Determination of Infrared Laser Energy Dose for Cancer Cells Inactivation as a Candidate of Photodynamic Therapy

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Abstract. The aim of this research is to find out the effect of GaAlAs diode laser 808 nm exposure on MCF-7 breast cancer cells *in vitro* with and without the addition of a photosensitizer. Methylene blue ($C_{16}H_{18}N_3SCl$) with concentration of 2 µM is used as the photosensitizer based on the preliminary results of cytotoxicity assay. Energy dose is set in the range of 23,043 to 322,062 J/cm^2. Laser exposure with the addition of the photosensitizer generates the highest percentage of cell death of 20.80% at energy dose of 184,344 J/cm^2 and cell death begin to decrease at energy dose above this value. This behavior is likely caused by photodegradation and photobleaching effect of the photosensitizer due to the longer exposure time. However, the percentage of cell death without the addition of photosensitizer is increased proportionally to the increase of energy dose and achieved 32,45% cell mortality at 299,559 J/cm^2. This study not only shows that the exposure of infrared laser can be used to inactivate cancer cells but also determines its optimum energy dose, makes it a possible candidate for photodynamic therapy in the future.

Keywords: diode laser, MCF-7 cells, methylene blue, photochemical, photodynamic therapy, photothermal

1. Introduction

Cancer is a leading cause of death worldwide. Based on the data from the World Health Organization in 2010, the number of cancer death reached 13% (7.4 million) of all death annually [1]. More than 70% of all cancer death occur in low-income and developed countries. Based on Health Research Association in 2007, the prevalence of death by cancer is 5.7% after stroke, tuberculosis, hypertension, injury, perinatal, and diabetes mellitus. Based on the same data, the prevalence of tumor was 4.3 every 1000 population. Cancer is a collection of abnormal cells characterized by out-of-control growth. It occurs due to the rise and proliferation of the surrounding tissue. It can spread to other parts of the body and can happen to anyone regardless of age or sex, but the risk of developing cancer increases with age [1].

Cancer treatment is done in various ways, namely chemotherapy, radiotherapy, surgery, and combination therapy. However, each method of treatment still has weaknesses. Treatment by surgery is generally not effective in cells that have already been metastasis. Treatment with radiation is often not selective and not safe for normal cells, while chemotherapy treatment has not provided optimal results due to the non-specific action of the drug, which can also damage the normal cells (Padmi, 2008). Currently, cancer diagnosis and therapy are focused on non-invasive methods, which means preventing damage to the surrounding tissue. Analysis of tissue pathology requires a biopsy and cancer diagnosis usually uses high energy or high-frequency equipment, such as MRI, CT scan, ultrasound, and X-ray tomography. Nowadays, an optical spectroscopic technique such as laser and photodynamic therapy becomes potential in medical applications due to its noninvasive and nondestructive effect.

Photodynamic laser therapy is a companion method for cancer therapy that involves activation of the photosensitizer, a laser light source, and free radicals which can cause damage to the target tissue selectively and noninvasively [2]. Photosensitizer in photodynamic therapy is a medium that allows the transfer and translation of photon energy (from the light source) into chemical reaction

[3]. Laser (Light Amplification by Stimulated Emission of Radiation) as a light source in laser photodynamic therapy is preferred because it has a focused, coherent beam, and high intensity, so only the desired cancerous tissue is affected without damaging the surrounding healthy tissue. Among the laser technology used for laser photodynamic therapy in the medical field is the infrared laser. This technique was developed for cancer therapy as a companion technique because of its photothermal and photochemical effects [4].

Research on the interaction of infrared laser on living tissues was performed by Maksimova [5] using gold nanoparticles combined with continuous wave diode laser with a wavelength of 808 nm. On the in vitro studies, it had been proved that the laser diode with a pulse duration of 1 microseconds, the light intensity of 2 W/cm^2, and energy dose of 100 – 200 J/cm^2 was able to effectively destroy cancer cells. Subsequent research had been carried out by Souza [2] using photodynamic therapy of continuous wave diode laser with a wavelength of 630 nm and 5-ALA (5-aminolaevulinic acid) as the photosensitizer to treat non-melanoma skin cancer in human. For the in vivo study, the results obtained that the energy dose of 100-200 J/cm^2 has a cure response rate of 90% on long-term therapy.

