Journal of Craniomaxillofacial Research

Vol. 10, Issue. 4 Autumn 2023

Does Low-Level Laser Therapy Affect Bcl-2 Gene Expression In Oral Squamous Cell Carcinoma

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ARTICLE INFO

Article Type:
Original Article

Received: 8 August 2023
Revised: 7 October 2023
Accepted: 19 November 2023

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ABSTRACT

Introduction: Oral squamous cell carcinoma, which is known as the most common cancer of the oral cavity, is associated with high morbidity and mortality rate. To date, many efforts have been made to find effective methods to stop the growth of cancer cells. One of the emerging methods in this field is the application of low-level lasers.

Materials and Methods: Human head and neck carcinoma cell lines were prepared and irradiated with four different wavelengths of low-level laser with a density of 1 j/cm^2 and a power of 100 mW. The expression of the Bcl-2 and Bax genes and the Bax/Bcl-2 ratio were investigated by real-time PCR.

Results: The highest percentage of Bcl-2 gene expression was related to 660nm wavelength and the highest percentage of Bax gene expression was related to 810 nm infrared wavelength. The lowest level of expression of the Bcl-2 gene related to the 810nm infrared wavelength and the Bax gene related to the 532nm wavelength was obtained. The lowest Bax/Bcl-2 ratio was obtained at the wavelength of 660nm and the highest ratio was obtained at the wavelength of 810nm.

Conclusion: Despite the extensive studies conducted in the field of low-level laser application in oncology, more studies are needed to investigate the effect of this technology on cancer cells.

Keywords: Low-level laser therapy; Low-level light therapy; Squamous cell carcinoma; Squamous cell carcinoma of head and neck.

Please cite this Article as:
Tonkaboni A, Tatar A, Hafezi Motlagh K, Fekrazad R. Does Low-Level Laser Therapy Affect Bcl-2 Gene Expression In Oral Squamous Cell Carcinoma. J Craniomaxillofac Res 2023; 10(4): 153-161. DOI: 10.18502/jcr.v10i4.15306

Introduction

It is a well-known fact that a staggering 90% of oral cancers are classified as oral squamous cell carcinomas (OSCC), undoubtedly the most prevalent form of cancer to afflict the mouth cavity. According to a World Health Organization (WHO) report, it is the eighth most pervasive form of cancer found to afflict individuals across the globe [1]. Globally the prevalence of OSCC has increased, particularly in younger age groups. The tongue is the most often affected area and has a greater death rate than other OSCC subregions such as the gingiva, retromolar region, and the floor of the mouth [2]. Oncogenic viruses like HPV, syphilis, irradiation, immunosuppression, candida, vitamin A deficiency, betel nut, phenol, tobacco, alcohol, betel nut chewing, betel nut, betel nut chew, candida, and syphilis can all be recognized to be risk factors or leading factors for the development of oral cancer [1].

Oral squamous cell carcinoma (OSCC) still has a high mortality rate despite significant advancements in oncology and cancer treatments, which calls for more research in the area of prognostic factors. The Tumor-Node-Metastasis (TNM) classification is the primary factor used to determine the OSCC treatment plan [4]. The prognosis and behavior of cancer are hardly predictable, and the best prognostic indicators for patients are the extent of metastasis, tumor size, and tumor grade at diagnosis [2].

