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# The Influence of Photodynamic Therapy on Tumor Cell S180 Pouran Sadat Tayebi<sup>1</sup>, Hamid Reza Zangeneh<sup>1</sup>, Bahman Aghahadi<sup>2</sup>\*

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## ABSTRACT

Today cancer is the second important factor of the death in the world. Most of the cancer patients are treated with standard therapies, including surgery, radiation and/or chemotherapy. These standard therapies are most efficient on the primary tumor, but in the case of disseminated disease, they are often not effective. Treatment of disease that has disseminated from the primary tumor and metastasized to distant sites has promoted the investigation of immunotherapeutic strategies for cancer, and has been a major area of research over the last couple of decades. Chemotherapy and radiotherapy, standard therapies, are the main treatments for majority of cancer patients. Our studies demonstrate that ALA-HMME-PDT has a role in enhanced the quality of life and lengthens survival in mice infected by sarcoma 180. The reported method is hardly implemented but it possible in any clinical situation where PDT is needed. These therapies are most efficient in bearing the tumor in its first process of formation. Currently, the hot topic of discussion and research in the cancer arena is photodynamic Therapy (PDT). This type of therapy is an emerging channel of treatment that is very successful in eradicating cancer, with few side effects. The effectiveness of photodynamic therapy on the sarcoma treating process in mice by using ALA and HMME photosensitizers is investigated by this study. Many factors help us determine effectiveness of PDT including concentration of the sensitizer, absorption of light energy and accessibility of molecular oxygen in the target tissue during light irradiation, besides intrinsic sensitivity of target tissue.

Keywords: Photodynamic therapy, Photosensitizer, Cancer therapy, ALA, HMME, Sarcoma 180

## INTRODUCTION

#### **Background and Significance**

Cancer is the second leading cause of death in the industrialized world. The majority of cancer patients are treated with standard therapies, which involve a combination of surgery, radiation and/or chemotherapy. These standard therapies are most efficient on the primary tumor, but in the case of disseminated disease, they are often not effective. Treatment of disease that has disseminated from the primary tumor and metastasized to distant sites has promoted the investigation of immunotherapeutic strategies for cancer, and has been a major area of research over the last couple of decades.

PDT (Photodynamic therapy) has been considered as a mean to target the host's immune defenses to cure radically tumors. PDT is an established therapy for the cure of different types of cancer. It consists of two elements: light and photosensitizing drug to inflict damage to the tissue of tumor. A possibility of the modern oncology has become richer with developing of PDT. PDT is a new way of treating dicey tumors, using photochemical reaction to induce damage to tumor cells.

PDT is a two-component method of treating. The first component is a photo- sensitizer accumulating in a tumor and delaying in it longer than in healthy tissues. Another component of PDT is a light action. Under the local irradiation of a tumor by light with the special wave length corresponding to a peak of the photosensitizer absorption, a photochemical reaction occurs producing the singlet oxygen and oxygen free radicals acting toxically on tumor cells. Connecting tissues devour the tumor and slowly replace it. Directed and precise radiation of laser can preserve the locality of photodynamical damage of a tumor. The locality of photodynamical damage of a tumor is provided by a photosensitizer ability to be accumulated in tumor tissues and by help of the directed, localized, precise laser irradiation. PDT method has advantages, as compared to traditional methods of treating malignant tumors (surgery, radiation and chemical therapy), by its precise damaging of tumors; by absence of risk of surgical interventions and local, and system complications; by possibility of repeating PDT procedures multiply if necessary; and by combination of fluorescent diagnostic with a healing effect in the same procedure.

During last several years PDT, with the use of different photosensitizers, has been successfully applied for the wide range of malignant tumors, mostly skin ones as well as for tumors of low lip, tongue, oral cavity, larynx, lungs, bladder, gastrointestinal tract, genitals and so on. Several trends of the PDT applications exist:

Due to harmlessness and a good endurance of PDT, it is possible to use it in combination with a surgical intervention, radiation, and chemical therapy. There are no doubts that PDT has obvious economical advantage as a result of the shortening time of treating patients mostly in outpatient conditions. The restricted light regime during a quite long period of time after administration of a photosensibilisator preparation is the only inconvenience of PDT. This is connected with the skin photo sensibility.

#### Photodynamic therapy

Photodynamic therapy (PDT) is a method of selectively depositing a cytotoxic agent, putatively identified singlet oxygen [1,2], in tissue. This result is accomplished by using a photosensitizing agent to transfer energy from light in the visible to near infrared spectrum to molecular (triplet) oxygen, exciting is to its highly reactive single state [3,4]. This method has been used to treat diseased tissue such as cancer, the antigenic vessels in the eye responsible for macular degeneration [5,6], premalignant diseases such as Barrett's oesophagus and skin aliments [6], arteriosclerosis plaques in major arteries [7], and the synovial tissue responsible for arthritis [8,9].

Many factors play role in determining the PDT effectiveness, including sensitivity of target tissue, sensitizer concentration and absorption of light energy and amount of molecular oxygen in the target tissue during irradiation of light. Without oxygen molecular, PDT has no effective cure [10-15].

Raab was the first one, who used light to destroy tissues of tumor. He realized that a combination of acridine orange and light could induce damage to living organisms [16]. Policard in 1920s reported that healthy tissue inherently was less fluorescent that tumor tissue [17]. Ronchese in 1950s [18] tried to distinguish boundaries between tumor tissues and healthy tissues more accurately, using the grade of fluorescent molecules. During recent decays, many scientists using fluorescent differences in tissues (tumor and healthy) and photosensitizing agents, has attempted to discover new method of destroying malignant tissues of tumors; listed below: Figge, et al. [19-21], Rasmussen-Taxdal, et al. [22], Winkelman [23-25], Tappeiner, et al. and Jesionek, et al. [26,27], Dougherty [28,29] and Weishaupt, et al. [30]. Since Photofrin has gained legal status many of the mechanisms by which PDT works have been elucidated, many new photosensitizers have been created [31] and research and development continues.

