Factors Determining the Increased Sensitivity of Cancer Cells to the Action of Optical Radiation in Blue Spectral Region

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Abstract. The majority of studies state that inactivating effect of blue light is more pronounced in relation to cancer cells. We show, for the first time, that one of the reasons for increased sensitivity of cancer cells to blue light is higher concentrations of endogenous porphyrin sensitizers in them, which is confirmed by fluorescence methods. This leads to higher levels of light-induced production of reactive oxygen species in cancer cells, detected by chemiluminescence, and higher rate of light-induced decrease in metabolic activity of cancer cells compared to normal cells. The decisive role of endogenous porphyrins is evidenced by higher rate of photoinactivation of cells and more intense chemiluminescent signal when cells are exposed to laser radiation with wavelength of λ = 405 nm, corresponding to the maximum of absorption spectrum of porphyrins, compared with λ = 445 nm, corresponding to the maximum of absorption spectrum of flavins.

Keywords: cancer cells; 405 nm; 445 nm; endogenous porphyrins; flavins; reactive oxygen species.

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1 Introduction

Currently, the ability of low-intensity (0.5–100 mW/cm^2 irradiance) optical radiation in blue spectral region (λ = 400–485 nm) to influence the functional characteristics of cancer cells *in vitro* is beyond doubt [1–3]. There are numerous reports on the inhibitory effect of blue light in the literature on colon cancer cells [4–11], melanoma [2, 12–22], carcinoma [18, 23–33], sarcoma [3, 5, 34–37], lymphoid cells [38–41], leukemia cells [42–46], bladder cancer cells [47, 48], pancreatic [49] and mammary [30, 50, 51] gland cancer, hepatoma (liver cancer) cells [52, 53], gliomas [54], glioblastomas [55, 56], neuroblastomas [30, 57], stem cells of tumor tissue [9, 58], prostate cancer cells [59], lung cancer [60–62], cancer-associated fibroblasts [10] and others.

An analysis of previously performed studies allows us to conclude that exposure of cancer cells *in vitro* to blue light in the energy dose range of 1–250 J/cm^2 leads to a dose-dependent decrease in their survival, suppression of proliferation, and a decrease in the ability to migrate and invade. Exposure to blue light causes apoptosis [4, 7, 8, 14, 19, 22, 23, 27, 30, 31, 36–39, 41, 43–45, 49, 52–54, 56], necrosis [16, 30, 56, 57], autophagy [4, 6, 10, 21, 35, 38, 41] and disruption (arrest) of the cell cycle [4, 19, 22, 27, 43, 47, 49, 54, 57]. The occurrence of these processes in cells is facilitated by increased production (under blue light) of reactive oxygen species (ROS) [7, 8, 16, 19–21, 23, 28, 30, 35, 36, 38, 41, 60] and DNA damage at relatively high energy doses [7, 20, 22, 31, 38, 41, 52, 63].

It is characteristic that in studies in which, *in vitro*, the effect of light of the same spectral and energy parameters on cancer and non-transformed cells was compared, the presence of pronounced differences in their response to the influence of the specified physical factor was noted [4, 8, 23, 28, 31, 33, 36, 39, 46, 48, 49, 57, 59, 60]. These differences are manifested in a higher degree of

photoinhibition of metabolic activity and cell proliferation [4, 8, 23, 31, 33, 39, 46, 48, 49, 57, 59, 60], as well as higher levels of light-induced ROS generation [23, 28, 60, 33, 39, 48]. Moreover, the difference in the reactions of cancer and non-transformed cells to light exposure is observed in almost the entire spectral range of blue light: 450 nm [31, 48], 456 nm [39], 460 nm [49], 465 nm [4, 8, 36], 460–470 nm [57], 470 nm [46], 473 nm [60], 485 nm [59], 400–500 nm [23, 28, 33], 450–500 nm [33] as when exposed to laser [33, 48, 60], and LED [4, 8, 31, 36, 39, 46 49, 57] and lamp (broadband) [23, 28, 33, 59] sources.

However, the reasons for the increased sensitivity of cancer cells to the action of blue light remain unclear. In our opinion, a complicating factor in resolving this issue is the lack of complete understanding of the mechanisms of photophysical and photochemical processes that determine the effects of photobiomodulation initiated by exposure to blue light. The question of the primary acceptor molecules responsible for the regulatory action of this physical factor remains the least studied and most debated. At the same time, the opinion has been repeatedly expressed about the multiplicity of molecular targets capable of absorbing light quanta in the blue spectral region and mediating the reaction of animal cells to such exposure [1, 64–66]. Traditionally, the respiratory chain enzyme (electron transport chain) cytochrome c oxidase (CCO), as well as flavoproteins ("cryptochromes") and nitrosated proteins are considered as main targets of blue light [1, 64–66]. In addition, evidence has been obtained of the important role of light-sensitive ion channels in the photoregulation of the metabolic activity of cells, the best known of which are channelrhodopsins, which absorb light in blue spectral region [1, 6, 54, 65–70]. Another family of broad group of light-sensitive cation channels are transient receptor potential channels (TRP) [1, 65, 71], which are found in most organisms, tissues, and cell types.