Oncogenes play a role in the initiation and development of a variety of neoplasms, including OSCC. On the other hand, because of mutation or inactivation, tumor suppressor genes are indirectly connected to the development of tumors. Aging is correlated with mutagenic and epigenetic alterations [6]. Activation of oncogenes increases gene expression, which drives the synthesis of new genetic material. There have been 4 identified apoptotic mechanisms: 1. The function of caspases 2. Mechanisms that start at the cell surface 3. The mitochondrial roles 4. The Bcl-2 gene family, includes pro- and anti-apoptotic proteins like Bax and bad, Bcl-2, and bcl-xl [7]. Bcl-2 overexpression causes changes in planned cell death as well as the prolonged inability of cells to die. The increase of Bcl-2 protein has been associated with the early stages of epithelial carcinogenesis. This protein has been found to express abnormally in a variety of solid tumors, including colon, lung, thyroid, breast, ovary, and breast carcinomas [9]. Bax and Bcl-2 are antagonistic to one another, and both of these proteins' expression levels are crucial for controlling the apoptotic process. It appears that the ratio of Bcl-2/Bax and other apoptotic proteins affects how sensitive cells are to apoptosis-initiating trigger [3]. Advanced stages of oral carcinoma are usually treated by a combination treatment plan, including surgery, radiotherapy, and even chemotherapy. However, surgical treatment is the first option for small and accessible oral carcinomas. In the presence of significant lymphatic metastases, radical neck dissection is the preferred treatment. The first line of treatment is the combination of EFGR inhibitors and chemoradiotherapy secondary to tumor recurrence [11]. In the medical field, lasers are currently involved in the treatment of various types of diseases.

Laser is one of the most efficient tools for making mankind for a variety of uses but there are doubts about using the laser in places where tumor cells can remain. Clinical applications of lasers were initially limited to ophthalmology and dermatology [12]. Photobiomodulation (PBM) is used to accelerate healing by stimulating ATP synthesis at the cell membrane and mitochondrial receptors [13]. Previous research has indicated that PBM can enhance cell bioactivities, such as cell migration, proliferation, and survival [4]. The impact of photobiomodulation on head and neck squamous cell cancer has been the subject of several investigations. The safest irradiation wavelength, according to Schalch TD et al., is 780nm, and it reduces the ability of tumor cells to migrate and sustain cell viability while inducing apoptosis [15]. According to a related investigation, oral SCC cell lines exposed to low-level laser radiation at three different wavelengths displayed lower cell survival than the control group [5]. Due to the lack of research in this field and the contradictory results of existing studies, this study aimed to investigate the influence of low-level laser therapy on the expression of apoptosis-associated genes (BCL-2, BAX) in oral squamous cell carcinoma cell lines.

Materials and Methods

HN5 cells (human head and neck carcinoma cell lines, NCBI code: C196) were prepared from Pasteur Institute (Tehran, Iran). The cells were kept in RPMI 1640 at 37°C with 5% CO2 after being transferred from the HANKS solution. This medium comprises 10% embryonic bovine serum (Gibco, New York, USA), DMEM (Sigma-Aldrich, St. Louis, USA), 100 IU/ml penicillin, and 100g/ml streptomycin (Figure 1). All cells were cultured on six-row plate (Greiner Bio-One, Germany). So, each row was considered for a specific dose of laser and a row for control (A negative control group was considered, which did not undergo laser

 DOI: 10.18502/jcr.v10i4.15306

irradiation). Using the RANDBETWEEN function in Microsoft Excel, sampling was carried out using a computer-generated basic random approach. Four diode lasers with wavelengths of blue 485(LASER SYSTEM Ltd, Iran) nm, green 532nm (LASER SYSTEM Ltd, Iran) (Figure 2), red 660nm (THOR Photomedicine Ltd, UK), and Infrared 810nm (THOR Photomedicine Ltd, UK) (Figure 3) were used. The cells were irradiated with a density of 1 j/cm^2 and the power of 100mW and in continuous wave.

