



Selective Response of Cancer and Normal Cells to 808 nm near Infrared Low Level Laser Irradiation: An *in Vitro* Study

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Abstract

Introduction: This study aims to investigate laser irradiation therapy as a potential non-invasive treatment for breast cancer, introducing a promising alternative with the potential for the selective inhibition of cancerous cells while minimizing damage to surrounding healthy tissues.

Methods: In this study, the utilization of cell culture stands out as an excellent methodology for evaluating both the effects and dosage of a given treatment. This study was conducted to evaluate the selective effect of low-level near-infrared (NIR) laser at an 808 nm wavelength, along with single and double irradiation with 5-minute intervals, on human breast cancer (MDA-MB-231) cells and human foreskin fibroblasts (Hs-27) cells using different laser powers and exposure times. The experiment involved exposing both cell lines to an NIR laser at 25, 43, 120, and 300 mW for 1, 5, 10, and 15 minutes. Cell viability was assessed via the MTT assay.

Results: The outcomes revealed disparate responses in both cell types used in this study. The double irradiation procedure with a 15-minute exposure at 25, 43, 120, and 300 mW displayed the most inhibition of cancer cell growth among the applied durations. Notably, using 300 mW during 15-minute double irradiation resulted in the highest cancer cell growth inhibition at 4.67%. Conversely, normal cells showed enhanced proliferation across most powers and exposure times applied. However, a considerable reduction in the cell viability of normal cells was evident following 15-minute double irradiation at 300 mW. This indicates that normal cells have been negatively affected by this power. Caution is advised.

Conclusion: The results suggest that NIR laser therapy at 808 nm with different output powers and exposure times significantly inhibits MDA-MB-231 cells, and this approach exhibits promising potential for inducing cancer cell death.

Keywords: Low-level laser therapy, Near infrared, Breast cancer, Cell viability



Introduction

Low-level laser therapy (LLLT) has been utilized in medicine since the late 1960s.¹ LLLT refers to applying light, typically a low-power laser or LED with a power range of 1 mW to 500 mW, to a pathology to help with pain relief and inflammation reduction and to promote tissue regeneration. Usually, the light has a spectral width within the red or NIR spectrum (600 nm – 1000 nm).² NIR light is used in laser therapy because its wavelengths fall within the so-called therapeutic window, where biological tissue has relatively low light absorption, allowing for deeper tissue penetration.³ This property makes NIR light especially useful in biomedical applications. NIR light is more suitable for deep tissue samples, as it helps reduce the scattering effect. Consequently, one of the main goals in optical imaging and light-based therapies is to achieve deep photon penetration. However, light penetration

depends on both the wavelength of the light and the optical properties of the tissue. Since tissues are heterogeneous, even within a single tissue, these inhomogeneity sites (such as nuclei, membranes, and other structures) cause light reflection, scattering, transmission, or absorption.⁴ Figure 1 illustrates the approximate depths at which different types of light can penetrate biological tissue and how they interact with it. Over time, LLLT has evolved into a sophisticated therapeutic tool, finding applications in various clinical fields and showing promising potential for diverse ailments.⁵ Recent studies have focused on photobiomodulation (PBM) therapy using infrared (IR) wavelengths, mainly within the NIR region.^{6,7} These include neural stimulation through direct activation of neural tissue,⁸ photoaging due to the biphasic effect of IR radiation. And anti-tumour actions, where IR radiation inhibits cancer cell proliferation and enhances

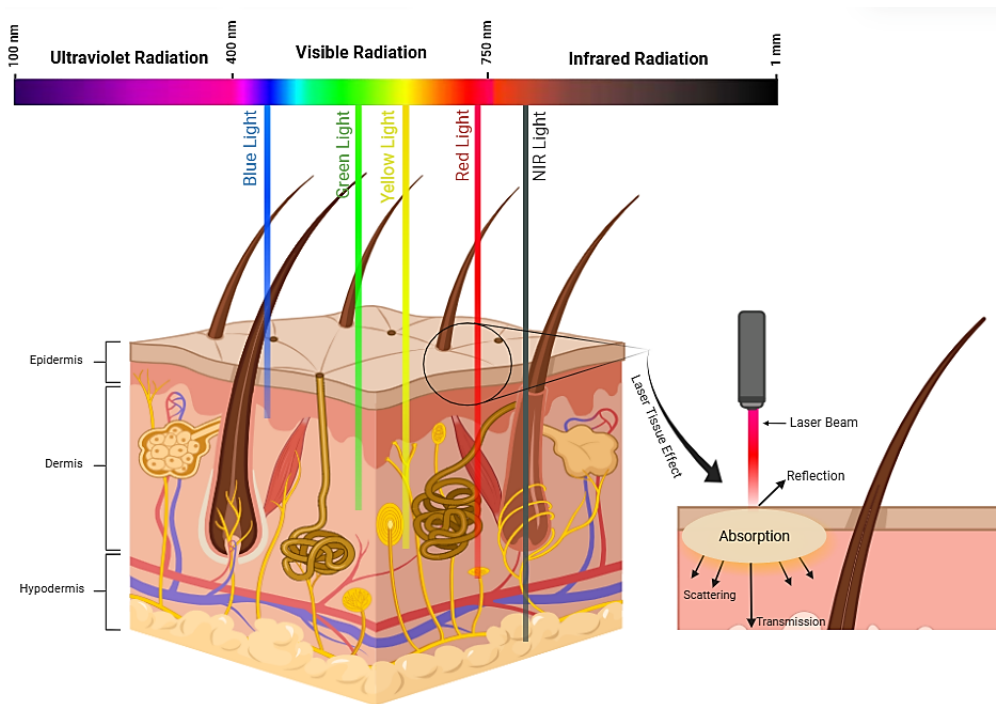


Figure 1. The approximate depths of light penetration in biological tissue by wavelength and interaction with tissue. This figure was created with [BioRender.com](https://www.biorender.com)

chemotherapy efficacy. Additionally, IR radiation provides brain neuroprotection, offering treatments for stroke and traumatic brain injury (TBI) *in vivo* models;^{8,9} as well as for neurodegenerative disorders like Alzheimer's and Parkinson's diseases. LLLT has been reported to enhance wound healing and promote the stimulation of bone cells, resulting in faster bone repair.¹⁰ Despite these potential benefits, some researchers and therapists have expressed concerns about the clinical advantages of laser treatment. These concerns are due to inconsistencies in methodological standardization and uncertainties regarding the applicability of LLLT in clinical settings.¹¹ Previous studies have often reached varying conclusions, making it challenging for clinical teams to determine the optimal parameters for treatment. Experimental investigations have reported both stimulatory and inhibitory impacts of low-level lasers on cell cultures.¹² Importantly, some *in vitro* studies indicate that LLLT can stimulate the growth of cancer cells,¹³ and potentially increase the aggressiveness of specific cancer cell types.¹⁴ It is essential to acknowledge that these effects are influenced by a range of factors, including the specific parameters employed by LLLT, such as wavelength, power density, and treatment duration in biological tissue.¹⁵⁻¹⁷ The earliest study investigating LLLT in cancer research was conducted by Mester et al., who applied LLLT to the shaved dorsal skin of mice in an attempt to cure cancer. Although the therapy did not cure the tumours, the researchers observed enhanced hair growth and improved wound healing. This observation provided the first indication that low-level laser light might confer