Referring to the research conducted by Maksimova [5] and Souza [2], this study uses a GaAlAs continuous wave diode laser at a wavelength of 808 nm with energy dose between 23.043 to 322.602 J/cm^2 combined with methylene blue as photosensitizer to determine the effective energy dose that can inactivate the MCF-7 breast cancer cell cultures. Methylene blue is a photosensitizer with absorption wavelength between 605 – 763 nm and the maximum at 670 nm [6] with a safe dose usage is at a concentration of 20 μM for treatment in vitro [7]. A cultured cancer cell is used as the sample for this study emphasizes on information regarding the mechanism of cancer cell inactivation due to photothermal and photochemical interactions caused by both photosensitizer and infrared laser exposure. The results of this study are expected to provide a reference in determining the dose of infrared laser energy in photodynamic therapy of breast cancer that is safe, painless, and noninvasive to the surrounding tissue. This study is also expected to assist in the development of laser technology in medical applications, particularly in the field of noninvasive and nonionizing cancer therapy. It is expected that the method developed in this study can be used as a companion therapy of cancer in the future, especially in laser photodynamic therapy.

2. Experimental Details

2.1 Samples preparation

Manufacture of washing medium is done by dissolving RPMI 1640 in 80 ml of aquabidest, adding 2.3 g of sodium bicarbonate, 2 g of Hepes, then diluting to 1000 ml, pH 7.2 and sterilizing by filter of 0.22 μm in diameter. 10% FBS (fetal bovine serum), 1% penicillin – streptomycin, and 0.5% fungsison is added to the RPMI 1640 medium. Media is stored in a refrigerator at 4ºC [8]. Cells are taken from the liquid nitrogen tank, immediately thawed in a 37ºC water bath, and then the ampoules are sprayed with 70% ethanol. The ampoules are opened and MCF-7 cells are then transferred to a sterile conical tube containing RPMI 1640 medium. The cell suspension is centrifuged for 5 minutes, the supernatant is discarded and replaced with new RPMI medium, then suspended slowly. The cell suspension is centrifuged again for 5 minutes and then washed once again. The supernatant is discarded, 1 ml of medium containing 10% grower FBS is then added to the pellets. After resuspending slowly until homogeneous, the cells are grown in tissue culture flasks and incubated in a small incubator with temperature of 37ºC and 5% CO_2 stream. After 24 hours, the medium is replaced and grown until confluent cells and the amount is enough [8]. After a sufficient number of cells achieved (approximately after 3 days old), RPMI 1640 medium is replaced again and then released from the cell wall using a Pasteur pipette. Cells are then transplanted into sterile conical tube, RPMI medium is added, and the cells are centrifuged at 3000 rpm for 5 min. The supernatant is discarded and the cell pellet is resuspended slowly in 1 ml medium. Cells are then calculated using a haemocytometer. Cell suspension is mixed with medium until the cell concentrations of 2x10^4/μl are

obtained, therefore it is ready to be used for research [8]. Cells are harvested, planted in a 96-well plate, and then incubated for 2-3 hours. In general, the sample was divided into two major groups, laser exposure is given to the first group and the combination of photosensitizer and laser exposure is given to the second group. After incubation for 2-3 hours, methylene blue solution is added and then incubated for 3-4 hours. Methylene blue dose used in this study is based on the results of the cytotoxicity assay. After incubation for 3-4 hours, the cell is ready for the treatment.