Time of irradiation (second):

1. Red (660nm) (5 s).
2. Infra Red1(IR1) (810nm,1 s).
3. Infra Red2 (IR2) (810nm,17s).
4. Green (21s).
5. Blue (22s).

For five days, the operation was carried out once every 24 hours. The laser head output was performed as closely to the plate's bottom as possible and was totally adhered to it (Figure 4). It was separated by a row to avoid the danger of the laser touching the next rows. A thick blackboard sheet was also positioned between the row's boundary and the radiation beam during the irradiation. The plate was placed in a dark room for the rest of the time. All of the plate's contents were transferred to a microtube and centrifuged at 12,000 rpm for 2–5 minutes after being neutralized with media containing 10% FBS. After removing the supernatant, 1 ml of the RNX PLUS ice-cold solution was added to the tube holding 2ml of the homogenized material. The tube was then centrifuged for 5–10 seconds and allowed to sit at room temperature for 5 minutes. To extract RNA, after filling 90% of the surface of the plate with cells and washing it with PBS, the cells were separated from the bottom of the plate using the trypsin enzyme.

In order to isolate RNA, 200µl of chloroform per 1000 ml of RNX PLUS was used, and after 15 seconds of manual mixing, it was placed on ice for 5 minutes and then centrifuged for 15 minutes at 4°C at 12000 rpm. After this step 2 distinct layers can be seen in the microtube, we carefully separated the upper colorless layer and poured 1.5ml of RNA ase-free into the microtube, and added the same volume of isopropanol. The liquid was gently mixed and incubated on ice for 15 minutes. Then the microtube was centrifuged at 12000 rpm for 15 minutes at 4°C.

The supernatant solution was taken out, 1ml of 75% ethanol was added to the microtube, and the microtube was centrifuged for 8 minutes at 4°C and 7500 rpm to wash the RNA with the ethanol solution. The supernatant was once more separated and discarded. For a few minutes, the microtube was left at room temperature to allow the plate to become comparatively dry. Following drying, the plate was dissolved in 50 l of DEPC water and the microtube was heated for 10 minutes to 55–60 degrees.

The expression of Bcl-2 and Bax genes, as well as the Bax/Bcl-2 ratio, were evaluated using real-time PCR on a Rotor-Gene 3000 (Corbett Robotics, Australia). For this purpose, 30µL of total RNA from each sample was obtained and 1µg was converted to cDNA with a reverse transcription kit. The reaction components for the real-time PCR consisted of a 100ng template, 12.5µL MasterMix (Applied Biosystems, Foster City, CA, USA), 10 pmol forward and reverse primers, and deionized water to make the final reaction volume 25µL. This study was approved by the Ethics Committee of Tehran University of Medical Sciences under the identification code of IR.TUMS.VCR.REC.1395.1191.

Statistical Analysis

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

Results

The results of ANOVA analysis indicated that Bcl-2 and Bax and Bax/Bcl-2 gene expression in cancer cells was significant after radiation with different wavelengths at a density of 1 j/cm^2 ($p<0.001$). The comparison of the expression of Bcl-2 and Bax gene and their ratio in the HN5 cell category with different wavelengths were investigated two-by-two through Games-Howell analysis. The results showed that after the irradiation with different wavelengths, the difference between the expression of the Bax gene and the Bax/Bcl-2 ratio was significant ($p<0.05$). There is a significant difference in the expression of the Bax gene in infrared wavelengths ($p=0.02$) (Figure 5). In the Bax/Bcl-2 ratio, a significant difference was observed between infrared wavelengths 1 ($p=0.048$), infrared 2 ($p=0.004$), and green ($p=0.04$). The red wavelength has a significant difference with infrared wavelengths 1 ($p=0.04$), infrared 2 ($p=0.005$), and green ($p=0.04$). Green wavelength with infrared

wavelength 2 (p=0.01) is significant (Figure 6). By evaluating the expression of both genes in cells after radiation, the highest percentage of Bcl-2 gene expression was related to 660nm (Red) wavelength and the lowest percentage was 810nm (IR1) at the energy density 1 j/cm^2 (Figure 7) (Table 2). In the Bax gene, the lowest was at the 532nm (Blue) wavelength, and the highest was at the 810nm (IR2) wavelength (Table 1). The Bax/Bcl-2 ratio was the lowest at 660 nm (Red) wavelength and the highest ratio was obtained at 810 nm (IR2) wavelength (Table 3).

