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In vitro toxicity of low-level green laser irradiation effects on human breast cancer cell lines

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Laser irradiation therapy on cancer cells is a promising alternative in providing a non-invasive treatment of breast cancer that has a possibility to inhibit cancerous cells selectively without damaging surrounding healthy tissues. This present study aimed to evaluate the effect of the low-level green laser of 532 nm wavelength with various laser power and irradiation time on MCF-7 cancer cell lines. In this work, the MCF-7 cells were seeded to a rate of fifty thousand cells/well in 96-well plate and incubated for 24 h. The cells were then irradiated with the green laser at different power from 0.002 to 0.1 W at 60 s, 540 s, and 900 s duration. The cell viability of the cells was measured by using Alamar Blue assay. From result, the laser irradiation on the cells was able to produce 25-40% inhibition of cell proliferation whereby the untreated cells exhibited a 93% cell viability. It was revealed that high power with longer exposure time increased cell bio-inhibition. Thus, this work using low-power green laser irradiation on cells demonstrated a significant effect on cells and was also demonstrated a promising non-invasive approach that can be used alone or in combination with several other therapies in cancer treatment.

Keywords: Cancer therapy, Cell inhibition, Cell proliferation, Non-invasive, Radio-resistance, Toxicity

Cancer is one of the major health issues and it is the second leading cause of death globally with 9.6 million deaths in 2018 that expected to increase up to 12 million in 2030 according to a press release by World Health Organization (WHO)[1]. The WHO press release also revealed the female breast cancer was the fifth leading cause of death amounting to 627000 deaths (6.6% of total deaths). The burden of cancer diseases on human life remains to be disastrous despite significant breakthroughs in our medical technology and cancer research attempts. Typical cancer treatments such as surgery, radiotherapy, and chemotherapy are usually performed to treat cancer patients offered a little contribution to the survival and often followed by serious long-term side effects as these treatments also induce damage on normal cells. However, applications which are commonly used in physics, such as laser, can help to develop an effective strategy for a cure. The application of laser devices in oncology is a promising alternative in providing a non-invasive therapy of breast cancer. The word of "LASER" is an acronym for light amplification by stimulated emission of radiation. In 1960, Theodore H. Maiman developed the first LASER or "Microwave Amplification by Stimulated Radiation Emission (MASER)"[2].

Interaction between laser irradiation and biological tissues has been an area of great interest[3]. Lasers are classified as hard and soft tissue lasers based on the type of laser–tissue interaction and not on the type of tissue exposed[4]. Photons emitted by the laser can either be absorbed or scattered by tissue whereby the scattered photons will eventually be absorbed or escape from tissue *via* diffuse reflection[5]. Meanwhile, the absorbed photons interact with photoreceptors or chromophores located within the tissue resulting in energy- boosting of the electrons in molecular orbitals and the hot electrons emit photoelectrons by losing its energy[6]. After that, the emitted photoelectrons transfer a photon or electron to form a free radical anion or radical cation and these radicals may further react with oxygen to produce Reactive Oxygen Species (ROS) that can cause significant toxicity leading to cell death *via* apoptosis or necrosis[7].

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Interestingly, the laser in the medical application have two general categories which are high intensity and low intensity. Each of these categories has very different therapeutic properties in their designs and applications. High power lasers cut tissue while low-power lasers stimulate various tissues and help the cells to function[8]. However, high doses of laser have certain disadvantages, and to overcome these matters, extensive research is going on with low-level laser therapy (LLLT) as this therapy has a stimulatory effect on cells at a low dosage and a suppressive effect at high dosage. LLLT involves an application of a light source (laser) that is set at low power known as "cold laser" due to its low thermal effect. In general, the power densities used for LLLT are lower than those needed to produce heating in tissue that depends on wavelength and tissue type[3]. The hyperthermia effect due to the laser thermal energy occurs when the heat is used to increase the body temperature from a normal 37°C to the temperature ranging from 41 to 45°C[9]. Therefore, the critical temperature at which cancer cells can be killed while normal tissues remain alive is 45°C as the heat reduced by blood flow protects the healthy tissue from overheating[9,10]. In spite of that, this laser thermal therapy with the aim to produce significant tissue destruction heating also have the risk to lead the cell death of normal tissues even it has positive effects on killing tumour cells, hence this therapy has to be applied with caution[11].

