

Cell lines and culture conditions

Leiomyosarcoma cell line (L20B) derived from the murine uterine Leiomyosarcoma (LMA) was cultured in minimum essential medium (MEM) (Gibco, UK) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in an atmosphere containing 5% CO₂. L20B cell lines were obtained from Iraqi central health laboratories.

Maintenance and subculture of cell lines

L20B cell lines (passage number 21) were subcultured according to Freshney's method (Freshney 1994).

The photosensitizer

Freshly prepared methylene blue (MB) solutions were chosen in the current study by dissolving the dye powder in maintenance medium MEM, MB diluted with MEM medium without adding (FBS). To get final concentrations between (0.1, 1, 10 mg/ml) then filtered through a sterile 0.22 μm Millipore filter membrane kept protected in dark bottle till being used.

Cytotoxicity Assay (cell growth inhibition assay)

The cell lines were treated firstly with methylene blue using a (96 wells) microtitration plate cell culture technique. The cells were exposed to a range of MB concentrations (0.1 mg/ml, 1 mg/ml and 10 mg/ml) after monoconfluent layer formation. The

following protocol was performed (Freshney 1994):-

Seeding After trypsinization, suspended cells were seeded in a (96 wells) microtitration plate by taking 0.2 ml cell suspension into each well that might contain 10⁴-10⁵ cell/well, growth medium supplemented with 10% (FBS) used for seeding.

Incubation All plates were incubated 24 hours for full cells attachment.

Exposure to the photosensitizer the plates were exposed to different concentrations of a photosensitizer (MB) dissolved at filtered MEM at serial concentrations of MB (0.1, 1, 10 mg/ml), 20 μl of MEM was added to each well of control group, 20 μl of MB + MEM for tested groups (Six replicate wells for each group). The plates sealed with self-adhesive transparent film then re-incubated at 37°C for 1 hr. Then MB+MEM in the tested groups were discarded and cells in the wells were gently washed by adding and removing 0.1 ml sterile PBS twice then MB solutions were replaced by standard growth medium, 20 μl of MEM was added to each well of tested plates.

Laser irradiations L20B cells were exposed to diode laser (Dream lasers, China) at wavelength (λ) 650 nm, continuous wave (CW), the output power 41 mW = 0.041 W. Laser irradiations were performed for different times of exposure (10, 20 and 30 min) respectively. This laser emits light in a collimated beam diameter about 3 mm, Convex lens at focal length 3 cm has been used as a beam expander to produce a diameter of 1 cm to cover the area for each well of plates seeded with L20B cells. The power density was 0.052 W/cm².

Experimental irradiation procedure

The irradiation experiments were done with and without photosensitizer that involved three exposure times (10, 20 and 30 minutes).

For each exposure time the irradiation experiments were done in dark room (six replicate wells for each group) including the following group:-

Group (A): control group cells cultured with growth medium (MEM only).

Group (B1): treated with 1% (MB) + irradiated with laser (L).

Group (B2): treated with 0.1% MB + L.

Group (B3): treated with 0.01% MB + L.

Group (B4): treated with 1% MB (only a photosensitizer).

Group (B5): treated with 0.1% MB.

Group (B6): treated with 0.01% MB.

Group (B7) this group was the one that treated with laser radiation only without adding the photosensitizers (only L).

After the irradiation stage the plates were sealed with self-adhesive transparent film then incubated at 37°C. Cells were then cultured in the dark until the day-5 (Freshney 1994). After 5 days the medium decanted off, finally 20 µl of crystal violet stain was added to the wells and the plates were incubated for 20 minutes at 37 °C then the plates were washed gently with distilled water and left to dry. At the end of the assay the plates of different cells culture were examined by ELISA reader at 492 nm transmitting wavelength. The cells growth inhibitory rate was measured according to (Gao, Dong and Luo 2003).

The optical density of each well was read by using a micro-ELISA reader at a transmitting wavelength on 492 nm (Mahoney; et al., 1989, Freshney 1994). The proliferation rate was measured as the formula according to as following formula (Chumchalova, and Smarda, 2003):-

$$PR\% = \frac{B}{A} \times 100$$

PR=proliferation rate, A= the optical density of control, B= the optical density of test.

