



The Cytotoxic Effect of 4 Different Wavelengths of Low-Level Laser Therapy on Squamous Cell Carcinoma

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ABSTRACT

Introduction: Low-level laser therapy is a noninvasive method with the potential ability to change the balance of cell mediators and gene expressions. It affects cellular function resulting in beneficial clinical effects. This study aims to assess the effect of low-level light therapy (LLLT) using four different laser wavelengths on oral carcinoma cell viability in vitro.

Materials and Methods: HN5 human head and neck squamous cell carcinoma cell lines (HNSCC) were cultured and irradiated using four wavelengths of blue (485nm), green (532nm), red (660nm), and near Infra-red (810nm) in a continuous mode with a dose of 1 J/cm² (0.1W, 10sec) every 24hours for five consecutive days. Cell viability was assessed by evaluating mitochondrial activity by MTT assay.

Results: All the wavelengths resulted in reduced viability of these cells compared to the controls. (P<0.05) There were statistically significant differences in cell viability between different wavelengths (P<0.001). The 810nm laser irradiation showed the highest percentage of cell survival (55.92%) while 660nm induced the lowest (36.02%).

Conclusion: Different laser wavelengths may result in different effects on irradiated cells and red irradiation showed the lowest cell viability and the infrared laser had the highest cell viability results.

Keywords: Low-level laser therapy; LLLT; Head and neck squamous cell carcinomas; HNSCC.

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Introduction

Low-level laser therapy (LLLT) or Photobiomodulation (PBM) refers to the use of photochemical or photophysical effects of light (laser or LED) at wavelengths typically between 600nm and 1200nm, known as the optical window the efficiency of laser penetration into target tissues is maximized [1]. The radiation intensity or power density for this can vary from 5W/cm² to 500W/cm² and output powers from 1mW to 500mW, which can be used in continuous or pulsed radiation mode. LLLT achieves its photochemical effects by penetrating the mucosa without overheating or having special adverse effects. After passing through the mitochondrial inner membrane, the emitted photons affect cytochrome-c oxidase [2]. This process leads to the production of adenosine triphosphate (ATP), reactive oxygen species (ROS), and nitric oxide (NO), which ultimately improves cell viability, induces the production of growth factors, and improves the viability of irradiated cells [3]. It has been shown that LLLT can have many different biological effects on cell activity, from proliferation to apoptosis depending on the irradiation parameters and the cells treated [4]. LLLT which stimulates these biological processes, has many applications in dentistry, including the healing of acute or chronic wounds and reducing the healing process with minimal pain [5].

Oral squamous cell carcinoma (OSCC) is considered the most common oral cancer and a public health problem with wide geographic distribution [6]. In recent years, the incidence of OSCC has increased significantly, especially among women and the younger population. This phenomenon is said to be related to an increase in alcohol and tobacco consumption [7]. To date, no screening marker has been shown to be effective, and careful physical examination remains the main approach for early detection [8]. Conventional therapy is surgical resection, adjuvant radiation, or chemotherapy plus radiation (known as chemoradiation or CRT), which has many side effects, such as oral mucositis (OM), which can affect the quality of life and negatively affect the prognosis of cancer treatment. Except for early cancers (which are treated only with surgery), the treatment of most cases of OSCC requires a multidisciplinary approach [9]. Low-level laser therapy seems to be a promising method in the treatment of these patients. In addition, many systematic reviews have reported a significant benefit of phototherapy or PBM in the treatment of side effects of (chemo)radiotherapy (CRT), such as oral mucositis, in patients with head and neck cancer [10,11]. In recent years, pho-

tobiomodulation has become the basis for many less invasive cancer treatments. Antitumor photodynamic therapies (PDT), which use low-level light and exogenous chromophores or carriers, are an effective form of less invasive therapy to precisely target these cells and help overcome some of the surgical limitations of cancer treatment in areas such as the head and neck [12]. Photoimmunotherapy and inducing a host antitumor response is another intriguing approach that uses PBM or low-dose biophotonic therapies to target tumors based on the high similarity between the healing wound and the tumor. This approach is implemented by simulating both the recreation of biological settings and the final stages of repair, including resolution and organization [13].