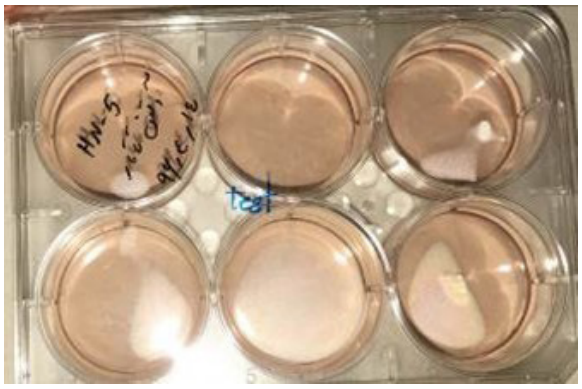

Figure 1. Preparation of the culture medium and human head and neck carcinoma cell lines.

Figure 2. Lasers of blue 485nm (Laser System Ltd, Iran) and green 532nm (Laser System Ltd, Iran) wavelength.

Figure 3. Laser of red 660 nm (THOR Photomedicine Ltd, UK), and Infrared 810 nm (THOR Photomedicine Ltd, UK) wavelength.

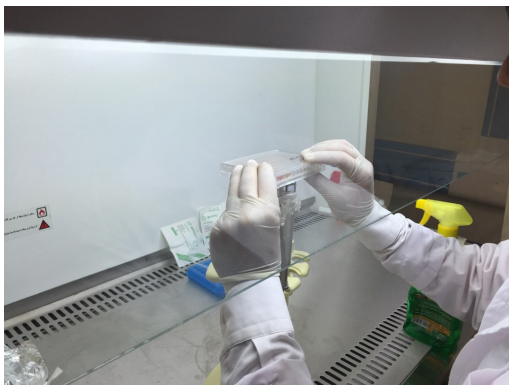

Figure 4. Laser irradiation on the head and neck squamous cell carcinoma cell line (HN5 cell).

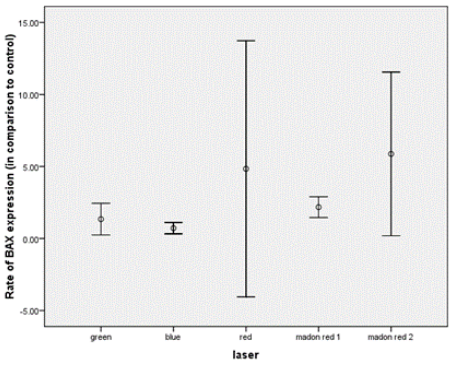

Figure 5. Comparison of Bax expression in HN5 cells in laser groups.

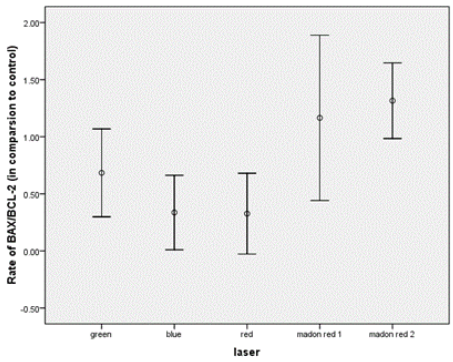

Figure 6. Comparison of Bax/Bcl-2 ratio in HN5 cells in laser groups.

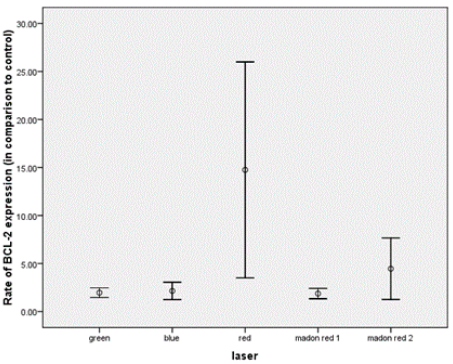

Figure 7. Comparison of Bcl-2 expression in HN5 cells in laser groups

DOI: 10.18502/jcr.v10i4.15306

Table 1. Comparisons of bax in the games-howell analysis.

