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Abstract

Modern radiation therapy of malignant tumors requires careful selection of conditions that can improve the effectiveness of the treatment. The study of the dynamics and mechanisms of tumor reoxygenation after radiation therapy makes it possible to select the regimens for optimizing the ongoing treatment. Diffuse optical spectroscopy (DOS) is among the methods used for non-invasive assessment of tissue oxygenation. In this work DOS was used for *in vivo* registration of changes in oxygenation level of an experimental rat tumor after single-dose irradiation at a dose of 10 Gy and investigation of their possible mechanisms. It was demonstrated that in 24 h after treatment, tumor oxygenation increases, which is mainly due to an increase in the oxygen supply to the tissues. DOS is demonstrated to be efficient for study of changes in blood flow parameters when monitoring tumor response to therapy.

1. Introduction

Hypoxia or oxygen deprivation (a decrease in partial oxygen pressure) is one of the characteristic features of solid tumors also responsible for their resistance to ionizing radiation. 'Oxygen effect' phenomenon determined by dependence of tissue radiosensitivity on their oxygenation was described in the middle of XX century [1]. The classic 'reoxygenation effect', i.e. increase in oxygen partial pressure after irradiation was described for conventional radiotherapy regimens at the same time [2]. By irradiation with standard conventional single doses (1.8–2 Gy), a minimal vessels' damage and a death of definite number of tumor cells appeared to be a main mechanism of reoxygenation due to decrease in oxygen consumption and increase in the perfusion [3, 4]. For many years, conventional irradiation has been a 'gold standard' of radiation therapy. However, as a result of the significant advances in imaging technologies, treatment planning, dosimetry and radiation delivery systems, currently high doses (more than 10 Gy) could be delivered to tumors in a single fraction or 2–5 fractions (stereotaxic radiosurgery or stereotaxic radiotherapy) [5]. In this situation, a relevant and important question is the fate of tumor blood vessels when tumors are exposed to high-dose hypofractionated radiation. Moreover, to optimize the treatment it is necessary to identify whether the well-known radiobiological phenomenon of conventional fractionated radiotherapy such as reoxygenation of hypoxic cells plays any role in the response of tumors to higher doses of stereotaxic radiation therapy (SRT) and radiosurgery (SRS).

The tumor reoxygenation process observed to occur within 24–72 h of irradiation has been most extensively studied by various methods. These studies were conducted both after split-dose (fractionated) irradiation [6, 7] and after single-dose irradiation [8]. Fujii *et al* [9] demonstrated, using the electron paramagnetic resonance (EPR) oximetry technique, that tumor oxygenation depends on the dose of ionizing radiation it was exposed to. The most pronounced reoxygenation effect being observed to occur in the

case of single 10 Gy exposure within 12–72 h of irradiation. Using an immunohistochemical analysis, Fenton *et al* [10] demonstrated a reduced uptake of the exogenous hypoxia marker by a tumor tissue in 24 h after a single 10 Gy irradiation. The early reoxygenation in tumors and the mechanisms governing this process were studied by Crokart *et al* [11] and Olive [12]. They showed that the oxygen partial pressure in the tumors under study started to rise immediately following irradiation and continued for 4–6 h thereafter. Not in all cases the tumor reoxygenation level was observed to rise on the first day after irradiation. Using the EPR oximetry technique, O'Hara *et al* [13, 14] demonstrated that within the first 12 h after irradiation there has been a temporary reduction of pO_2 in the tumor under study, the dynamics of its subsequent reoxygenation being independent of the tumor model and irradiation dose used, be it 10, 20, or 40 Gy.

Various methods are being now used to measure the oxygen status of neoplasms allowing to assess both the chronic hypoxia component (due to inadequate diffusion) and the acute hypoxia component (due to inadequate perfusion) [15]. Most widely used in clinic at this time is the positron emission tomography (PET) imaging [16]. Optical techniques provide simultaneous assessment of several parameters characterizing the mechanisms underlying changes in neoplasm oxygenation level. The diffuse optical spectroscopy (DOS) allows one to reveal differences in component composition (oxyhemoglobin, deoxyhemoglobin, lipid, and water concentrations) between healthy and pathological tissue areas [17–19]. The determination of the oxygenation status of tumor tissues by this technique is based on the fact that the visible and near-infrared absorption spectra of hemoglobin strongly depend on the degree of its oxygen saturation. With the oxyhemoglobin/deoxyhemoglobin known ratio, one can calculate the blood oxygen saturation level of tissues, which is an indirect indicator of their oxygenation [20]. Moreover, finding the concentration of each hemoglobin form, which reflect the delivery of oxygen to tissues (oxygemoglobin) and its consumption or outflow therein (deoxygemoglobin), allow one to reveal the mechanisms governing changes of tissues oxygenation status [21, 22].

