The Photobiomodulation of Vital Parameters of the Cancer Cell Culture by Low Dose of Near-IR Laser Irradiation

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***Abstract*— The mechanisms underlining the cell adaptive and/or activating oxidative stress called low level light (LLLT) or photobiomodulation therapies (PBMT) still remain unclear for near-infrared (NIR) spectrum range (750-3000 nm) especially for 1265-1270 nm range (highest absorption by molecular oxygen). It is most probably that the mitochondria may also appear to be the main target for these wavelengths. It is known that mitochondria can generate ROS under visible and 800-1060 nm spectrum range irradiation which in turn control voltage-dependent anion channels (VDAC). Here we investigated cellular damage caused by low doses of 1265-70 nm laser radiation regarding to VDAC activity, the level of oxidative stress, malondialdehyde (MDA) content, cell viability, mitochondrial potential and mass, GSH level, mitochondrial and nuclear DNA damage in the cancer cell culture exposed to low-level laser irradiation at 1265 nm. We used continuous wave laser with output power 4 mW, the energy densities employed were 0.3-9.45 J/cm^2. We observed that the laser radiation at 1265 nm can induce the oxidative stress, enhance apoptosis and disturb mitochondrial functioning at the energy density of 9.54 J/cm^2. In addition, inhibition of VDAC enhances the observed effects. It has been shown that the laser irradiation at 1265 nm damages mitochondrial DNA but does not affect the nuclear DNA. The performed experiments bring us to the conclusion that the laser irradiation at 1265 nm can affect cells through mitochondrial damage and the inhibition of VDAC enhances effects of PBMT.**

***Index Terms*— biophotonics, cancer cell, near-infrared lasers, photobiomodulation therapy**

I. Introduction

Last decade advance in development of the compact efficient and reliable lasers in visible and especially in near infrared spectrum ranges significantly accelerated the laser applications in the different fields of the biology and medicine [1].

One of the most demanding area of the laser use is photobiomodulation therapy (PBMT) widely applied in modern medicine. Extensive studies have already demonstrated high potential of PBM therapy for various fields of surgery, oncology and cosmetology. The main mechanism of PBM action is associated with the effect of low-level laser or light irradiation (LLLI) on intracellular processes by activating intracellular signaling pathways through their interaction with endogenous photosensitizers. It has been shown that PBMT can affect oxidative stress related pathways, PI3-K / Akt signaling cascade and nuclear receptors [2], NFkB [3] and others [4], [5].

Most of intracellular endogenous photosensitizers are localized in mitochondria making them main acceptors of laser radiation [6], [7]. LLLI affects the cytochrome *c* oxidase changing its absorption spectra [6]. Changes in the cytochrome *c* oxidase state causes fluctuations of mitochondrial redox state, which, in turn, affects the mitochondria dependent generation of reactive oxygen species (ROS) [7], [8]. Interacting with photoactive molecules in mitochondria, in particular with the cytochrome *c* oxidase, LLLI at the wavelengths in the range 600-1070 nm can increase superoxide anion radical (O_2^{*-}) production in both cytoplasm and mitochondria leading to intracellular oxidative stress [9], [10]. Apart from this wavelength range, there are ranges at

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which laser irradiation is able to induce intracellular oxidative stress. IR range of 1265-1270 nm is the most studied among them [11]-[15].

The mechanism of cellular effects induced by laser irradiation at the wavelengths of 1265-1270 nm is still unclear. Some researchers believe that 1265-1270 nm lasers affect cells due to the oxygen absorption line coinciding with this range. When the laser energy is absorbed in the wavelength range of 1265-1270 nm, oxygen from triplet state ($^3O_2$) can transform into the singlet state ($^1O_2$). It is assumed that laser irradiation of living objects at these wavelengths can induce intracellular generation of the singlet oxygen [12]. As expected, the singlet oxygen generated under laser exposure at 1265-1270 nm induces oxidative stress, which can cause cell damage and death [12]-[14]. Several authors report singlet oxygen generation in cell cultures under laser exposure at the wavelengths of 1265-1270 nm. Since apoptosis induction is the main challenge in these studies, high-power lasers with high energy density from 60 J/cm^2 up to 400 J/ cm^2 have been employed [12], [13]. Theoretically, in the isolated molecule direct $^3O_2 \rightarrow {}^1O_2$ transition is forbidden according to the selection rules of spin and orbital symmetry highlighting small chance of singlet oxygen generation without photosensitizers [16]. However, surprisingly, such a transition has been recently observed in inhomogeneous media [17].

Under irradiation at 1265-1270 nm mitochondria seem to be the most probable source of the oxidative stress. LLLI at the wavelengths in the range 600-1070 nm has demonstrated that LLLI can affect the redox state of mitochondria and modulate intracellular oxidative stress [18], [5]. Our previous study demonstrates that increase in the intracellular ROS concentration induced by mitochondrial damage contributes to the damaging effect of the laser irradiation at 1265 nm [11].

Mitochondrial functioning is mainly dependent on VDAC (voltage-dependent anion channel) which is involved both in metabolic processes and mechanisms of programmed cell death [19].

Voltage-dependent anion channels regulate the metabolite flux through the outer mitochondrial membrane. Also, they are responsible for the ROS transport from the mitochondrial intermembrane space to the cytoplasm and participate in regulation of the redox status of the cell [20], control the flow of ADP/ATP, NADP and Ca2+ from outer mitochondrial membrane [19], participate in the glycolysis regulation [21]. VDAC inhibition impedes transport of metabolites through the outer mitochondrial membrane and leads to ROS accumulation in the intermembrane space of mitochondria [22]. In the case of mitochondria serving as ROS source under laser irradiation at 1265 nm, VDAC inhibition has to potentiate the effects of LLLI at this wavelength.

To study the mechanisms of the 1265 nm laser irradiation and to estimate their potential for LLLI we have irradiated cancer cells in the presence of 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) that is an inhibitor of VDAC. We have studied the dynamics of oxidative stress, cell viability, change of mitochondrial potential and mass, reduced glutathione level, mitochondrial and nuclear DNA damages under low-level laser irradiation.

II. Materials and methods

1265 laser parameters

A semi-conductor laser (Yenista Optics, OSICS T100 Tunable Laser Module T100 1310) with a tuning range from 1260-1360 has been used as the irradiation source. The average output power is 4 mW with the linewidth less than 1 nm and the wavelength stability of 0.1 nm/h. The irradiation output is made with the help of fiber patch cord with an air-spaced doublet collimator at the end. The fiber collimator has Non-Magnetic Stainless Steel Housing. It is pre-aligned to collimate a laser beam propagating from the tip of an FC/PC-connectorized fiber with diffraction limited performance at the design wavelength.

The surface dose (energy density) of laser radiation absorbed by a biological tissue (E, J/ cm^2) is calculated as follows:

$$E=Pt/S, \quad (1)$$

where P is the average output power (W), t is the exposure time (sec), S is the laser spot area on the cell culture (cm^2).

Cell culture and conditions

Experiments have been performed with colorectal cancer HCT116 cells (ACCT ® CCL-247TM) obtained from American Type Culture Collection (Manassas, VA, USA). The cells are maintained in DMEM/F12 medium, supplemented with 10% fetal bovine serum and gentamycin at a final concentration of 50 μg/ml at 37°C, 95% and 5% CO_2. Twenty four hours before irradiation the cells are made a passage in the 8-well slide chamber (SPL LifeSciences) at a concentration of 10^5 cells/ml. The volume in the slide chamber is 500 μl.

Laser treatment of cell culture

Irradiation of cells has been performed in the middle of the exponential growing phase using an incubator microscope (UNO, OkoLab) prepared for culture maintenance during 5-30 minutes. The laser light source is fixed at the bottom side of the plate at a distance of 0.5 cm from the slide chamber. While one cell is irradiated, another (control) is shielded by a steel foil. Laser energy density between 0.3 and 9.45 J/cm^2 is set depending on the exposure time. Each experiment includes at least three irradiation sessions. A solution of 4,4'-diizotiotsiano-stilbene-2,2'-disulfonic acid (4,4'-diisothiocyano-2,2'-stilbene-disulfonic acid) (DIDS) in dimethyl sulfoxide (DMSO) with the final concentration of 100μM is added to cells 30 minutes before irradiation. Rotenone solution in DMSO is added at a final concentration of 50 μM 20 minutes before adding 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Sodium azide (NaN_3) solution in ddH_2O is added at the final

concentrations of 0.5, 5 and 20 mM 1 hour before laser irradiation.

Fluorescent microscopy

Cell viability is evaluated 24 h after irradiation (9.45 J/cm^2). The cells are stained by a mixture of fluorescent dyes YO-PRO-1 and propidium iodide and kept in a thermostat for 20 minutes. Analysis is performed with the fluorescence microscope as described in [23].

Intracellular ROS concentration is determined using DCFH-DA. 5 minutes and 3 hours after irradiation the medium is replaced with the equivalent volume of sodium-phosphate buffer (PBS, pH=7.4) and the cells are labeled by solution of DCFH-DA in 96% ethanol (final concentration is 30 μM). 20 minutes after PBS with the dye is replaced with the equivalent PBS volume. Cell fluorescence is captured with microscope during 10 minutes after incubation at 4°C (filter 480/529) [24].

To assay the net cellular Ψm cells are stained by adding tetramethylrhodamine ethyl ester (TMRE) into the growth medium at a final concentration of 50μM for 20 min at 37°C. To determine the mitochondrial mass cells are incubated with 100 nM 10-N-nonyl acridine orange (NAO, Invitrogen) for 20 min at 37°C [25]. Images are captured with filter 480/529.

To assay the reduced glutathione cells are irradiated at 9.54 J/cm^2, 5 minutes and 3 hours after irradiation they are incubated in a growth medium with 5 μM monochlorbimane at 37°C for 20 min [26]. Images are captured with filter 380/450.

To assay the intracellular nitric oxide (NO) concentration cells are incubated with 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF-FM Diacetate) at the final concentration of 1 μM for 20 min at 37ºC. Images are captured with filter 495/515.

In each experiment using the microscope, fluorescence of more than 300 cells has been analyzed with each technique. All images are captured using an optical system comprising Nikon Ti-S microscope, DS-Qi1MC camera, Nikon S Plan Fluor ELWD 20×0.45 lens and appropriate filter and PC with NIS-elements 4.0 package.

Quantitative image analysis is performed using Image J software. The corrected total cell fluorescence (CTCF) = Integrated Density – (Area of selected cell x Mean fluorescence of background readings) [27].

Spectrophotometry

Malondialdehyde (MDA) content was assayed using thiobarbituric acid (TBA) as described in [28]. Absolute MDA concentration (micromol/L) is normalized to protein concentration per sample and expressed in nanograms (ng). Protein concentrations are measured using The Qubit® 2.0 Fluorometer (Invitrogen, USA) supplemented with Qubit Protein Assay Kit.

Mitochondrial and nuclear DNA damage assay

Total DNA is extracted with CTAB buffer containing no phenol. DNA samples for qPCR are stored for 24 hours at +4ºC in TE-buffer to prevent freezing induced lesions.

We use a qPCR-based assay for DNA damage described by Santos et al. [29] with some modifications. Primers for qPCR are taken from the Santos protocol. We have been using the primers for the following human gene targets: beta-globin region (13.5-kb), mitochondrial DNA (8.9-kb and 222-bp) during our study. We designed our own primers for the short beta-globin region target (245-bp) because of their absence in the protocol. Before qPCR setting up, primer pairs and reaction conditions are checked by gel electrophoresis. DNA concentration in each qPCR reaction is calculated using the standard curve. The resulting values are converted into relative lesion frequencies per 10 kb DNA as described in [30].

Statistical analysis

Each test has been performed in triplicate and results have been expressed as mean ± SD. Differences between irradiated and control cells are regarded as statistically significant when P calculated by the two-sided Student t-test is <0.05.

III. Results

Oxidative stress after 1265 nm laser irradiation in presence of VDAC inhibitor

To evaluate mechanisms of ROS generation under irradiation at the wavelength of 1265 nm a series of experiments has been performed. Fig.1A shows the dependence of intracellular ROS generation on the laser energy density at the wavelength of 1265 nm. ROS concentration has been determined 5 minutes after irradiation. As the diagram shows, this dependence is linear. Significant differences with the control group have been observed at the energy densities of 3.15 J/cm^2 and 9.45 J/cm^2.

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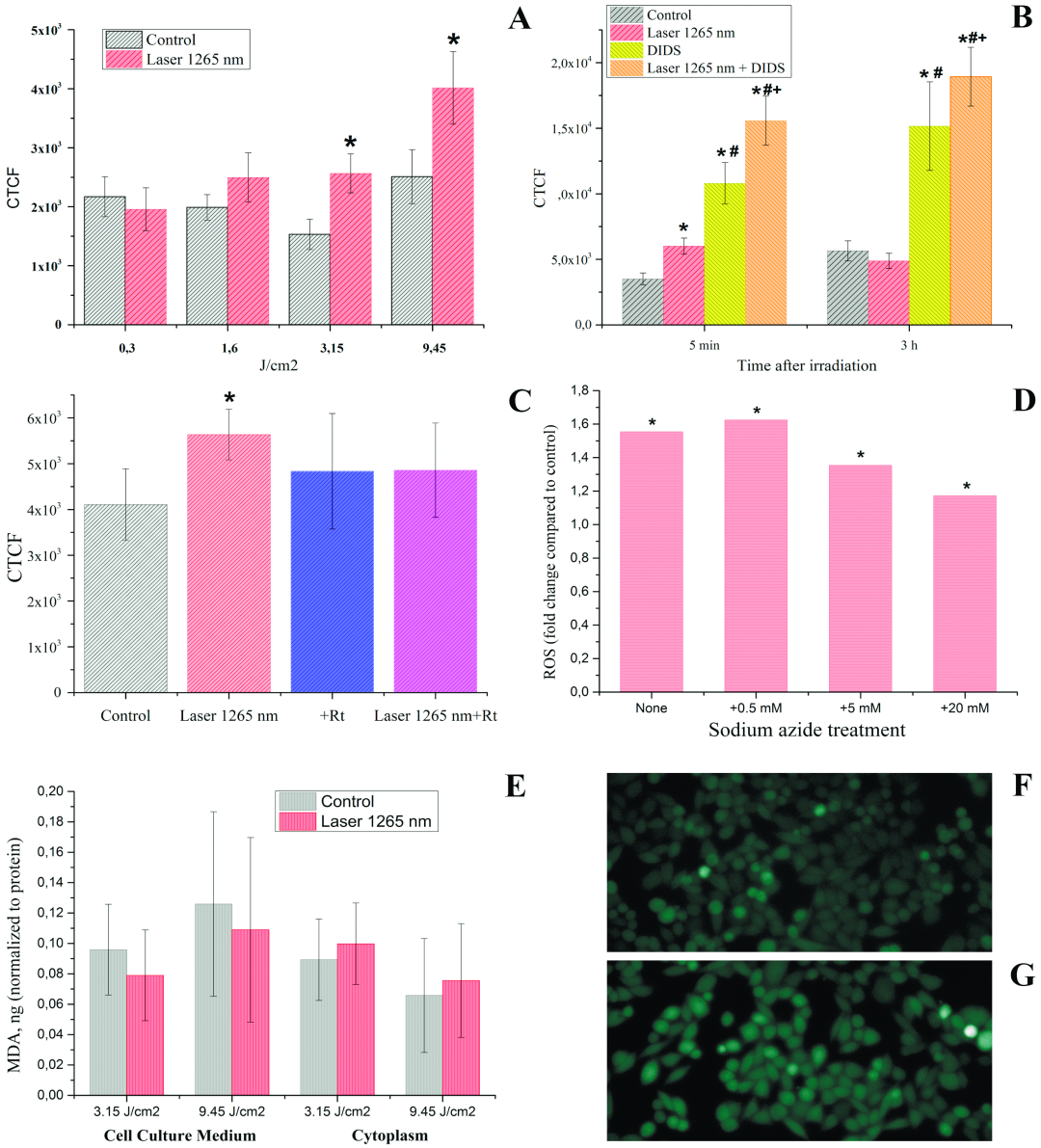

Fig. 1. Mechanisms of ROS generation under LLLI. Fig. 1A. The dependence of intracellular ROS concentrations on the energy density of the 1265 nm laser. Fig. 1B. Level of ROS in HCT116 cells exposed to the 1265 nm laser with or without DIDS. Control group cells are unirradiated and are not treated with DIDS. Energy density is 9.45 J/cm^2. Fig. 1C. Level of ROS in HCT116 cells exposed to the 1265 nm laser irradiation with or without rotenone (Rt). Control group is not irradiated by the laser and is not treated with Rt. Energy density is 9.45 J/cm^2. Fig. 1D. Level of ROS in HCT116 cells exposed to 1265 nm laser and treated with sodium azide. The respective control and experimental groups are treated with equal sodium azide concentrations. Energy density is 9.45 J/cm^2. Figs. 1A, 1B, 1C show the ROS concentration expressed as DCFH-DA corrected total cell fluorescence (CTCF). Fig. 1D show the CTCF fold change compared to unirradiated control. Fig. 1E. Normalized content of malondialdehyde in cytoplasm of HCT116 cells exposed to 1265 nm laser irradiation or in cell culture media after irradiation. Energy density is 9.45 J/cm^2. All results are given as mean values ± S.D. * - statistically significant difference between control and other groups. # - statistically significant difference between irradiated cells and other groups. + - statistically significant difference between irradiated DIDS treated cells and DIDS treated cells ($p<0.05$). Fig. 1 F, G. DCFH-DA fluorescent staining of unirradiated and exposed to the 1265 nm laser irradiation HCT116 cells, respectively. Energy density is 9.45 J/cm^2.

To reveal mitochondrial contribution to ROS generation under laser irradiation 4,4'-diisothiocyano-2,2'-stilbene-disulfonic acid (DIDS) and rotenone have been used. DIDS is a blocker of mitochondrial membrane voltage-dependent anion channel (VDAC), rotenone is an inhibitor of NADH-CoQ reductase. Fig. 1B shows the experimental results on irradiation of the HCT116 cells at the wavelength of 1265 nm and energy density of 9.45 J/cm^2 in the presence of DIDS.

Intracellular ROS concentration has been defined 5 minutes and 3 hours after irradiation. As one can see, an increase in ROS concentration is registered immediately after the laser irradiation. Three hours after laser exposure the intracellular ROS concentration recovers to the control group level. Adding of DIDS into the culture medium causes an increase of ROS concentration. Irradiation of cells in the presence of DIDS makes this effect more pronounced. Fig. 1B shows that DIDS prevents normalization of the intracellular ROS concentration 3 hours after irradiation. Fig.1C shows the experimental results on irradiation of the HCT116 cells at the wavelength of 1265 nm and energy density of 9.45 J/cm^2 in the presence of rotenone. In this experiment, ROS concentration is determined 5 minutes after irradiation. Adding of rotenone into the medium inhibits ROS generation under laser exposure (Fig. 1C). Figs. 1F, 1G show the result of DCFH-DA staining of HCT116 cells in unirradiated control group and experimental group exposed to 1265 nm laser irradiation, respectively.

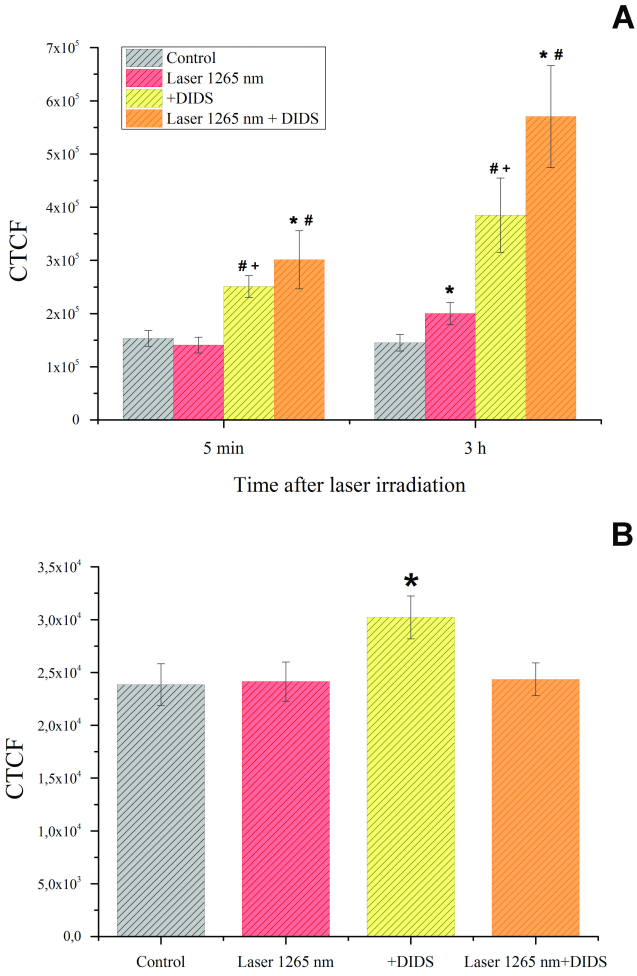

Fig. 2. Fig. 2A. Changes of intracellular reduced glutathione level in HCT116 cells exposed to the 1265 nm laser with or without DIDS. Energy density is 9.54 J/cm^2. Intracellular reduced glutathione level is expressed as monochlorobimane corrected total cell fluorescence (CTCF). Fig. 2B. Level of intracellular nitric oxide (NO) concentration in HCT116 cells exposed to the 1265 nm laser. Control group cells are unirradiated. Energy density is 9.45 J/cm^2. The RNS (NO) concentration is expressed as DAF-FM corrected total cell fluorescence (CTCF). Results are given as mean values ± S.D. * - statistically significant differences between control and laser irradiated cells ($p<0.05$).

Fig. 2A shows changes of intracellular GSH level in HCT116 cells exposed to 1265 laser at the energy density of 9.54 J/cm^2 with or without DIDS. Intracellular GSH concentration is an important indicator of a cell metabolism. GSH is the source of hydrogen atoms in intracellular chemical reactions and contributes to cell resistance to ROS. According to Fig. 2A, 3 hours after irradiation, the laser exposure increases intracellular GSH in HCT116 cells and it is 1.37 times higher than in the control group.

Similar to ROS, reactive nitrogen species (RNS) are also able to initiate intracellular damages. So, concentration of nitric oxide (NO) has been found to estimate the effects of the 1265 nm laser irradiation on the RNS level. Fig. 2B demonstrates no differences between NO level registered for the experimental groups and the control.

Effect of 1265 laser irradiation in presence of VDAC inhibitor on cells viability

Fig. 3A shows the amount of cells with apoptotic and necrotic signs one hour and 24 hours after laser irradiation with or without DIDS treatment. One hour after laser irradiation the number of cells with apoptosis is 35593.02 that is less than in the control group where this value is 41272.78. Twenty-four hours after irradiation the number of cell with apoptosis in the experimental group is 1.26 times higher than in the control group. One hour after irradiation the number of cells with necrosis is the same as in the control group. Twenty-four hours after exposure the number of cells with necrosis is 1.15 times greater in the group of cells irradiated at the wavelength of 1265 nm and energy density of 9.45 J/cm^2 than in the control group (Fig. 2A).

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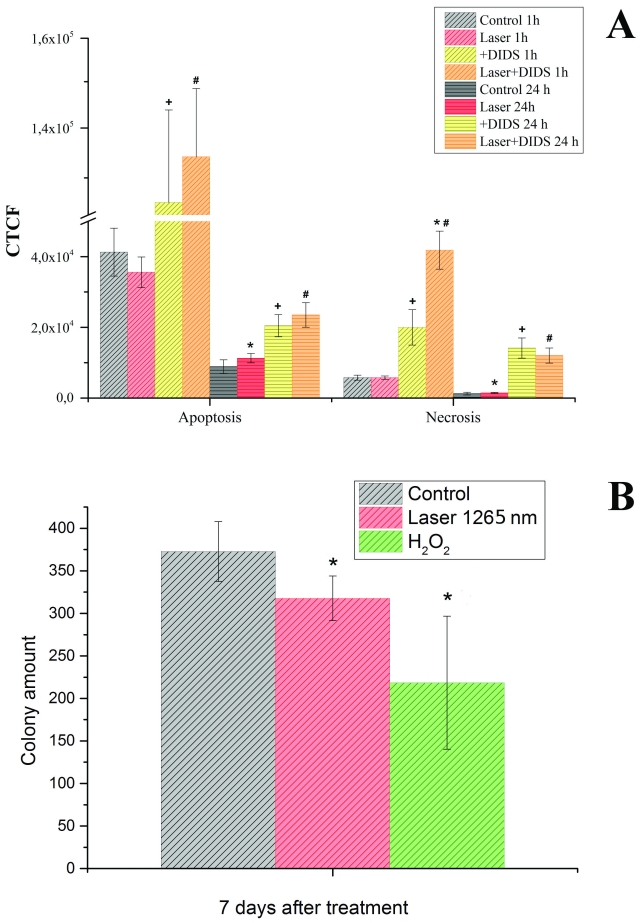

Fig. 3. Effect of the laser irradiation at the wavelength of 1265 nm on the HCT-116 cell survival rate. Fig. 3A. Amount of cells with apoptotic and necrotic signs 1 hour and 24 hours after irradiation with or without DIDS. Energy density is 9.45 J/cm^2. Apoptosis is expressed as YO-PRO-1 corrected total cell fluorescence (CTCF). Necrosis is expressed as propidium iodide corrected total cell fluorescence (CTCF). Fig. 3B. Clonogenic assay of HCT-116 cells 7 days after irradiation at the wavelength of 1265 nm and energy density of 9.45 J/cm^2. * - statistically significant differences between control and irradiated cells.

Fig. 3B shows the results on clonogenic assay. In the control group, the number of cell colonies comprising more than 20 cells is 372.68. On the seventh day after irradiation at 1265 nm and energy density of 9.45 J/cm^2 the number of cell colonies is 317.78. For a positive control, HCT116 cells are incubated for 15 minutes in the presence of hydrogen peroxide at a final concentration of 500 µM. Seven days after irradiation the number of colonies in the presence of hydrogen peroxide is 218.5 that is 99.28 colonies less than the number of cell colonies after laser exposure. After irradiation, cancer HCT116 cells have not lost the ability to form colonies. However, as the graph shows, after irradiation a significant decrease in the number of colonies has been observed.

Changes in mitochondrial status

Fig. 4 shows changes of mitochondrial potential and mitochondrial mass in HCT116 cells exposed to 1265 nm laser at the energy density of 9.45 J/cm^2. As Fig. 4A shows, decreases in the total cellular mitochondrial potential have been registered in HCT116 cells exposed to laser irradiation. A decrease in mitochondrial potential has been observed immediately after irradiation and 3 hours after exposure. Immediately after irradiation mitochondrial potential is 28% lower than in the control group, 3 hours after irradiation mitochondrial potential is recovering and it is 12% lower than in the control group (Fig. 4A). DIDS treated HCT116 cells exposed to the 1265 nm laser also exhibit a decrease of mitochondrial potential in all time intervals. The maximal decrease of mitochondrial potential in HCT116 cells is recorded 3 hours after exposure and it is 2.3 times less than in the control group. Mitochondrial potential in unirradiated DIDS treated HCT116 cells is 1.2-1.62 times less compared to the control group (Fig. 4A). Fig. 4B shows the experimental results on irradiation of the mitochondrial mass at the energy density of 9.45 J/cm^2 by the 1265 nm laser. Effects of DIDS treatment also have been analyzed. As the diagram shows, the laser irradiation causes a decrease in mitochondrial mass during the whole experiment. Five minutes and three hours after irradiation mitochondrial mass is 1.95 and 1.65 times less than in the control group, respectively.

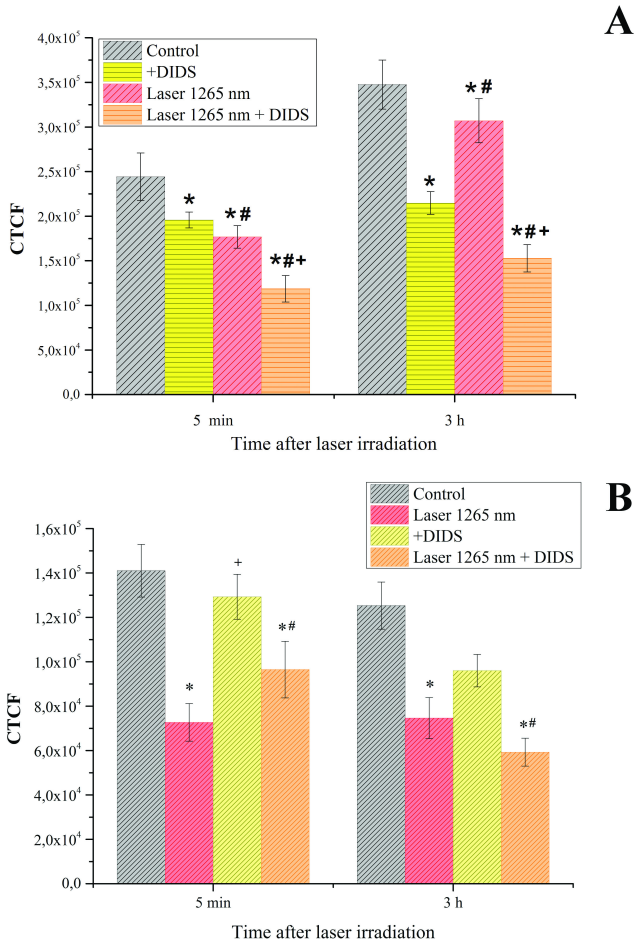

Fig. 4. The laser effect at 1265 nm on the total

mitochondrial potential and mitochondrial mass. Fig. 4A. Changes of mitochondrial potential in HCT116 cells exposed to the 1265 nm laser with or without DIDS. Control group is not irradiated by laser and is not treated with DIDS. Energy density is 9.45 J/cm^2. Mitochondrial potential is expressed as TMRE corrected total cell fluorescence (CTCF). Fig. 4B. Changes of mitochondrial mass in HCT116 cells exposed to the 1265 nm laser with or without DIDS. Mitochondrial mass is expressed as nonyl-acridine orange corrected total cell fluorescence (CTCF). All results are given as mean values ± S.D. * - statistically significant difference between control and other groups. # - statistically significant difference between laser irradiated cells and other groups. + - statistically significant difference between laser irradiated DIDS treated cells and DIDS treated cells ($p<0.05$).

Mitochondrial and nuclear DNA damage

Fig. 5 shows the calculations of the relative amount of lesions for 10 kb DNA. No significant differences have been observed between the damage levels of DNA localized in nucleus and mitochondria for the cells in the control groups. However, in the group subjected to the 1265 nm laser irradiation at the energy density of 9.45 J/cm^2 a significant increase of mitochondrial DNA damage has been recorded compared to the nuclear DNA damage. After irradiation, the relative amount of mitochondrial DNA lesion increases in 6.37 times compared to the control value, respectively.

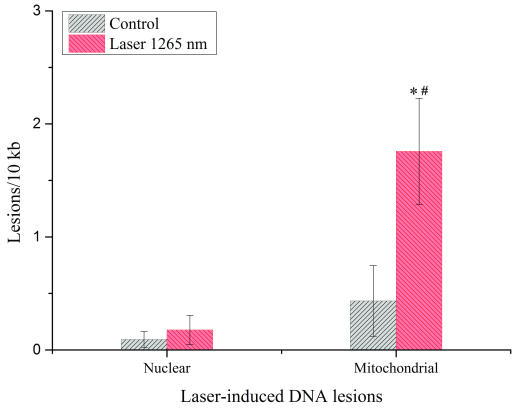

Fig. 5. 10 kb nuclear and mitochondrial DNA lesions in HCT116 cell line after laser irradiation at the wavelength of 1265 nm. * - statistically significant difference between the control group cells and irradiated cells. # - statistically significant difference between the nuclear and mitochondrial values ($p<0.05$).

IV. Discussion

In our studies, we have used cancer cells since they have more VDAC in the outer mitochondrial membrane than normal cells [31]. Cancer cells have normal mitochondria but their ability to synthesize ATP is inhibited by an activation of glycolysis through increased expression of hexokinase. Hexokinase is associated with VDAC and its overexpression suppresses VDAC conductivity, thereby limiting mitochondrial ability to synthesize ATP [21]. Thus, VDAC of cancer cell mitochondria is more sensitive to inhibitors and can produce more pronounced effects.

In the present study, the 1265 nm LLLI has been used to explore its effect at the cell level to assay the possibility of its application in cancer photobiomodulation therapy. For a typical photosensitizer (e.g. porphyrins), in the near-IR range (including 1260-1270 nm) the absorption cross-section is about 10-18-10-19 cm^2 [17]. For a direct transition $e_g \rightarrow {}^1\Delta_g$ in water (or water solution) the absorption cross-section is below 10-22 cm^2 [32] and this value is apparently overrated. Taking into account that in the presence of photosensitizers the intensities ensuring significant generation of singlet oxygen (SO) are more than 10 mW/cm^2 [32], one can conclude that the intensities much more than 102 W/cm^2 are required to obtain the significant singlet oxygen generation in inorganic media under laser irradiation without photosensitizers. Noteworthy, in our case, the oxidative stress has been registered under irradiation at the wavelength of 1265 nm at the intensities of about 10 mW/cm^2, i.e. at the intensities employed for photodynamic therapy (Fig. 1A). Moreover, our experiments show that the oxidative stress has been observed at the intensity as low as 2 mW/cm^2 (the energy density is 3.15 J/cm^2 (Fig.1A)). We have also determined the levels of malonic dialdehyde, the end-product of the polyunsaturated fatty acid oxidation [33], in culture medium and in the cell cytoplasm. The MDA levels have not been significantly changed neither in cell cytoplasm nor in the cell culture medium after laser applied. This observation points on the fact that 1265 nm laser-induced ROS generation has rather short life effects (disappeared by 3rd hour after the irradiation (Fig. 1A,B) unable to significantly oxidize the lipids maybe due to consequent activation of the cell antioxidant defense system showing the increase of intracellular glutathione level after 1265nm laser pulse (Fig. 2A).

All this indirectly indicates the presence of endogenous photosensitizer capable of generating free radical molecules.

Studies on the absorption spectra of natural porphyrins report on their presence in the wavelength range of 1100-1700 nm [34], [35]. Some synthetic porphyrins have absorption spectra in the range of 1260-1270 nm [36]. Studies on the absorption spectra of natural and synthetic porphyrins indirectly confirm our assumption that the effects from laser radiation at 1265 nm are due to the presence of endogenous photosensitizers in mitochondria whose nature is still unknown.

To determine whether laser-induced oxidative stress initially comes from mitochondria (complex I or cytochrome c oxidase) we disrupted its functioning with rotenone, sodium azide and DIDS [37, 38]. The decrease of intracellular ROS concentration (Fig. 1C, D) in HCT116 cells after 1265 nm under application of the rotenone or sodium azide clearly demonstrated that mitochondrial respiratory chain is a

potential source of ROS generation inducing cell oxidative stress by the 1265 laser with prevalent activation of the cytochrome c oxidase.