Group		Mean difference	95% Confidence Inverted	p-Value
Blue	Red	-4.12	-22.30_14.06	0.22
	IR 1	-1.46	-2.16_0.75	0.02
	IR 2	-5.15	-16.62_6.31	0.11
	Green	-0.62	-2.07_0.82	0.14
Red	Red	4.12	-14.06_22.30	0.22
	IR 1	2.66	-15.13_6.81	0.33
	IR 2	-1.03	-8.88_20.46	0.75
	Green	3.50	-13.63_20.62	0.25
Green	Red	0.62	-0.82_2.07	0.14
	IR 1	-3.50	-20.62_13.63	0.25
	IR 2	-0.83	-1.79_0.12	0.06
	Green	-4.53	-14.52_5.46	0.12
IR 1	Red	1.46	0.75_2.16	0.02
	IR 1	-2.66	-20.46_15.13	0.33
	IR 2	0.83	-0.12_1.79	0.06
	Green	-3.70	-14.59_7.20	0.15
IR 2	Red	5.15	-6.31_16.62	0.11
	IR 1	1.03	-6.81_8.88	0.75
	IR 2	4.53	-5.46_14.52	0.12
	Green	3.70	-7.20_ 14.59	0.15

Table 2. Comparisons of bcl-2 in the games-howell analysis.

Group		Mean difference	95% Confidence Inverted	p-Value
Blue	Red	-12.60	-35.13_9.92	0.09
	IR 1	0.28	-0.56_1.11	0.27
	IR 2	-2.31	-7.13_2.51	0.12
	Green	0.18	-0.69_1.06	0.45
Red	Blue	12.60	-9.92_35.13	0.09
	IR 1	12.88	-10.08_35.84	0.09
	IR 2	10.29	-6.68_27.26	0.09
	Green	12.79	-10.21_35.78	0.09
Green	Blue	-0.18	-1.06_0.69	0.45
	Red	-12.70	-35.78_10.21	0.09
	IR 1	0.09	-0.36_0.54	0.61
	IR 2	-2.50	-8.42_3.42	0.12
IR 1	Blue	-0.28	-1.11_-0.56	0.27
	Red	-12.88	-35.84_10.08	0.09
	Green	-0.09	-0.54-0.36	0.62
	IR 2	-2.59	-8.41_3.23	0.12
IR 2	Blue	2.31	-2.51_7.13	0.12
	Red	-10.29	-27.26_6.68	0.09
	Green	2.50	-3.42_8.42	0.12
	IR 1	2.59	-3.23_8.41	0.12

Table 3. Comparisons of the two Bax / Bcl-2 in the analysis of Games-Howell.

Group		Mean difference	95% Confidence Inverted	p-Value
Blue	Red	0.01	-0.28_0.3	1.00
	IR 1	-0.83	-1.6_-0.01	0.05
	IR 2	-0.98	-1.26_-0.69	0.004
	Green	-0.35	-0.66_-0.03	0.04
Red	Blue	-0.01	-0.30_0.28	0.00
	IR 1	-0.84	-1.60_-0.07	0.04
	IR 2	-0.99	-1.28_-0.69	0.005
	Green	-0.36	-0.67_-0.03	0.04
Green	Blue	0.35	0.03_0.66	0.04
	Red	0.36	0.03_0.67	0.04
	IR 1	-0.48	-1.19_0.23	0.09
	IR 2	-0.63	-0.94_-0.31	0.01
IR 1	Blue	0.83	-1.11_-0.56	0.05
	Red	0.84	-35.84_10.08	0.04
	Green	0.48	-0.54-0.36	0.09
	IR 2	-0.15	-8.41_3.23	0.04
IR 2	Blue	0.98	-2.51_7.13	0.004
	Red	0.99	-27.26_6.68	0.005
	Green	0.63	-3.42_8.42	0.01
	IR 1	0.15	-3.23_8.41	0.44