By the present time the DOS technique has found application in clinical oncology to diagnose neoplasms of various localizations (breast cancer, brain, head and neck tumors) [23]. A promissing application of this technique is monitoring of tumor reaction to theraphy and prediction its efficacy [24–27]. Moreover, optical techniques have already been used in clinic to assess radiation-induced changes [28, 29]. A number of reports can be found in the literature on the use of DOS in experimental oncology for studies of oxygenation level variations in tumor models subject to various therapeutic treatments [30–33]. Using experimental tumors, long-term dynamics of oxygenation level after irradiation has been studied [34–37].

In our work, we used a previously developed frequency-domain DOS setup [38] in trans-illumination configuration to study the dynamics and possible mechanisms of the oxygenation level changes in a rodent tumor model over a period of 24 h following its single 10 Gy irradiation.

2. Methods

2.1. Animals and tumor models

The experiments were carried out on male outbred rats (220 ± 10 g) inoculated with the Plyss' lymphosarcoma (PLS) [39]. Cell line of PLS was obtained from the N. N. Blokhin Russian Cancer Research Center (Moscow, Russia). PLS is a connective tissue metastasizing tumor characterized by a rapid growth and early occurrence of necrotic areas. The bloodstream of the tumor is represented by numerous small thin-walled vessels. The tumor was transplanted subcutaneously into the right lower third of the abdominal wall. The experiments were conducted in accordance with the requirements of codes and enactments ruling research works as to the safety and efficiency of pharmaceuticals (Regulation by Ministry of Health and Social Development of Russian Federation No. 708-n from 23.08.2010), and international legal and ethical codes of experimental use of animals (NIH Publications No. 8023, revised, 1978). A total of 8 tumor-bearing animals (4 irradiated and 4 untreated) were enrolled.

2.2. Diffuse Optical Spectroscopy system

The DOS studies were carried out using the system with trans-illumination configuration (figure 1) developed at the Institute of Applied Physics of the Russian Academy of Sciences (Nizhny Novgorod, Russia) [38]. The use of high-frequency (140 MHz) amplitude modulation allows determination of reduced scattering and absorption coefficients separately. The illumination source comprised three lasers operating at wavelengths of 684 nm (close to the absorption maximum of deoxyhemoglobin), 850 nm (close to the absorption maximum of oxyhemoglobin), and 794 nm (close to the isobestic point at which the absorption coefficients of these two hemoglobin forms are equal). The DOS images were acquired by scanning the object of interest with the coaxial light source and detector located from the object opposite sides, synchronously moving along the transversal axis with a 1-mm steps. At each source–detector position, trasmittance value was registered for all the three sources and a single pixel of the resultant image is produced from each measurement. Acquisition time of one image is about 10 min

The source-detector pair geometry used in the system requires the consistency of the thickness of the scanning region and the homogeneity of the medium refractive index and scattering properties. To obtain a scanning region of the same thickness, the