LLLT has shown remarkable results in a wide range of medical technologies due to its wavelength and biphasic dose- effect at a cellular level[2]. LLLT has been thoroughly studied to understand its action mechanisms on the metabolism of benign cells[12]. It is reported that LLLT could improve the process of wound healing and also has stimulating effects on bone cells and can hasten the repair process of the bone[8]. Unfortunately, LLLT is also known to increase cell proliferation, leading to the stimulation of undesired risk of cancer cell growth[13]. Laser light may induce the number of genomically altered cells with greater proliferative activity in malignant cells, indirectly accelerating the mutations during the natural carcinogenesis process[14]. Due to many variables in the use of laser light source and treatment procedures, previous studies always came out with different conclusions which difficult to the clinical team to select the optimum parameters. Thus, researchers and therapists have questioned the clinical benefits of laser therapy caused by divergent results in the literature due to lack of methodological standardisation in studies as well as by its clinical applicability[15]. In addition, the wavelengths, dosage schedules, and appropriate conditions of laser irradiation are also not well established.

The first study used the LLLT for cancer study was done by Mester and his team. Their aim of the research was to study the effect of LLLT on the shaved dorsal skin of mice. Despite LLLT did not cause any tumours development, they observed a higher rate of hair development and better wound healing after the irradiation. This was the first sign that low-level laser light could have its own useful medical applications[16]. However, a study found that after 635 and 670 nm irradiation on H.Ep.2 cells could significantly increase the proliferation of laryngeal cancer cells[17]. Another study observed the percentage of lung cancer cell proliferation were higher in the treated group by using Nd:YAG laser compared to the control groups and concluded that LLLT promote cancer cell proliferation depending on the power of the laser and the number of treatments[18]. Their study proved this therapy using Nd:YAG laser does not inhibit lung cancer cell proliferation. Their results can also be supported by previous research in 2015, the human leukemic cells that were irradiated (810 nm) with a different dose, 20 J/cm^2 (high dose) showed a significant increase in cell proliferation after two exposures but there were no changes in the growth rate of cells treated with lower doses at 5 J/cm^2 and 10 J/cm^2 [19]. In the same year, the breast cancer line of MDA-MB-231 cell viability increased after being treated by laser with 248 nm but slightly decreased after irradiated with both 1064 and 532 nm lasers were found in the study by[20]. Because LLLT has been shown to stimulate the development of cancer cells and may also enhance the aggressiveness of some cancer cells, some researchers have claimed that LLLT may be contraindicated in clinical use in cancer patients[21]. Fortunately, it was found that LLLT was very effective at minimizing many distressing side effects that occur as a result of a range of different cancer treatments[22].

In the meantime, the exact action mechanism of LLLT are not well understood and several theories exist. As stated by[23], most of the study and the best understood in the mechanism of this therapy being that of cytochrome-c oxidase (Complex IV) in the cellular mitochondrial respiratory chain. Complex IV

appears to be a chromophore or photoreceptor that absorbs energy from photons moving on wavelengths in the near- infrared spectrum which accelerates electron transfer rate[8]. The more photons being absorbed by cytochrome c oxidase, the more oxidized state cytochrome c oxidase will be. On the other hand, since cytochrome c oxidase is modulated by the LLLT, the production of ROS will then be activated[24,25]. The photon absorption by photoreceptor can also transfer the energy to other molecules that leading photochemical reactions in surrounding tissue and give rise to observable biological impacts[26]. The effectiveness of laser therapy is characterized by a biphasic dose response curve. The biphasic dose-response curve or Arndt – Schulz curve is a crucial part of LLLT. This principle specifies that optimum parameters provide an advantage to the specific disease, and if these parameters are significantly surpassed, the advantages will disappear and may even result in harmful outcomes when the dose is extraordinarily high[27]. In an attempt to better understand the effect of laser therapy on cancer cells, this work investigated the effect of low-level green laser 532 nm on breast carcinoma cell line, MCF-7 cell and to find the optimum laser dose for appreciable cell inhibition.

Materials and Methods

Cell Culture

Human breast cancer cell line, MCF-7, was used in this research. MCF-7 cell line was grown in RPMI 1640 medium with Stable Glutamine (Capricorn Scientific GmbH) supplemented with 10% heat-inactivated Fetal Bovine Serum, FBS (Capricorn Scientific GmbH) and 1% penicillin-streptomycin (Merck Millipore). The cells were maintained in an incubator at 37°C in an atmosphere of 5% CO_2 in air at a relative humidity of 80%. At 80 to 90% confluence, cells were harvested by using 1 mL trypsin and were subcultured into 75 cm^2 flasks and 96-well plates according to experiments. Cells were allowed to attach to the surface for 24 h prior to treatment.