Potential preferences of PDT are:

- No scarring.
- Highly target tissue necrosis:
  - Localizing the drug to the proliferating tissue.
  - > Selective uptake of photosensitizes to particular tissue layers.
  - > Precise directing of laser light using optical fibers.
- Repeated treatment entails no resistance to treatment.

However, there are some limitations to PDT:

- Light must be directed appropriately to target tissue while we must consider the tissue depth.
- Before any use of PDT, diagnosis must be made.
- It is complex requiring optimal light delivery with laser and collaboration and coordination between clinicians.
- Persistent skin photosensitivity lasting weeks with some photosensitizes limits use.

• Availability of the necessary light sources was a problem. Now low-cost portable light sources are more readily available.

The experience of PDT will vary from person to person. How the treatment is given and the side effects produced vary according to:

- What part of the body is treated?
- The type of photosensitizing drug given.
- The time between giving the drug and applying the light.
- The skin sensitivity to light following treatment.

#### Photosensitisers

Photosensitisers are molecules which have the special property of absorbing light energy and using this energy to carry out chemical reactions in cells and body tissues. The photosensitiser must be able to absorb light in order to work and it places a requirement on its absorption spectrum or colour. For most purposes, we wish to use red light because it penetrates tissues better than blue light.

An ideal PDT photosensitizer should meet several criteria [32,33] : (1) have a known chemical composition and high purity; (2) selectively localize in tumor cells; (3) fast accumulation in target tissues and rapid clearance; (4) maximize tissue penetration by absorbing light strongly at wavelengths 600 nm to 850 nm to increase tissue penetration of light.; (5) possess minimal toxicity toward cells before irradiation and (6) exhibit maximum toxicity toward cancer cells during irradiation. Unfortunately, most of the PDT sensitizer meet these criteria partially.

The only systematic photosensitizer which its indications has been approved is porfimer sodium (Photofrin®). Its main indications are observed in oesophageal and endobronchial cancer [34]. Two days after the medication is used via intravenous injection of 2 mg/kg, irradiation with monochromatic laser light ( $630 \pm 3$  nm) at the dose of 200-300 J/cm<sup>2</sup> would be applied. 4-5 days after the initial injection of porfimer, treatment by light can be repeated if necessary. Patients, being treated by porfimer-PDT avoid light during 2 days after injection. Usually, residual photosensitivity remains for a month after injection; however some photoreaction was described after 9 week [35]. This is the major problem with HPD group sensitizers. Nausea, vomiting, shivering and hypotension are other side effects. Polyhematoporphyrin (Photosan®) is another HPD medication. Both products share the same etherized hematoporphyrin monomer but differ in the concentration of dimeric and monomeric molecules which are almost absent in Photosan® [36]. These molecules have the strongest affinity to skin [37], and thus could be responsible for long lasting skin photosensitivity. The second generation of porphyrin-related photosensitizer is hematoporphyrin monomethyl ether (HMME) has higher selective uptake by tumor tissue, stronger photodynamic effect, lower toxicity, and shorter term of skin photosensitization than HpD according to experimental studies and clinical trials [38,39]. HMME is a porphyrin-related photosensitizer [40], and porphyrin-related photosensitizers are lipophilic and have a high propensity to accumulate in the membranes of intracellular organelles like endoplasmic reticulum and mitochondria [41-44].

#### Mechanism of action of photosensitisers

The mechanisms of cellular destruction induced by the combination of a photosensitizer and light irradiation include several reactions initiated by the absorption of light photons by the sensitizer, causing it to be promoted from ground to the excited singlet state. It then either decays to the ground state or undergoes intersystem crossing to the triplet excited state: in this process, the spin of an excited electron is reversed, and a change in multiplicity of the molecule results. The excited sensitizer can react with surrounding oxygen molecules in two ways. Type I photooxidation involves direct reaction with a substrate by a mechanism mediated by hydrogen or electron transfer, yielding radicals that may react with oxygen to form free oxygen radicals, initiating radical chain reactions. Type II pathway involves energy transfer from the excited sensitizer to nearby molecular oxygen to produce  ${}^{1}O_{2}$ , which initiates oxidation of susceptible substrates. Type I and II pathways occur simultaneously and competitively. Type II reaction; however, appears to play the central role in cytotoxicity, because of the highly efficient interaction of the  ${}^{1}O_{2}$  species with various biomolecules. Singlet oxygen is believed to be the main cytotoxic agent in PDT.

PDT combines the preferential accumulation of the photosensitiser in the target tissue with precise illumination, to provide the selectivity of the treatment. The light penetrates the tissue and causes excitation of the photosensitiser. Activation of the photosensitisers by light is a pre-requisite to successful PDT. The transmission of light through tissue is low at 400 nm because of scattering and absorption by natural chromophores. Light penetration increases with increasing wavelength up to 800 nm. A particular wavelength of light is needed for each photosensitiser to maximise penetration through the tumor and excitation of the photosensitiser.

The therapeutic principle behind PDT lies within the generation of a reactive oxygen species, usually singlet oxygen [45]. The generation of singlet oxygen occurs via Type II photosensitization reactions. The process leading up to these photosensitization reactions begin with the activation (excitation) of the photosensitizer (chromophore).

There are two types of mechanism by which the triplet state photosensitiser can react with biomolecules: Type I consists of electron/hydrogen transfer directly from the photosensitiser, producing ions, or electron/hydrogen abstraction from a substrate molecule to form free radicals. These radicals then react rapidly, usually with oxygen, resulting in the production of highly reactive oxygen species (e.g. the superoxide and the peroxide anions). These radicals then attack cellular targets as described below.

Type II reactions produce the electronically excited and highly reactive state of oxygen known as singlet oxygen. Direct interaction of the excited triplet state photosensitiser with molecular oxygen (which, unusually, has a triplet ground state) results in the photosensitiser returning to its singlet ground state and the formation of singlet oxygen.