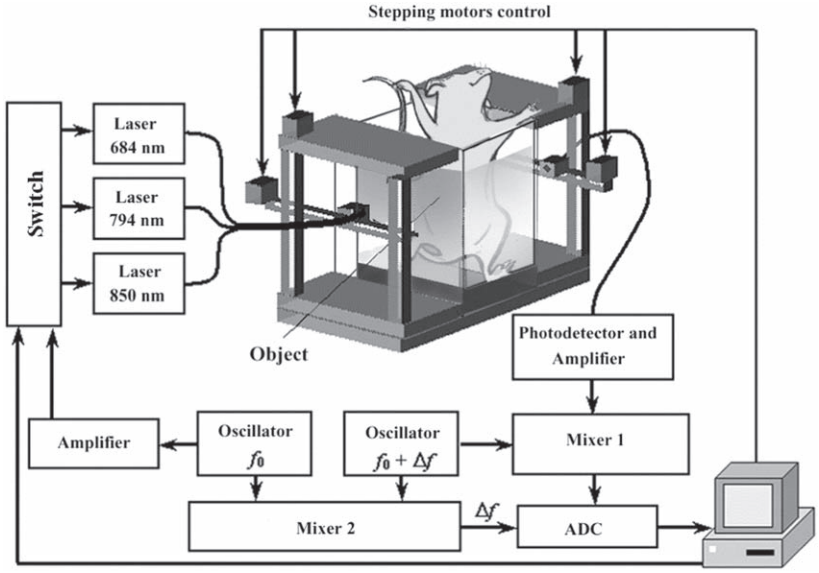

Figure 1. Schematic (block diagram) of DOS setup [40].

Table 1. Optical properties of the immersion liquid compared to those of murine biotissues.

	Immersion liquid		Murine brain		Murine muscle	
λ, nm	μ_a, mm^{-1}	μ_s', mm^{-1}	μ_a, mm^{-1}	μ_s', mm^{-1}	μ_a, mm^{-1}	μ_s', mm^{-1}
685	0.03	1.96	0.09	1.96	0.110	1.13
795	0.02	1.67	0.05	1.74	0.087	0.99
850	0.02	1.44	0.05	1.57	0.088	0.96

experimental animals were placed in a cuvette filled with an immersion liquid with optical parameters similar to that of animal tissue [41]. As an immersion medium, 2% suspension of Lipofundin MST/LST 20% (B. Braun Melsungen, Germany) with the addition of 0.008% India ink (Gamma, Russia) was used. Optical properties of the immersion liquid at the probing wavelengths are shown in table 1.

The properties are reconstructed from the spectrophotometry measurements with a Specord 250 PLUS device (Analytik Jena, Germany) equipped with an integrating sphere. The reconstruction is performed employing inverse Monte Carlo technique.

Determination of tissue components distribution from DOS measurements was performed using a custom-developed Mathcad software, as described in [40]. Spatial distributions of absorption and reduced scattering coefficients were reconstructed from the raw data acquired by the DOS setup: amplitude and phase of the photon density waves at three wavelengths. Concentrations of deoxyhemoglobin (HHb) and oxyhemoglobin (HbO_2) were reconstructed from the determined absorption coefficients. Total hemoglobin concentration was calculated as $[tHb] = [HbO_2 + HHb]$ and blood oxygen saturation as $StO_2 = [HbO_2]/[tHb] \times 100\%$.

2.3. Irradiation and measurement protocol

The study was started on the 7th day after tumor inoculation when tumors were exposed to a single 10 Gy irradiation by a Cobalt-60 (1.25 MeV) unit. Gamma-irradiation was performed by a local field that included a visually detected tumor area with a symmetric margin of 3 mm. Irradiation field was formed by special shielding blocks, so irradiation did not influence surrounding normal tissues. As a control, non-irradiated animals were used. The DOS study was performed before and 24 h post-irradiation. Before the beginning of the experiment, animals were anesthetized by the injection of 50 mg kg^{-1} of Zoletil 100 (Virbac, France), fixed on a support plate and placed in a cuvette with immersion liquid. The cuvette thickness was 35 mm. In the obtained DOS images, each tumor region was contoured manually and averaged values of concentrations of tHb, HHb, HbO_2 as well StO_2 level were calculated using ImageJ program (NIH, USA).

Before each scanning, tumors were measured in two mutually perpendicular directions with a caliper, and tumor volumes were calculated as follows: $v = (a^2 \times b)/2$, where v is the volume, a is the short diameter, and b is the long diameter [42]. The tumor growth inhibition factor was defined as (1—(mean v of treated tumors)/(mean v of control tumors)) $\times$ 100% [43].