2.2 Cytotoxicity test of Methylene Blue on MCF-7 Cells

Cytotoxicity test is performed to determine the safe dose of the methylene blue. One type of cytotoxicity assay is acute toxicity test, which is a total effect of the dose within 24 hours. Acute toxicity test can serve as a preliminary test to determine the dose that should be used in testing or further research. After the various concentration of methylene blue solution are prepared, cytotoxicity assay is performed with the following procedure. A total of 100 ml suspension of MCF-7 cells with density of $2x10^4$/100 ml is put in a 96-well plate, which already contains 100 ml of methylene blue solution with a different kind of concentration in each well from 1 μM to 50 mM. As a control, 100 ml of cell suspension was added to the wells containing RPMI 1640 medium and 5 mM sodium phosphate buffer pH 7.2. The 96-well plate is then incubated for 24 hours at 37ºC in an incubator with 5% CO_2 stream [8]. At the end of incubation, medium grower and a solution of methylene blue are removed from the wells and washed twice. 10 mL MTT 5 mg/ml in RPMI 1640 medium is added and incubated again overnight at 37ºC in an incubator with 5% CO_2 stream. Living cells will react with MTT and form a purple color. The reaction is stopped by adding 100 mL reagent stopper and incubated overnight at room temperature. The absorption spectra is taken by ELISA reader at 550 nm. The amount of absorption is proportional to the number of living cells.

2.3 Treatments and data retrieval

GaAlAs diode laser output beam has a tunable diameter or adjustable magnitude. Beam diameter setting is done by tuning the diode laser head cover parts manually to achieve the appropriate value. In the microcontroller laser setup, there are two types of position settings: manual and automatic mode. The procedure is as follows. Cells that had been harvested are incubated for 3-4 hours and 2 μM of methylene blue is given to the wells (ie wells A1 - A6, B1 - B6, C1 - C6, D1 - D6, E1 - E6, F1 - F6, G1 - G3, H1 - H3). Cells are then incubated again for 3 – 4 hours. Before treatment, the laser setup should be sterilized with alcohol and all works must be done in a laminar flow cabinet to prevent any kinds of contaminations. Laser is positioned 2.5 cm above the sample [9]. Cells then are exposed by laser with energy dose variations between 23.043 to 322.602 J/cm^2 with three times iterations for each dose. After exposure, the cover plate installed back then cells are incubated for 24 hours. After incubation, the results are collected using ELISA reader.

3. Results and Discussion

3.1 Laser Characterization

Characterization of laser intensity is performed using digital powermeter and Optical Spectrum Analyzer (OSA). The power generated in dBm is equivalent to 180.884 mW. The result obtained from OSA (Figure 1) also proves that the wavelength is 806 nm and lies in the near infrared spectrum.

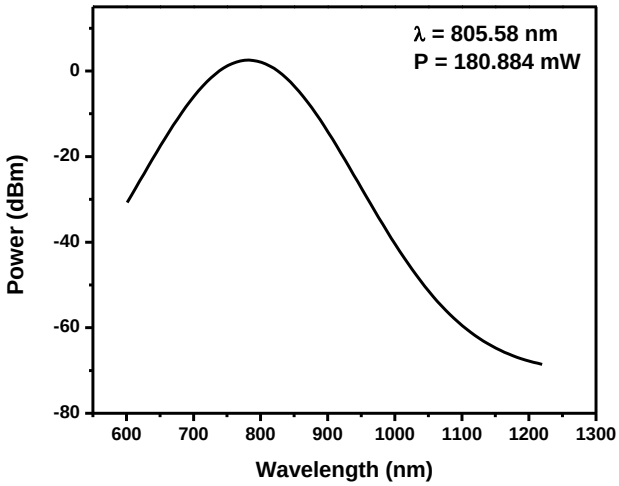

Figure 1 The results of the characterization of the laser with OSA

3.2 Cytotoxicity test of Methylene Blue on MCF-7 Cells

The effect of methylene blue as a photosensitizer is determined through an acute toxicity test. The acute toxicity test is the total effect of dosing within 24 hours, which is used a preliminary test to determine the dose that should be used in testing or further research. After the first incubation (See Section 2.2), cell cultures are photographed and some darker cells (Figure 2b) are observed, compared to the first day before the administration of methylene blue (Figure 2a). This change is because the cells absorb and react to the methylene blue. Absorption is expected to occur in photodynamic therapy. Methylene blue absorption into the cells will cause increasing cell sensitivity to light, make photodynamic therapy more selective and effective.