VDAC inhibition produces a multiple effect on mitochondria. ROS accumulation in the intermembrane mitochondrial space is among them [22].

In our experiments, the oxidative stress has been registered immediately after irradiation. Three hours after exposure ROS concentration in the irradiated cells is the same as in the control group (Fig. 1B). Laser radiation at the wavelength of 1265 nm in the presence of DIDS leads to a more intensive oxidative stress compared to the stress observed in the cells exposed to irradiation only (Fig. 1B). In addition, in the presence of DIDS the oxidative stress has been maintained for 3 hours after laser exposure (Fig. 1B). DIDS increases ROS generation due to inhibition of ROS flux through VDAC into cytoplasm, where ROS are neutralized by antioxidant enzymes. PBMT at 1265 nm induces ROS generation and, in the presence of DIDS, ROS concentration in mitochondria demonstrates further increase due to the blocking of ROS release through VDAC into the cytoplasm. PBMT at 1265 nm does not increase the intracellular concentration of nitric oxide (Fig. 2B). This result is different from the data obtained with LLLI employing other wavelengths [39].

As expected, DIDS increases the intracellular concentration of RNS by blocking their release from the mitochondria [19]. The combined use of DIDS and LLLI at 1265 nm does not increase RNS concentration, indirectly indicating that an acceptor of the 1265 nm laser irradiation is not the cytochrome *c* oxidase.

VDAC inhibition not only disturbs the mitochondrial metabolic balance but also leads to depolarization of mitochondrial membrane and decreasing of mitochondrial potential. In our experiment, laser irradiation at 1265 nm causes a decrease of mitochondrial potential. In the presence of DIDS, mitochondrial potential demonstrates a stronger decrease than under laser irradiation without DIDS (Fig. 4A). A similar effect has been produced by the laser on mitochondrial mass (Fig. 4B). As the experiments show, mitochondrial disturbance due to VDAC inhibition can be responsible for the mitochondrial mass decrease. Also, the mitochondrial mass decreases due to cardiolipin oxidation resulted from ROS generation in a mitochondrial respiratory chain. It has been shown that the cardiolipin is bound with 10-N-Nonyl acridine orange in an unoxidized intact state [40]. Thus, the laser effect at 1265 nm on mitochondrial potential is similar to the effect produced by DIDS but the mechanisms of action may be different and associated with formation of mitochondrial permeability transition pores (MPTP) in the inner mitochondrial membrane. Noteworthy, VDAC is known to be the integral part of MPTP.

Induction of MPTP takes place in the case of oxidation of thiol groups in mitochondrial proteins by ROS. MPTP formation decreases the mitochondrial potential by equalizing gradients of proton concentration between the intermembrane space and internal mitochondrial space [41]. MPTP leads to mitochondrial destruction and cytochrome c escape into the cytoplasm, which in turn, initiates antioxidant defense mechanisms, or, in the case of a large amount of degraded mitochondria, apoptosis is triggered [42].

In mitochondrial function glutathione plays an important role. A decrease of the GSH concentration and increase of the concentration of oxidized glutathione both cause hyper S-glutathionylation of mitochondrial proteins, including cytochromes and ATPases, and, as a result, decreasing of their activity [43]. A key role of the glutathione system is to decompose intracellular peroxides and to prevent oxidation of –SH moiety of proteins. Hence, with the ROS concentration increase the GSH concentration should decrease [44]. Surprisingly, in our experiments the concentrations of ROS and GSH increase simultaneously. In the presence of DIDS, these effects are substantially potentiated. This effect could be considered as a specific feature of mitochondrial metabolism in cancer cells that still requires its explanation.

ROS can damage DNA, in particular, mitochondrial DNA [45]. In this study, we estimate the damage intensity of nuclear and mitochondrial DNA after laser irradiation (Fig. 5). LLLI at 1265 nm causes a significant damage in mitochondrial DNA and has no effect on nuclear DNA. All this supports our assumption that the laser light at 1265 nm interacts with endogenous photosensitizers included into mitochondria.

V. Conclusion

In our study, we have demonstrated that the low-level near infra-red laser irradiation at 1265 nm can induce the oxidative stress and disturbance of mitochondrial functioning at the energy density 9.54 J/cm^2 and VDAC contributes to enhancement of this effect. LLLI at 1265 nm damages the mitochondrial DNA but produces no effect on the nuclear DNA. The experimental results have brought us to conclusion that the 1265 nm laser irradiation affects intracellular processes through interaction with mitochondrial photoactive molecules. Inhibition of VDAC enhances the damage effect of PBMT under irradiation at 1265 nm. This property has the potential in treatment of malignant skin neoplasms employing PBMT.

Appendix

Ethical aspects. Cell cultures used in the research have been obtained from ATCC (American Type Culture Collection (Manassas, VA, USA)). No human/animal directly participated in biomaterial sampling process during this research. Local Russian regulations require no approval for using cell line biomaterials for scientific research purposes (Federal Law of June 23, 2016 No. 180-FZ).

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