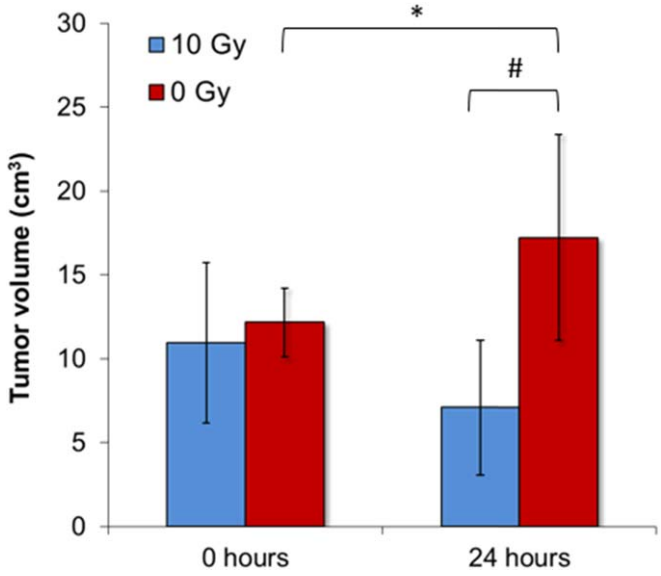

Figure 2. Tumor volume of radiation-treated and untreated Plyss lymphosarcoma. Asterisk (*) indicates statistically significant difference between the current and the initial values ($p < 0.05$). Hash (#) indicates statistically significant difference between values obtained for irradiated and untreated animals ($p < 0.05$).

2.4. Statistical analysis

For tumor volumes and all parameters obtained by DOS means (M) and standard deviations (SD) were calculated. Paired *t*-test was used to reveal statistically significant differences between the initial values and the same values determined in 24 h. Unpaired *t*-test was used to establish differences between corresponding parameters measured in treated and untreated animals. The threshold of statistical significance was taken at $p < 0.05$.

3. Results

From 7th to 8th days after the PLS inoculation, the tumor volume in the untreated animals increased by 1.4 times (from 12.2 ± 2.13 cm^3 to 17.2 ± 6.1 cm^3). The differences were statistically significant ($p < 0.01$). In the irradiated group, the corresponding volumes were 10.9 ± 4.8 cm^3 and 7.1 ± 4.0 cm^3 (figure 2), with the statistical significance between treated and untreated group ($p = 0.03$). The tumor growth inhibition index after irradiation was 59%.

Figure 3 shows the examples of the distribution of blood oxygen saturation (figure 3(a)) and changes in its average values (figure 3(b)) in the PLS tumor in untreated and radiation-treated animals before (0 h) and after (24 h) treatment, on the 7th and 8th day after tumor inoculation correspondently. The level of oxygen saturation in the PLS on the 7th day after the transplantation was lower than in the surrounding normal tissues. In untreated PLS there were no changes in the level of StO_2 from 7th to 8th days of tumor growth. In 24 h after irradiation, StO_2 increased in 1.2 times and became comparable with the surrounding normal tissues (figures 3(a), (b)).

The differences of the values of StO_2 post-irradiation (figure 3(b)) were statistically significant both in comparison with the baseline ($p = 0.01$) and with the values obtained for untreated animals ($p = 0.03$).

PLS tumor is characterized by a lower HbO_2 content in comparison with the surrounding normal tissues (figure 4(a)). In untreated animals, from 7th to 8th days after inoculation, there were no significant changes in the values of HbO_2 concentration. In 24 h after irradiation (figure 4(b)), this parameter increased in comparison with the baseline level ($p < 0.01$), and with the level of untreated animals ($p = 0.04$). The observed increase amounted 1.7 times. Radiation therapy also caused significant changes ($p = 0.02$) in the content of total hemoglobin in the PLS (figures 6(a), (b)).

The concentration of HHb in the tumor on the 7th day after the transplantation was higher than in the surrounding normal tissues and did not change significantly during the experiment either in the course of the natural tumor development or after exposure to ionizing radiation (figures 5(a), (b)).

For all animals, initial tumor StO_2 ($54.2 \pm 10.1\%$), HbO_2 (39.1 ± 14.4 μM), HHb (30.2 ± 5.7 μM), and tHb (69.3 ± 16.4 μM) demonstrated in our work are in the physiological range of concentrations and agree with the results obtained by Pham *et al* [30] using an diffuse optical method for experimental tumor in rodents. The values in normal tissues obtained in our work were $67.2 \pm 7.4\%$ for StO_2, 47.8 ± 13.0 μM for HbO_2, 22.5 ± 3.2 μM for HHb, and 70.3 ± 14.2 μM for tHb with statistically significant differences with tumor values estimated for StO_2 ($p = 0.01$) and HHb ($p < 0.01$). No statistically significant differences were revealed in normal tissues