While the inhibition rate measuring according to the following formula (Gao , Dong, and Luo 2003):-

$$IR\% = (A-B)/A \times 100$$

IR= inhibition rate, A= the optical density of control, and test respectively.

Statistical analysis

The calculations were carried out according to program statistical package for social sciences SPSS (Version 19) and Microsoft Office Excel (for examination of the specific significant differences among groups). The results (values of PR or IR) were analyzed statistically by analysis of variance (ANOVA) followed by post-hoc Tukey's test. P values < 0.05 they were used for the statistical analysis of the results and to look for the differences which statistically significant at the level (0.05) (Negi, 2008)

Results and Discussion

The diode laser 650nm at 0.052 W/cm² power density was fixed throughout all experiments. The combination of MB with diode laser provides a new PDT protocol that is inexpensive, safe and efficient. Statistical significant differences (p<0.05) in the inhibition rate IR and proliferation rate PR were detected between the 7 test groups (B1,B2,B3,B4,B5,B6,B7) in comparison with control group (A). This experiment showed that the highest IR was detected in the group B1 of which involve cells treated with (1%MB+L) at 30 min of exposure time. This indicates that the irradiation with diode laser in the presence of 1%MB was able to reduce the viability of cancer cells.

The highest concentration, 10 mg/ml or (1%MB+L) produced the lowest percentages of cell viability thus reaching 19.54% PR (proliferation rate) while IR (percentages of inhibition) 80.46% was recorded after 30 minute of exposure time. There is a significant difference was observed in PDT groups in the inhibition rate at 20 and 30 minutes exposure time. This fact indicates that time of exposure is vital in deciding the outcome. However group B1 (irradiated with laser in the presence of MB1%) showed a significant reduction (p<0.05) in the number of viable cells with statistical significance differences in comparison with control group and MB1% group (without irradiation) at 30 min of exposure time, Figure (1 and 2) and Table(1).

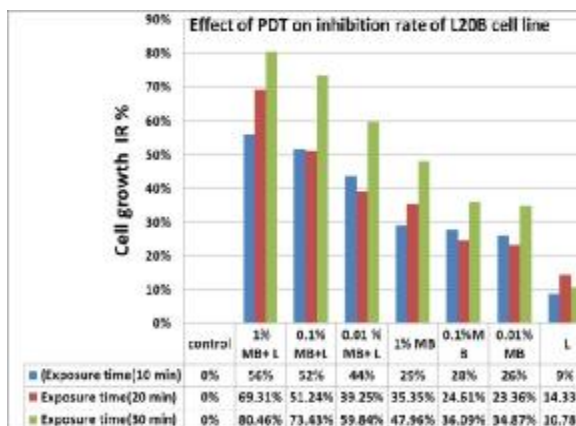


Fig. (1): Cytotoxic effect of diode laser with and without photosensitizer on inhibition rate of L20B cell line.

The cytotoxic effect of the photosensitizer MB in connection with the radiation of a diode laser was shown in Table(1). Statistical analysis shows non-significant differences between experimental groups at 10 minute of exposure time, while there is a significant differences between PDT groups (B1,B2,B3) and control group at 30 min of exposure time. After subjecting the data to statistical analysis

indicated non-significant differences between 20 and 30 minutes of exposure time, while there is a significant difference between (10 min and 20min)and between (10and 30 min)of exposure time, there is a significant difference between PDT group (B1) and control group at 20 min of irradiation but no significant differences were recorded with the other PDT groups B2,B3 as showing in Table (1).

Table (1): Cytotoxic effect of diode laser 650nm with different concentrations of MB at different exposure time(10,20 and 30min)on growth of L20B cell line.

| Exposure Time (Min.) | 10 | 20 | 30 |
|----------------------|-----------------|-----------------|-----------------|
| GROUPS | Inhibition rate | Inhibition rate | Inhibition rate |
| | mean ±SD | mean± SD | mean± SD |
| CONTROL | 0.360 ± 0.146 | 0.642 ±0.086 | 0.640 ±0.162 |
| | | ac | a |
| 1%MB+L | 0.158 ± 0.080 | 0.198 ±0.092 | 0.125 ±0.014 |
| | | bc | bc |
| 0.1%MB+L | 0.174 ± 0.033 | 0.313 ± 0.147 | 0.170± 0.014 |
| | | ac | bc |
| 0.01MB+L | 0.202 ±0.078 | 0.390± 0.161 | 0.257 ± 0.200 |
| | | ac | bcd |
| 1%MB | 0.255± 0.137 | 0.415 ± 0.308 | 0.333 ± 0.196 |
| | | ac | ac |
| 0.1%MB | 0.260± 0.127 | 0.484 ±0.198 | 0.409 ± 0.131 |
| | | ac | ac |
| 0.01%MB | 0.266 ± 0.132 | 0.492 ±0.093 | 0.420± 0.180 |
| | | ac | ac |
| L | 0.330 ± 0.116 | 0.55 ± 0.312 | 0.571 ± 0.275 |
| | | ac | ad |

Mean values ± SD (optical density of L20B cells) followed by different small letters vertically differed significantly (P<0.05) among experimental groups.

The results showed that increasing the exposure time increases the cytotoxic effect of MB against cancer cells growth. In addition to that there is time and concentration- dependent inhibition of cell lines L20B, the effect of PDT increased with increasing the concentration of

the photosensitizer. Based on the data from the in vitro PDT experiments, a likely sequence of events triggering apoptosis is as follows: internalization of MB to endosomesor lysosomes, production of singlet oxygen and reactive oxygen species (ROS) upon irradiation

damage to endosome or lysosomes membranes with concomitant release of proteinases activating a cascade pathway toward apoptosis (Caruso, Mathieu, Reiners, Brunk, et al., 1997).

Cell proliferation tests were performed to further evaluate the effects of PDT, the highest PR was recorded in using laser alone at 10min, 20min and 30 min of exposure time in comparison with the lowest PR which recorded at 30 min exposure time for PDT groups (treated with MB1%+L & MB 0.1%+L) as showing in the Figure(2), these results are in accordance with those reported by Guido Seitz et al 2007 (Guido Seitz et al., 2007) that revealed a reduction of cancer cell proliferation with higher concentrations of the photosensitizer.

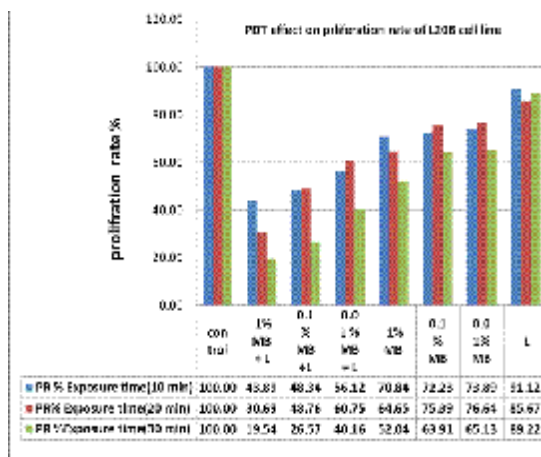


Fig.(2): Cytotoxic effect of diode laser with and without photosensitizer on proliferation rate of L20B cell line

The inhibition rate depends on the concentration of the MB and the time of the exposure, the highest toxic effect was recorded ($P \leq 0.05$) in L20 B cell lines with the highest concentration of MB (10mg/ml) which caused significant inhibition rate (IR) for cell viability. The photosensitizer MB mediated photodynamic effect on mouse LMS cell line L20B. The photosensitizer can be focally excited by laser light in the presence of oxygen using laser light of a wavelength matched to an absorption peak of the photosensitizer, it transfer energy from photons to oxygen molecules, direct killing of tumor cells (China papers 2010) our results goes in line with agreement with (China papers 2010) to determine the cytotoxicity of different concentrations of 5-ALA with different laser dose.

Methyl crystal violet was used to count the number of viable cells, and the optical density was measured under a wavelength 492 nm. The stain was referred to the number of viable cells and the cytotoxic effect was manifested from the inhibition of the growth rate (IR) of the treated cells in comparison to the non-treated cells showing in the Figure (3) and Figure (4).

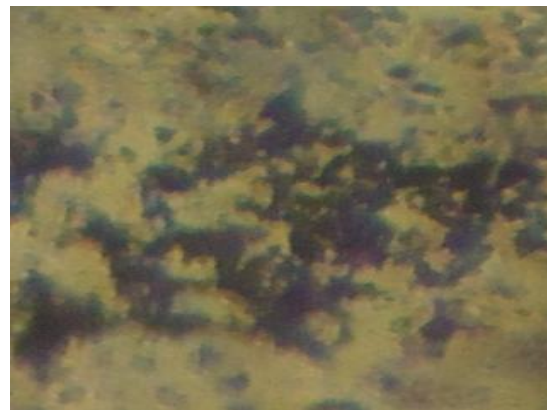


Fig. (3): Microscopic appearance of L20B cell line group (B1) which is exposed to (PDT with 1 %MB) showed patchy of necrotic cells area & few no. of cancer cells (100 X).

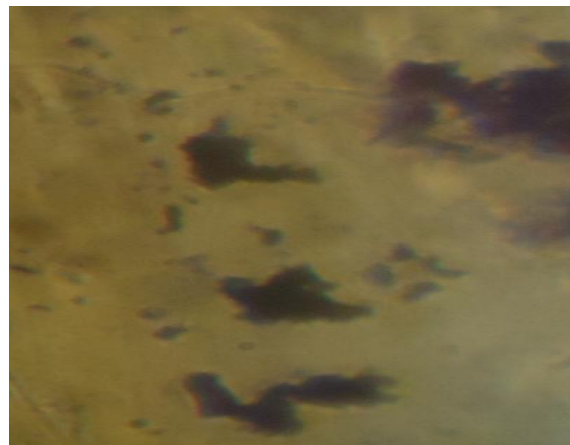


Fig.(4): Microscopic appearance of Confluent monolayer of L20B cell line which is not treated (control) (100 X).

Photodynamic therapy (PDT) is currently studied as a novel treatment approach in various malignancies producing relevant cytotoxic effects by the photochemical generation of reactive oxygen after light activation (Hendrickx, et al., 2003), methylene blue is a potential photosensitizer for PDT with diode laser at power density 0.52 W/cm².

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العلاج الديناميكي الضوئي لخلايا غرن العضلوم الاملس (دراسة خارج الجسم الحي)

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الخلاصة غرن العضلوم الاملس هو نوع نادر من الاورام الخبيثة في العضلات الملساء , فهو سرطان مقاوم , وعموما لا يستجيب للغاية للعلاج الكيميائي ,والاشعاع والجراحة. تهدف الدراسة لتقييم العلاج الديناميكي الضوئي كاساس ممكن للعلاج باستخدام ليزر (650 نانومتر) ليزر الثنائي الصمام مع المثيلين الأزرق كمتحسس ضوئي على خط خلايا سرطان العضلات الملساء ال20 بي خارج الجسم الحي. المواد والطرق:التاثيرات الخلوية السامة لليزر الثنائي الصمام650نانومتر, بكثافة قدرة 0.052 واطم سم2 وفي وقت تعريض مختلف(10,20,30 دقيقة) مع المثيلين الأزرق بتركيز مختلفة(1%,0.1%,0.01%) قد حقق فيها على خط خلايا غرن العضلوم الاملس ال 20 بي. النتائج: التاثير الخلوي السام لليزر يزداد مع زيادة وقت التشعيع لتركيز معين من المثيلين الازرق. الاستنتاجات: وقت التعريض ل 30 دقيقة من ليزر الثنائي الصمام مع 1% من المثيلين الازرق يحتوي نسبة تثبيطية كبيرة على نمو خلايا سرطان العضلات الملساء والتي تصل الى 80.46%مع تخفيض لمعدل الانتشار للخلايا يصل الى 19.54%.