In PDT, it is difficult to realize the two reactions types from each other. Both of them are useful and contributing. Damage is dependent on photosensitizer concentration and oxygen tension.

#### Objectives

The purpose of thesis work is to assess the therapeutic effect of PDT in treating sarcoma tumor process in mice. The aims of our research work are to investigate on the following four aspects:

• In the present work to uptake the influence of PDT, we want to study that whether the therapeutic will successful to healing of animal when the tumor is in early stage especially when the diameter of tumor is less than 1 cm.

• To investigate the tumor recurrence occurs or even the mice die during our observation period.

• To explore which PDT conditions are most effective to healing of animals (where least number of mice died, least number of recurrent tumors occur).

• To survey which group treatment the recurrent tumors were smallest and which PDT conditions are least effective.

The current research includes five steps: in the first step, we chose two "Kunming mice" with S180 tumor cells line for use their tumor cells line for groups' mice excrement. In the second step, inject S180 into body of 100 healthy "Kunming" mice will randomly divide into ten groups (n=10). Step three includes ALA and HMME inject to groups treatment as photosensitizers in different dosage. In step four, to survey tumor necrosis we will do histopathological examination, 24 h after PDT as 5 mice in each group will sacrifice. And in the final step in order to evaluation conditions and quality of life of mice we will keep another groups of mice which receive the same photodynamic treatment for six months after PDT.

#### MATERIALS AND METHODS

#### Instruments

In present work, the laser driver/temperature controller model HPD 7404-S (made in U.S.A.) was used (Figure 1a). The laser is designed to provide a simple and reliable method of controlling HPD's semiconductor laser. The HPD7404 is integrated laser system offers a complete solution to visible laser diode. In the HPD 7404 source model it also contains the laser module with a front panel connection. The HPD 7404 can produce the wavelength from 635 nm or 655 nm to 500 mW and from 670 nm or 690 nm to 700 mW. In our experience for measurement of power of laser emit the laser power meter model Coherent FieldMate (made in U.S.A.) was used (Figure 1b).



Figure 1 A) Laser driver/temperature controller model HPD 7404; B) Laser Power Meter Model Coherent FieldMate

Fiber-optic spectrometers are optical instruments designed to measure light intensity in the ultraviolet, visible, and infrared spectral regions. Spectroscopic measurements are used to measure colour, to determine the type and concentration of chemical components in a sample, and to analyze electromagnetic radiation, along with many other applications. A spectrometer generally consists of an entrance slit or aperture, a collimator, a dispersive element such as a grating or prism, focusing optics, and a detector array. In our experiments for finding the spectrum of sample groups a miniature fiber optic spectrometer model USB2000 (made in U.S.A.) was used.

#### Mice

Totally 120 healthy adult white male Kunming mice weighing  $20 \pm 2$  g and aged 2 to 3 weeks were purchased from Department of Experimental Animals, first affiliated Tumor Hospital of Harbin Medical University. They were randomly divided into ten groups (n=10) and also, we considered two mice more in each group, because some mice could die, (for example for bad development of tumors or for bad received irradiated laser and etc.).

The animals were housed five per plastic cages with wood chip bedding in an animal room with a 12 h light and 12 h dark cycle at room temperature ( $25 \pm 2^{\circ}$ C) and allowed free access to standard laboratory diet (purchased from Animal Center, Cancer Research Institute of first affiliated Tumor Hospital of Harbin Medical University, China).

When the sarcoma 180 (S180) tumor cells line in all mice groups treatments reached to around 1 cm in diameter (6 to 8 mm is ideal) we intravenously injected the ALA and HMME to 90 mice divided randomly in 9 groups (Group 2 to 9 (n=10)). Group 1 is control group which no received any photosensitizers. The list of all group mice and specifications in below are listed:

Group 1: No medicine, No laser (Control Group, untreated group).

Group 2: No medicine, Laser

Group 3: ALA (50 mg kg<sup>-1</sup>), No laser

Group 4: ALA (50 mg kg<sup>-1</sup>), laser

Group 5: HMME (10 mg kg<sup>-1</sup>), No laser

Group 6: HMME (10 mg kg<sup>-1</sup>), laser

Group 7: ALA (50 mg kg<sup>-1</sup>), HMME (15 mg kg<sup>-1</sup>), No laser

Group 8: ALA (100 mg kg<sup>-1</sup>), HMME (10 mg kg<sup>-1</sup>), No laser

Group 9: ALA (100 mg kg<sup>-1</sup>), HMME (10 mg kg<sup>-1</sup>), laser

Group 10: ALA (50 mg kg<sup>-1</sup>), HMME (15 mg kg<sup>-1</sup>), laser

After 4 h, injected ALA and 24 injected HMME, the subcutaneous tumors were exposed to 630 nm light delivered by a diode laser at a total light dose of 30 J/cm<sup>2</sup> at a fluence rate of 200 mW/cm<sup>2</sup> with duration irradiation 170 second were induced. This therapy system employs a diode laser as light source and has support fiber which is convenient for clinical application.

#### Implantation of sarcoma 180 tumor cells line

The tumor system studies include Sarcoma 180 (S180) tumor cells line which purchased from Cancer Research Institute of first affiliated Tumor Hospital of Harbin Medical University. Tumor cell S180 inoculations were harvested exponentially growing cultures. S180 is an abbreviation for mouse sarcoma 180, a cell line of mouse cancer cells derived from a soft tissue tumor of a Swiss mouse. S180 is well known for its aggressiveness when transplanted into mice, since the cells can grow everywhere in the mouse and invariably kills the host in a few weeks, if not months. It can be transplanted into many mouse strain and grow very fast. S180 is a sarcoma, a tumor originated from non-epithelial soft tissue. A sarcoma is a cancer of the connective or supportive tissue (bone, cartilage, fat, muscle, blood vessels) and soft tissue. Soft tissue sarcomas are more common in adults than in children [46,47].