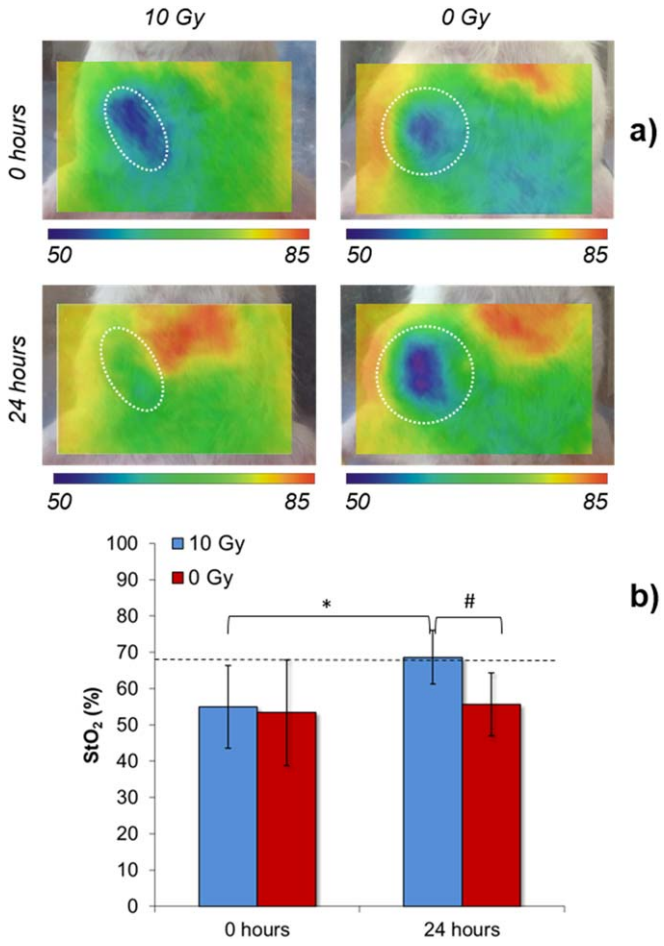

Figure 3. Blood oxygen saturation (StO_2) level of radiation-treated and untreated Plyss lymphosarcoma: (a) DOS images of StO_2 distribution (%) overlay on the animal photo; (b) values of tumor StO_2 level (M $\pm$ SD). DOS image size 80 $\times$ 60 mm. White dotted lines contour the tumor area. Black dotted line shows the averaged over all animals StO_2 level in normal tissue on the 7th day of tumor growth. Asterisk (*) indicates statistically significant difference between the current and the initial values ($p < 0.05$). Hash (#) indicates statistically significant difference between values obtained for irradiated and untreated animals ($p < 0.05$).

during tumor growth and after treatment (data not shown).

4. Discussion

Understanding the mechanisms of the ionizing radiation effect on the microcirculatory bed and oxygen state of the tumor is important for improving the effectiveness of radiotherapy. Most studies dedicated to radiation-induced changes of tumor vasculature and oxygenation have been performed in the middle and the second half of the XX century [3]. A significant part of them concerned the influence of a single irradiation at relatively higher doses (more than 5 Gy) on these parameters. The results of these studies became of high demand nowadays, since hyperfractionation (the increase in a single dose and decrease in a number of fractions) is widely used now for radiation therapy of malignant tumors allowing maximize its effect against tumor and minimize a damage of surrounding normal tissues.

Reoxygenation (an increase in an oxygen partial pressure comparing to the initial one) appeared to be a typical response of a tumor tissue to conventional irradiation [44]. In our work, it was demonstrated that single irradiation of Pliss' lymph sarcoma at a dose of 10 Gy leads in 24 h after exposure to a statistically significant inhibition of tumor growth comparing to non-irradiated control (figure 2), and to a significant change of its oxygenation. Blood oxygen saturation that is consistent to tumor oxygenation [40] increased as compared to the initial level as well as to the non-irradiated control (figure 3). The results were in a good accordance with the data obtained in the study of radiation induced changes of tumor oxygen state using the EPR-oximetry [9].