Laser system

The experiments were conducted with a low-power green laser DPSS (Diode-Pumped Solid-State) laser system in Medical Physics Laboratory in School of Physics, Universiti Sains Malaysia, that emitted laser rays at 532 nm and the laser was set at several power outputs up to 0.1 W. The Table 1 shows the list of different power, time exposure and dose energy of green laser used in the treatment. Dose was calculated following the equation: Irradiance (J/cm^2) = time (s) × [power (W)/surface (cm^2)][28]. This laser was calibrated before the irradiation procedure.

Treatment

Laser irradiation on MCF-7 cells with different dose energy was conducted in sterile culture hood to avoid any contamination. This test also have performed in dark surrounding to eliminate influences from other light source[14]. In 96-well plates, the cells were seeded at a density of 5×10^4 cells/well with complete media and incubated for 24 h before irradiated with the green laser system. The cells were irradiated by laser beam with a 5 mm diameter at different power and time exposure as shown in (Table 1). The distance between each well and laser system was 1 cm. The unused wells were covered up with media without cells to prevent unintentional light scattering during laser application. Hence, the 2nd, 4th,

Table 1 — List of different power, time exposure and dose energy for 532 nm laser irradiation used in this experiment

Power (W)	Exposure time (s)	Dose (J/cm^2)
0.002	60	0.24
	540	2.16
	900	3.60
0.02	60	2.40
	540	21.60
	900	36.00
0.06	60	7.20
	540	64.80
	900	108.00
0.1	60	12.00
	540	108.00
	900	180.00

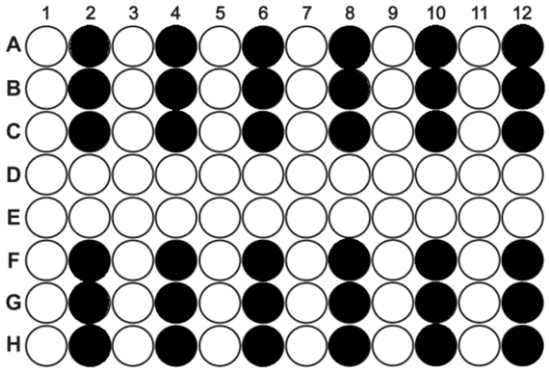

Fig. 1 — 96-well plate template for laser irradiation therapy. The black color indicated wells filled with the media only (without cells)

6^{th}, 8^{th}, 10^{th}, and 12^{th} columns of these plates are filled with 100 µL of media as indicated in black colour as shown in (Fig. 1). The experiments were carried out in triplicates for each dose. After laser irradiation, the treated cells were incubated again at 37°C and 5% CO_2 in a humidified atmosphere incubator for 24 h. The untreated and treated cells were observed under Phase Contrast Inverted Microscope (Olympus).

Cell viability test

After 24 h incubation, cytotoxicity of irradiated cells was performed with Alamar Blue Reagent to check the cell viability according to the Bio-Rad AbD Serotec protocol. This assay was selected because it is not toxic to cells and does not necessitate killing cells during the experiment. Furthermore, this assay also does not change the viability of the cell, unlike that which happens by trypan blue exclusion. The medium from the wells was removed carefully after incubation. Each well was rinsed with Phosphate Buffered Saline (PBS) three times and 10% of the Alamar Blue solution with 90 µL of fresh media were added to each well. The cells then were incubated for another 6 h at 37°C. The absorbance value at 570 nm and 600 nm were measured by a microplate reader (Model BioTek Power Wave XS).The assays were carried out five times for each dose with the same methodology and condition to investigate either there were major or minor changes in the results.

Statistical analysis

The values were expressed as the mean ± SD. Statistical analysis was performed using ANOVA. Student's *t*-test were used to determine the significant difference between groups. Statistically, significance was accepted at $P < 0.05$.

Results

Laser dose effects on MCF-7 cell lines

The effects of 532 nm laser irradiation on cell growth at different doses were tested using the Alamar Blue assay. The results of the untreated and irradiated cells were presented in (Fig. 2). The data obtained as a result of the percent reduction occurs in MCF-7 cancer cell viability tests were also demonstrated in (Table 2). Generally, it was proven that the cell proliferation rates were lower in the treatment groups than in the control groups. However, the lowest cell proliferation was detected in those cell irradiations with the highest power of 0.1 W significantly ($P < 0.001$) at all exposure time with the lowest rate of cell viability (approximately 53 to 63.5%) after 24 h incubation if compared to other laser power values. At the power of 0.002 W, the cells had a significantly increased ($P < 0.005$) survival rate with 70.3% viability after 60 s exposure whereby the cell viability for the control (untreated) group had the highest percentage of 93.48%. Meanwhile, at the same time exposure of 60 s but at different power values (0.02, 0.06 and 0.1 W), the cell viability decreased significantly ($P < 0.001$ to $P < 0.005$) at 70.04% (25.07% reduction), 65.41% (30.03% reduction), and 64.04% (31.49% reduction), respectively. As the power of laser light increases, the more cells will reduce.

In other case with the same power for 0.002, 0.02, 0.06 and 0.1 W at 540 s exposure, the number of viability cells reduced significantly ($P < 0.001$ to $P < 0.005$) as shown in the graph with the viability percentage of 61.40% (32.08% reduction), 65.17% (28.31% reduction), 60.1% (33.38% reduction), and lastly 57.29% (36.19% reduction), respectively. The cell growth inhibition for 900 s exposure time for all power of 0.002, 0.02, 0.06, and 0.1 W had the following cell viability percentages: 63.22% (30.23% reduction), 63.61% (29.87% reduction), 62.02% (31.43% reduction), and finally is 52.88% (40.6% reduction). Based on the cell viability trend, cancer cell growth inhibition increased with higher laser

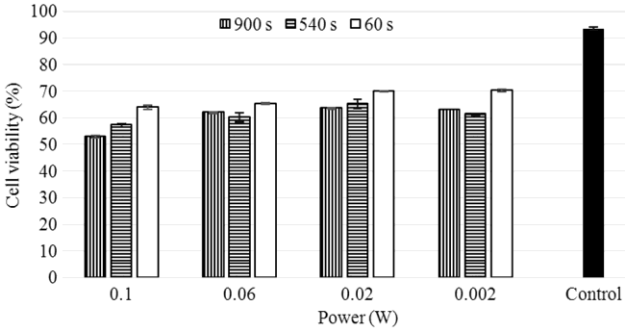

Fig. 2 — The graph shows the percentage of cell viability of MCF-7 line, irradiated with a laser from 0.002 to 0.1 W at 60 s (1 min), 540 s (9 min) and 900 s (15 min) exposure time

Table 2 — List of the% reduction for the cell treated with different power and time exposure

Power (W)/ Exposure time	60 s	540 s	900 s
	Reduction (%)	Reduction (%)	Reduction (%)
0.002	24.79 ± 2.39	32.08 ± 1.89	30.23 ± 0.63
0.02	25.07 ± 1.25	28.31 ± 4.58	29.87 ± 2.12
0.06	30.03 ± 1.68	33.38 ± 4.46	31.43 ± 1.95
0.1	31.49 ± 3.23	36.19 ± 2.73	40.60 ± 3.93

power and long time exposure. It was evident that the highest cancer cell growth inhibition occurred at the maximum power (0.1 W) and the longest exposure time (900 s). A possible explanation for the low cell viability is hyperthermia effects experienced by the cells after being heated by the laser beam that caused a localised temperature exceeding 45°C at which cancer cells can be damaged while normal human cells remained alive[9,10]. Thermal energy was deposited in cancer tissue upon absorption of light released by laser and cancer tumours are much more heat-sensitive compared to healthy tissues because of the high acidity of the cancer cells as a consequence of the high glycolytic activities inside cancer cells[29].

Discussion

The general result of this work was that laser irradiation caused a 25-40% reduction in MCF-7 cell proliferation which was below the desired 50% reduction. A probable reason for this relatively low% reduction was its multifactorial radio-resistance whereby the surviving cells could have acquired radio-resistance. Small populations of cancer cells might have survived after treatment and repopulated with advanced malignant phenotypes[30]. Cancer Stem Cells (CSCs) within tumours are one of the factors of radio-resistance and this property contributes to the poor therapeutic outcome of cancer patients[31]. This CSC harbour low reactive oxygen species (ROS) formed following irradiation which leading the reduction of DNA double strand breaks and expressing a high level of free radicals scavenger system. Free radical scavenger is an antioxidant that protects cells from free radical that cause damage[32].

Furthermore, overproduction of MicroRNAs (miRNAs) might have been one of the reasons the MCF-7 survived after irradiation of low-level laser making them more resistant to the irradiation[33]. Numerous researches have shown that miRNAs could function as radiosensitizers to strengthen the radiosensitivity of cancer cells but unfortunately, it also can cause radio-resistance when overexpressed in cancer cells[34]. A previous study found that miR-205[35], miR-200c[36], and miR-155[37,38] act as tumour radiosensitizers while miR-95[39] is promoting tumour radio-resistance in breast cancer cells. However, the overexpression of miR-200c in MCF-7 cells increased the survival fraction in MCF-7 cells post-irradiation[36].

Another factor for the cell resistance to radiation was attributed to hypoxia that refers to oxygen deficiency in cancer cells. Cancer cells are known to provide insufficient oxygen because of their abnormal vasculature, which leads to severe hypoxia in tumour cells that are away from capillaries[40]. This hypoxic condition of cancer cells protects them from radiation making them to be two or three times more resistant to irradiation[41]. In contrast, cells will be sensitive to irradiation when they have enough oxygen supply causing DNA damage by generated of free radicals involving oxygen[32].

By analyzing using the dose, the viability of cells that increased and decreased depending on the power and exposure time followed the biphasic-dose response that explained the cell behavior after laser irradiation. Inadequate power density or short exposure time may have no impact on the cell, but inhibitory effects will occur if too much power or long exposure time. There is an optimal relationship between power and exposure time that results in maximum stimulating action[42]. Based on the viability trend in (Fig. 2), the graph shows the linearly decreasing of viability trend as the doses increased. However, at 21.6 J/cm² (0.02 W, 540 s) the viability is higher than the cell treated with dose 2.16 J/cm² (0.002 W, 540 s). In addition, the same doses do not apply the same results. For example, at dose 108 J/cm², the cell experienced more reduction as viability percent is 57.29% for 0.1 W, 540 s but not for 0.06 W, 900 s with 62.02% of viable cells. Thus, the positive effect can be observed using high power with short exposure time which is safest to use in cancer therapy as the irradiation exposure duration reduced. The lowest exposure time is better to provide minimal undesired effect and offer less time-consuming during treatment which is comfortable to the patient. Overall, the result shows that at dose 180 J/cm² the cell viability of the MCF-7 is the lowest, followed by 108 J/cm² compare to the other doses. This can be assumed that dose is not the key parameters to be tested, but the time of exposure and the power density used[3]. The cell will be inhibited differently by different times and power exposure. From the result, it reveals that high power with long exposure time increased laser cell bio-inhibition. In addition, no major changes occurred in the results as the treatment were repeated for five times including trials to ensure the reproducibility of the experiment.

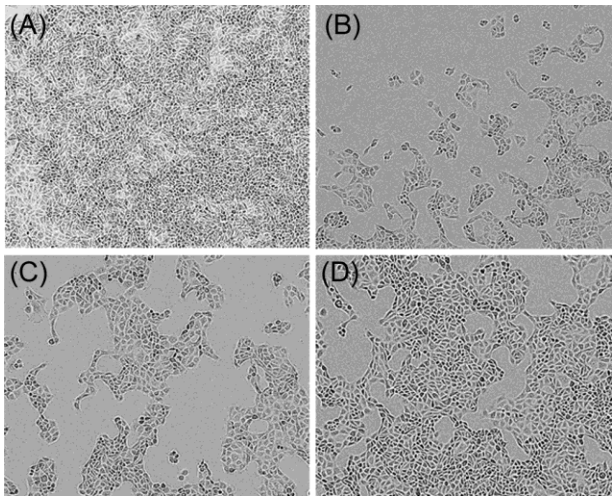

Fig. 3 — Image of MCF-7 Cell lines (A) Untreated cells (control); (B) The cells treated with 0.1 W for 15 min exposure; (C) The cells treated with 0.1 W for 9 min exposure; and (D) The cells treated with 0.1 W for 60 s exposure. Magnification was 40x

The microscopic images of both untreated and treated MCF-7 cells are shown in (Fig. 3). Based on (Fig. 3A), the untreated MCF-7 cells maintained their original morphology and appear to be in close contact with each other even when the incubation was prolonged to 48 h. The shape for control cells without any treatment is an epithelial cell line. However, some cancer cells in (Fig. 3B-D) treated with laser irradiation have changed in shape into circular morphology in that the circular shape is an indicator of the dead cell[18]. Nevertheless, the small number of circular cells suggested that there was not much apoptosis and necrosis occurred in this work. But one thing is clear – increasing the laser power and lengthening the irradiation exposure time can cause higher cell growth inhibition.

Conclusion

In conclusion, MCF-7 breast cancer cell lines possessed appreciable resistance toward green laser irradiation set at low power up to 0.1 W as the cell viability not reduced by half of percentage. The 532 nm laser irradiation slightly inhibit proliferation of MCF-7 cells at any doses in the range of 60 to 70% of cell viability compared to untreated cells which maintained their survival with 93% of viability. The data also showed that the highest cancer cell growth inhibition occurred at the maximum power (0.1 W) and the longest exposure time (900 s). Increasing the laser power strength and lengthening the irradiation time had a clear impact on inhibiting the MCF-7 cell proliferation and optimal laser parameter observed at high power with short exposure time.

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Conflict of interest

All authors declare no conflict of interest.

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