The blue color is caused by a highly concentrated solution of methylene blue, therefore at the end of the first incubation before reacted with MTT, each wall needs to be washed repeatedly with washing medium (PBS) to remove it. Cells then reacted with MTT and allowed to stand for 4 hours in an incubator. Dead cells will be light yellow while the living cells will be purple. The amount of absorption is proportional to the number of living cells.

$$\%\ \text{cell death} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100\% \quad (1)$$

What ELISA reading is captured is the optical density of the sample. Optical density is the logarithm of the ratio of the light intensity into the sample to the intensity of the light transmitted by the sample. The greater the value of optical density, the more cells are alive.

Based on the analysis of the optical density, the highest and the lowest percentage of cell death is obtained at concentration of 90 μM and 2 μM respectively. At concentrations between 2 to 50 mM, the color produced by methylene blue becomes too dense and part of it remain stuck on the wall surface although it has been washed three times. As a result, the yellow color of the MTT reacts with the blue color of methylene blue produces a yellowish green that will interfere with the reading of ELISA, so the optical density information on that range of concentration becomes inaccurate. Figure 3 resumes the cell death percentage over the variations of methylene blue concentration from 1 – 150 μM.

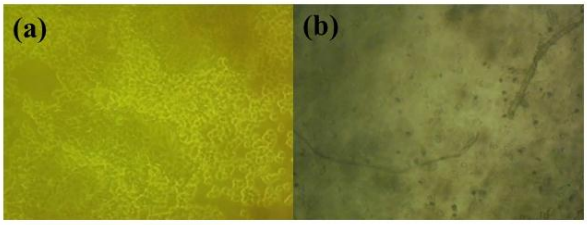

Figure 2 MCF-7 cells (a) before treatment and (b) on the second day after the administration of methylene blue (dark dots indicates the cells that absorbed the methylene blue)

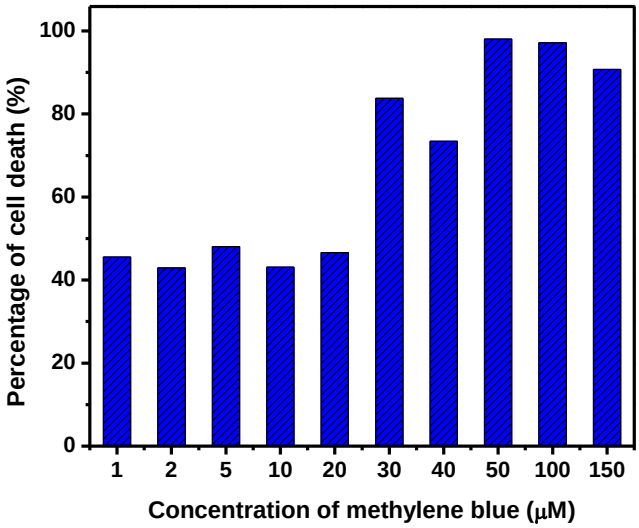

Figure 3 Percentage of MCF-7 cell death as a result of the concentration of methylene blue administration 1-150 μM

At concentration of 60 and 70 μM, the optical density results are negative. Since the optical density is the logarithmic ratio of the light intensity emitted to the light intensity transmitted back, the negative result indicates that the optical density of light intensity transmitted by the sample is higher than the intensity of the light emitted by the source. We assume that it is due to bacterial contamination leading to the false negative result. Figure 3 also shows that above 20 μM the cell death percentage is increased to more than 70% compared to the 50% cell death obtained at concentration between 1 – 20 μM. This observation also supports the results from Tardivo [7] which found that the safe dose of methylene blue for the in vitro treatment lies at concentrations of 20 μM.

3.3 Effect of Energy Dose on MCF-7 Cells

Figure 4 summarizes the results of laser exposure to the breast cancer cells and the combination of laser exposure and photosensitizer. It is found that the treatment using photosensitizer-assisted achieves the highest mortality percentage of 20.8% at energy dose of 184.344 J/cm^2. It can be observed that the energy dose of 184.344 J/cm^2 is more effective in increasing the percentage of cell death. However, at energy dose above 184.344 J/cm^2, the percentage of cell death begins to decrease. This is likely due to the longer exposure time which causes the photosensitizer loses its efficiency. The use of photosensitizer at low concentrations combined with a long exposure time can cause photodegradation or photobleaching effect [10]. It is expected to be resolved by repeated therapy.