Discussing the mechanisms of tumor reoxygenation, two factors should be taken into account. The first one is oxygen consumption, which depends on the number of viable tumor cells, and the second one is the state of perfusion, which reflects the state of tumor microvasculature [11, 42, 45, 46]. In our study, we observed a significant increase in tumor oxygenation in 24 h after irradiation due to increase in oxygemoglobin concentration (figure 4). This fact could indirectly testify for an increase of tumor perfusion.

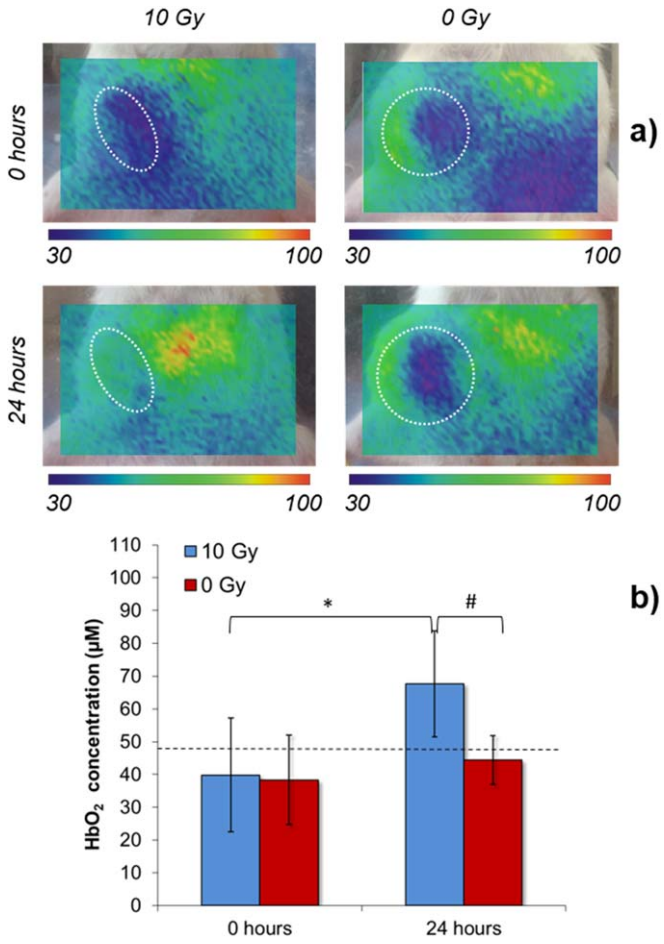

Figure 4. Oxyhemoglobin concentration (HbO_2) of radiation-treated and untreated Plyss lymphosarcoma: (a) DOS images of HbO_2 distribution (μM) overlay on the animal photo; (b) values of tumor HbO_2 concentration (M $\pm$ SD). DOS image size 80 $\times$ 60 mm. White dotted lines contour the tumor area. Black dotted line shows the averaged over all animals HbO_2 concentration in normal tissue on the 7th day of tumor growth. Asterisk (*) indicates statistically significant difference between the current and the initial values ($p < 0.05$). Hash (#) indicates statistically significant difference between values obtained for irradiated and untreated animals ($p < 0.05$).

Irradiation with 5 Gy caused conjoint increase in both vascular density and perfusion during 24–72 h post-irradiation, although the degree of change was variable between individuals [47]. The degree of change in vascular density was inversely related to median pretreatment diameter [47]. The results of our work are in good agreement with the data showing radiation-induced increase in the level of tumor oxygenation due to improved perfusion [11]. In [48] it was shown that the effect of irradiation at a dose of 6 Gy on the tumor vasculature is mediated by proangiogenic effects that can affect the effectiveness of radiotherapy through increased regulation of the endothelial pathway of nitric oxide (NO).

At the same time, some studies have demonstrated a decreased or a stable tumor perfusion in 24–72 h after single irradiation at the appropriate doses. Irradiation with 4–16 Gy in a single dose increased the vascular permeability in 24–72 h and decreased the functional vascular volume in 24 h [49]. The density of perfused vessels decreased in 24 h after 10 Gy irradiation and recovered to the control level in 72 h [10]. Other studies demonstrated that intratumor mean pO_2 and pO_2 fluctuation were not altered by irradiation with 5 or 10 Gy, indicating that tumor microenvironment was not affected [50]. Irradiation with 20 Gy in a single dose caused no changes in vascular density whereas apoptosis of tumor cells was significant in 10.5 h post-irradiation. Blood perfusion increased in 1 h post-irradiation. Hypoxic area in the tumors decreased in 30.5 h after irradiation [51].

