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The impact of optical radiation of femtosecond duration on human glial cells

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ABSTRACT

The paper presents the results of the studies of influence of optical radiation with wavelengths of 520 and 780 nm on human glial cells (U251) at the range of exposure times ~ 1-15 min. It was found that after the first minute of irradiation at the wavelength of 780 nm, the relative number of apoptotic cells significantly increased. The result corroborates the concept of biological hazard of optical radiation for tumor cells, and suggests that the approach has a great potential in clinical application for the treatment of human glioma.

Keywords: Irradiation, glioma, cell line U251, cytochrome C oxidase, apoptosis, pulse IR radiation, resonance effect

1. INTRODUCTION

Glioma is the most aggressive and difficult for treatment type of cancer. Malignant glioma cells of the U251 line were originally detected in a 75-year-old patient with multiple glioblastomas in the 70s of the last century [1]. These cells evolved into glioblastomas, histochemically and genetically repeating human disease, when introduced to animals [2]. Malignant gliomas represent one of the most aggressive forms of brain cancer [3]. They are difficult to treat and often resistant to conventional radio- and chemotherapy [4]. Despite application of common treatment procedures, surgery is included [5], the survivability of patients with tumors of the type remains low [6].

High-grade glioma features are rapid proliferation, angiogenesis, and invasive growth. Eradication or inhibition of infiltrating glioma cells poses a significant clinical challenge, unlikely to be solved using conventional treatment regimens consisting of ionizing radiation and chemotherapeutic agents [7]. The most promising treatment technique is the photodynamic therapy (PDT). Though its use in treating brain tumors dates back to 1980 [8-11], the results of clinical trials are ambiguous, partly because of their limited scope. Almost universally, the PDT is administered as a single treatment immediately after the surgery. In most cases, the treatment failure ensues due to local recurrence. This indicates that a more aggressive local treatment may prove more efficient as about 80% tumors recur within 2 cm of the resection cavity [12].

The radiation therapy is believed to be the most advanced, though having specific limitations, since both soft brain and cranial osseous tissues absorb the radiation. In the paper [13] it was shown that the radiation at wavelengths of 670 and 830 nm stimulated proliferation of Schwann cells (the glial cells of peripheral nervous system) [14], but the radiation at wavelength of 780 nm inhibited these.

The effect of continuous wave radiation with wavelength of 635 nm was studied earlier [7]. In this study, the effects of 5-aminolevulinic acid (ALA)-mediated photodynamic therapy (PDT) on the invasiveness of human glioma cells migrating from implanted multicell human tumor spheroids were evaluated. Measurement of cell survival, and results from cultures with blocked cell proliferation, indicated a direct migratory inhibition effect on the invading cells.

The article [15] reports the phototoxicity effects of a novel photosensitizer ZnPcS4-BSA on human U251 glioma cells in vitro at wavelength of 670 nm with energy density of 25, 50, 100, 150 and 200 J/cm².

Also, the influence of THz and IR radiation on human brain tumor was studied in [16, 17]. Since liquids absorb THz and IR radiation easily, the method cannot be used in non-invasive manner.

Recently, the increasing interest in studying cells, tissues, and embryos using femtosecond laser pulses took place. This is related to the fact that for multiphoton absorption, femtosecond pulses in the visible and near-infrared region can be used, where transparency window for biological objects is situated [18]. In addition, dissection, ablation, and microsurgery on various biological objects can be conducted with minimal heating and sometimes even without it. This circumstance is of fundamental character, since heat leads to uncontrollable destructive processes. Moreover, the optimal mode for femtosecond pulses in microsurgery consists of pulses with a small peak power and high repetition rate.

In this paper we have used optical radiation of femtosecond duration at wavelengths of 520 nm and 780 nm with exposure time of 1-15 min to control the number of glial cells effectively.

2. SAMPLE PREPARATION AND ANALYSIS

The study of U251 human glioma cell line included the cultivation of cells and used DMEM medium supplemented with 10% fetal calf serum (FCS), 40 µg/ml of gentamicin, and 2 mM of L-glutamine.

During experiments, the cells were seeded into wells of a 24-well flat-bottomed plate (Sarstedt AG & Co. KG, Germany). 20 wells of the plate were exposed, whereas samples in 4 wells were used as control ones. The irradiation was performed at a cell monolayer density of 50%. After irradiation, the cells were incubated at 37 °C in atmosphere of 5% CO₂ for 24 hours.