therapeutic biological effects.¹⁸ Subsequent research, however, demonstrated that LLLT can also promote cancer cell proliferation and may activate precancerous cells, with these effects varying according to laser power and treatment frequency. Notably, the study reported a higher percentage of lung cancer cell proliferation in the group exposed to the Nd:YAG laser compared with the control groups. The study demonstrated that LLLT using Nd:YAG does not inhibit lung cancer cell proliferation.¹⁹ Their findings are consistent with earlier research from 2015, which reported that human leukemic cells exposed to 810 nm exhibited a significant increase in proliferation at a dose of 20 J/cm² after two exposures, while no changes in the growth rate were observed at 5 J/cm² and 10 J/cm².²⁰ Similarly, another study reported that the viability of MDA-MB-231 breast cancer cells increased following irradiation with a 248 nm laser, whereas a slight reduction was observed after exposure to 1064 nm and 532 nm lasers.²¹ Additionally, a study using a He-Ne (632 nm) laser found that in the MCF-7 breast cancer cell line exposed to 5 mJ/cm², the treated group showed the highest number of dead cells at all times except at 48 hours. For the group irradiated with 28.8 mJ/cm², the percentage of dead cells was significantly higher at 24 and 72 hours.²² Gao et al. studied the effects of low-power laser irradiation (LPLI) using a He-Ne laser at 632.8 nm and 5 mW with a fluence of 0.8 J/cm² and high-fluence LPLI (HF-LPLI) using the same laser at 40 mW with a fluence of 60 J/cm² on ASTC-a-1 cells. The study found that low-fluence LPLI increased cell proliferation and activated protein kinase Cs (PKCs) in the cells. However, HF-LPLI decreased cell

viability and PKCs activity while increasing cell apoptosis.²³ In addition, 808 nm LPLI from 18 to 54 J/cm² inhibited the growth of A-172 human-derived glioblastoma cells in a dose-dependent manner.²⁴ In a previous work, it was shown that HF-LPLI, which uses 632.8 nm LPLI at 60 J/cm², may trigger cancer cells' apoptosis, as demonstrated by the activation of caspase-3.²⁵ Furthermore, the cytotoxic effects of various LLLT wavelengths were examined on MCF-7 breast cancer cells. The lasers were delivered at different wavelengths of 473 nm, 660 nm, and 780 nm, with powers of 10, 25, 45, and 65 mW and exposure times of 60, 300, 600, and 900 seconds. The results showed that the blue laser produced the minimal adverse effect, yielding the highest 24-hour survival rate of 107.62%. In contrast, the most pronounced toxicity occurred at 45 mW for 900 seconds, where cell viability ranged from 81.85% to 107.62%. Additionally, the study found that red laser irradiation, measured at a power of 45 mW for 60 seconds, achieved a cell survival rate of 147.62%. Whereas the most substantial toxic effect was observed at a power of 10 mW for 60 seconds, which led to a reduction in cell viability to 91.56%. In contrast, when using infrared laser irradiation, the highest cell survival of 109.37% was observed after 24 hours at a power of 10 mW for 600 seconds. The 780 nm infrared laser, operating at 25 mW power for 900 seconds, had the most noticeable cytotoxic impact, resulting in the lowest survivability of 32.53%.²⁶ Another finding indicates that Low-Level Laser Irradiation (LLLI) at 808 nm suppresses the proliferation of human hepatoma cells *in vitro*. This inhibition is linked to the disturbance of cytoskeletal architecture and the distribution of intermediate filament-associated proteins induced by photoradiation.²⁷ Numerous factors influence the biological effects of PBM on the exposed tissues, such as the cells' location in the exposure field, the type of cell, their molecular and redox states, the tissue microenvironment, PBM parameters like wavelength and power density, the delivery method (continuous or pulsing), the size of the beam or spot, and the exposure duration.²⁸ While several studies have demonstrated that PBM may increase the development of cancer cells in cell culture,²⁹ relatively few indicate that PBM may worsen or promote cancer growth in animal tumour models *in vivo*. In a study by Frigo et al., the impact of PBM (660 nm, 2.5 W/cm²) administered once daily for 3 days at low (150 J/cm²) or high (1050 J/cm²) doses was examined in subcutaneous melanoma in mice.³⁰ High-dose treatment markedly increased the tumour size, whereas the low dose decreased it (not statistically significant). However, this research faced several issues, including the claim that a C57BL/6 tumour (B16F10) was developed in a nonsyngeneic mouse strain (BALB/c). In a different study, Rhee et al. investigated PBM (650 nm, 100 mW/cm²) given as a single dose to an orthotopic mouse model of anaplastic thyroid cancer.³¹ Nevertheless, the

immunodeficient nude mice model that these researchers employed does not accurately represent the majority of human patients. In the PBM groups, tumour growth was faster; TGF- β 1 expression decreased, while HIF-1 α and p-Akt increased. Another study examined the use of PBM in a Syrian hamster cheek pouch model of chemically induced carcinogenesis using dimethylbenzanthracene (DMBA).³² Treatment with PBM (660 nm, 424 mW/cm²) was applied every other day for a duration of 4 weeks, starting immediately following the completion of the 8-week DMBA exposure. The PBM group had more histologically classified tumours as "poorly differentiated," which is likely to indicate a poorer prognosis. It is essential to realize that there are three distinct ways in which the potential benefits of PBM on cancer might occur. The first pertains to the direct effect of light on tumour cells, representing a deliberate application of the biphasic dose-response curve to "overdose" the cancer cells.³³ The Chinese laboratory of Da Xing has promoted this potential approach.³⁴ This method is known as HF-LPLI, implemented by this team frequently using a 632 nm HeNe laser, delivering 1200 J/cm² at an irradiance of 500 mW/cm² over a 40-minute exposure.³⁵ After publishing several *in vitro* studies, the team advanced to an *in vivo* investigation involving BALB/c mice with EMT6 breast tumours.³⁶ Complete tumour regression was achieved with a single dosage of 1200 J/cm², but rho-zero EMT6 tumours (which lack functional mitochondria) did not experience this effect. Furthermore, the cancer-cured mice had some long-term immunological memory since EMT6 tumours are known to be immunogenic. The second strategy is based on exploiting a difference in PBM's effects between cancer cells and normal cells. Combining PBM with another cytotoxic anticancer treatment enhances the number of cancer cells killed while protecting normal cells at the same time. Even while this might seem "too good to be true," there are some scientific reasons why it might be. The third possible way that PBM may benefit cancer patients is by stimulating their immune systems to combat the disease. In a mouse model of melanoma, Ottaviani et al.³⁷ demonstrated that PBM given once daily for 4 days using three different protocols, such as 660 nm with 50 mW/cm² providing 3 J/cm² and 800 nm or 970 nm with 200 mW/cm² delivering 6 J/cm², could all decrease tumour growth and enhance the recruitment of immune cells, specifically T lymphocytes and dendritic cells secreting type I interferons. In addition, PBM promoted vessel normalization and decreased the number of highly angiogenic macrophages inside the tumour, which serves as another strategy to control tumour growth.