Cell solution during the logarithmic growth phase had a density of circa.  $5 \times 10^6$  cells mL<sup>-1</sup>. After a week later, ascites (liquid in the abdominal cavity) will be developed in the mice. We can see that the abdomen is enlarged.

When the abdomen is obviously enlarged (but not extremely enlarged, because it will be too late, and there will be intra-abdominal haemorrhage), we killed the mice, (we can open or not open the abdominal cavity) aspirate the liquid

out from the abdominal cavity. If the ascites is clear and not bloody, we can use it for injection to the other mice. If the ascites is reddish or red, it means that there are many blood cells mixed in the ascites; we should discard that mouse, and take the other mouse. Even we need to repeat the experiment once again (for this reason, we considered two mice, just for prevention of the worse situation).

After obtained the tumor cells line, we dilute the liquid with saline (Sodium chloride 0.9%) in a ratio 1:4 (1 volume of ascites, 4 volumes of saline). Injected 0.2 ml of this diluted suspension subcutaneously into new all healthy group mice on the back of the right groin area (the axillary region). That is very important which we injection tumor cells subcutaneously in the back of mice, closer to the buttocks and thigh, and try to get a tumor far from the important visceral organs in the abdomen. Because if we made a transplanted tumor in the front side of abdomen, especially if the tumor is growing in an area close to the liver or intestine, it is very easy to get serious photodynamic injury to those important organs, and sometimes, leading to un-wanted death of the animal.

#### Statistical evaluation

All measured values are presented as mean  $\pm$  SE. Student's t test with Welch's correction was used for comparison between groups in all the experiments with P values of 0.05 representing statistical significance.

#### **RESULTS AND DISCUSSION**

#### Groups 1 to 4

### Assessment of tumor growth for Group 1 to 4

Beginning the first week after the implantation of the S180 sarcoma cells line, tumor size was assessed in the mice every other day measuring by a slide gauge. The tumor's volume (V) was then computed using the formula [48] (1):

$$V = \frac{1}{2} a b^2 \tag{1}$$

Where, a is the maximum horizontal diameter of tumor and b is the maximum vertical diameter of tumor.

About one week later (6-8 days) after injection S180 sarcoma cells line, the tumor was growing in back area. We chose the mice with a tumor in appropriate diameter less than 1 cm (not too small, and not too large), divide them randomly into experimental and control groups. The mean tumor size values for animal Groups 1 to 4 which calculated. The results present the average tumor size before PDT, ranging from 0.22 cm<sup>3</sup> (at Groups 1 and 2) to 0.26 cm<sup>3</sup> (at Group 4).

For PDT treatment in all group experiments the setup system are designed (Figures 3-9) as the following three aspects a) Sarcoma 180 was injected on back area of healthy mice, b) after one week, photosensitizers ALA was administered for Groups 3 and 4, c) diode laser irradiated on tumor area after 4 h injected ALA (this setup also for HMME in next stage applied and diode laser irradiated on tumor area after 24 h injected HMME).

#### ALA administration for Groups 3 and 4

Prior to PDT, ALA was purchased from Beijing Yingfa Kangmei Technological Development Co., Ltd. ALA was obtained as a powder and reconstituted to a concentration of 354 mg/1.5 ml in a 9% solution of sterile saline kept in dark place at 4°C to 8°C according to the manufacture's recommendations. ALA was administered in a dose 50 mg kg<sup>-1</sup> body weight (BW) by injected 0.2 ml of diluted suspension on the back of the right groin area of Groups 3 and 4. The animals were then kept in dark room to prevent the development of sunlight-induced dermatitis during the experiment period with access to food and water for 4 h.

Before PDT treatment to prevent movement during irradiation and optical measurements, the animals were mechanically fixed on a specially designed Plexiglas holder and restrained with tape without anaesthesia during the treatment.

#### Histopathological examinations for Groups 2 to 4

To evaluate the photodynamic killing effect on tumors (mainly, the extent of necrosis of the tumors), each groups treatment (Group 2 to 4) were divide two-part group which each part group was included 5 mice. So, in this stage, after 24 h PDT; 5 mice from the first part group treatment (Group 2 to 4) were sacrificed and taken a small sample to provide the microscopy slides.

The second part group treatment 2 to 4 which received the same photodynamic treatment, we kept them for six month to follow if tumor recurrence occurs or even the mice die during our observation period. During this period, the mice free access to food and water and record all the measurement data carefully for statistical analysis, and to summarize which PDT conditions are most effective (where least number of mice died, least number of recurrent tumors occurred, and the recurrent tumors were smallest), and which conditions are least effective. The results of condition of mice after PDT will be explain fully in future chapters.

#### Groups 5 to 6

#### Assessment of tumor growth for group 5 to 6

After one week injection S180 sarcoma cells line to animals, we divide them randomly into experimental groups 5 and 6 for PDT treatment with HMME. The mean tumor size values for animal Groups 5 and 6 which showed that the average tumor size before PDT, ranging from 0.18 cm<sup>3</sup> (at Group 5) to 0.27 cm<sup>3</sup> (at Group 6).

#### HMME administration for groups 5 and 6

The photosensitizer HMME was obtained from Shanghai Red and Green Photosensitizer Institute Co, Ltd. The concentration of HMME was 100 mg/10 ml and it was dissolved in sterile saline 9%. A stock solution kept in dark with storage condition -18°C to -20°C according to the manufacture's recommendations. HMME was administered at a dose of 10 mg kg<sup>-1</sup> BW by injected 0.2 ml of diluted suspension on the back of the right groin area of Groups 5 and 6. After injection the animals were kept in darkness for 24 h with accessible to food and water.

Similarly, like PDT treatment for Groups 2 to 4; in this stage, also before PDT treatment for Groups 5 and 6 to prevent movement of mice during experiments, the animals were mechanically fixed on a specially designed Plexiglas holder and restrained with tape without anaesthesia during the treatment. In all cases, the irradiation area was on back in tumor area, posterior to the right groin area. This location was selected to avoid possible damage to internal organs and to provide repeatable positioning of the probe. As described in last chapter, for PDT treatment of all animal groups' treatment; the effective dose 30 J/cm<sup>2</sup> with duration irradiation 170 second were considered.

Only Group 6 has received diode laser and like last stage the optical probe was placed 8 mm above the skin and fixed in place. The treatment area was irradiated with 635 nm light from a laser diode driver HPD 7404 as mentioned in 3.3.1. The power output of the laser was monitored with power laser meter to ensure that the desired irradiance at the skin surface was maintained. Each animal was used for one experiment on each rear flank.

#### Histopathological examinations for group 5 and 6

In this part of our experiment, like before each groups treatment (Group 5 and 6) were divide two-part group (each part group was included 5 mice). After 24 h PDT; 10 mice from Group 5 and 6 were sacrificed and taken a small sample to provide the microscopy slides.

To assess the photodynamic killing effect on tumors, the microscopy slides by a pathologist who was strangled with the control and test groups were reviewed. The outcomes of histopathological examinations for Groups 5 and 6 after PDT were explained in next chapters.

As mentioned above, the subsequent mice in group treatment 5 and 6 which received the same photodynamic treatment, we kept for six month to follow if tumor recurrence occurs or even the mice die during our observation period. During this stage, the mice free access to food and water and record all the measurement data carefully for statistical analysis, and to summarize which PDT conditions are most effective or which conditions are least effective. The results of condition of mice after PDT completely will be explained in next chapters.

### Groups 7 to 10

In this part of research, we combination two photosensitizes ALA and HMME in different dose and administrated for Groups 7 to 10.

#### Assessment of tumor growth for group 7 to 10

One week later after injection S180 sarcoma cells line, the tumor was growing in back area. Like before experiment we chosen the mice with a tumor in appropriate diameter less than 1 cm and divided them randomly into treatment Groups 7 to 10 (n=10). The mean tumor size values for animal Groups 7 to 10 which with standard deviation (SD) showed that the average tumor size before PDT, ranging from 0.17 cm<sup>3</sup> (at Groups 1 and 2) to 0.24 cm<sup>3</sup> (at Group 7).

#### Combination ALA and HMME administration for mice groups 7 to 10

One of most important aim of present work is that to investigation the quality of life and affects the photodynamic therapy by combination two type of photosensitizers (ALA, HMME) in animals. In this step of our experiment, we combined two photosensitizes ALA and HMME in difference dose and difference time to injection in animal groups seven to ten.

For administration two photosensitizers ALA and HMME, first we injected 0.2 ml of diluted suspension HMME in a dose of 10 mg kg<sup>-1</sup> BW on the back of the right groin area of Groups 8 and 9 and kept them in dark room with free access to food and water for 20 h. After 20 h since injection HMME, ALA was administered in a dose of 100 mg kg<sup>-1</sup> BW by injected 0.2 ml of diluted suspension on the back of the right groin area of Groups 8 and 9. The animals in these groups again were then kept in dark conditions with access to food and water for 4 h. Similarly, for Group 7 and 10, HMME were injected in a dose of 15 mg kg<sup>-1</sup> BW by injected 0.2 ml of diluted suspension on the back of the right groin area and kept in dark room with free access to food and water for 20 h and then ALA was injected in a dose of 50 mg kg<sup>-1</sup> BW by injected 0.2 ml of diluted suspension on the same area and kept them in dark place with free accessible to food and water for 4 h. The parameters were chosen to fall within the range of doses and drug-light intervals reported in the literature as effective in preclinical and clinical studies.

Similarly, last chapter, in this stage also before PDT treatment for Groups 7 to 10 to prevent movement of mice during experiments, the animals were mechanically fixed on a specially designed Plexiglas holder and restrained with tape without anaesthesia during the treatment. In all cases, the irradiation area was on back in tumor area, posterior to the right groin area. As described in before, for PDT treatment of all animal group treatment; the effective dose 30 J/cm<sup>2</sup> with duration irradiation 170 second were considered.

In this part of examination, Groups 7 and 8 have no received diode laser and Groups 9 and 10 irradiated with effective dose 30 J/cm<sup>2</sup> and duration irradiation 170 second. The optical probe was placed 8 mm above the skin and fixed in place. The treatment area was irradiated with 635 nm light from a laser diode driver HPD 7404 as mentioned in 3.3.1. The power output of the laser was monitored with power laser meter to ensure that the desired irradiance at the skin surface was maintained.

#### Histopathological examinations for mice groups 7 to 10

In this stage for histopathological examinations like before, each groups treatment (Group 7 to 10, n=10) were divide two-part group (each part group was included 5 mice). After 24 h PDT, 5 mice from each Group 7 to 10 were sacrificed and taken a small sample to provide the microscopy slides. The processing method to provide microscopy slides described in 3.5.9.1.

To evaluate the photodynamic killing effect on tumors, all the microscopy slides from Groups 7 to 10 by a pathologist were reviewed. The results of histopathological examinations for these groups after PDT wholly were explained in next chapter.

As mentioned above, each groups treatment (Group 7 to 10, n=10) were divide two-part group which the first part group treatment 7 to 10 (n=5) were applied for histopathology examinations and the second part group treatment which received the same photodynamic treatment, we kept them for six month to follow if tumor recurrence occurs or even the mice die during our observation period. During this period, the mice free access to food and water and record all the measurement data carefully for statistical analysis, and to summarize which PDT conditions are most effective (where least number of mice died, least number of recurrent tumors occurred, and the recurrent tumors were smallest), and which conditions are least effective. The results of condition of mice after PDT will be explain fully in next chapter.

#### Influence of photodynamic therapy on all mice groups treatment

In present work, we studied the effect of two photosensitizers ALA and HMME on body of mice and the spectrum of the endogenous fluorescence of tissue when illuminated by blue light (LED) with used spectrometer model USB2000 were obtained. All basis spectra are derived from measurements performed *in vivo*. The fluorescence of skin of mice Group 2 (no received medicine) after received diode laser was shown in Figure 2. As shown in this figure, the first intensity is belonging to blue light in 405 nm and second is related to fluorescence of body of mice in normal tissue at 483 nm. The comparison of spectra of normal tissue area and tumor area of mice group 2 after received diode laser were shown in Figure 3. This spectrum clearly shows that the intensity of tumor area (489 nm) is bigger than normal tissue (483 nm).



Figure 2 The fluorescence of normal skin of mice Group 2 after received diode laser



Figure 3 The comparison of spectra of normal and tumor tissue of mice group 2 after PDT



Figure 4 The fluorescence of tumor tissue of mice group 4 after PDT



Figure 5 The fluorescence of normal tissue of mice after PDT received HMME



Figure 6 The comparison of spectra of normal and tumor tissue of mice group 6 (after PDT)



Figure 7 The comparison of spectra of normal and tumor tissue of mice group 8 (before PDT)



Figure 8 The comparison of spectra of normal and tumor tissue of mice group 9 (after PDT)



Figure 9 The comparison of spectra of normal and tumor tissue of mice group 7 (before PDT)



Figure 10 The comparison of spectra of normal and tumor tissue of mice group 10 (after PDT)

The spectrum which was shown in Figure 4 is belonging to Group 4 (received ALA=50 mg kg<sup>-1</sup>) in tumor area after PDT (received ALA). The maximum culmination in 488 nm represented tumor area which the concentration of ALA after 4-h injection was the maximum rate and the minimum peak (635 nm) represented the other normal tissue area where the concentration of ALA in that area was at least. Also, the spectrum of treatment group 6 (received HMME=10 mg kg<sup>-1</sup>) in normal tissue area after PDT shown in Figure 5. In this figure, the small peak was appeared in 622 nm which is belong to low concentration of HMME in normal tissue. In order Figure 6 represented the comparison of spectra of normal and tumor tissue of mice group 6 after PDT (received HMME). In this figure, it is clearly that two peak 625 nm and 685 nm are resulting of concentration of HMME in tumor area of mice.

The spectrum Group 8 (received ALA=100 mg kg<sup>-1</sup> and HMME=10 mg kg<sup>-1</sup>) before PDT was shown in Figure 7 and the fluorescence of Group 9 after PDT was shown in Figure 8. The Figure 7 clearly shows that normal and tumor area of skin mice Group 8 which exactly after 24 h and 4 h injected HMME and ALA (before irradiated diode laser) was obtained respectively. Also, the Figure 8 shows that normal and tumor area of skin mice Group 9 (received ALA=100 mg kg<sup>-1</sup> and HMME=10 mg kg<sup>-1</sup>) after 2 h irradiated diode laser (after PDT).

The fluorescence of skin of mice Group 7 (received ALA=50 mg kg<sup>-1</sup> and HMME=15 mg kg<sup>-1</sup>) before received diode laser was shown in Figure 9. Also, the spectrum of skin of mice group 10 (received ALA=50 mg kg<sup>-1</sup> and HMME=15 mg kg<sup>-1</sup>) after PDT was shown in Figure 10.

Normal tissue area of healthy mice was shown in Figure 11 a). Red regions represented red blood cells in the vascular without any tumor cell. Figure 11 b) show that tumor tissue area of mice Group 1 which no received any medicine or irradiated laser. In this figure, as can be seen almost all of tumor cells are circularity and vital tumor cells were observed. The tumor cells are black and in all area fully distributed.



Figure 11 Normal tissue of healthy mice and tumor cell of mice group 1 before PDT stained with hematoxylin-eosin. a) Normal tissue area of healthy mice with red blood (RB) cells in the vascular. b) Tumor tissue of mice group 1, vital tumor (VT) with lager dark cell nuclei (NT score 1)

Microscopic section of tumor in Groups 2, 3 and 4 were shown in Figure 12 a) Group 2 (No medicine, Laser), VT cells fully distributed in surface with lager dark cells nuclei (NT score 1). b) Group 3 (ALA (50 mg kg<sup>-1</sup>), No laser), VT cells clearly can be seen with lager and small dark cells nuclei and also, we can see a few Necrotic tumor (NT) nearly less than 10% of total cells (NT score 1). c) Group 4 (ALA (50 mg kg<sup>-1</sup>), laser) large VT cells showing in the upper right part and some small VT cells in the lower left part, also the NT cells can be seen in the medium part which is more than 20% of total cells (NT score 2). This amount of NT cells may be resulting of ALA after PDT.

Microscopic section of tumor in Groups 5 and 6 were illustrated in Figure 13 a) Group 5 (HMME (10 mg kg<sup>-1</sup>), No laser), large VT cells showing in the upper part and small vital tumor (sVT) cells in the lower left part. In the medium right part, we can see the NT cells about 20% of total were observed (NT score 1). In some area, red blood cells in the vascular were observed. Also, the tumor cells with blood cells were mixed. b) Group 6 (HMME (10 mg kg<sup>-1</sup>), laser), the microscopic section of this group showing sVT cells in whole area and tumor cells are in the early stage of necrosis. Nucleus becomes shrunken, dense, and deeply basophilic mass. The psychotic nucleus may break up into numerous small basophilic particles. Also, NT cells in overall were diffused (NT score 2).



Figure 12 Tumor cells of groups 2, 3 and 4 after 24 h PDT. a) Group 2, VT cells completely spread in surface with lager dark cells nuclei with NT score 1. b) Group 3, VT cells showing with lager and small dark cells nuclei with NT score 1. c) Group 4, large VT cells presentation in the upper right part and small VT cells can see in the lower left part. NT cells in the middle part were observed with NT score 2. Arrow indicates border between vital and necrotic tumor.



Figure 13 Microscope sections of a tumor of groups 5 and 6 after 24 h PDT. a) Group 5, lager VT and sVT in Upper and lower part with lager and small dark cells nuclei respectively. NT cells can be seen in middle right part with NT score 1. Arrow indicates border between vital and necrotic tumor. b) Group 6, sVT cells are in the early stage of necrosis and NT in on the whole of surface were observed with NT score 2

Figure 14 the microscopic section of tumor in Groups 7 and 10 were shown. a) Group 7 (ALA (50 mg kg<sup>-1</sup>), HMME (15 mg kg<sup>-1</sup>), No laser), it is clearly that the VT cells were diffused on the whole of tumor and the medicine without laser can't to necrosis of tumor. We can see plenty of tumor cells with large and deeply basophilic nucleus (NT score 1). b) Group 10 (ALA (50 mg kg<sup>-1</sup>), HMME (15 mg kg<sup>-1</sup>), laser), this figure clearly show that combination of two photosensitizers ALA and HMMH with dose 50 and 15 mg kg<sup>-1</sup> (respectively) play important rule in necrosition of tumor cells. As we can see in this figure plenty of necrosis cells after 24 h PDT (NT score 5). Normal cell appearance with coagulated cytoplasm, nucleuses break up into numerous small basophilic particles. Vascular tissue and inflammatory cells are not being observed. Still we can see some sVT cells on some part of surface. The white area represented adipose tissues.



Figure 14 Microscope sections of a tumor of groups 7 and 10 after 24 hours PDT. a) Group 7, VT cells on the whole of tumor were diffused. The photosensitizers without used laser didn't kill the tumor cells and there are no any necrosis cells on the tumor. b) Group 10, necrosition of tumor cells fully on whole of tumor clearly were observed (NT score 5). Arrows indicates some small part sVT cells which still on the tumor were existed.

Microscopic section of tumor in groups 8 and 9 after 24 h PDT were illustrated in Figure 15. a) Group 8 (ALA (100 mg kg<sup>-1</sup>), HMME (10 mg kg<sup>-1</sup>), No laser), in this figure we can see plenty of tumor cells with large and deeply basophilic nucleus with sVT cells (NT score 1). b) Group 9 (ALA (100 mg kg<sup>-1</sup>), HMME (10 mg kg<sup>-1</sup>), laser), the microscopic section of this group showing lager VT cells in upper part and in lower part clearly, we can see necrotic tumor with NT score 3. This NT score is resulting of PDT after 24 h which is more than half of tumor cells necrosis were observed.



Figure 15 Microscope sections of a tumor of groups 8 and 9 after 24 hours PDT. a) Group 8, sVT cells on the whole of tumor were diffused. The photosensitizers without used laser didn't killed the tumor cells and there are no any necrosis cells on the tumor (NT score 1). b) Group 9, necrosition of tumor cells after 24 hours PDT in half lower part clearly were observed (NT score 3).

Table 1 Schema of extent of necrosis groups before and after PDT

Group	1	2	3	4	5	6	7	8	9	10
NT Score	1	1	1	2	1	2	1	1	3	5

The NT score for Groups 2 and 4 by the pathologist was defined as value 2 and it means the PDT for these groups can necrosis of cells in 20 to 40 percentages of total cells. The maximum of necrosis in Group 10 was observed. In this group after 24 h PDT, more than 80% was killed. It means the animal in this group compare with the other groups can living for long time. Results of histopathological examinations for Group 10 (ALA (50 mg kg<sup>-1</sup>), HMME (15 mg kg<sup>-1</sup>), laser) clearly demonstrated that compare to other dose groups the combination of ALA and HMME in dose 50 mg kg<sup>-1</sup> and 15 mg kg<sup>-1</sup> (respectively) for killing the tumor was significant. But still we can't to predict that the animal of this group can live in normal situation because of inside of body of mice the tumor cells are existent and from point of pathology and genetic science if the animal only has one tumor, it maybe will kill the mice in future time. For this reason, we follow up other part mice groups for proper period six months and the living conditions of mice after PDT were studied.

#### Living conditions of mice after PDT

The other part groups of mice which received the same photodynamic treatment, we kept them for six months to evaluation of tumor recurrence occurs or even the mice die during observation period. We kept the mice groups in a normal animal room and far from windows to avoid direct irradiation and strong lamps. All measurement data such as recurrent tumors, tumor size, weight of mice, and number of mice died carefully were recorded. The animals of part groups after PDT (Group 2-10, each Group 5 mice) were housed five per plastic cages with wood chip bedding in an animal room with a 12 h light and 12 h dark cycle at room temperature ( $25 \pm 2^{\circ}$ C) and allowed free access to standard laboratory diet.

Results showed that all mice in control group and treatment groups were dead approximately in date of fifth weeks after PDT except Groups 9 and 10. In Group 9, all 5 mice were dead as the last mice was dead in thirteenth weeks after PDT whereas in Group 10, only 2 mice were dead as the last one was dead in twenty first weeks after PDT. The other 3 mice of Group 10 extant after six months PDT were no any tumor on the surface of body of mice observed and its look as healthy mice with normal activation. We sacrificed the extent mice of Group 10 after end of PDT pried.

In fact, in present work, the influence of photodynamic therapy on group 10 clearly was observed as only 2 mice after six month were dead whereas the other group all mice because of the tumor gradually growth up was dead. The results presented in this study indicate the tumor therapeutic for Group 10 was much better than other group and combination of ALA and HMME in dose 50 mg kg<sup>-1</sup> and 15 mg kg<sup>-1</sup> was highly effective respectively. In order the condition of mice Group 10 compare with other group was improved as mice of this group was long lived. So, with performance of this methods at least we able to improve the life condition and delay of mice death.

The average weight of mice Group 2 to 10 after PDT weekly recorded and results for period six months were shown

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in Table 2. Results in this table show that the minimum weight is belong to Group 2 with an average  $34 \pm 2.3$  g and the maximum weight is belonging to Group 10 with an average  $58 \pm 2.7$  g. In fact, the average weight of mice Group 1 to 8 approximately was for 35 days after PDT and only mice Group 9 and 10 the average weight was maximum respectively which was resulting of PDT.

			0	0		,		0		
Group	1	2	3	4	5	6	7	8	9	10
Weight (g)	$35 \pm 2$	$34 \pm 2.3$	$35 \pm 1.8$	$39 \pm 2.1$	$34 \pm 2.6$	$41 \pm 2.3$	$36 \pm 2.8$	$35 \pm 2.8$	$47 \pm 3.1$	$58 \pm 2.7$

All mice in control group and treatment groups were dead approximately in date of fifth weeks after PDT except Groups 9 and 10. So the tumor size of animals in all group weekly till fifty weeks after PDT were measured. The last one of mice in Group 9 in thirteenth weeks after PDT was dead which the tumor size finally reached to 0.83 cm<sup>3</sup> whereas in group 10 only 2 mice were dead which the last one was dead in twenty first weeks after PDT. The tumor size for last one dead mice of group 10 lastly reached to 0.12 cm<sup>3</sup>. The other 3 mice of this group no tumor observed after six months PDT and it seemed that was rustling of PDT, so we can say at least the PDT for group10 can successful to healing of 3 of total 5 mice of this group. So, it means the PDT for group 10 improves the condition of life and recovered of healthy of mice as amount 60%.

#### CONCLUSIONS

In the present work, we studied effect of Photodynamic therapy on sarcoma 180 processes in mice when two photosensitizers 5-Aminolaevulinic acid (ALA) and Hematoporphyrin monomethyl ether (HMME) were applied. The research was focused on these two photosensitizers especially when ALA and HMME were combined and injected to mice in different dose. This study first work in the clinical research results for ALA-HMME-PDT in china. It has indicated that ALA- HMME-PDT is an effective treatment of sarcoma cancer such as S180 tumor cell disease. It has some special advantage, including non-traumatic for healthy tissues, easy implementation, and suitability for healing of sarcoma when the tumor is in early of stage. It is a new alternative modality for sarcoma cancer therapy and of course it needs further studying. The important conclusions that can be drawn from this dissertation and the results for all group treatment are as follows:

1) The effectiveness of PDT is determined by many factors. Besides intrinsic target tissue sensitivity, the other important factors include concentration of the sensitizer, absorption of light energy and availability of molecular oxygen in the target tissue during light irradiation. In this thesis work for each photosensitizer we consider two concentrations as ALA in dose 50 mg kg<sup>-1</sup> and 100 mg kg<sup>-1</sup> and HMME in dose 10 mg kg<sup>-1</sup> and 15 mg kg<sup>-1</sup> were used.

2) The influence of photodynamic therapy on groups treatment 2 (No medicine, Laser), 3 (ALA=50 mg kg<sup>-1</sup>, No laser), 5 (HMME=10 mg kg<sup>-1</sup>, No laser), 7 (ALA=50 mg kg<sup>-1</sup>, HMME=15 mg kg<sup>-1</sup>, No laser) and group 8 (ALA=100 mg kg<sup>-1</sup>, HMME=10 mg kg<sup>-1</sup>, No laser) shown that PDT can't affected on these groups because of Group 2 no received medicine and the other groups no received diode laser. The necrosis tumor score system for these groups defined by pathologist as NT score=1 and it mean the PDT for these groups can't kill the tumor cells. In order, one month after PDT all mice of these groups were dead.

3) Results of groups treatment 4 (ALA=50 mg kg<sup>-1</sup>, laser) and Group 6 (HMME=10 mg kg<sup>-1</sup>, laser) shown that PDT for these groups were killed only in maximum ranging 21% to 40% of total tumor cells as NT score=2 defined by pathologist for these group. Unfortunately, PDT can't improve quality of life of group 4 and 6 and tumor recurrences were occurred. All mice of these groups, six weeks after PDT were dead.

4) The effects of PDT on Group 9 (ALA=100 mg kg<sup>-1</sup>, HMME=10 mg kg<sup>-1</sup>, laser) demonstrated photodynamic therapy for group treatment 9 in maximum ranging 41% to 60% of total tumor cells were killed as NT score=3. The quality of life compare with previous group was better but unfortunately again tumor recurrence was occurred. The last mice of this group, thirteenth weeks after PDT were dead. So, we can to understudying which the combination of ALA and HMME in dose 100 mg kg<sup>-1</sup> and 10 mg kg<sup>-1</sup> (respectively) were not successful to improve the quality of life but we can say in some dose like this PDT can only delay of mice death for few weeks later.

5) The special effects of PDT on Group 10 (ALA=50 mg kg<sup>-1</sup>, HMME=15 mg kg<sup>-1</sup>, laser) was observed. The quality of life for this group was much better than other groups as in this group ranging from 81% to 100% of total tumor cells were killed. The NT score=5 for this group was defined by pathologist. Furthermore, in this group totally two mice were dead which the last one on twenty first weeks after PDT was dead. Although from this group, two mice

were dead but three mice recovered and end of six months after PDT, no any tumor observed. So, the effect of PDT on Group 10 was successful and it improved the conditions of life and lengthens survival.

#### RECOMMENDATIONS

The recommendations for future works to make more progress in the related research field are listed below:

1) For destroy of tumor cells it would better to consider the tumor in early stage as less than 1 cm in diameter (6-8 mm is idea).

2) Light power density shout not exceed 200 mW/cm<sup>2</sup>, because too high power density will produce heat injury to the irradiated tissue. Induce radiation with power density 100 mW/cm<sup>2</sup>  $\sim$  200 mW/cm<sup>2</sup> in research experiment or clinical treatments are useful.

3) The treatment schedule of such combination ALA-HMME-PDT will have to be determined, as well as we can use these photosensitizers in different dose with light power density less than 200 mW/cm<sup>2</sup>.

4) Finally, many other new photosensitizers like  $\text{TPPS}_4$ , m-THPC and  $\text{SnET}_2$  will useful to treat S180 or any other solid tumor which the future studies are request.

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