The assessment of changes in mitochondrial membrane potential was performed after the stage of samples preparation. The technique bases on the use of two fluorescent dyes, methyl tetramethyl-rhodamine (TMRM) and DNA-binding dye DRAQ7 [19]. The effect causes a change in the cell fluorescence intensity in the red-orange portion of the spectrum with maximum emission wavelength of 573 nm, which is recorded with a cytofluorometer. For detect different stages of apoptosis (in addition to TMRM), cells are additionally stained with DRAQ7, a dye being capable of interacting with cell nucleic acids. DRAQ7 cannot diffuse through the bilipid membranes and, hence, contact with DNA of the cells. A consequence of the interaction is the dye accumulation in the cytoplasm and nucleus, whereby the cell takes on fluorescence in the red part of spectrum. It should be noted that a diode laser with an emission wavelength of 638 nm was used to efficiently excite the dye, whereas the detection of the signal from DRAQ7 after its binding to the ligands was recorded at a wavelength of 725 ± 20 nm.

Thus, live cells have bright fluorescence through a channel designed to detect TMRM, but do not accumulate DRAQ7 (the phenotype TMRM^{high}/DRAQ7⁻). Cells at the early stages of apoptosis when the mitochondrial potential decreases, but the plasma membrane still retains its integrity and impermeability for DRAQ7, have the TMRM^{low}/DRAQ7⁻ phenotype. However, the late apoptotic and necrotic cells do not efficiently accumulate TMRM, but are stained DRAQ7 (the phenotype TMRM^{low}/DRAQ7⁺).

To evaluate the mitochondrial membrane potential the cell labeling procedures were performed. It was taken 20× solution of TMRM with added to 100 µl cell suspension (2-3×10⁶ cells/ml) at the final concentration of 150 nM. The working solution prepared ex tempore, adding 9990 µl of PBS and 10 µl of the stock solution. The incubation completed, 200 µl of PBS were added to the samples, and cytometric analysis was performed. It was classical method of cell labeling presented in work [20].

The next stage of experiment was to perform cytofluorometry analysis. The Navios Flow Cytofluorometer was employed for sample analysis. For each sample, at least 10000 single cells were analyzed. The results were analyzed with Navios software. The result was expressed as a percentage of the total number of cells analyzed and was presented as an average with an error in the form of mean error (Mean \pm SEM). The samples were compared using the Student t-test. Differences were considered significant at $p < 0.05$.

3. EXPERIMENTAL SETUP AND PROCESS

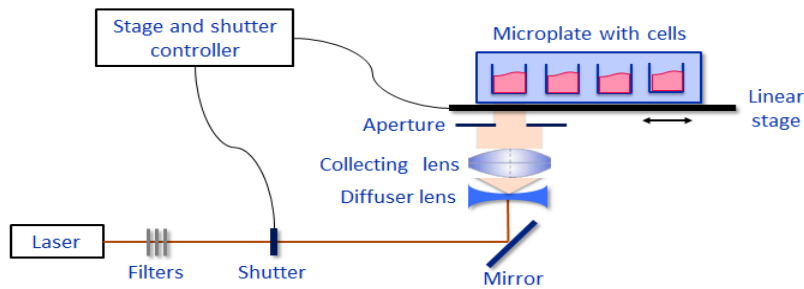


Fig.1. Experimental scheme of setting.

The scheme for setting of irradiation has shown in Fig. 1. The beam of IR laser radiation passes through the filters and comes to the shutter, which is controlled by the stage and shutter controller via PC. To control the power and duration of irradiation, a special software was written in LabView. When the shutter is opened, the IR beam passes through it to the system of mirrors and enters the diffuser and collecting lenses. Afterwards, the light beam propagates through the aperture of the same diameter as wells, 1.7 cm. This helps to avoid irradiation of other wells. Every line includes one control well, not subjected to the irradiation.

Two IR femtosecond lasers were used in the experiment. The first one is Fiber-based “Avesta project EFOA-SH” with wavelength of 1560 nm/780 nm (2H), average power up to 130 mW, pulse duration of 120 fs. The second one is “SolarLS FL-1” with wavelength of 1040 nm/520 nm (2H), average power up to 1000 mW, pulse duration of 200 fs. Repetition rates are the same, 70 MHz. The exposition of the cell line U251 was performed in both cases at the average power of 45 mW.