An important issue with direct irradiation of cancer cells has always been the safety of this procedure. It is known that the low-level light used in PBM is non-ionizing and does not cause DNA damage. Some international cancer treatment guidelines have even found that PBM therapy is an effective adjunctive treatment for oral mucositis [14]. In addition, the safety of PBM in cancer patients has been shown to be safe in the treatment of oral mucositis in recent long-term human clinical trials. These reports showed a positive effect without recurrence [15,16]. However, there has always been concern about the potential adverse effects of PBM on residual malignant and nonmalignant cells at these sites, and many studies have focused on these effects. Although some studies have reported an increase in the proliferation of these cells, there are also signs of a possible inhibitory and apoptotic effect of phototherapy on malignant cells. An improvement in the host response and an increase in antitumor immunity has also been observed in some live animals [17]. However, further research is needed.

Another interesting finding was the ability of PBM to increase the sensitivity of cancer stem cells to therapy. Based on these findings, PBM therapy may help in oncology by inducing cell cycle changes and promoting the differentiation of cancer stem cells, which would make them more sensitive to routine cancer treatment [18,19]. It should be taken into account that PBM is a sensitive procedure, and the effect of laser treatment depends on parameters such as wavelength, exposure time, and dose. The dose is considered one of the most important parameters of laser treatment. Different cell lines may even respond differently [20]. A systematic review focused on the effects of PBM therapy in squamous cell carcinoma of the head and neck only. They found wide variations in study designs,

PBM treatment protocols, and reported findings and outcomes. While one in vivo study reported increased progression of oral squamous cell carcinoma (OSCC), another found decreased tumor progression and reported that no clear conclusions could be drawn based on the current literature [21]. Therefore, it seems that in vitro studies are still necessary to investigate the effects of PBM on cancer cells and to find an effective therapeutic dose for future well-designed in vivo studies. In the present in vitro study, we aim to evaluate the effect of wavelengths used in low-level laser PBM on oral carcinoma cell line viability at different wavelengths with similar radiation parameters.

Materials and Methods

HN5 -cells (human head and neck carcinoma cell lines, NCBI code: C196) grown in Dulbecco Modified Eagle Medium (DMEM) (Sigma-Aldrich, St Louis, MO, USA) 10% FBS (Gibco, UK) and 2mM l - glutamine with pH 7.2-7.5 was obtained from the Pasteur Institute (Tehran, Iran). All cells were kept frozen at the temperature of -80°C and a medium containing 10 % bovine serum (FBS), 1% L-glutamine, and 1% antibiotic solution (250 $\mu\text{g}/\text{ml}$ streptomycin and 80 $\mu\text{g}/\text{ml}$ gentamicin sulfate). After thawing at 20°C and pH maintained at 7.8 using 10% sodium bicarbonate solution, cells were grown in plastic culture flasks. Cell cultures were reseeded twice a week. Before experiments, all cultures were observed by light microscopy and culture viability was confirmed by trypan (T8154-20 ml, Sigma-Aldrich, USA) exclusion test. Twenty-four hours before irradiation, a cell suspension (5 x 10⁴ cells/well) was prepared and inoculated into 96-well plates (Greiner Bio-One, Germany). A standard deviation of 0.095 was taken when calculating the sample size to investigate the main effect of different wavelengths of diode lasers on viability, according to De Lima et al. [22], who obtained an SE value of approximately 0.03 with a sample size of 10. The smallest sample size to test for a difference of 0.1 unit between the studied groups was 12 cases. Six wells were counted for each group, and empty wells were cultured between them (Figure 1).

Sampling was done by a computer-generated simple random method using the RANDBETWEEN function in Microsoft Excel. The wells were irradiated from below, and the other wells were covered with dark paper during irradiation to avoid accidental exposure. 6 wells were cultured as a control group without irradiation. The cells were then irradiated in a dark room with doses of 1 J/cm² for five consecutive days (every 24 hours). Four diode lasers with wavelengths of blue 485

nm (LASER SYSTEM Ltd, Iran), green 532nm (LASER SYSTEM Ltd, Iran), red 660nm (THOR Photomedicine Ltd, UK) and infrared 810nm (THOR Photomedicine Ltd, UK) were used (Figure. 2,3). All devices were operated at continuous wavelengths with an output power of 100 mW for 10 s and a spot size of 1cm covering each well (Figure 4). Each session was irradiated at an energy density of 1 J/cm², resulting in a total energy density of 5 J/cm². A similar group was considered as the control without laser application (Table 1).

MTT ASSAY

MTT assay is a widely used colorimetric assay that is used to measure cell viability and proliferation. MTT stands for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which is a yellow tetrazolium salt. In the MTT assay, tetrazolium salt is added to cells in the culture. The tetrazolium salt is taken up by living cells and is converted by mitochondrial enzymes into a purple formazan product. The formazan product is insoluble and is trapped within the cells. It can be extracted from the cells using solvents such as dimethyl sulfoxide (DMSO) and the intensity of the resulting color is measured using a spectrophotometer [23]. This study was approved by the ethics committee of the Tehran University of Medicine with reference code IR.TUMS.VCR.REC.1395.2.