Materials and Methods

Cell Culture

Two cell lines, MDA-MB-231 and Hs-27, were obtained

from the American Type Culture Collection (ATCC). Both cell types were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) complete media supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin solution (all from GIBCO, Invitrogen, USA). Cultures were incubated at 37 °C in a humidified environment containing 5% CO₂. For the experiment, a volume of 100 µl of cell suspension, containing a density of 2×10^4 cells per well, was introduced into clear-bottom 96-well culture plates, and these plates were then incubated for 24 hours. To prevent interference from different laser powers, the experiment was conducted in wells surrounded by media without cells.

Laser Irradiation Parameters

The experiments utilized a continuous low-power NIR (808VI200) diode laser device operated at an 808-nm wavelength, reaching a maximum of 500 mW output. Emitting a narrow spectral band of optical radiation, it covered a 4-mm-diameter spot, while the laser-to-culture well distance remained 3 cm. The study encompassed powers of 25, 43, 120, and 300 mW, with exposure durations of 1, 5, 10, and 15 minutes. The laser irradiation occurred in both single and double irradiation with 5-minute intervals between the regimes. A sterile culture hood ensured contamination-free conditions, and the irradiation was conducted in dark surroundings to eliminate external light effects. Moreover, the shape and morphology of MDA-MB-231 and Hs-27 cells were observed using an inverted microscope (Olympus, Japan). For every combination of output power (W), radiation time (s), and area (cm²), the energy density (J/cm²) was computed by:

$$\text{Energy Density} \left(\frac{\text{J}}{\text{cm}} \right) = \frac{\text{Power (W)} \times \text{Time (s)}}{\text{Area (cm)}} \quad (1)$$

The list of different power outputs, time exposures, and dose energies of the NIR (808 nm) laser utilized in this study is displayed in Table 1.

Cell Viability

To assess the cell viability of both MDA-MB-231 and Hs-27 cell lines, a microculture tetrazolium (MTT) assay was employed. After the 24-hour incubation period, MTT (all from GIBCO, Invitrogen, USA) was prepared at a concentration of 10% in DMEM, which was done in a dark environment. After removing the medium from each well, a mixture of 110 µl of MTT solution and a 1:10 ratio of DMEM was added. After that, the plates were transferred to an incubator at 37°C for 4 hours. Once the 4-hour incubation was completed, the media in each well were carefully extracted, and 100 µl of dimethyl sulfoxide (DMSO) (all from GIBCO, Invitrogen,

Table 1. List of different powers and exposure times with dose energies for NIR (808 nm) laser irradiation.

Power (W)	Exposure time (s)	Dose (J/cm ²)	Double Dose (J/cm ²)
0.025	60	11.94	23.88
	300	59.71	119.42
	600	119.42	238.85
	900	179.14	358.28
0.043	60	20.54	41.08
	300	102.70	205.41
	600	205.41	410.82
	900	308.12	616.24
0.12	60	57.32	114.64
	300	286.62	573.24
	600	537.24	1146.49
	900	859.87	1719.74
0.3	60	143.31	286.62
	300	716.56	1433.12
	600	1433.12	2866.24
	900	2149.68	4299.36

USA) was introduced to dissolve the water-insoluble purple formazon crystals. The readings were conducted using a microplate reader (Thermo Scientific Multiskan Spectrum, USA) at a wavelength of 540 nm. An overview of the cell viability procedures (cell culture, treatment, incubation, and viability analysis) is illustrated in Figure 2. The entire assay procedure was repeated three times for each dose under the same experimental methodology and conditions to assess any significant variations in the results.

Results

The Single Irradiation

The effect of varying laser power values on the viability of MDA-MB-231 cells after 24 hours of incubation is illustrated in Figure 3. At one minute of exposure with 43 mW, the cells showed a survival rate of 104.01%. As the laser power increased, cell viability decreased to 90.72%, 86.46%, and 80.99% for 25, 120, and 300 mW, respectively. The trend in cell viability indicated that higher laser power and longer exposure times led to increased inhibition of cancer cell growth. For exposure durations of 5 and 10 minutes, cell viability decreased across power levels of 25, 43, 120, and 300 mW, resulting in cell viability rates of 89.07%, 97.20%, 78.40%, and 70.14%, respectively. Correspondingly, with a 10-minute exposure, cell viability percentages for the same power settings stood at 87.91%, 98.55%, 70.96%, and 35.93%. Moreover, when cells were subjected to 300 mW laser power for 15 minutes, a significant reduction in viability reached 24.69%. In contrast, at the same 15-minute exposure, cell viability percentages for 25, 43, and 120 mW laser powers were 85.20%, 79.10%, and 75.92%, respectively. The comparison among outputs at 25, 43, and 120 mW revealed that 300

