EFFECTS OF DIFFERENT PHOTOBIOMODULATION MODES ON THE BJ-5ta-hTERT FIBROBLASTS EXPOSED TO IONIZING RADIATION

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Abstract. The aim of the work was to study the effects of photobiomodulation (PBM) in the red spectrum (640 nm) with fluences from 3 mJ/cm^2 to 2 J/cm^2 in combination with ionizing radiation (IR) at doses of 2–6 Gy against human BJ-5ta-hTERT – postnatal fibroblasts. The cells were exposed to low-intensity red light before or after their exposure to IR, the viability of the cells was determined by MTT 24 hours after the last exposure. It is shown that the effects of PBM depend on the fluence of PBM, the dose of IR and the sequence of the actions of these physical factors on cells. The adaptive effect of PBM was observed only for high fluences-1 and 2 J/cm^2 when exposed to PBM and subsequent (after 1 hour) irradiation of IR. At the same time, the stimulating effect of PBM was observed only for low fluences from 3 to 300 mJ/cm^2 under IR irradiation and subsequent (after 1 hour) exposure to PBM. These data should be taken into account when using PBM for the correction of adverse events of radiation therapy in an oncological clinic.

Keywords: gamma-irradiation; photobiomodulation; BJ-5ta-hTERT; low-intensity red light.

List of Abbreviations
PBM – Photobiomodulation
LILR – Low-intensity laser/LED radiation
ROS – Reactive Oxygen species
IR – Ionizing radiation

Introduction

Changes in normal tissues that occur at various times after radiation therapy for malignant neoplasms are one of the unsolved problems of modern radiation oncology (Mothersill & Seymour, 2002; Sedova *et al.*, 2018), as a result, the search for methods for preventing and correcting side effects that occur during radiation therapy of malignant tumors does not lose its relevance. One of the methods of prevention and correction of side effects of radiation is PBM (with exposure to low-intensity laser/LED radiation (LILR)).

This method has been used for more than two decades for the prevention and treatment of complications of radiation therapy (Cowen. *et al.*, 1997), because it has numerous biological effects, including anti-inflammatory, immunocorrective and analgesic effects, and also promotes the stimulation of cell proliferative activity (Avci *et al.*, 2013; Lins *et al.*, 2010; Hamblin, 2017). One of the main mechanisms for triggering the cellular response to the effect of LILR is photosensitization of endogenous cellular chromophores, which results in an increase in the concentration of reactive oxygen species (ROS) and further activation of a large number of signaling pathways (Sonis *et al.*, 2016; Hamblin, 2018). First of all, we are talking about the enhancement of cell proliferation through Src kinases with further activation of the kinase cascade, including MAPK, Akt, PKC and EGFR. One of the most studied signaling pathways influenced by PBM is the activation of PI3K/Akt/mTOR associated with cell growth, proliferation, differentiation and cell viability (Zhang *et al.*, 2008). PBM-induced activation of PI3K/Akt/mTOR was detected in

normal, dysplastic and tumor, as well as mesenchymal stem cells (Bellacosa *et al.*, 2014). When studying the effects of PBM on various cell types, it should be taken into account that it leads to the formation of ROS and/or the development of oxidative stress (Sun *et al.*, 2010). LILR, as a rule, slightly increases the amount of ROS over a relatively short period of time and is able to stimulate cell differentiation, proliferation and increase their viability (Huang *et al.*, 2011; Freitas & Hamblin, 2016).

In the prevention and treatment of radiation reactions from normal tissues that fall into the volume of radiation during the course of (chemo)radiation therapy, the effect of LILR may occur directly in the area of the location of the tumor focus (for example, in the treatment of patients with tumors of the oral mucosa and pharynx or tumors of the pelvic organs) (Migliorati *et al.*, 2013). For many years, the LILR has been used empirically in cancer patients, basing on positive clinical experience. At the same time, by default, it was assumed that due to the absence of a thermal effect, it does not carry the risk associated with a stimulating effect on «live» tumor cells. Recent studies have shown, however, that PBM in combination with IR causes multidirectional effects, including those associated with an increase in the number of viable tumor cells (Sonis *et al.*, 2016; Cherkasova *et al.*, 2020). Based on this, it is necessary to choose the modes of PBM (parameters and sequences of exposure to LILR and IR), which will promote the proliferation of cells of normal tissues, without increasing (and ideally suppressing) the proliferation of tumor cells. In this work, human postnatal fibroblasts BJ-5ta-hTERT (ATCC® CRL4001™) were used as a model of normal tissue cells.

The first publications on the effects of LILR therapy on cell cultures appeared more than 30 years ago (Voskanyan, 1985). In the work of 2011, the influence of low PBM fluences (energy densities) was studied for epithelial cells (BEAS-2B) and human fibroblasts. During the experiment, the cells were exposed to PBM for 15 minutes (the power varied from 0.39 to 63.7 MW/cm^2). Increased fibroblast proliferation and decreased BEAS-2B cell activity were shown (Schartinger *et al.*, 2011). In another study, the effect of different PBM fluences on mouse embryonic fibroblasts (NIH/3T3, CCl-226), human skin fibroblasts and neoplastic cells (RIF-1 and EMT-6) was studied. The effect of PBM was carried out at λ = = 632.8 nm with a laser with a power density of 1.25 mW/cm^2. The fluence range was from 60 mJ/cm^2 to 600 mJ/cm^2. It was found that the optimal fluence for stimulating proliferation at λ = 632.8 nm is 180 mJ/cm^2, and at higher fluences, proliferation inhibition occurs (Watban *et al.*, 2011). In a 2014 study, the effect of PBM on keratinocytes, fibroblasts, endothelial cells and osteoblasts was studied. The exposure was carried out by laser radiation with λ = 670 nm, with a power of 280 mW and an exposure of 60 s. It was shown that the exposure to PBM had a positive effect on the viability of all cell groups (Walter *et al.*, 2014). The authors (Huang *et al.*, 2013) studied the effect of PBM on the cells of the periodontal ligament of the tooth. They were exposed to radiation with λ = 670 nm, with a power of 500 mW for 2.5 or 5 seconds, with fluences of 1.25 J/cm^2 and 2.5 J/cm^2.

It was found that PBM significantly increases cell proliferation, reduces inflammation and increases the activity of the paradontal ligament cells under unfavorable conditions.

It is reported that PBM is able to increase the resistance of cells of normal tissues to the effects of ionizing radiation (Chen *et al.*, 2011). Thus, in a 2009 study, the effect of PBM on the proliferation of Achilles tendon fibroblasts and gene expression was studied. Four groups of identically cultured fibroblasts were exposed to PBM before exposure to ionizing radiation. The control group was not exposed. The other three groups were affected by PBM with different fluences: 1 J/cm^2, 2 J/cm^2 and 3 J/cm^2, respectively. A day later, the level of cell proliferation, the expression of type I collagen mRNA and decorin were determined. Compared with the control group, the proliferation of fibroblasts irradiated with LILR significantly increased by (13±0,8)%, (30±0,4)% and (12±0.6)%, respectively (Chen *et al.*, 2011).

In a 2015 study, the effect of PBM on NIH/3T3 fibroblast-like cells and HeLa tumor

cells was studied. The cells were exposed to laser radiation with λ = 685 and λ = 830 nm, the laser fluences were 1 J/cm^2 and 5 J/cm^2. After PBM, gamma irradiation of cells was carried out at a dose of 2 Gy, 4 Gy and 6 Gy. The analysis of cell survival showed that the use of a laser with a wavelength of 685 nm before IR could significantly inhibit the survival of HeLa cells compared to cells, which were not exposed to PBM. On the contrary, the use of PBM at a wavelength of 830 nm was able to protect NIH/3T3 cells from IR and increase the percentage of surviving cells (Gholamreza *et al.*, 2015).

In 2016, the effect of PBM on fibroblasts and tumor cells after exposure to IR doses of 2.5 Gy and 10 Gy was investigated. After a day, the cells were exposed to PBM with fluences of 30 J/cm^2, 90 J/cm^2 and 150 J/cm^2. Viability, cell cycle phases, cell proliferation index, and aging were evaluated on the 1st and 4th days after exposure to PBM. It was found that for fibroblasts, PBM contributes to an aging decrease, increase in cell viability and proliferation with dependence on the fluence. No statistically significant effect of PBM on their viability were revealed for tumor cells, but a decrease in proliferation and aging of the population were registered. These results show that fibroblasts and tumor cells react differently to PBM after IR (Silva *et al.*, 2016). Thus, the currently presented literature data on the response of normal tissue cells to the combined effects of PBM and IR are incomplete and quite contradictory. The studies were performed with different modes of PBM, doses of IR and their combinations, which makes it difficult to compare and interpret the results.

The aim of the study was to study the effects of low-intensity radiation of the red range of the red spectrum on fibroblasts of the BJ-5ta-hTERT line exposed to IR at doses used in the oncology clinic, depending on the PBM fluence and the sequence of effects.

Materials and Methods

Cell line. Fibroblasts represent large, flat, fusiform cells. They play an important role in maintaining the constancy of the connective tissue matrix and in wound healing: they migrate to the site of injury, where they synthesize new collagen (Weissmanshomer & Fry, 1975).

BJ-5ta-hTERT cell line which was used in the work was provided by the Shemyakin-Ovchinnikov Institute of bioorganic chemistry, Russia. The cells were cultured in a DMEM medium (PanEco, Russia) containing 10% embryonic calf serum (HyClone, USA) and 2 mM L-glutamine (PanEco, Russia). Cultivation was carried out in a CO2 incubator at 37°C and an atmosphere of 5% CO_2, at each stage of passaging the cells were treated with 0.25% trypsin-EDTA solution (PanEco, Russia). The cells were counted by the standard method using the Goryaev chamber. For the experiments, the cells were sown on 96-well plates (2000 cells per well) and grown without changing the nutrient medium for 24 hours.

PBM. BJ-5ta-hTERT cells were exposed to LED light with λ = 640±11 nm, with an intensity of 1 mW/cm^2 (for low fluences of 3 mJ/cm^2, 30 mJ/cm^2, 300 mJ/cm^2) or 16 mW/cm^2 (for high fluences of 0.5 J/cm^2, 1 J/cm^2, 2 J/cm^2)). The LED device «REIR-4» (FRC «Crystallography and photonics», Russia), specially designed to ensure uniform irradiation of cell tablets, was used for PBM exposure to low fluences. For exposure to high fluences of PBM, the CDM-08 device was used (FRC «Crystallography and photonics», Russia). Summary information about the PBM parameters is presented in Table 1. BJ-5ta-hTERT cells were exposed to low-intensity red light with the specified fluences 24 hours after sowing.

Gamma-irradiation. Irradiation of cells with IR (hard gamma radiation) was performed on a remote radiation therapy device «Terabalt 80» (UJP Praha, Czech Republic, Co60, beam energy 1.25 MeV, dose rate 1 Gy/min). To achieve a uniform dose distribution inside the tablet, the method of irradiation with two opposite fields was used. Calculations of the parameters of the irradiator and the dose distribution were carried out in the PlanW 2000 dosimetric planning system based on computed tomogram-

Table 1

PBM parameters

Device	Wavelength, nm	Intensity, mW/cm^2	Time, s	Fluence, J/cm^2
REIR-4	640±11	1	3	0.003
			30	0.03
			300	0.3
CDM-08	640±11	16	30	0.5
			62	1
			125	2

phy images. Irradiation was carried out in doses of 2 Gy, 4 Gy and 6 Gy.

MTT test. Cell viability was determined by the MTT test based on the ability of mitochondrial dehydrogenases to convert water-soluble dimethylthiazolyl-diphenyl-tetrazolium bromide (MTT, PanEco, Russia) into insoluble formazane. The MTT solution (5 mg/ml in DMEM) was added to the wells of a 96-well tablet in a ratio 1:10 to the volume of DMEM, incubated for 3 hours at 37 °C in a CO_2 incubator. The liquid was removed, 100 ml dimethyl sulfoxide was added to each well (DMCO, PanEco, Russia), the optical density was calculated on a Synergy Mx tablet reader (Biotek, USA), the main filter was 540–590 nm, the correction filter was 630–690 nm.

Experiment design. First, the effect of low-intensity red light on BJ-5ta-hTERT cells was studied (Fig. 1). For this, PBM of attached cells (2000 in the well) with the above parameters was performed (Table 1). When calculating the number of viable cells, the number of cells in the sample without exposure was taken as 100%.

At the second stage of the experiment, the effects of PBM on BJ-5ta-hTERT cells that were further exposed to ionizing radiation were studied (adaptive effect). Initially, the attached cells (2000 in the well) were exposed to low-intensity light with the above parameters (Table. 1), and after 1 hour, IR irradiation was performed. Cell viability was determined 24 hours after gamma-radiation exposure for each PBM mode. The number of cells in the samples irradiated with IR at doses of 2 Gy, 4 Gy and 6 Gy without PBM was taken as 100%. The experiment design is presented in Figure 2A.

At the third stage, the effects of PBM on BJ-5ta-hTERT fibroblasts previously irradiated with IR (stimulating effect) were studied. To do this, the initially attached cells (2000 in the well) were irradiated with IR, and after 1 hour, PBM was performed with the above parameters (Table 1). Cell viability was determined 24 hours after exposure for each PBM parameter. The number of cells in the samples irradiated with IR at doses of 2 Gy, 4 Gy and 6 Gy without PBM was taken as 100%. The design of the experiment is shown in Figure 2B.

The experiments were carried out in the amount of at least three repetitions for each of the PBM fluences, doses and control samples. The data were processed using the statistical software packages Microsoft Excel and GraphPad Prism 4.00 for Windows (GraphPad Prism Software Inc., USA). The results are presented in the form of M±σ (mean and standard deviation). The statistical significance of the differences in the mean values compared to the control was determined by the Student's criterion at a significance level of $p < 0.05$.

Results

The effect of PBM on cell viability. A day after PBM with fluences of 0.003, 0.03, 0.3 and 0.5, 1, 2 J / cm^2, the cell viability was, respectively, 95±10%, 116±7% and 118±8%, 101±12%, 99±13% and 97±13% (Fig. 3). Statistically significant differences compared to

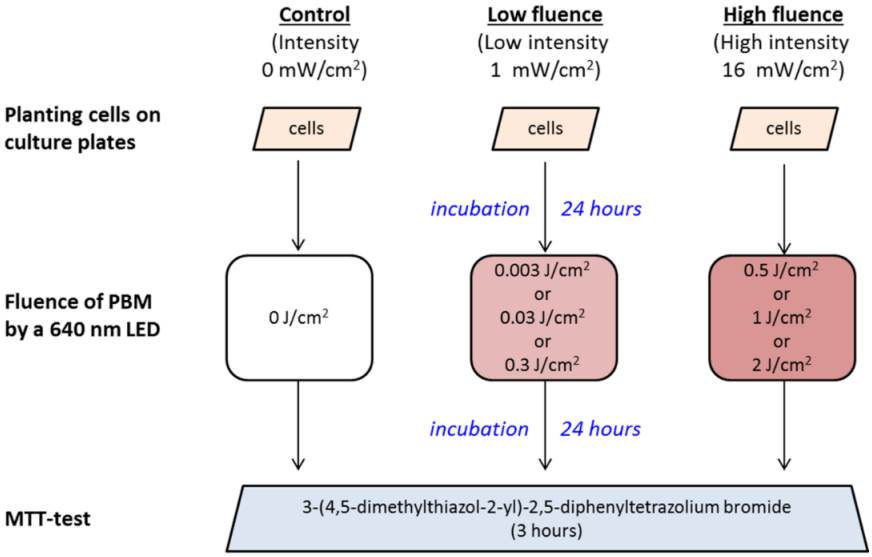

Fig. 1. Design of an experiment to study the effects of PBM on to BJ-5ta-hTERT

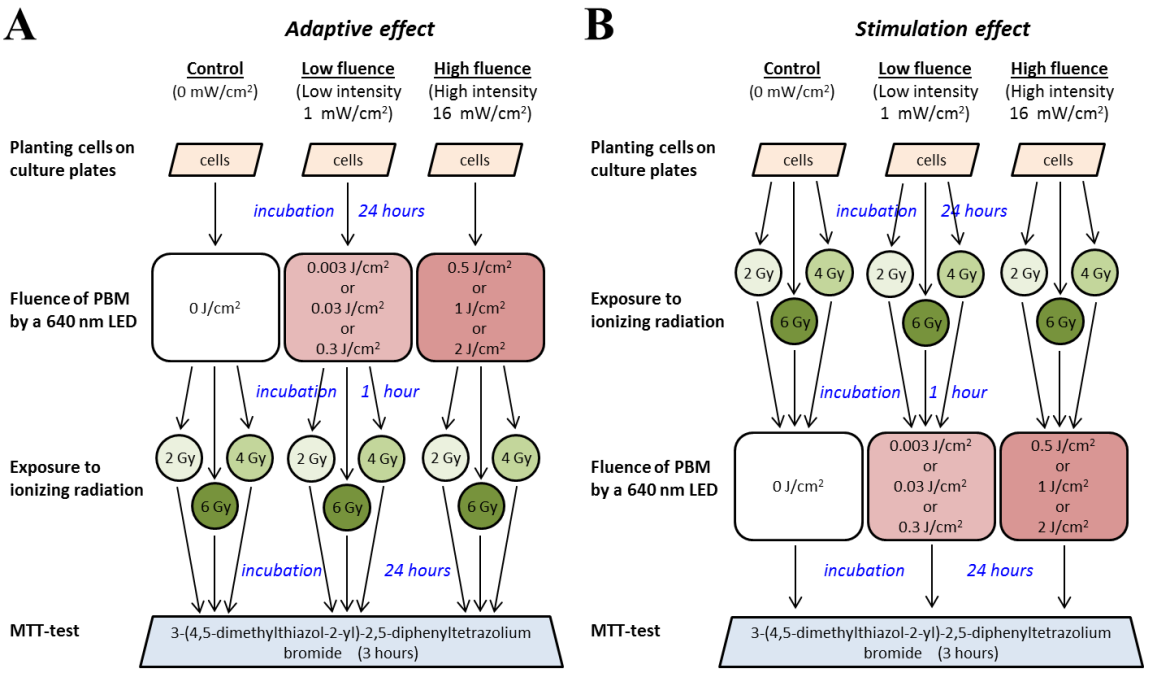

Fig. 2. Design of an experiments to study various effects of PBM in combination with exposure to IR in relation to BJ-5ta-hTERT cells

the control corresponded to fluences of 0.03 and 0.3 J/cm^2.

Adaptive effect of PBM. When alternating the effects of PBM with fluences of 0.003, 0.03, 0.3 and 0.5, 1, 2 J/cm^2 and IR at a dose of 2 Gy, the viability of BJ-5ta-hTERT cells was 105±9%, 98±10%, 99±9%, 96±10%, 102±±13%, 114±13%, accordingly (Fig. 4A). The statistically significant difference compared to the control corresponded only to the fluence of 2 J/cm^2.

When alternating the effects of PBM with fluences of 0.003, 0.03, 0.3 and 0.5, 1, 2 J/cm^2

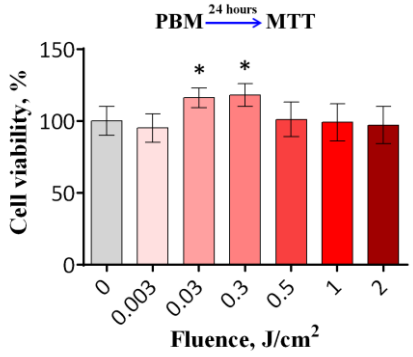

Fig. 3. Viability of BJ-5ta-hTERT after FBM of various fluences.
* statistically significant differences

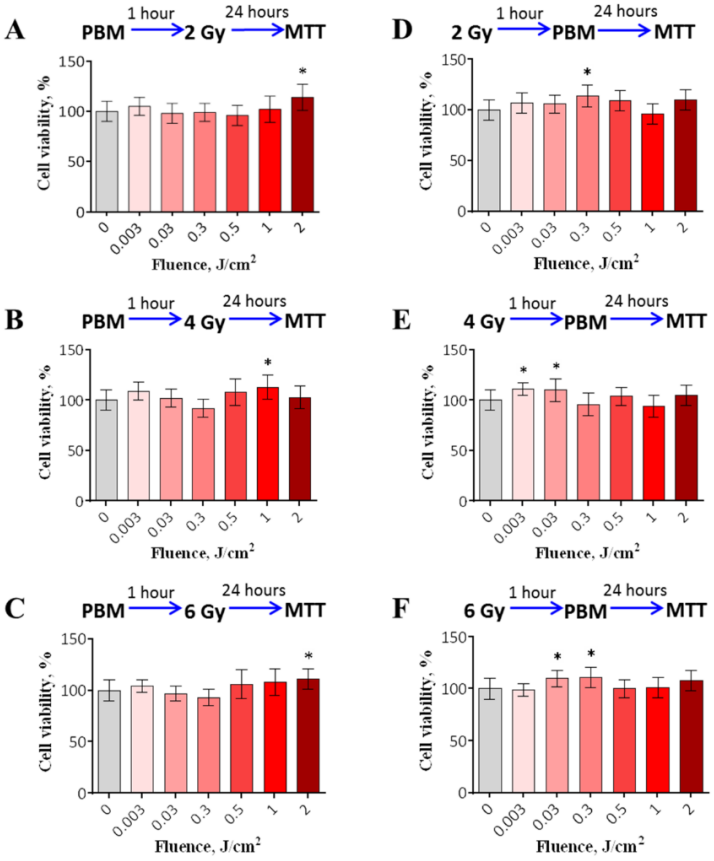

Fig. 4. Viability of BJ-5ta-hTERT after alternation of FBM with various fluences and IR at doses of 2, 4 and 6 Gy.
* statistically significant differences

and IR at a dose of 4 Gy, the viability of BJ-5ta-hTERT cells was 109±9%, 102±9%, 92±9%, 108±13%, 113±12%, 103±11% accordingly (Fig. 4B). The statistically significant difference compared to the control corresponded only to the fluence of 1 J/cm^2.

When alternating the effects of PBM with fluences of 0.003, 0.03, 0.3 and 0.5, 1, 2 J/cm^2 and IR at a dose of 6 Gy, the viability of BJ-5ta-hTERT cells was 104±6%, 97±7%, 93±8%, 106±14%, 108±13%, 111±10% accordingly (Fig. 4C). The statistically significant difference compared to the control corresponded only to the fluence of 2 J/cm^2.

The stimulating effect of PBM. When alternating the effects of IR at a dose of 2 Gy and further PBM with fluences 0.003, 0.03, 0.3, 0.5, 1, 2 J/cm^2 viability of BJ-5ta-hTERT cells was 107±10%, 106±9%, 114±11%, 109±10%, 96±10% and 110±10%, respectively (Fig. 4D). The statistically significant difference compared to the control corresponded only to the fluence of 0.3 J/cm^2.

When alternating the effects of IR at a dose of 4 Gy and PBM with fluences 0.003, 0.03, 0.3, 0.5, 1, 2 J/cm^2 viability of BJ-5ta-hTERT cells was 111±6%, 110±11%, 96±11%, 104±9%, 94±11%, 105±10% accordingly (Fig. 4E). A statistically significant difference compared to the control corresponded to fluences of 0.003 and 0.03 J/cm^2.

When alternating the effects of IR at a dose of 6 Gy and PBM with fluences 0.003, 0.03, 0.3, 0.5, 1, 2 J/cm^2 viability of BJ-5ta-hTERT was 99±6%, 110±8%, 111±10%, 100±9%, 101±10%, 108±10% accordingly (Fig. 4F). A statistically significant difference compared to the control corresponded to fluences of 0.03 and 0.3 J/cm^2.

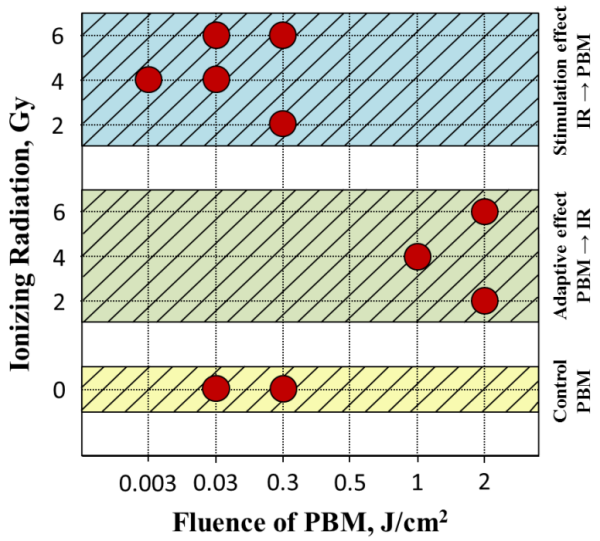

Fig. 5. Diagram of the dependence of the obtained positive effects of FBM on the dose and fluence of FBM and the sequence of actions

As can be seen from Figure 4, the adaptive or stimulating effects of PBM were manifested in all the studied groups. Figure 5 shows the dependence of the obtained positive effects of PBM on its parameters, the dose of IR and the sequence of two modalities on cells. The adaptive effect was achieved at high fluence values (1–2 J/cm^2), the stimulating effect – at low fluence values (0.003–0.3 J/cm^2). In the absence of IR, the viability of fibroblasts increased when exposed to fluences of low values – 0.03–0.3 J/cm^2.

Discussion

Photobiomodulation (with the influence of LILR) has been actively used in clinical medicine over the past few decades. One of the actively developing areas of its application is the prevention and correction of side effects of radiation therapy for malignant neoplasms (Avci *et al.*, 2013; Lins *et al.*, 2010; Sedova *et al.*, 2018). Currently, it is recommended to use low intensity light with λ = 635 nm, an output power of less than 100 mW and a fluence of at least 1 J/cm^2 to correct and prevent undesirable consequences of radiation therapy (Lalla *et al.*, 2014). In our study, the effects of PBM on human fi-broblast cells BJ-5ta were studied at an energy density of PBM both significantly less and more than 1 J/cm^2.

The study showed that PBM in the range of energy densities from 0.03 J/cm^2 to 2 J/cm^2 does not cause a decrease in the viability of BJ-5ta-hTERT fibroblasts (Fig. 3). A day after exposure to PBM with fluences of 0.03 J/cm^2 and 0.3 J/cm^2, the number of viable cells increases by 16–18%. These results correspond to studies that note that PBM in small doses does not have a negative effect on normal tissues (Watban & Bernard, 2011).

When studying the response of normal tissue cells to the combination of PBM and IR, our study revealed multidirectional effects depending on the sequence of actions and the energy density of PBM. Preliminary PBM of BJ-5ta-hTERT cells using low fluences did not lead to the development of an adaptive effect (Fig. 4). On the contrary, adaptive effects were detected when using high PBM fluences (1 and 2 J/cm^2). PBM of fibroblast, which preceded IR, led to a significant increase in their proliferation by 13–30%. These results generally correspond to the data obtained in 2011 by Chen et al., 2011. In the study of Chen et al., PBM fluences from 1 to 4 J/cm^2 were used, while the highest efficiency was obtained for 2 J/cm^2, which correlates with our results.

Regarding the stimulating effect of PBM on fibroblasts previously irradiated in doses 2 Gy, 4 Gy and 6 Gy, it was shown that PBM an hour after IR exposure, under certain parameters, led to a statistically significant increase in the number of viable cells compared to the control. Thus, the effect of LILR with 0.3 J/cm^2 fluences contributed to an increase in the number of viable cells previously irradiated with IR at a dose of 2 Gy. PBM with fluences of 0.003 and 0.03 J/cm^2 led to an increase in the number of surviving cells (up to 10%) irradiated with IR at a dose of 4 Gy. Exposure to LILR with fluences of 0.03 and 0.3 J/cm^2 increased the number of viable cells after gamma irradiation at a dose of 6 Gy. Our results are consistent with the study of Silva et al., 2016, in which PBM with an output power of 40 mW promotes the growth of viability and proliferative activity of fibroblasts

of the FMM1 line with preliminary irradiation of IR in 2.5 and 10 Gy.

Conclusions

A statistically significant increase in the number of human fibroblast culture cells hTERT-BJ5ta was revealed compared to the control with their PBM with fluences of 0.3 and 0.03 J/cm^2. PBM using higher fluences without additional IR irradiation did not lead to an increase in cell proliferation.

An adaptive effect of PBM against BJ-5ta-hTERT was revealed, in which the viability of cells irradiated with IR after PBM was higher after a day compared to the control (without preliminary PBM). PBM of fibroblasts with fluences of 2 J/cm^2 for 2 and 6 Gy, as well as 1 J/cm^2 for 4 Gy, led to a statistically significant increase in the number of viable cells. At the same time, the PBM of cells with lower fluences could not protect the cell culture from IR.

The stimulating effects of PBM against hTERT-BJ5ta fibroblasts were revealed. The PBM of these cells an hour after irradiation with IR led to a statistically significant increase in the number of viable cells compared to the control at low fluence values: from 0.003 to 0.3 J/cm^2. At the same time, the PBM of cells with higher fluences did not lead to cell stimulation.

The obtained data indicate that the effects of PBM can vary depending on the modes: the energy density of the LILR, the dose of IR and the sequence of exposure to these physical factors on cells. This suggests that in order to protect normal tissues from IR or to correct the undesirable consequences of IR in clinical practice, the PBM regimens (parameters and sequence of exposure) must be selected individually, depending on the dose of IR and the tasks set.

Acknowledgements

The work was carried out with the financial support of the Russian Foundation for Basic Research of the RFBR, project No. 20-02-00531 “Studying the effects of photobiomodulation in relation to tumor cells, normal tissue cells and stem cells exposed to ionizing radiation” and project No. 19-34-60034 “Monitoring of the molecular-genetic characteristics of circulating tumor cells in patients with metastatic lung cancer using innovative microfluidic system”, as well as with the partial support by the Ministry of Science and Higher Education within the State assignment FSRC “Crystallography and Photonics” RAS in part of creation and calibration of LED devices.

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