4. EXPERIMENTAL SETUP AND PROCESS

To analyze the results, two-dimensional histograms of TMRM fluorescence intensity were plotted. According to the data shown in Table 1, Table 2 and Fig.2, the number of cells with actively functioning mitochondria (phenotype TMRM high/DRAQ7-) in samples exposed to radiation remains practically unchanged. An increase in the irradiation time leads to a significant decrease in the number of such cells in the sample in one of the three experiments performed with the maximum irradiation time (15 minutes). The number of cells with a reduced ability to accumulate the mitochondrial dye (the "TMRM low" phenotype) significantly decreased in the corresponding experiment at the same point of time. It should be noted that the population of cells with the phenotype "TMRM low/DRAQ7 +" exhibited no significant changes in the test samples against the control ones.

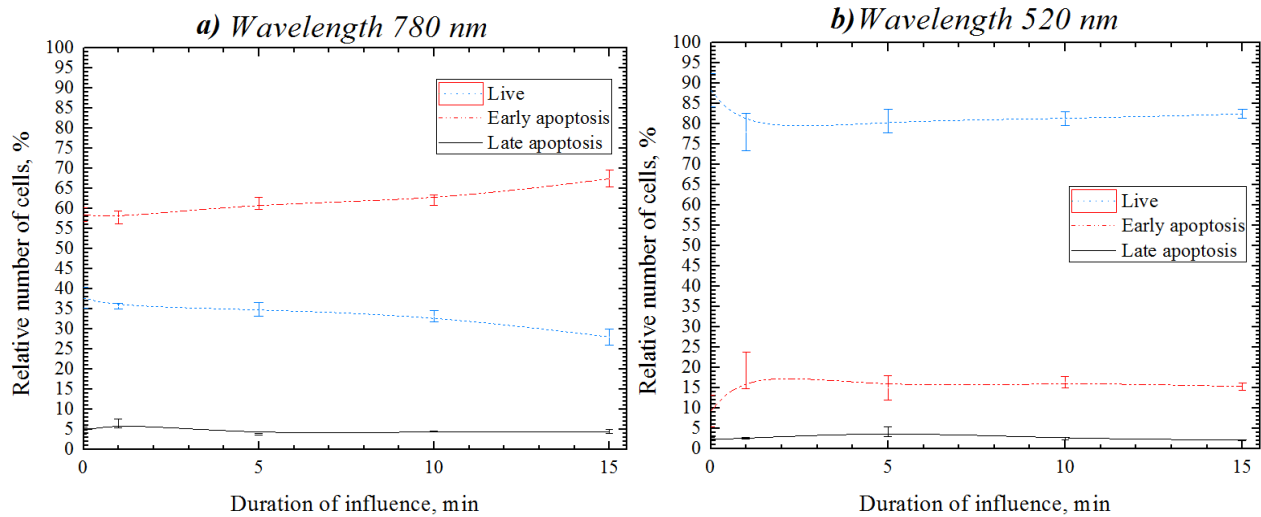


Fig.2. The percentage of cells in solution (live, early apoptosis and late apoptosis / necrosis) vs. the exposure time at wavelengths of 780 nm (a) and 520 nm (b)

The quantities in both figures (Fig. 2) tend to stabilize after 1 minute. The number of early apoptosis cells starts to increase in the experiment with the wavelength of 780 nm.

Table 1. Percentage of live cells and cells at different stages of apoptosis in samples. The irradiation was performed at wavelength of 520 nm for different durations (Mean \pm SEM, $n \geq 4$)

Exposure, min.	Live cells	Early apoptotic cells	Late apoptotic/necrotic cells
	(TMRM high/DRAQ7-)	(TMRM low/DRAQ7-)	(TMRM low/DRAQ7+)
0	88,27 \pm 4,02	9,30 \pm 3,90	2,37 \pm 0,41
1	77,96 \pm 4,55	19,38 \pm 4,56	2,62 \pm 0,17
5	80,72 \pm 2,96	15,08 \pm 2,99	4,20 \pm 1,22
10	81,26 \pm 1,63	16,40 \pm 1,43	2,56 \pm 0,21
15	82,46 \pm 1,04	15,36 \pm 0,95	2,18 \pm 0,14

Table 2. Percentage of live cells and cells at different stages of apoptosis in samples. The irradiation was performed at wavelength of 780 nm for different durations (Mean \pm SEM, $n \geq 4$)

Exposure, min.	Live cells	Early apoptotic cells	Late apoptotic/necrotic cells
	(TMRM high/DRAQ7-)	(TMRM low/DRAQ7-)	(TMRM low/DRAQ7+)
0	37,75 \pm 2,61	58,48 \pm 1,68	4,73 \pm 0,86
1	35,66 \pm 0,70	57,74 \pm 1,61	6,58 \pm 1,11
5	34,88 \pm 1,68	61,28 \pm 1,54	3,84 \pm 0,26
10	33,30 \pm 1,38	62,08 \pm 1,31	4,56 \pm 0,11
15	28,06 \pm 2,06	67,42 \pm 2,13	4,54 \pm 0,50

The present study has convincingly demonstrated the increase of apoptotic cell number along with the reduction of the number of living cells in the sample under the application of radiation at a wavelength of $\lambda = 780$ nm. We attribute this selective effect of optical radiation to the influence on molecules of cytochrome C at the absorption frequency [21] (Fig. 3). Cytochrome C in complex with the cardiolipin is known to be located on the inner surface of the mitochondrial membrane. As Tiina Karu notes [22], there are enough chromophores in the cytochrome C oxidase molecule (Cu and Mg atoms, heme groups). We suggest that the influence by radiation with $\lambda = 780$ nm can launch a cascade of intramitochondrial reactions. This is triggered by a change in the conformational-physical state of cytochrome C oxidase and/or its electron transportation function due to the absorption and, possibly, the resonance effect (temporal changes in hydrogen bond structure, change in charge and possibly secondary structure). This leads to a drastic change in peroxidase activity of Cytochrome C, an increase of hydroperoxides on cardiolipin and subsequent dissociation of the cytochrome C-cardiolipin complex. Further reactions in the mitochondria and cytoplasm of the cell can follow several metabolic pathways. Somehow, each of them is connected with the development of early apoptosis: formation of NO and active forms of oxygen (mitochondrial ROS), increase of mitochondrial membrane permeability, release of cytochrome C into the cytoplasm and its binding with the protein Apaf-1 and ATP, as well as with pro-caspase 9 (apoptosome formation) followed by its activation (pro-caspase 9 transition into the active form of caspase 9) and activation of effector caspase 3.

Thus, we suggest that a resonance effect on the U251 cell culture triggers the metabolic cascade of spontaneous apoptosis. The trigger is a change in the physical properties of the cytochrome C oxidase and, possibly, a local change in its secondary structure.

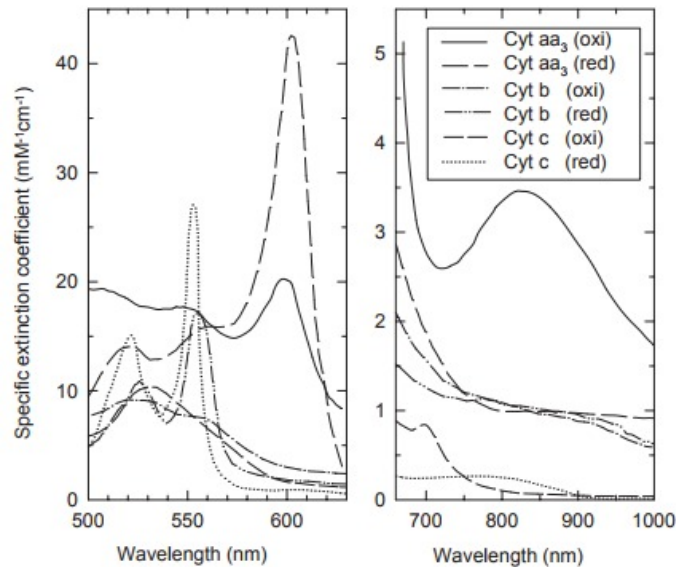


Fig.3. Specific extinction coefficient spectra of cytochrome C oxidase, cytochrome b_5 and the C (per function unit) [25].

5. CONCLUSION

The influence of optical radiation (the wavelengths of 520 and 780 nm and exposure times 1-15 min.) on human glial cells (U251) was studied in the research. As a result of the study it was found that number of apoptotic cells increases by approximately 10% upon irradiation at the wavelength of 780 nm after 1 minute of influence.

The hypotheses are assumed, why the observed effect takes place. Thus, we suggest that a resonance effect on the U251 cell culture triggers the metabolic cascade of spontaneous apoptosis. The trigger is a change in the physical properties of the cytochrome C oxidase and, possibly, a local change in its secondary structure.

The result corroborates the concept of biological hazard of optical radiation on oncology cells.

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