At the same time, animal cells also contain other chromophores that are potentially capable of acting as molecules-acceptors of optical radiation in various regions of the visible spectrum and initiating a change in the redox state of cells due to the generation of reactive oxygen species [72–81]. Thus, a number of studies argue for the participation of endogenous flavins [29, 78, 79] and lipofuscin [80, 81] in the effects of photobiomodulation in cells under *in vitro*. Our laboratory has obtained evidence of the participation of endogenous porphyrin photosensitizers in the implementation of the regulatory effect of light in various types of cells: microorganisms [73], sperm [74, 75], human blood cells [76], somatic cells [29, 77].

The purpose of this work is to substantiate the important role of endogenous porphyrins in the sensitization of photobiological processes in cancer and normal cells when their suspension is exposed to blue light and to clarify the role of these tetrapyrroles in the manifestation of increased sensitivity of cancer cells exposed to blue light.

2 Materials and Methods

2.1 Cell Culture

Human cervical epithelioid carcinoma cells (HeLa) as well as non-transformed (non-tumor) green monkey kidney cells (BGM) were selected as objects of study. The cells were obtained from Republican Scientific and Practical Center for Epidemiology and Microbiology of the Ministry of Health of the Republic of Belarus (Minsk) in the form of a suspension in the Dulbecco's Modified Eagle's Medium (DMEM) with 5% fetal serum. Cell monolayers were grown in disposable Petri dishes with a diameter of 35 mm on the same nutrient medium at 37 °C and 5% CO_2 in an incubator. An amount of 3 ml of growth medium with cells was added to each dish. The seed cell concentration was 130,000 ml^{-1}. After a period of 48 h following seeding, cell monolayers were exposed to blue light.

2.2 Effect of Optical Radiation on Cell Cultures

Cells in culture were exposed to blue light using semiconductor GaN lasers with radiation wavelengths of λ = 405 nm and λ = 445 nm. According to Ref. [82], the emission spectrum of semiconductor lasers of this type is formed by narrow lines with a total spectral width of ~1.5 nm. The lasers operated in continuous-wave mode. To obtain a more homogeneous light spot on the surface of cell monolayers, laser radiation was introduced into a monofilament quartz-polymer light guide with a light-conducting core diameter of 125 μm. Petri dishes were irradiated from below. Light was defocused onto the entire surface of the bottom of Petri dish. The irradiance at the level of the surface of Petri dish bottom was varied in the range of I = 5–100 mW/cm^2 and was controlled using a power meter PM100D with an S121C photodiode sensor (Thorlabs GmbH, Germany). Irradiation time was varied in the range of 1–10 min. After irradiation, the cells were placed for 24 h in a CO_2 incubator, which maintained the temperature at 37 °C and 5% CO_2 content. Similar manipulations (except for irradiation) were performed with control cell monolayers.

2.3 Influence of Light on Metabolic Activity of Cells

The biological effect of optical radiation was assessed photocolorimetrically using MTT test. This test is based on the ability of dehydrogenases in living metabolically active cells to convert the pale yellow water-soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into blue formazan crystals, which are insoluble in water [83]. Non-viable dead cells do not have this ability. MTT produced by Applichem (Germany) was used in the studies. The MTT solution was prepared according to the standard procedure: 5 mg of MTT was dissolved in 1 ml of H_2O (concentrated solution). After 24 h of cell incubation after irradiation, the growth

medium was spilled out and a new one without serum, containing 50 μl of concentrated MTT per 1 ml of medium, was added. The cells were incubated with new medium for 3 h at 37 °C and 5% CO_2. After 3 h of incubation, the supernatant was removed and the cell monolayer was left in Petri dishes overnight to dry. Then, 1 ml of dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to each Petri dish and incubated for 30 min at room temperature. Analysis of the metabolic activity of cells was carried out by monitoring the absorbance of extract at a wavelength $\lambda = 570$ nm. A quantitative measure of the metabolic activity of cells after exposure to light in comparison with control samples was the value $\gamma = (A_{ir}/A_c)\cdot 100\%$, where A_{ir} and A_c are the absorbance of formazan solution from experimental (irradiated) and control Petri dishes, respectively.

2.4 Chemiluminescence Assay for Monitoring the Formation of Reactive Oxygen Species upon Exposure to Optical Radiation

To determine the contribution of reactive oxygen species to the photobiological effects initiated by exposure of cells to blue light, chemiluminescence assay was used. As is known [84, 85], the chemiluminescence is quite sensitive, since it allows simultaneous (total) detection of various reactive oxygen species (including superoxide anion radical O_2•-, hydrogen peroxide H_2O_2 and hydroxyl radical OH•), localized both in the intracellular and in extracellular space. Chemiluminescence parameters were measured using a Lum 5773 chemiluminometer (DISoft, Russia), operating in photon counting mode with a spectral sensitivity range of 300−650 nm. