1265 nm laser irradiation activates antioxidant system in B16-F10 and CHO-K1 cells

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ABSTRACT

Photodynamic therapy is an effective modality for treating advanced melanoma. However, melanoma's inherent resistance to laser radiation hinders its widespread clinical application. The near-infrared laser radiation range of 1264-1270 nm offers unique properties: firstly, its ability to penetrate melanin-producing cells, and secondly, its capability to generate singlet oxygen without xenobiotics. We assess the impact of continuous wave 1265 nm laser radiation on an antioxidant defense system in melanoma B16-F10 and normal CHO-K1 cells. We observe a time-dependent increase in superoxide dismutase and glutathione-S-transferase activities, fluctuations in reduced glutathione levels, as well as a simultaneous increase in melanoma cell proliferation and cell death. We hypothesize that the differential activation of cellular antioxidant defense mechanisms contributes to melanoma cells' resilience to laser radiation.

1. INTRODUCTION

Currently, melanoma is recognized as a significant challenge in the field of oncoimmunology, characterized by its high aggression and heterogeneity. The primary skin melanoma is located close to the blood vessels, which increases the likelihood of the metastasis spread throughout the body, unlike most other types of malignancies that undergo the stage of inhibition in the lymph nodes. The most frequent organs for melanoma metastasis are the liver, bone, and brain. [1, 2].

The treatment approach for melanoma varies depending on its stage. More advanced treatment options for metastatic melanoma include photodynamic therapy, immunotherapy, and targeted therapy, each tailored to the specific characteristics of the tumour [3, 4]. While photodynamic therapy is often less invasive than surgical methods, it is not without drawbacks, including side effects such as toxicity, allergic reactions, and sensitization [5, 6], as well as challenges related to cost and regional availability [7].

In recent decades, studies have demonstrated that the generation of reactive oxygen species (ROS) in living cells can occur without the need for exogenous substances, such as xenobiotics, due to the presence of $^3O_2$. When $^3O_2$ interacts with laser radiation in the 1264-1270 nm wavelength range, it induces the formation of singlet oxygen and trigger oxidative stress [8-11].

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Depending on the parameters and modes of the laser radiation, this wavelength range could serve as either the primary or an auxiliary method for melanoma therapy, pending successful clinical trials. However, multiple studies indicate an unusual resistance of melanomas from various origins to laser radiation [12, 13].

Given that, mitochondria are believed to be the primary acceptors of 1265 nm laser radiation, and the predominant effect of this exposure is the induction of oxidative stress [14, 10, 15], understanding the cell's antioxidant defense system and associated processes, such as lipid peroxidation is particularly relevant.

2. MATERIALS AND METHODS

2.1. Source of laser radiation

A Bragg grating-stabilized semiconductor laser diode, model LD-1265.5-FBG-350 (Innolume, Germany), was employed as the source of laser radiation.

2.2. Cell culture

Experiments were conducted using murine melanoma cell line B16/F10 (*Mus musculus*; ATCC CRL-6475™) (Fig. 1A). As a non-cancerous control, a Chinese hamster ovary epithelial cell line CHO-K1 (*Cricetulus griseus*; ATCC CCL-61™) was used (Fig. 1B).

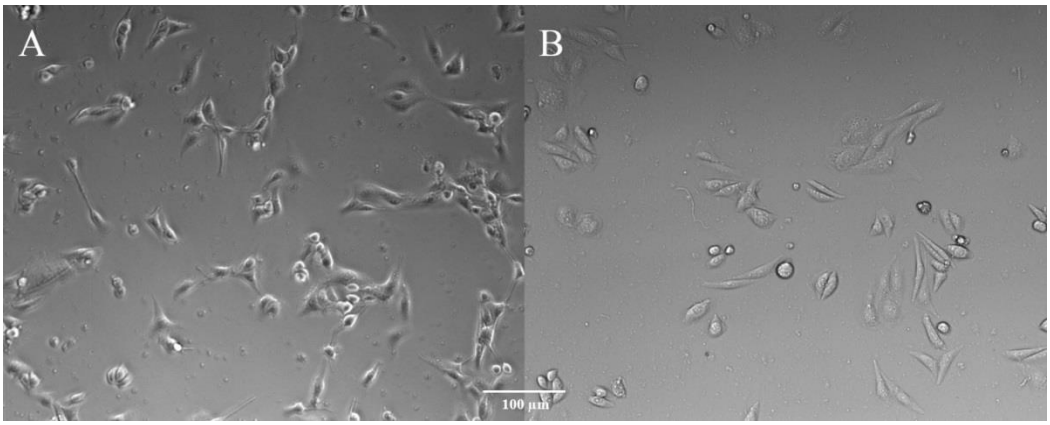

Figure 1. Cell cultures before experiments, early exponential phase, ×100. A. B16-F10. B. CHO-K1.

Cells were cultured in a Sanyo MCO-18AIC CO_2 incubator (Japan) with 5% CO_2, using RPMI-1640 medium supplemented with glutamine (Paneko, Russia). Additionally, the medium contained 10% fetal bovine serum (Biosera, France) and 80 µg/ml gentamicin (Paneko, Russia).

Twenty-four hours prior to the experiments, cells were seeded into 8-well slide chambers (SPL LifeSciences, South Korea) at a density of 50,000 cells per well. Upon achieving adherence and transitioning to the exponential growth phase, cells were subjected to laser irradiation.

2.3. Laser irradiation of cell culture

Irradiation was performed in a UNO tabletop incubator (OkoLab, Italy) for a duration of 30 minutes. The laser radiation source was positioned 0.5 cm away from the bottom of each well. The laser source had a power output of 250 mW.

The energy density of laser radiation (E, J/cm^2) absorbed by the cells was calculated as follows:

$$E = \frac{P \times t}{S}, \quad (1)$$

where P is the average output power (W); t is the exposure time (s); S is the irradiation area (cm^2).

The intensity of laser radiation (I, W/cm^2) was calculated as follows:

$$I = \frac{P}{S}, \quad (2)$$

The parameters of laser radiation during irradiation of B16-F10 and CHO-K1 cell cultures are presented in Table 1.

Table 1. Laser radiation parameters during irradiation of B16-F10 and CHO-K1 cell cultures

Wavelength of laser radiation (Λ, nm)	1265
Laser output power (P, mW)	250
Irradiation time (t, min)	30
Irradiated surface area (S, cm^2)	0.8
Laser radiation intensity (I, mW/cm^2)	312.5
Energy density of laser radiation (E, J/cm^2)	562.5

After irradiation, we determined the activity of superoxide dismutase, glutathione transferase, and the concentration of reduced glutathione and malondialdehyde in control and experimental wells.

2.4. Cell Proliferation

The proportion of proliferating cells was determined by directly calculating the cell concentration using a Goryaev chamber as follows:

$$X = \frac{A}{10} \times 2{,}5 \times 10^5 \times V, \quad (3)$$

where X is the cell concentration in 1 ml, A is the number of cells observed in 10 large squares of the Goryaev chamber, and, V is the volume of the sample in mL.

Subsequently, the calculated values for the experimental and control wells were compared.

2.5. Cell Survival

The proportion of non-viable cells was quantified in at least 300 cells per sample using trypan blue staining (Paneko, Russia) and observation under a Nikon Ti-S inverted light microscope (Nikon, Japan). The resulting values were then expressed as percentages.

2.6. Superoxide Dismutase Activity

Post-irradiation, cells were detached using a 0.25% trypsin-EDTA solution (Paneko, Russia), and subsequently washed three times with a 1× sodium phosphate buffer solution at pH 7.4. A cell lysate was prepared through a freeze-thaw cycle and diluted 1:10 with distilled water.

Superoxide dismutase activity was quantified using an enzyme-linked immunosorbent assay (ELISA) (Cytokin, Russia) and analyzed with a Multiskan FC spectrophotometer (Thermo Scientific, USA). Optical density was measured at a wavelength of 450 nm. Data were presented in pg/mL and normalized to the number of cells in each sample.

The activities of glutathione-S-transferase, as well as the concentrations of reduced glutathione and malondialdehyde, were measured in the cell lysate using a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, USA).

2.7. Glutathione-S-Transferase Activity

A 0.1 ml aliquot of cell lysate was added to a 10 mm path length spectrophotometer cuvette containing 1.2 ml of a 2 mM solution of reduced glutathione in 0.1 M potassium phosphate buffer (pH 6.5). The reaction was initiated by adding 1.2 mL of a 2 mM solution of 1-chloro-2,4-dinitrobenzene in absolute methanol and 0.1 M potassium phosphate buffer (pH 6.5) to the cuvette. The control sample contained an equivalent volume of distilled water.

Optical density was measured at a wavelength of 340 nm relative to distilled water, both immediately after mixing the solutions in the cuvette and 3 minutes later.

The activity of glutathione-S-transferase was calculated using the following formula:

$$A = \frac{\Delta E \cdot V_{r.m.} \cdot 10^6 \cdot 11}{V_{samp.} \cdot l \cdot \varepsilon \cdot t}, \quad (4)$$

where A is the enzyme activity in μmol/(min × L); ΔE is the difference in extinction of samples before and after incubation, excluding the control value; $V_{r.m.}$ is the reaction mixture volume (2.5 ml); $V_{samp.}$ is the cell lysate sample volume (0.1 ml); l is the optical path length (1 cm); ε is the molar extinction coefficient of the resulting product (9600 M-1 × cm-1); t is the incubation time (3 min) [16].

2.8. Reduced Glutathione Concentration

To a 0.6 ml aliquot of cell lysate, 0.2 ml of sulfosalicylic acid was added. The mixture was then centrifuged at 3000 g and +2°C for 10 minutes. A 0.2 ml portion of the supernatant was transferred to tubes containing 2.55 ml of 0.1 M Tris-HCl buffer with 0.01% EDTA at pH 8.5. A 25 µl aliquot of a 0.4% solution of 5,5'-dithio-bis (2-nitrobenzoic acid) in methanol was added to this mixture [16].

Photometric measurements of the sample were performed at a wavelength of 412 nm using a 10 mm path length cuvette, against distilled water as the reference.

The concentration of reduced glutathione was determined using a calibration curve, which was prepared using solutions of reduced glutathione ranging from 0.02 to 2 mmol/l.

2.9. Malondialdehyde concentration

0.3 ml of cell lysate was mixed with 3.7 ml of a 1% orthophosphoric acid solution and 1 ml of a 0.6% thiobarbituric acid (TBA) solution. The mixture was boiled in a water bath for 45 minutes. To the samples, 3 ml of butanol was added, followed by mixing and centrifugation at 3000 rpm for 10 minutes until phase separation occurred. Samples were taken from the upper (butanol) layer for photometric analysis at wavelengths of 535 and 580 nm, using a cuvette with a 10 mm optical path length, compared to butanol as the reference [17].

The concentration of TBA-reactive products was calculated using the formula:

$$\text{TBA } (\mu\text{mol / l}) = (E535 - E580)\ 53.2, \quad (5)$$

where *E535* and *E580* are the extinction values of the samples at 535 nm and 580 nm, respectively; and *53.2* is the conversion factor.

2.10. Statistical Methods

Each experiment was conducted a minimum of three times. Statistical analysis for determining the activity of superoxide dismutase, glutathione-S-transferase, as well as the concentrations of reduced glutathione and malondialdehyde, was performed using the Mann-Whitney U test in Statistica 13.0 software.

3. RESULTS

3.1. Cell proliferation and survival after exposure to 1265 nm laser radiation

The proliferation rate of B16-F10 cells was evaluated 24 hours post-exposure to 562.5 J/cm² laser radiation. As shown in Figure 2A, there was a statistically significant increase in cell proliferation compared to the non-irradiated control group.

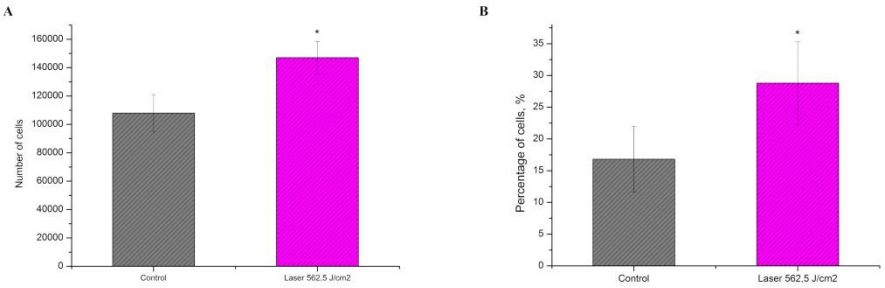

Figure 2. Proliferation and survival of B16-F10 cells 24 hours after exposure to 1265 nm laser radiation. A. The number of cells in the experimental and control groups. B. Percentage of dead cells (%) in control experimental and experimental groups. * - statistically significant difference between irradiated and non-irradiated cells.

The proportion of cell mortality was assessed 24 hours post-exposure to the same energy density. Figure 4B reveals that the cell mortality rate was statistically significantly higher in the irradiated group compared to the non-irradiated control.

The survival of CHO-K1 cells post-1265 nm laser exposure was studied separately by Saenko et al. [14].

3.2. Dynamics of Cellular Antioxidant Defense Systems and Lipid Peroxidation After 1265 nm Laser Exposure

The activity of the key enzyme involved in ROS detoxification, Mn-SOD, which serves as the first line of cellular defense, was evaluated in the lysate of B16-F10 melanoma cells. After exposure to 562.5 J/cm² laser radiation, the activity of Mn-SOD demonstrated a clear time-dependent trend during subsequent cell incubation (Figure 3A, Table 2).

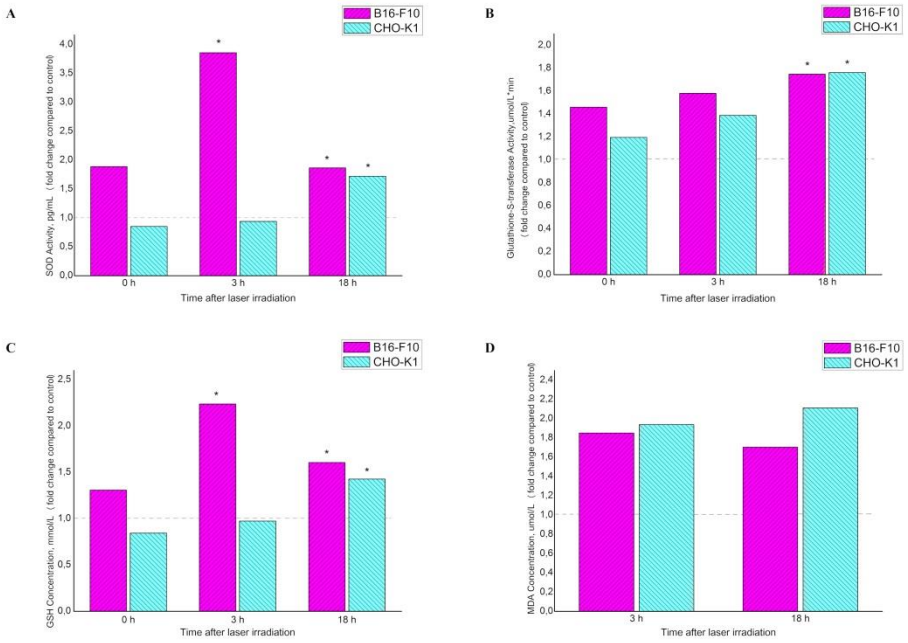

Figure 3. Effect of 1265 nm laser radiation on dynamics of the antioxidant system and lipid peroxidation in both cell lines. A. Superoxide dismutase enzyme activity in B16-F10 and CHO-K1 cells at 0, 3, and 18 hours post-irradiation. B. Glutathione-S-transferase enzyme activity in B16-F10 and CHO-K1 cells at 0, 3, and 18 hours post-irradiation. C. Concentration of reduced glutathione in B16-F10 and CHO-K1 cells at 0, 3, and 18 hours post-irradiation. D. Malondialdehyde concentration in B16-F10 and CHO-K1 cells at 3 and 18 hours post-irradiation. The dotted line in each graphic represents the baseline were experiment and control values are equal * - statistically significant difference between irradiated and non-irradiated cells.

B16-F10 cell culture didn't indicate statistically significant difference between the control and experimental groups immediately after irradiation (616.13±122.72 pg/ml and 1163.24±402.61 pg/ml, respectively). However, 3 hours post-irradiation, enzyme activity surged to 6971.5±4592.44 pg/ml, a value statistically significantly higher than the activity in non-irradiated cells (1806.88±245.98 pg/ml). Moreover, the elevated level of Mn-SOD in B16 melanoma cells persisted even after 18 hours of incubation (7446.03±1261.85 pg/ml) compared to the control group (3990.3±426.78 pg/ml).

CHO-K1 cell culture did not reveal significant differences in SOD activity between the control and experimental groups neither immediately after irradiation nor 3 hours later it. However, an elevation in SOD activity to 9367.7±1430.79 was observed 18 hours after 1267 laser application up to 1.7-fold higher than in the control groups (5437.8±813.88).

Another key enzyme in the cellular detoxification system, glutathione-S-transferase (GST), exhibited no alteration in activity immediately after or 3 hours post-irradiation in both B16-F10 and CHO-K1 cell lines (Fig. 3B, Table 2). However, 18 hours after irradiation, a significant increase in GST activity was observed in the experimental groups of both cell lines.

Concentrations of reduced glutathione (GSH) were measured in B16-F10 and CHO-K1 cells at 0, 3, and 18 hours after 1265 nm laser radiation exposure (Fig. 3C, Table 2). No immediate differences in intracellular GSH concentrations were noted found be-tween experimental and control groups. However, 3 hours after the irradiation, B16-F10 cells exhibited a significant 2.24-fold increase in GSH levels compared to the non-irradiated controls. In CHO-K1 culture, GSH concentration remained relatively stable compared to control levels. Notably, 18 hours after irradiation, both cell lines showed a statistically significant rise in GSH levels in the experimental groups.

Lastly, the concentration of malondialdehyde (MDA), an end-product of lipid peroxidation and a marker of oxidative stress, was assessed in B16-F10 and CHO-K1 cells 3 and 18 hours after exposure to 1265 nm laser radiation (Fig. 3D, Table 2). The analysis revealed no statistically significant differences in intracellular MDA concentrations between the experimental and control groups across both cell lines.

Table 2. Antioxidant System Activity and Lipid Peroxidation Product Concentration in B16-F10 and CHO-K1 Cells Following Irradiation with a 1265 nm Laser at an Energy Density of 562.5 mJ/cm²

	0 h		3 h		18 h	
Superoxide Dismutase Activity (pg/ml)	**B16-F10**					
	experiment	control	experiment	control	experiment	control
	1163.24± 402.61	616.13± 122.72	6971.5*± 4592.44	1806.88± 245.98	7446.03*± 1261.85	3990.3± 426.78
	CHO-K1					
	experiment	control	experiment	control	experiment	control
	1631.03± 152.33	1910.89± 157.01	1534.73± 360.01	1633.39± 344.24	9367.7*± 1430.79	5437.8± 813.88
Glutathione-S-Transferase Activity (µmol/l·min)	**B16-F10**					
	experiment	control	experiment	control	experiment	control
	121.2± 24.84	83± 12.76	363.7± 129.18	229.94± 30.03	658.69*± 195.34	376.73± 66.95
	CHO-K1					
	experiment	control	experiment	control	experiment	control
	232.06± 36.12	193.86± 18.89	185.46± 40.71	133.,5± 20.63	694.06*± 118.59	393.93± 52.49
Concentration of Reduced Glutathione (mmol/l)	**B16-F10**					
	experiment	control	experiment	control	experiment	control
	2389.97± 581.75	1826± 389.83	11504.8*± 7206.61	5142.09± 883.52	13496.19*± 3066.91	8402.12± 1423.72
	CHO-K1					
	experiment	control	experiment	control	experiment	control
	4929.87± 618.7	5836.73± 1270.01	4011.61± 1106.93	4117.59± 493.45	17964.06*± 2764.26	12584.16± 1927.75
Malondialdehyde Concentration (µmol/l)			**B16-F10**			
			experiment	control	experiment	control
			28910.25± 18768.17	15613.41± 4043.53	29426.9± 5361.18	17258.92± 3281.6
			CHO-K1			
			experiment	control	experiment	control
			11815.7± 3412.6	6097.1± 989	26710.71± 4269.76	12643.93± 3606.76

* - indicates a statistically significant difference between irradiated and non-irradiated cells.

4. DISCUSSION

In this study, we observed a controversial dual effect: enhanced proliferation and a statistically significant level of cell death in B16-F10 melanoma cells (Fig. 2). This suggests that multiple mechanisms may be involved in directing the fate of individual cells within this population, especially given the substantial genetic and metabolic heterogeneity of the cell line in question.

Several studies have indicated that melanoma cell proliferation can be enhanced under the influence of laser irradiation [18-21]. It's worth noting that these studies employ not only different types of laser sources and parameters but also vary significantly in their experimental designs, including the use of in vivo tumour transplantation models in mice [19, 22]. In contrast, other research shows that elevated doses of laser irradiation can result in melanoma cell death [13, 23, 24], or even a reduction in tumour size [24], culminating in cytotoxic effects [13]. Additionally, some studies suggest implementing repeated laser irradiation cycles, varying in the number of repetitions and the intervals between them [13, 19, 22]. Contrastingly, most in vitro studies observing the inhibition of growth or apoptosis in melanoma cells typically involve a single application of laser radiation with varying parameters.

Figure 3A indicates that the activity of superoxide dismutase (SOD) in B16-F10 cells significantly increases three hours post-irradiation and sustains this elevated level for up to 18 hours. This suggests that laser irradiation at 1265 nm and a dose of 562.5 J/cm² prompts a more rapid and sustained response to an elevated concentration of superoxide anions in melanoma cells as compared to normal CHO-K1 cells. In the study by Saenko et al. [14], oxidative stress in CHO-K1 cells was significantly elevated after irradiation with a 1265 nm laser at a dose of 400 J/cm², resulting in a dose-dependent decrease in cell viability 24 hours post-irradiation.

Reactive oxygen species (ROS) in cancer cells play a role in the regulation of signaling molecules during various cellular processes, including cell cycle progression, proliferation, survival, apoptosis, intercellular adhesion, cell motility, and angiogenesis [25, 26]. This is particularly intriguing because ROS are implicated in modulating signaling pathways during apoptosis. Antioxidants such as N-acetylcysteine (a glutathione precursor) or MnSOD overexpression can block or arrest apoptosis. In anaplastic thyroid cancer cells, high levels of SOD3 are associated with cell growth arrest through p53-p21 signaling. Conversely, decreased mRNA expression of SOD3 correlates with enhanced malignant cell proliferation, mediated by Ras oncogene signaling [27, 28]. Therefore, it can be posited that the observed increase in overall SOD activity in our study was a driving factor leading to significant cellular death 24 hours post-irradiation (Figure 2B).

Given that enzymes in the SOD family catalyse the dismutation of the superoxide anion into hydrogen peroxide, elevated levels of these enzymes could drive the progression of specific apoptotic pathways, particularly when superoxide dismutase activity is heightened. Consequently, the proliferative effects induced by ROS should not be ascribed to hydrogen peroxide, which activates apoptosis, but more likely to the generation of superoxide ions [25].

Figure 3B and 3C illustrate the impact of 1265 nm laser radiation at a dose of 562.5 J/cm2 on the glutathione system in B16-F10 and CHO-K1 cells. Notably, the rise in GST activity in melanoma cells does not precisely correspond with levels of reduced glutathione. Three hours post-irradiation, GST activity significantly increases in B16-F10 cells, while the GSH levels remain comparable to those in non-irradiated controls [25]. The data reveal a marked time-dependent elevation in glutathione transferase (GST) activity in both cell lines following irradiation. Therefore, the cellular glutathione system exhibits a unidirectional and time-dependent response, indicative of a triggering effect by 1265 nm laser radiation at 562.5 J/cm^2. This specifically promotes the generation of superoxide anions and engages the glutathione system as part of the cellular defense mechanisms.

It is plausible to speculate that targeted disruption of the glutathione system could prove beneficial in devising treatment strategies for melanoma, particularly when used in conjunction with infrared laser radiation.

Our data show no impact of 1265 nm laser radiation at 562.5 J/cm2 on lipid peroxidation, as levels of its end product, malondialdehyde, remained constant at both 3 and 18 hours post-irradiation. It may be inferred that 1265 nm laser radiation has no effect on the lipid peroxidation (LPO) process. However, we believe that such a conclusion necessitates further data concerning the targets and parameters of laser radiation than what is currently available [15].

5. CONCLUSIONS

The 1264-1270 nm laser radiation deployed cellular antioxidant mechanisms, exerting both positive and negative outcomes in cellular defense to oxidative stress. The activation dynamics of the cellular antioxidant defense system differ between B16-F10 cancer cells and normal CHO-K1 cells, with more pronounced effects observed in the cancer cell line as early as 3 hours post-irradiation. We think that independently to observed melanoma cells resistance to oxidative stress induced by lower doses of the near infrared laser radiation this approach could be considered as a therapeutic in melanoma treatment after further studies of epigenetic changes induced by the near infrared laser irradiation in melanoma key tumour suppressor expression, namely NFkB, PI3K/AKT, RAS/RAF, p53 [29-33] should be considered to reveal their roles in regulation of the cell cycle, cell death mechanisms [32, 34-36].

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