Photodynamic therapy allows treatment of recurrent (repeated treatments) without cumulative side effects and risks damaging the surrounding tissue.

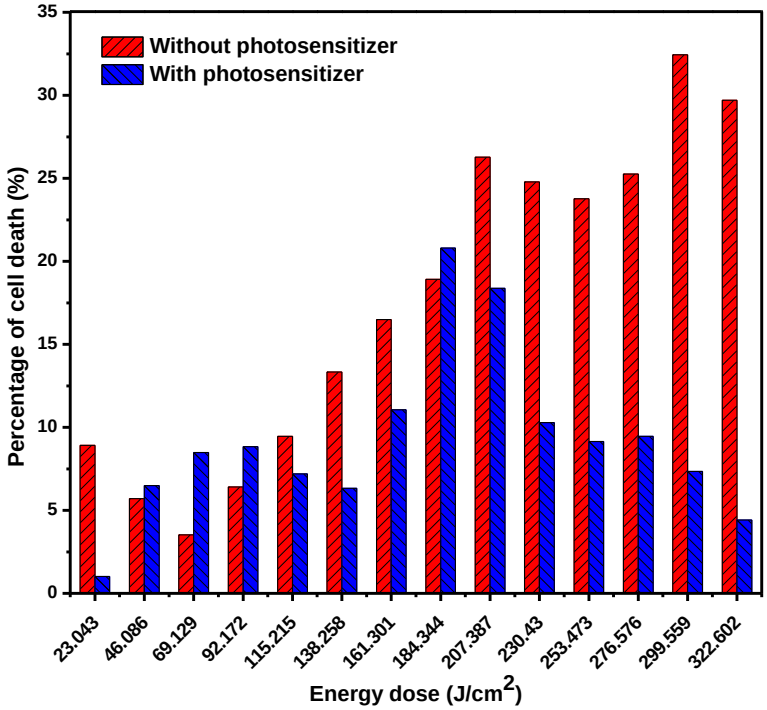

Figure 4 Graph of the percentage of cell death on exposure dose variation in laser energy with and without the administration of a photosensitizer

Photodegradation is the decomposition of a compound (usually organic compound) with the presence of the photon energy. The process requires a photocatalyst, which is generally a semiconductor material. The process begins when the electron jumps from the valence band to the conduction band of the semiconductor. The jumped electron creates a hole that can interact with the solvent (water) to form OH radicals, which is strong oxidizing agents. Electrons in the conduction band will react with oxygen in the environment, produce superoxide radical (•O2-) which acts as a reducing agent. Radicals can continue to react and form that outlines the target organic compounds due to its active properties. Photodegradation reaction of methylene blue can be written as follows [11]:

$$C_{16}H_{18}N_3SCl + 51/2\ O_2 + HCl + H_2SO_4 + 16CO_2 + 6H_2O\ 3HNO_3$$

Photobleaching, on the other hand, is the photochemical destruction of dye or fluorophore. Problems caused by photobleaching effect can be controlled by either reducing the intensity or exposure time, increasing the concentration of the fluorophore, or using lower frequency of light source so that the incoming photon energy will be lower.

Photodynamic therapy is a therapeutic technique that combines the accumulation of the photosensitizer in the irradiated target cell, make it selective. It is characterized by the accumulation of the photosensitizer reacting cells. According to Odhav [10], this reaction can occur because methylene blue ($C_{16}H_{18}N_3SCl$) is able to bind covalently to the corresponding carrier molecule, for example, lipoprotein, which leads the photosensitizer directly to the cancer cells without affecting the

surrounding tissue. However, the use of methylene blue with high concentration without laser exposure can be deadly to MCF-7 cell culture due to the nature of its toxicity. Therefore, the use of lower concentrations can be a solution to minimize cell death caused by the photosensitizer. Moreover, the incubation time that was given for methylene blue before laser exposure is 3 – 4 hours. According to Henderson and Gollnick [12], longer incubation time of more than one hour can lead to cell death by apoptosis. The incubation time should be reduced to minimize cell death due to the toxicity of the photosensitizer. Laser exposure and the use of photosensitizer cause the cells become starved of oxygen because oxygen molecule tends to form an excited singlet oxygen ($^1O_2$). Cells that lack oxygen would be too acidic (overacidify) so it can cause nutritional deficiencies. This is a metabolic disorder that would induce cell death by apoptosis.