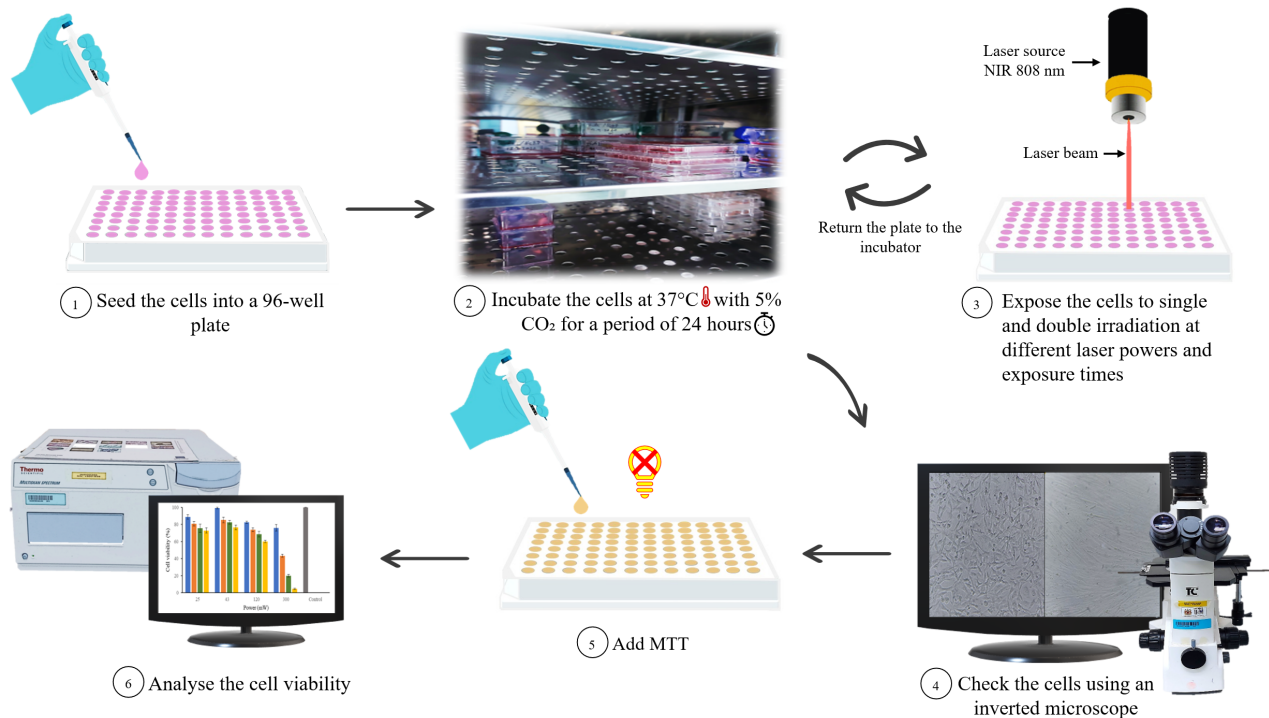


Figure 2. An overview of the procedure employed in this study, from culture to analysis

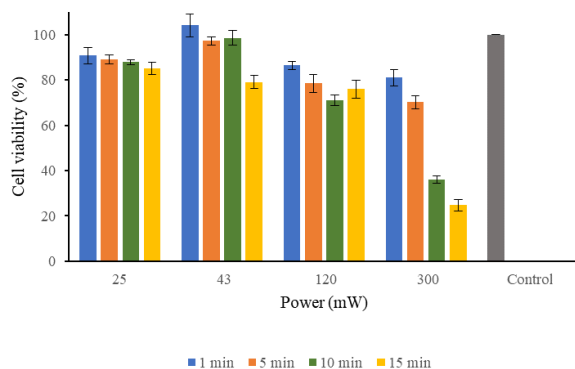


Figure 3. Cell viability percentage of MDA-MB-231 cells after single irradiation laser treatment for 1-, 5-, 10-, and 15-minute exposure time. Data are presented as the (mean $\pm$ SD, n=3) using one-way ANOVA (multiple comparisons). Statistically, all the groups are highly significant compared to their control groups: $1\text{ min } P=0.0003$, $5\text{ min } P=0.00001$, $10\text{ min}, 15\text{ min } P<0.00001$, and $P\leq 0.05$

mW irradiation yielded the lowest cell viability, indicating maximal inhibition of cancer cell growth. Notably, the highest power at 300 mW and the extended 15-minute exposure resulted in the largest decrease in cell viability, reaching 24.69%. This result can be ascribed to the effective absorption of photons, causing damage to select cells and increasing cell apoptosis (cell death).

An unexpected finding emerged when exposed to 43 mW power for one minute: cell viability exceeded 100%, a surprising outcome marked by proliferation instead of cell death, reaching 104.01%. The one-minute exposure duration was insufficient to cause cell damage; instead, it triggered a proliferation response. Furthermore, it did not

exhibit any toxic effect of low-level light energy on MDA-MB-231 cells. Instead, the prevailing mechanism revolved around cellular stimulation through absorbed light energy. Mitochondria leveraged this energy absorption to generate ATP, consequently prompting elevated vasodilation. This process led to increased oxygen utilization and enhanced cytoplasmic enzyme activity involving nucleic acids, ultimately fostering the stimulation of cell mitosis.¹⁹ Consequently, cell proliferation occurred rather than cell death. The NIR 808 nm laser with an output power of 45 mW and a one-minute exposure time proved unsuitable for the MDA-MB-231 cell line, as it unexpectedly stimulated cell growth instead of causing cell death. However, extending the irradiation time to 15 minutes at the same power of 43 mW led to increased cell death, reducing viability to 79.10%. This increased cell death was due to the longer exposure time, which impaired the cells' ability to multiply. Further extending the exposure time could additionally decrease the percentage of viable cells, demonstrating the potential of this method for targeted cancer therapy.

Conversely, Figure 4 shows that the effects of irradiation on Hs-27 cell viability after exposure at power levels of 25, 43, and 120 mW for durations from 1 to 15 minutes consistently surpassed those of the control group. It is important to highlight that there was no observed reduction in normal cell viability at these three power levels. Instead, the cells exhibited proliferation upon single irradiation across most times applied. The lowest cell viability for normal cells was 78.19%, achieved through single irradiation using a 300 mW output power

for 15 minutes. Over 10-, 5-, and 1-minute exposures, cell viability percentages were 89.29%, 97%, and 98.04%, respectively. These findings suggest that the normal cells experienced minimal impact from laser irradiation at the specified powers and exposure durations. In comparison, MDA-MB-231 cancer cells subjected to the same 300 mW laser power in a single exposure exhibited a notable reduction in cell viability, with percentages of 80.99%, 70.14%, 35.93%, and 24.67% after 1, 5, 10, and 15 minutes, respectively. This contrast highlights the targeted nature of the laser therapy approach, where cancer cells experienced notable effects while normal cells demonstrated resilience and continued to proliferate under similar conditions. This selective impact on cancer cells underscores the promising potential of laser therapy as a focused and safe strategy for cancer treatment, warranting further investigation and optimization for potential clinical applications.

The Double Irradiation

In this study, a method of double irradiation was utilized to investigate the effect of exposing MDA-MB-231 and Hs-27 cell lines to low-level NIR 808 nm laser light. The experiment was conducted at 5-minute intervals, subjecting the cells to double irradiation using different output powers and exposure durations. The primary objective was to comprehend and distinguish the outcomes of these exposures on cell apoptosis and induced effects in both MDA-MB-231 and Hs-27 cells. The double irradiation approach and post-exposure incubation were investigated to comprehend the influence of irradiation and post-exposure incubation. As depicted in Figure 5, the percentage of viable cells consistently dropped below 100% during the double irradiation process for all four exposure times. The most significant decrease in cell viability was noted at 4.67% following a 15-minute exposure to 300 mW radiation. Conversely, the highest cell viability value of 99.26% was observed, resulting from a double irradiation procedure with an output power

of 43 mW for 1 minute of exposure time. This result contrasted the upward trend observed with 1-minute single irradiation (104.01%). In the context of a 1-minute exposure period, cells that encountered a double dose at 25 mW exhibited a viability of 88.73%. This percentage decreased to 82.69% and 76.03% when subjected to 120 and 300 mW laser powers, respectively. Upon extending the exposure period to 5 minutes, cells exposed to 25 mW exhibited a reduced viability of 80.81%. Similarly, exposure to 43, 120, and 300 mW resulted in viabilities of 85.28%, 73.88%, and 43.30%, respectively. Notably, after 10 minutes of exposure, the viability percentage at 25 mW dropped to 75.58%. It is worth mentioning that the output powers at 43, 120, and 300 mW decreased to 82.43%, 68.77%, and 19.83%, respectively. A significant difference in cell viability percentage was evident among the applied output laser power values, specifically at the 300 mW output power. Extending the irradiation duration to 15 minutes resulted in a cell viability of 4.67% for the 300 mW output power. Simultaneously, with the same 15-minute exposure period but a power of 25 mW, cell viability decreased to 72.91%. Similar trends were seen with the 43 and 120 mW powers, yielding cell viability percentages of 76.46% and 59.97%, respectively. When comparing the four output laser power values used for cell exposure, it was evident that the 43 mW power had the least impact, resulting in the lowest cell death. Notably, the cell viability for all four output laser powers and exposure durations fell below 100%. This suggests that subjecting MDA-MB-231 cells to an additional radiation dose after a 5-minute interval intensified cell inhibition. These findings are consistent with those reported by Peidaee et al., who observed varying percentage levels of cell viability under different exposure regimes.³⁸

In the case of normal cells, the viability of Hs-27 cells was examined under different conditions involving double irradiation. Various output powers and exposure durations were investigated, with the outcomes depicted

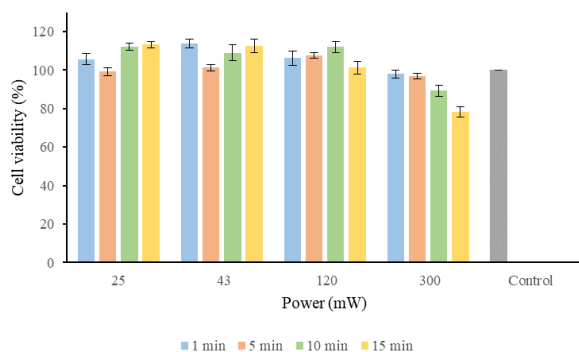


Figure 4. Cell viability percentage of Hs-27 cells after single irradiation laser treatment for 1-, 5-, 10-, and 15-minute exposure time. Data are presented as the (mean±SD, n=3) using one-way ANOVA (multiple comparisons). Statistically, all the groups are highly significant compared to their control groups: $1 \text{ min } P=0.001$, $5 \text{ min } P=0.0003$, $10 \text{ min } P=0.00004$, $15 \text{ min } P<0.00001$, and $P\leq 0.05$

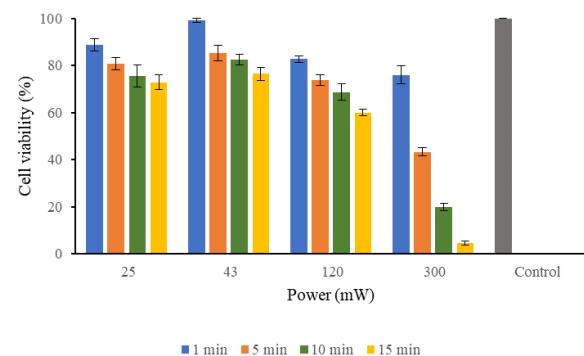


Figure 5. The percentage cell viability of MDA-MB-231 cells after double irradiation laser treatment for 1-, 5-, 10-, and 15-minute exposure time. Data are presented as the (mean±SD, n=3) using one-way ANOVA (multiple comparisons). Statistically, all the groups are highly significant compared to their control groups: $1 \text{ min } P=0.00001$, $5 \text{ min } P<0.00001$, $10 \text{ min } P<0.00001$, and $P\leq 0.05$

in Figure 6. Remarkably, the most notable increase in cell viability was observed after 1 minute of exposure to 120 mW power, yielding a substantial increase in the survival rate to 119.48% compared to the control group. Similarly, at 43 mW, the cell viability increased to 107.61%. In contrast, viability percentages were 99.20% and 92.41% at 25 and 300 mW, respectively. Interestingly, exposure for 5 minutes led to heightened viability percentages of 103.19% at 43 mW and 116.58% at 120 mW. However, upon exposure to 25 and 300 mW for the same duration, the viability reached 96.51% and 87.38%, respectively. Moving to a 10-minute exposure, the highest cell viability of 133.23% was attained at 43 mW. Conversely, cells subjected to 25 mW and 120 mW displayed similar viability percentages of 98.67% and 98.04%, respectively. At this duration, at 300 mW, the cell viability dropped to 66.12%, indicating the presence of dead cells. Extending the irradiation to 15 minutes at 43 mW resulted in continued cell proliferation, elevating cell viability remarkably to 141.96%. Meanwhile, at 25 mW and 120 mW, the cell viability percentages reached 98.09% and 96.65%, respectively. However, employing a 300 mW power for the same duration yielded a viability percentage of 50.55%. Out of all the normal cell viabilities, this represents the lowest viability observed. This suggests that prolonged exposure to this high dose leads to an increased cell death rate in normal cells.

Figure 7a and Figure 7b represent the untreated MDA-MB-231 and Hs-27 cell lines. As seen in Figure 8e and Figure 8f, it is evident that MDA-MB-231 cells experienced the most inhibition after double irradiation at the highest power of 300 mW for 10 and 15 minutes. These cells took on a circular shape, indicating cell death.³⁹ In contrast, cells in (a), (b), (c), and (d) were treated with 43 mW for 1, 5, 10, and 15 minutes, respectively. Furthermore, as shown in Figure 9f, the most significant inhibition of Hs-27 cells occurred with the highest power of 300 mW during an extended exposure time of 15 minutes. Similarly, cells in (a), (b), (c), and (d) were subjected to treatments with 43 mW for 1, 5, 10, and 15 minutes, respectively. Additionally, (e) depicts cells treated with 300 mW for 10 minutes.