Statistical Analysis

Analysis of variance was used for statistical evaluation. Data were expressed by SPSS software Version 25.0. Armonk, NY: IBM Corp and a two-way ANOVA test was used followed by the Tukey test for the cell viability assays. A significant difference of $P < 0.05$ was considered acceptable.

Results

The average cell viability was calculated in different wavelengths (Table 2). The results of ANOVA analysis showed that the viability of cancer cells after irradiation with different wavelengths at the same energy density is not the same ($p < 0.001$). (Figure 5). The comparison of viability of the HN5 cell line with different wavelengths was evaluated by two Tukey tests. The results showed that after irradiation with different wavelengths, the difference in viability was significant ($p < 0.001$) and it was somewhat significant in the case of blue and green wavelengths as well as blue and IR. The details related to the comparison of wavelengths with each other and their p-values are given in Table 3. By examining the viability of cells after irradiation-

among the wavelengths used, the highest percentage of cell survival was obtained for the wavelength of 810nm (IR) at an energy density of 21 j/cm^2 . In other words, more cells survived in this wavelength, and the lowest viability was related to the wavelength of 660nm (Red) in the same energy density, which compared to other wavelengths, fewer cells survived. This means that the 660nm wavelength had the greatest effect in destroying cancer cells. We finally concluded that as a result of irradiating the diode laser with an energy dose of 21 j/cm^2 and 4 different wavelengths, the percentage of cancer cells destroyed was the lowest with the infrared wavelength (810nm) and the highest with the red (660nm) wavelength.

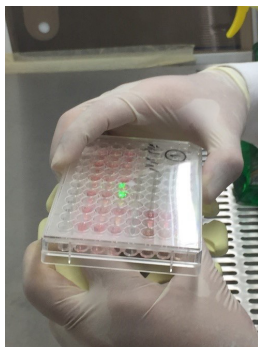


Figure 1. HN5 (oral squamous cell carcinoma) cell line prepared and seeded in 96 well plates.



Figure 2. Low-level diode laser (THOR Photomedicine Ltd, UK) with wavelengths of 660nm and 810nm.

Table 1. Irradiation parameters.

Wavelength	660nm, 810nm, 485 nm, 532nm
Wave type	Continuous wave
Power density	100 mW/cm^2
Application time	10 s
Energy density	1 J/cm^2
Number of applications	1-time per day for 5 days



Figure 3. Low-level diode laser (LASER SYSTEM Ltd, Iran) with wavelengths of 485nm, green 532nm.

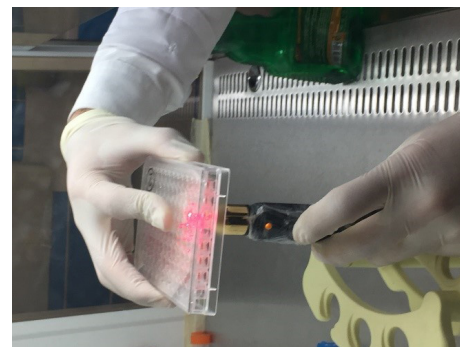


Figure 4. Laser irradiation on well plates.

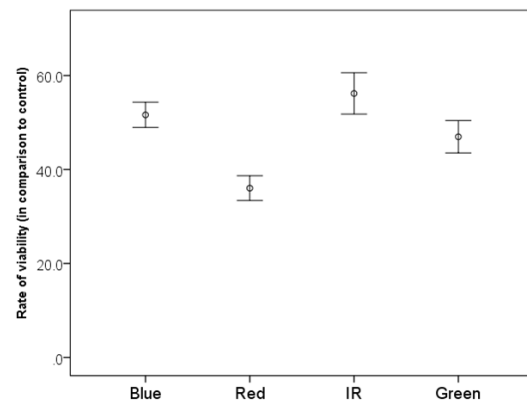


Figure 5. Percentage of viability of HN5 cells with different laser irradiations.

Table 2. Viability of irradiated and control groups.

Wavelength	Control (Mean±SE)	OD (Mean±SE)	Percentage compared to control
532nm	0.561±0.022	0.289±0.06	3.28±46.96
485nm	0.591±0.039	0.277±0.10	2.56±51.62
660nm	0.560±0.027	0.201±0.008	4.18±56.17
810nm	0.559±0.041	0.312±0.003	2.51±36.02

Table 3. Comparison of cell viability between different wavelengths.

Group		Mean difference (I-J)	p- Value	95% Confidence Interval	
				Lower Bound	Upper Bound
Blue	Red	0.15	<0.001	0.10	0.20
	IR	-0.04	0.09	-0.09	0.006
	Green	0.04	0.08	-0.005	0.09
Red	Red	-0.15	<0.001	-0.20	-0.10
	IR	-0.20	<0.001	-0.25	-0.14
	Green	-0.10	<0.001	-0.16	-0.05
IR	Red	0.04	0.09	-0.006	0.09
	IR	0.20	<0.001	0.14	0.25
	Green	0.92	<0.001	0.04	0.14
Green	Red	-0.04	0.08	-0.09	0.005
	IR	0.10	<0.001	0.05	0.16
	Green	-0.09	<0.001	-0.14	-0.04

Discussion

Mester was the first to introduce low-level laser therapy (LLLT) for the treatment of open wounds in mice [24]. LLLT can modify cell behavior depending on the irradiation settings. In vitro cultured cancer cells are the safest and easiest way to assess the viability and growth of neoplastic cells and to test the effect of laser radiation on these cells; however, it does not represent the complex biological environment of a tumor growing in the host. Therefore, it must be taken into account that the results of studies such as the present study may be different in an in-vivo situation. Based on the results of this study and the parameters used (100mW, 1J/cm²), all four different wavelengths of 485nm blue, 532nm green, 660nm red, and infrared 810nm diode laser radiation showed a reduction in the mitochondrial activity of cells compared to non-irradiated control SCC cells. Previous studies of these cells used different wavelengths and study designs [17,25]. In an in-vitro study on human oral carcinoma cell

lines, non-malignant epithelial cells, and fibroblasts, Schartinger et al. [23] evaluated the effects of low-level laser treatment on proliferation, cell cycle division, and apoptosis at the power of 350mW and the wavelength of 660nm for three days. Interestingly, their findings showed that LLLT caused increased proliferation levels of fibroblast cells and decreased proliferation levels of SCC and non-malignant epithelial cells. Their result was similar to this study and also to the study by De Castro and others [26] where LLLT was used with an energy of 4j/cm² at wavelengths of 830nm and 685nm. They assessed cell viability at 0, 6, 12, 24, 48, and 72 hours after laser application. After 6 hours, cell viability decreased at both wavelengths, but after 12 hours of treatment, an increase in proliferation was observed with 830 nm near-infrared radiation. Previous reports have observed differences in the effect of laser wavelength similar to our study. A study by Pinheiro et al [27] evaluated the effect of LLLT on laryngeal squamous cell carcinoma (SCC). Two 635nm and 670nm diode laser doses ranging from 0.04 J/cm² to 0.48 J/cm²

were applied to the cultured cells for seven consecutive days at an output power of 5mW. The 635nm laser did not significantly stimulate SCC cell proliferation, while the 670nm laser irradiation increased cell proliferation compared to both control and 635nm irradiated cells. They also report that SCC-irradiated cell cultures behave differently from unirradiated cell cultures, and a dose/wavelength dependence is observed. In a similar study, Shirazian et al. [28] studied the effect of low-level 660nm and 810nm lasers with an energy density of 4 j/cm² and different powers on tongue squamous cell carcinoma (TSCC-1). Laser irradiation was performed 4 times (0, 24, 72, and 168 h) and cell proliferation was evaluated by MTT assay. The authors concluded that 660nm laser radiation at 80mW and 810nm laser radiation at 200mW power have a significant inhibitory effect on tongue SCC cell proliferation. They also suggested timekeeping as an important factor in PBM efficiency.

In a study conducted by Mansourian et al. [29], oral squamous cell tumorigenic cells were irradiated with three different wavelengths of low-level lasers including 660nm, 810nm, and 940nm, and compared with a control group. Laser irradiation was done at the power of 200mW and energy density of 4j/cm². Cell viability, IL-6, and VEGF expression were analyzed by MTT assay, ELISA, and RT-P. Finally, they reported a significant reduction in cell viability in all laser-irradiated groups compared to controls.

Sperandio et al [30] investigated the effects of two energy densities, 660nm, and 780nm, with a power of 40mW and an energy density of 2.05, 3.07, or 6.15 J/cm² on dysplastic oral cells and oral cancer cells (SCC9 and SCC25) and reported a different behavior in the cell lines studied. Protein analysis was performed by Western blot and immunofluorescence. Although dysplastic oral cells showed an improvement in cell viability at all test periods and all laser wavelengths and doses used. The cancer cell line SCC9 showed a different behavior, with a general increase in cell viability at the infrared wavelength and a strong growth inhibition at the red wavelength, similar to the results observed in this study. The SCC25 cell line also showed significant growth stimulation at some of both wavelengths, while both SCC9 and SCC25 tended to show lower levels of cell viability at the last time point assessed. However, in a recent study by Schalch et al., the SCC9 cell line was irradiated with the same parameters as Sperandio et al. but their viability was reduced compared to the control [31].

Some studies also reported a result different from our report, for example, Bamps et al [25] found that LLLT with 830nm wavelength and 150mW power, increased cell proliferation in a dose-dependent manner in head and neck cancer. They found that LLLT at 1 j/cm² stimulated mitogenic pathways by increasing pAkt and perk protein levels. However, no significant effect was observed using 2 j/cm². In our study, when we used LLLT at all four different wavelengths with an energy of 1 j/cm² for 5 days, we observed a decrease in the viability of neoplastic cells compared to the control groups. On the contrary, Gomez et al. [32] also showed that LLLT with a 660nm laser at an energy density of 1 J/cm² can induce a stimulatory effect on the proliferation and invasion of a tongue squamous cell carcinoma cell line. Kara et al [33] evaluated the effect of LLLT on osteoblast-like osteosarcoma cells and human lung cancer cells at an infrared wavelength of 1064 nm, performed 1.2 and 3 times at 0.5, 1.2, and 3 W power output. According to these results, proliferation also increased in cell cultures treated with LLLT, but the increase was greater at 1 W compared to 2 and 0.5 watts, and proliferation was lowest in samples from the 3 W power group. 2 and 3-times applications resulted in the highest proliferation. Overall, 1 W of power and 2 times usage resulted in the highest proliferation. In contrast, the 3W power and 3-times applications sample groups had the lowest prevalence.

LLLT effects can be expected to depend on potency and the number of applications. These differences in results may be due to differences in irradiation methods and equipment used and irradiation parameters, as well as different evaluation methods and incubation times, and even differences in cell lines. A study on the effect of laser therapy on gene expression in patients with squamous cell carcinoma reported that, although laser therapy was able to suppress some dysregulated genes in patients, it simultaneously activated other unfavorable genes, such as the LCE3D gene, which are and thus can be both beneficial and possible side effects [34].

A recent study by Tabosa et al [35] evaluated the cellular response of oral squamous cell carcinoma after low-level laser treatment before radiotherapy. Cells were irradiated with a 660nm low-level laser at a single energy density (300J/cm²) before ionizing radiation at various doses. The authors report a significant reduction in cell proliferation and clonogenic cell survival. Analysis of cell death and migration also showed a lower migration rate and higher cell death in the treatment group. In addition, an increase in intracellular

ROS levels was observed. However, further *in vivo* studies are needed to confirm the results. In a review study by Del Vecchio et al. [36], the aim was to investigate the results of studies of Photobiomodulation applied to the neoplastic site. The authors emphasized that it is clear that the stimulatory or inhibitory effect of PBM is based on parameters such as light transmission and wavelength. This fact is consistent with the Arndt-Schultz law [37] because weak stimuli can increase physiological activity, moderate stimuli suppress activity, and very robust stimuli suppress activity. The authors concluded that Photobiomodulation can be expected to have a beneficial role in cancer treatment, but more research is needed to identify safe and correct clinical protocols.

In the present study, cell viability varied at different wavelengths. 810 nm laser induced the highest cell survival, while 660 nm had the lowest cell survival, and this superior stimulatory effect observed with infrared radiation is consistent with previous studies. However, due to differences in study protocols and the use of different doses, a clear summary of the effects of LLLT on cancer cell behavior is not yet possible. The results of this study confirm possible wavelength-dependent effects. Although different wavelengths resulted in different cell viability in all groups, no proliferation was observed. Despite the variability of many different results in this field, it seems that the application of laser PBM in cancer treatment has great potential, and with the correct understanding and application of laser parameters and wavelengths, we can achieve the desired effect. Therefore, further studies are needed to correctly explain the effect of different laser parameters on processes related to cancer growth, such as the apoptosis of these cells, the regulation of tumor suppressor genes, and the expression of molecules involved in cell adhesion and migration to help better control these malignant cell types.

Conclusion

Based on the results of this study, cancer cell viability decreased after laser irradiation with the lowest cell viability in the red laser (660nm) and the highest cell viability in the infrared laser (810nm). *In vivo* prospective studies are very necessary to consider LLLT as a promising strategy that could be implemented in the clinic.

Conflict of Interest

There is no conflict of interest to declare.

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