In treatment without the administration of photosensitizer, it is found that the percentage of cell death is proportional to the energy dose. The highest percentage of cell death of 32.5% is obtained at energy dose of 299.559 J/cm^2. This linear relationship has several drawbacks, including the following. First, the condition means that there is a linear dose optimum energy to the therapy, meaning that the higher dose of a given energy, the greater the percentage of cell death. The absence of an optimum energy dose can be an obstacle in therapeutic applications. Second, the greater the energy dose, the more harmful effects on the target cells. The effect is the heat that can trigger pain. In addition, the long laser exposure time would damage the surrounding tissue. The pulsed laser produces high-energy dose in the order of nanoseconds so that respondents would not feel pain. However, the type of laser used in this study is a type of continuous wave laser so the high-energy dose is achieved by long exposure time. Although the characterization of the laser produces a laser beam temperature values between 37 – 38ºC, a long exposure time will trigger a burning pain that is not recommended in therapeutic applications. The use of continuous wave laser in this study considers several factors, like the ease and economic value. The pulsed laser has a large and complicated equipment setup, making it impractical for mobile applications and its difficulty level tends to be high. Because of the complicated setup, the price of the pulsed laser is relatively high compared to continuous laser. Based on these factors, the use of continuous laser would be more appropriate than the pulsed laser in the field of medical therapy.

In contrast to the treatment using methylene blue as a photosensitizer, the mechanism of cell inactivation due to laser exposure without photosensitizer does not require the presence of radical singlet oxygen ($^1O_2$) to destroy target cells. The energy of the laser exposure will excite the molecules in the cell including endogenous photosensitizer (natural photosensitizer present in the cell) which will then release the vibrational energy in the form of heat. According to Niemz [4], thermal effects on the microscopic level can be divided into two interrelated processes. First, the absorption of photons with a specific energy causes the molecules to move into a state of excitation. Second, no elastic collision between an excited molecule with surrounding molecules lead to the deactivation of the excited molecules and increase the kinetic energy of molecules in the vicinity simultaneously. In other words, the temperature in the cell increases due to the conversion of photon energy into kinetic energy.

In conclusion, we prove that the use of an infrared diode laser combined with methylene blue as photosensitizer effectively killed the breast cancer cells in vitro due to the photodynamic effect. However, one drawback of a diode laser is its beam that is not perfectly circular but is a function of the distance to form an ellipse. The optimum energy dose received by the sample is based on the assumption that the beam is circular. Based on the theoretical review by Sun [13], at a distance of 2.5 cm [9] used in this study, a diode laser beam diameter is 18.482 mm on the z-axis (horizontal direction) and 3,080 mm on the y-axis (vertical direction). Therefore, to further improve the accuracy of diode laser photodynamic therapy in the future, the energy dose calculations based on diode laser beam that is elliptical, not circular, is expected.

4. Conclusions

The effect of infrared laser exposure together with 2 μM methylene blue as photosensitizer on MCF-7 breast cancer cells has been studied. 20.80% cell death is obtained by irradiating cancer cells with 184.344 J/cm^2 energy dose along with the addition of photosensitizer. This value marks the optimum energy dose since the mortality begins to decrease at energy dose above this value due to the occurrence of photodegradation and photobleaching effect of the photosensitizer for longer exposure times. However, the percentage of cell deaths without the addition of photosensitizer is increased proportionally to the increase of energy dose due to the photothermal effect and achieved 32,45% cells mortality at 299,559 J/cm^2. Based on the results, it can be concluded that the combination of photosensitizer and diode laser can be used to kill MCF-7 breast cancer cells as a candidate for photodynamic therapy.

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