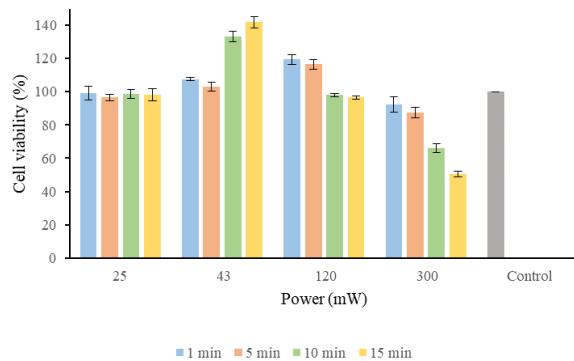


Figure 6. The percentage of the cell viability of Hs-27 cells after double irradiation laser treatment for 1-, 5-, 10-, and 15-minute exposure time. Data are presented as the (mean $\pm$ SD, $n=3$) using one-way ANOVA (multiple comparisons). Statistically, all the groups are highly significant compared to their control groups: $1\text{ min } P=0.00005$, $5\text{ min}, 10\text{ min}, 15\text{ min } P<0.00001$, and $P\leq 0.05$

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Discussion

Both low-energy and high-energy lasers have been the subject of several studies. These lasers have photochemical effects on various tissue cells, encouraging fibroblast growth and suppressing cancer cell proliferation. An 808-nm laser with output powers of 25, 43, 120, and 300 mW was utilized in this study. Significant changes in the proliferation of human fibroblasts and cancer cells were observed with increasing energy density and exposure time. The results show that the most effective reduction of cancer cell growth occurred with double irradiation, utilizing the highest power (300 mW) and the longest exposure time (15 minutes). This approach allowed for efficient thermal energy absorption in cancerous tissue through emitted NIR laser light. Importantly, tumours were demonstrated to have a higher sensitivity to

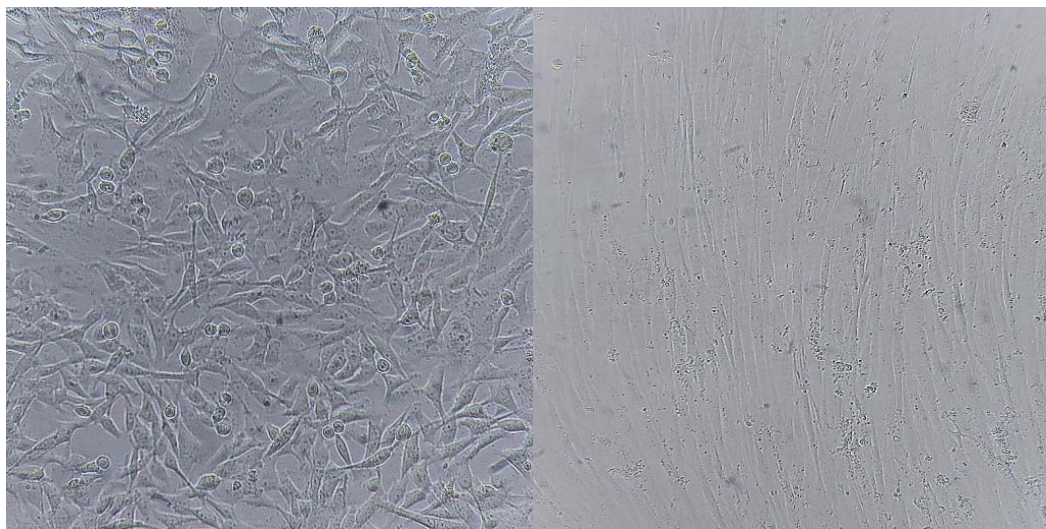


Figure 7. Inverted microscope images of untreated cells for (a) MDA-MB-231 cells and (b) Hs-27 cells. Images are at 10X magnification