There is an opinion that the sensitivity of the tumor vascular system to the action of ionizing radiation is one of the main factors determining the overall radiosensitivity of the tumor [52]. In response to ionizing radiation, tumors secrete cytokines that inhibit apoptosis of endothelial cells, reducing the effect of irradiation by minimizing damage of the vascular bed. It is shown that the hypoxia-induced factor HIF-1 plays the main role in the mechanism of such protective effect [45]. It is possible that the reoxygenation originated from improved tumor perfusion and increase in oxyhemoglobin concentration after

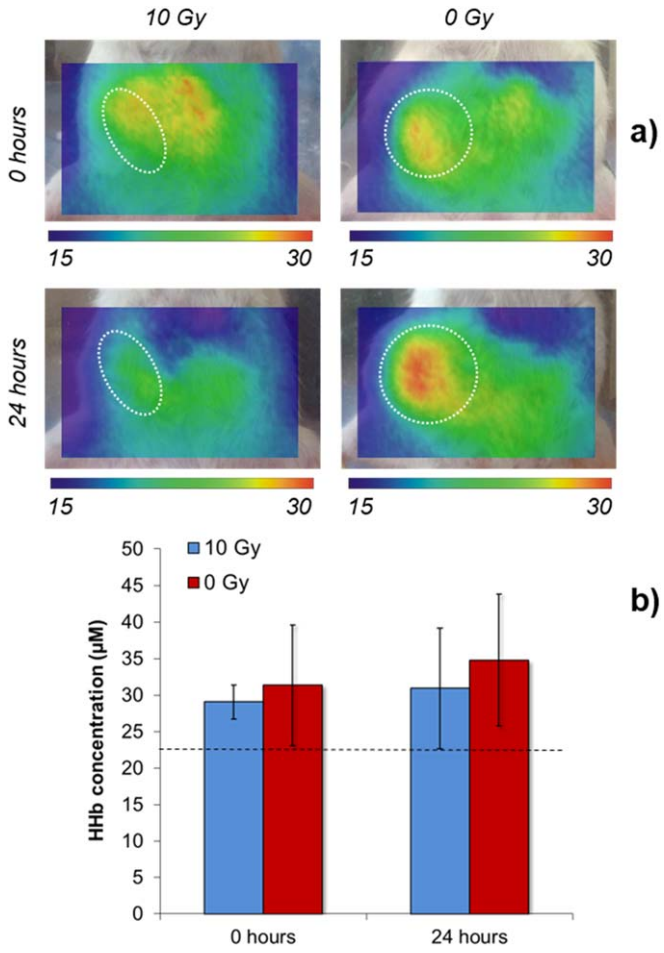

Figure 5. Deoxyhemoglobin concentration (HHb) of radiation-treated and untreated Plyss lymphosarcoma: (a) DOS images of HHb distribution (μM) overlay on the animal photo; (b) values of tumor HHb concentration (M $\pm$ SD). DOS image size 80 $\times$ 60 mm. White dotted lines contour the tumor area. Black dotted line shows the averaged over all animals HHb concentration in normal tissue on the 7th day of tumor growth.

irradiation, since a large number of blood vessels is a characteristic feature of Pliss' lymphosarcoma [40].

Another well-established mechanism of tumor reoxygenation after irradiation is a decrease in oxygen consumption due to decrease of the number of viable tumor cells [43]. It was noticed that despite the decrease in the perfusion, O_2 availability appeared to be increased due to reduction in O_2 consumption, nevertheless, the radiobiologically hypoxic cell fraction did not change. Since hypoxic cells are more resistant to ionizing radiation than well-oxygenated ones, and the proportion of the former ones in the tumor population becomes larger. Simultaneously, the tumor increases the access of oxygen to previously hypoxic cells, which again increases their sensitivity to radiation [10]. It should be mentioned that involvement of changes in oxygen consumption and blood flow to reoxygenation processes can be different [4].