Discussion

Low-level laser therapy (LLLT) employs the utilization of coherent monochromatic light at low energy levels which do not elevate the tissue temperature beyond 0.1°C. Research has indicated that low-level lasers are associated with mechanisms such as enhanced cell proliferation, alterations in cell cycle control, and apoptosis [6]. Light in low-level lasers exerts its photochemical effects by penetrating the mucosa without significant temperature increase or specific side effects. Penetration of emitted photons to the mitochondrial membrane and stimulating the production of ATP induces the production of growth factors and improves the cellular motility and viability of the irradiated cells [7]. Previous academic research has demonstrated that the investigation of Photobiomodulation dates back to the 1960s. Furthermore, a significant body of literature has focused on examining its beneficial impact in treating various medical ailments, still, many clinical aspects of the application of this method are not fully understood and there are still many unsaid in this field. The application of LLLT in cancer patients has been much debated due to the proliferative effects induced on the cells, although some researchers believe that PBM can directly damage tumoral cells and increase the effectiveness of cancer therapy and also increase the survival rate in OSCC patients [8]. In addition, some other authors stated this hypothesis that the beneficial effects of PBM in neoplastic areas through 3 possible mechanisms including 1. Selective inhibition of malignant cells 2. Stimulating the host's immune system 3. Its direct effect on neoplastic cells [9]. Among the studies that have been conducted to investigate PBM on the proliferation of malignant cells, Sroka et al. [10] have investigated the effect of different wavelengths of 410, 488, 630, 635, 640, 805, and 1064 nm and different energy densities of low-level laser on the mitosis rate of different cell lines, including normal cells and SCC cells of human gingival mucosa. They determined the mitosis rate by Orcein-staining and the proliferation index by the BrdU incorporation during the DNA synthesis. The authors observed that the rate of mitosis in human squamous cell carcinoma cells of the gingival mucosa exhibited a minor decline in response to an increase in irradiation energy, regardless of the wavelength used. Schartinger et al.[11] also conducted a study using 660nm lasers to examine the effect of PBM

 DOI: 10.18502/jcr.v10i4.15306

on human SCC cells and compare them with normal human cells. The authors examined cell proliferation by MTT assay, cell cycle assessment by FACS analysis, and cell apoptosis by flow cytometry. The findings indicate that photobiomodulation leads to a considerable reduction in cell proliferation and G1 phase, while significantly increasing the proportion of cells in the S phase, as compared to the control group. Prior research has demonstrated that low-level laser therapy (LLLT) can stimulate reactive oxygen species (ROS) production, thereby inducing apoptosis of cancer cells via the intrinsic mitochondrial/caspase-3 pathway [12]. In the present study, we aimed to evaluate the effect of 4 different wavelengths of low-level laser with the same energy density on the expression of Bcl-2 and Bax genes which are the apoptotic-related proteins. The results of this study showed that the highest percentage of Bcl-2 gene expression was related to 660nm (Red) wavelength and the highest percentage of Bax gene expression was related to 810 nm infrared wavelength. The lowest level of expression of the Bcl-2 gene related to the 810nm infrared wavelength and the Bax gene related to the 532nm wavelength (Blue) was obtained. Regarding the ratio of Bax/Bcl-2, the lowest amount was obtained at the wavelength of 660nm (Red) and the highest amount of this ratio was obtained at the wavelength of 810nm of infrared. As far as our understanding extends, this is the inaugural research inquiry into the impact of low-level laser therapy on the transcriptional activity of Bcl-2 and Bax genes in squamous cell carcinoma cell lines. To discuss the expression level of apoptotic Bcl-2 and Bax proteins in oral squamous cell carcinoma, Camisasca DR et al. [13] performed immunohistochemical analysis for 53 cases of primary oral SCC and reported that immunoreactivity for Bcl-2 was detected in 86.8% of the cases. Elevated levels of Bax and Bcl-2 expression were identified as independent prognostic indicators of increased overall survival (Bax) and Cancer-specific survival (Bax and Bcl-2). In a similar study conducted by Alam et al [14], Bax gene expression was investigated by immunohistochemistry and Western blot analysis in OSCC patients. The authors concluded that a progressive and significant decrease in Bax gene expression is associated with tumor progression and drug resistance. The role of Bcl-2 in OSCC and drug resistance has been recently reported [15]. These findings are in line with Zhang et al. [16] suggested that the downregulation of the Bax gene plays an important role in the progression of OSCC and is known to be the initiator of a more aggressive phenotype of OSCC.

A gradual decline in Bax expression is associated with tumor development however, the offsetting increase in its expression may be an early response to oral tumorigenesis [17]. Within the Bcl-2 family of proteins, certain members have been identified as pro-survival, including Bcl-2 and Bcl-xL. Conversely, others such as Bad, Bak, Bid, and Bax are commonly acknowledged to promote cell death [18]. Activation of oncogenes, inactivation of tumor suppressor genes, and changes in apoptosis-regulating genes that lead to dysregulation of cell proliferation and reduction of apoptosis are considered the basis of carcinogenesis [19]. Bcl-2 is present in areas rich in stem cells, such as the basal layers of epithelium undergoing proliferation, where it plays a role in inhibiting apoptosis. However, excessive expression of Bcl-2 has been observed during the initial stages of epithelial cancer development oral potentially malignant lesions [31,32].

In normal mucosal cells, the NF-κB (nuclear factor—kappa B) pathway is considered one of the critical pathways in the regulation of cell apoptosis and the production of factors related to apoptosis including Bcl-2 and Bax. Any disruption in this pathway leads to changes in normal mucosal cells. Radiotherapy can disrupt the NF-κB pathway by overexpressing proapoptotic BAX proteins and putting normal mucosal cells on the path of programmed cell death. This is considered one of the possible mechanisms of radiation-induced oral mucositis in cancer patients [20]. To date, multiple molecular mechanisms have been postulated to elucidate the benefits of laser radiation, with Karu's theory being the most widely accepted [21] that believed that mitochondrial signaling is the basis of the theory of cell-light interaction. Alterations in the mitochondrial membrane and its permeability and ultimately the increase in ATP synthesis caused by it, as well as changes in calcium ion homeostasis and augmentations in reactive oxygen species (ROS) production initiate cellular signaling cascades and molecular responses, which are involved in gene expression [22]. The absorption of light by mitochondria is essential for apoptotic processes, leading to cell death via both extrinsic and intrinsic pathways. The extrinsic pathway is initiated when fas-L ligands attach to receptors in the plasma membrane, activating cytoplasmic signaling. The intrinsic pathway is directly dependent on the mitochondria and specifically on the apoptotic proteins Bax and Bcl-2 these proteins facilitate the release of cytochrome-c from the inner membrane of mitochondria to the cytoplasm where cytochrome-c binds to caspase activating proteins [23]. Equilibrated

Bcl-2/Bax ratio, acts as a regulatory effect in apoptosis [24]. In our results, the highest Bax/Bcl-2 ratio amount was obtained after irradiation with an 810nm low-level laser, which may indicate stimulus to cancer cell death. Since the cell response to laser radiation is a biphasic-dose response and depending on the parameters of the laser used, a stimulatory or inhibitory response will occur [6], further studies to evaluate the response of OSCC cells to laser radiation with parameters different in terms of energy and power required.

Conclusion

To the best of our knowledge, the present study is the first study that investigated the effect of four different wavelengths of low-level laser on the expression of Bcl-2 and BAX genes in squamous cell carcinoma cell lines. Despite the extensive studies conducted in the field of low-level laser application in oncology, more studies are needed to investigate the effect of this technology on cancer cells.

Conflict of Interest

There is no conflict of interest to declare.

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