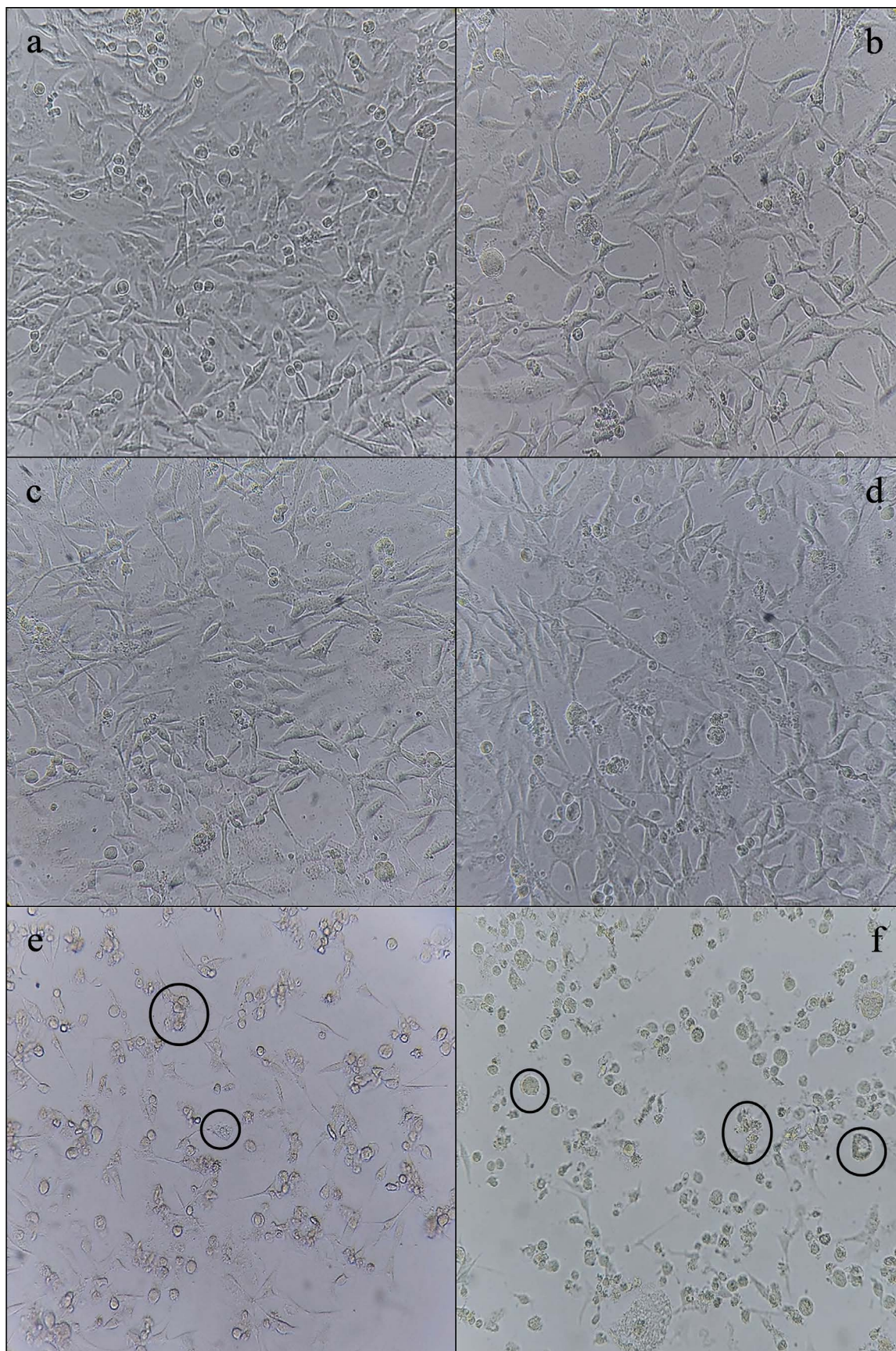


Figure 8. Inverted microscope images of MDA-MB-231 cells after double irradiation laser treatment. (a) the cells treated with 43 mW for 1-minute exposure; (b) the cells treated with 43 mW for 5-minute exposure; (c) the cells treated with 43 mW for 10-minute exposure; (d) the cells treated with 43 mW for 15-minute exposure; (e) the cells treated with 300 mW for 10-minute exposure; (f) the cells treated with 300 mW for 15-minute exposure. Images are at 10X magnification

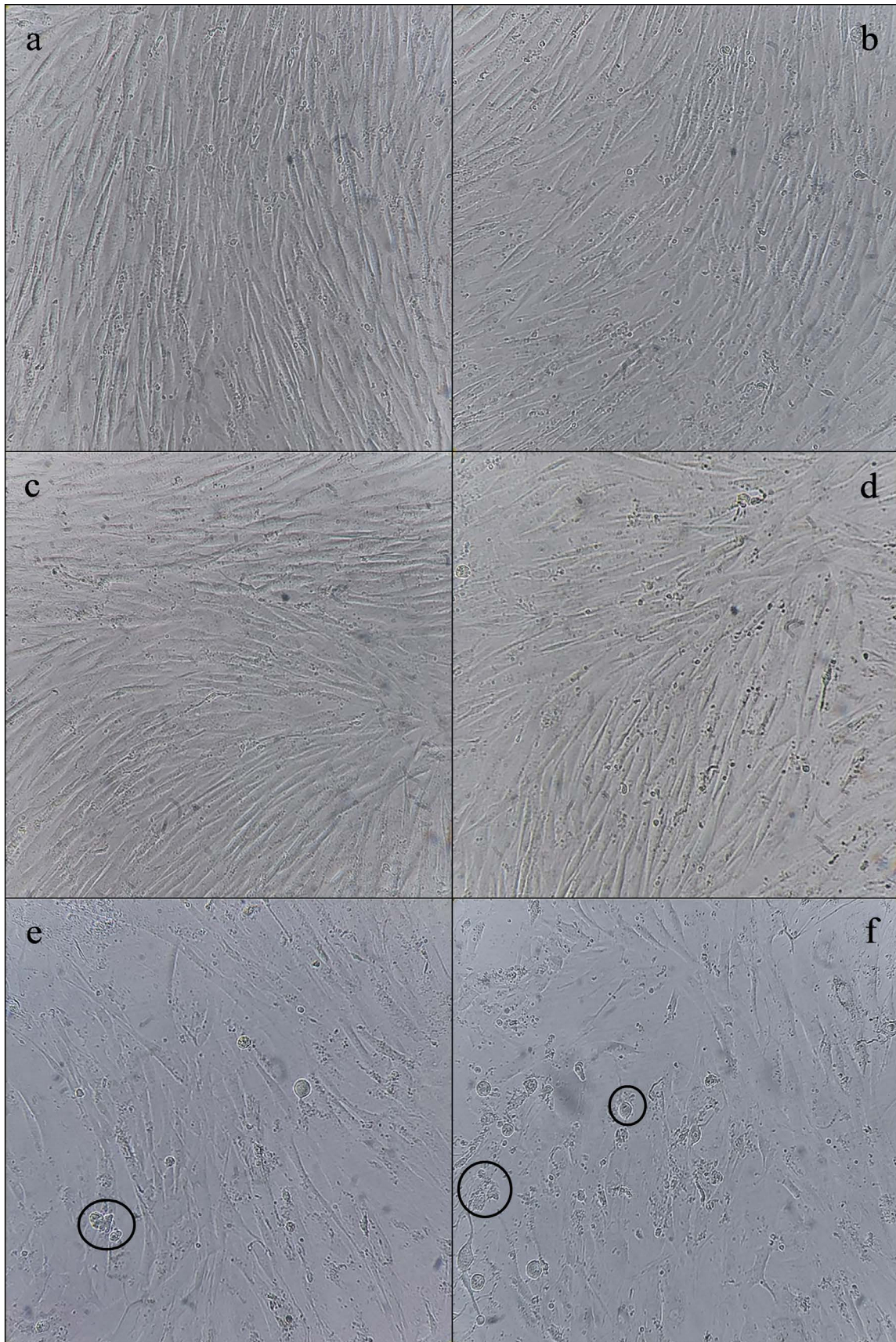


Figure 9. Inverted microscope images of Hs-27 cells after double irradiation laser treatment. (a) the cells treated with 43 mW for 1-minute exposure; (b) the cells treated with 43 mW for 5-minute exposure, (c) the cells treated with 43 mW for 10-minute exposure; (d) the cells treated with 43 mW for 15-minute exposure, (e) the cells treated with 300 mW for 10-minute exposure; (f) the cells treated with 300 mW for 15-minute exposure. Images are at 10X magnification