Deoxyhemoglobin content reflects an oxygen consumption by the tissues [10, 22]. PLS appeared to be a sufficiently hypoxic tumor as compared to normal tissues, and its reduced oxygenation level is determined by high oxygen consumption (high HHb content). In our study, a single irradiation at a dose of 10 Gy did not lead to a significant decrease of deoxyhemoglobin concentration (figure 5) that indirectly indicates a stable number of viable tumor cells of this model in 24 h after irradiation. At the same time, increase of tumor blood content was confirmed by the increase in the concentration of total hemoglobin comparing to the initial level (figure 6). This shows the possibility of a radiation-induced influx of oxygen-enriched blood to the tumor.

Thereby, using DOS technique only, indirect information about tumor oxygen delivery, tumor oxygen consumption, tumor oxygenation and blood content was obtained. It should be mentioned that this technique has several limitations that could affect the results. In transmittance configuration of DOS, absorption and scattering coefficients are averaged over the entire body thickness. The error in tissue-component content estimation in tumor depends on the fraction of tumor volume and the volume of surrounding normal tissue and immersion liquid in the measurement volume. The error reaches the highest values at the edge of the investigated object (due to large content of immersion liquid in the measurement

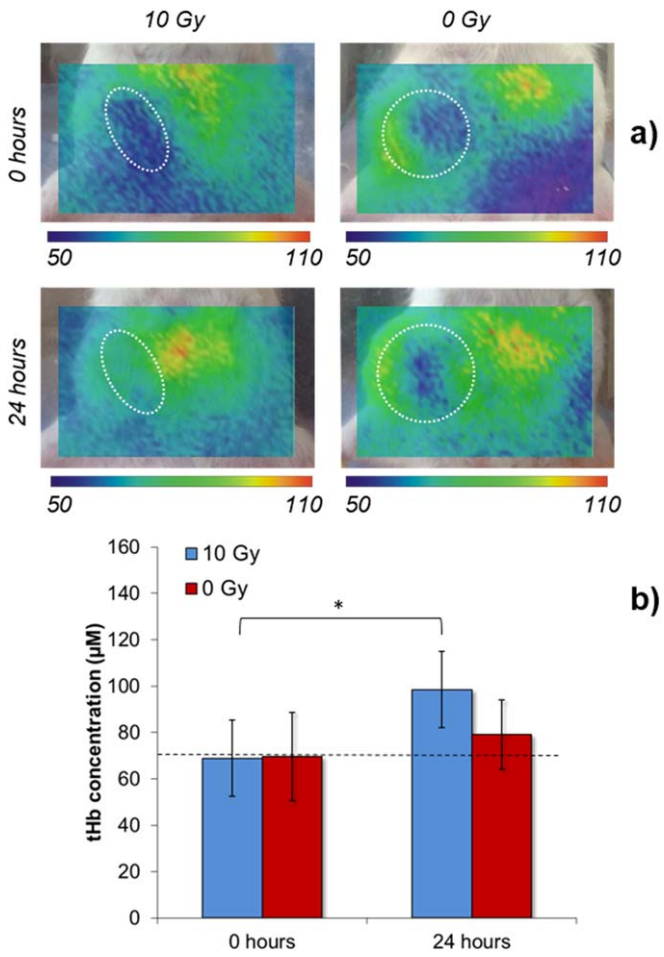

Figure 6. Total hemoglobin concentration (tHb) of radiation-treated and untreated Plyss lymphosarcoma: (a) DOS images of tHb distribution (μM) overlay on the animal photo; (b) values of tumor tHb concentration (M $\pm$ SD). DOS image size 80 $\times$ 60 mm. White dotted lines contour the tumor area. Black dotted line shows the averaged over all animals tHb concentration in normal tissue on the 7th day of tumor growth. Asterisk (*) indicates statistically significant difference between the current and the initial values ($p < 0.05$).

volume) and for small tumors (due to large content of normal tissues in the measurement volume).

5. Conclusion

The study demonstrated that SRT relevant dose irradiation (10 Gy) leads to tumor reoxygenation and its main mechanism in the case of a rapidly growing poorly oxygenated tumor with a large number of blood vessels (Pliss' lymph sarcoma) is an improved perfusion of tumor tissue. Diffuse optical spectroscopy provided indirect information about tumor perfusion, tumor oxygen consumption, tumor oxygenation and blood content. DOS is useful not only for study of changes in tumor blood flow parameters in response to different types of therapy but also for revealing the mechanisms of these changes.

Acknowledgments

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