thermal exposure than healthy tissues, likely due to the high acidity of cancer cells caused by elevated glycolytic activity inside the cancer cells.⁴⁰ Nonetheless, NIR laser irradiation decreased the proliferation of MDA-MB-231 cells, particularly at 25, 43, and 120 J/cm², under both single and double irradiation; however, the reduction in cell viability did not reach the 50% threshold typically associated with significant cytotoxic activity. MDA-MB-231 is an aggressive triple-negative breast cancer cell line known for its high invasiveness and resistance to many therapies. Following various doses of NIR radiation in this study, the survival of MDA-MB-231 cells suggests stress adaptation or level of treatment tolerance. This indicates the presence of intrinsic defense mechanisms that help the cells withstand photothermal stress. These mechanisms may include the activation of cellular defense pathways, such as the upregulation of heat shock proteins (HSPs), which have been shown to protect MDA-MB-231 cells under thermal stress.⁴¹ Another factor could be an enhanced antioxidant response to mitigate reactive oxygen species (ROS) generated by NIR exposure.⁴² Additionally, metabolic adaptations support cell survival under stressful conditions.^{43,44} An independent study also found that the cancer stem cells (CSCs) within tumours harbour low ROS generated after irradiation, resulting in a reduction of DNA double-strand breaks and high expression of free radical scavenging systems. These systems serve as antioxidants, crucially protecting cells from the harmful impacts of free radicals.⁴⁵ Furthermore, in response to radiation-induced damage, survival signalling pathways such as PI3K/AKT are activated to protect against cell death.⁴⁶ Additionally, NF- κ B has been recognized as a crucial factor in cancer development by influencing cell invasion, migration, and proliferation.⁴⁷ Upon radiation exposure, the activation of NF- κ B in pro-survival signaling pathways not only increases resistance to radiation but also increases the potential of repopulating tumours to exhibit malignancy.⁴⁸ A further possible explanation for the resistance to the laser radiation observed in the results is the existence of hypoxic conditions in the cellular microenvironment. Hypoxia is another factor influencing cell radiation resistance, a condition marked by low oxygen levels within cells. When cells have ample oxygen, they become more sensitive to radiation since radiation-induced DNA damage creates free radicals that involve oxygen.⁴⁵ Conversely, cells situated in hypoxic environments gain protection from radiation-induced damage. Cancer cells tend to generate oxygen-deficient zones within tumours due to abnormal vasculature, resulting in severe hypoxia in regions distal from capillaries.⁴⁹ Insufficient oxygen in the hypoxic tumour regions curtails the generation of DNA-damaging free radicals, diminishing their responsiveness to radiation. Consequently, elevated radiation doses are imperative in hypoxic tumour regions to inflict the same harm as in normoxic areas.⁵⁰ In the

present work, an 808-nm NIR light was utilized. Since the 808-nm light activates cytochrome c oxidase (COX), this wavelength is absorbed by COX in mitochondria, and the interest in mitochondrial functions has increased as a result of gaining insight into the contributions of ROS to normal biological function and pathological conditions. The fact that ROS can have both positive and negative effects is well-known.⁵¹ If it can be shown that the generation of ROS is dose-dependent on the given energy fluence, the stimulation and inhibition seen with low and high light fluences may be explained. Previous studies have revealed that HF-LPLI at doses of 80 and 120 J/cm² induces apoptosis through the mitochondrial signaling pathway (mitochondria/caspase-3), leading to substantial ROS production in both transformed African green monkey kidney fibroblasts (COS-7) and ASTC-a-1 cells.⁵² Moreover, *in vivo* findings showed that HF-LPLI could exhibit good antitumour effectiveness with rational dosimetry. Remarkably, the highest tumour-killing efficiency was achieved with HF-LPLI at 500 mW/cm² for 40 minutes, indicating a crucial window of irradiation fluence.³⁵ Besides, it was shown that high doses of LLLT induced apoptosis through a mitochondrial caspase-3 pathway, and the release of cytochrome c was linked to the opening of the mitochondrial permeability transition pore resulting from elevated levels of intracellular ROS production.³⁴ HF-LPLI was found to increase intracellular generation of ROS.⁵² Given the use of single and double irradiation approaches across different power levels and exposure times in this study on both MDA-MB-231 and Hs-27 cells, it is important to highlight the variability and effectiveness observed following 10 and 15 minutes of irradiation. The findings show that, even after 15 minutes of exposure, there were only slight effects on MDA-MB-231 cells and no significant reduction in the viability of Hs-27 cells at lower power levels (25 and 43 mW). This suggests that, at these power levels and durations, thermal effects are likely minimal. The viability of MDA-MB-231 cells was 85.20% (single) and 72.91% (double) at 25 mW for 15 minutes, while at 43 mW (15 minutes), it was 79.10% (single) and 76.46% (double). Under the same conditions, Hs-27 cells proliferated while maintaining high viability throughout. However, a more noticeable decrease in MDA-MB-231 cell viability was observed at higher power levels (120 and 300 mW), especially with extended exposure and double irradiation. For example, viability dropped to 24.69% (single) and 4.67% (double) at 300 mW for 15 minutes. Under similar conditions, Hs-27 cells also showed some reduction in viability (78.19% and 50.55%, respectively). These findings suggest that thermal effects may be causing cell damage at higher powers and longer durations, particularly when effects on Hs-27 cells also become evident. Consequently, overexposure heating, especially in the 300 mW groups, might be a secondary factor contributing to cell damage at higher powers and

longer exposure times.^{53, 54}

Importantly, one must also consider that in assessing the impacts of NIR and red laser irradiation in cancer cells, the reported results show less consistent outcome findings compared with findings in fibroblast models. Specific treatment conditions have shown that red and NIR laser radiation could potentially heighten fibroblast proliferation and inhibit the growth of cancer cells, as most findings of this study suggest. Adding to the complexity, comparing different studies becomes difficult due to the wide variation in sources, as the cellular response to irradiation is impacted by factors such as wavelength,⁵⁵⁻⁵⁷ energy dosage or fluence,^{55,58} treatment methodology,^{55,58,59} culture conditions, and cellular physiological state (activated/not activated).^{55,58}

Conclusion

This study evaluated the response of MDA-MB-231 and Hs-27 cell lines following irradiation with an 808-nm low-level NIR laser. The results showed different responses between these two cell types. Notably, using a double irradiation method with a 15-minute exposure at power settings of 25, 43, 120, and 300 mW led to the greatest reduction in cancer cell growth, lowering cell viability to 4.67% compared to untreated cells. In contrast, the effects on normal cells varied significantly, with increased proliferation compared to the control group at most power levels and exposure times. Additionally, a substantial decrease in normal cell viability was observed when they were subjected to double irradiation for 15 minutes at 300 mW. This study proposes a non-invasive method that can be used alone or in conjunction with other cancer treatments. Future research should focus on understanding the biological mechanisms involved and optimizing laser parameters. Moreover, to better represent the tumour microenvironment and enhance the accuracy of laser-cell interaction analysis, researchers should utilize more physiologically relevant models, such as 3D cultures or ECM-mimicking systems.

Statistical Analysis

To compare the means of the treated and untreated groups, a one-way analysis of variance (ANOVA) was used, followed by Tukey's post-hoc test. The SPSS software, version 27.0, was used to analyze the data. Error bars were represented as the mean $\pm$ standard deviation (SD), and the significance level was set at $P \leq 0.05$, $n = 3$.

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Competing Interests

None.

Ethical Approval

Ethical approval was not required for this study, as it did not involve human participants or animal subjects.

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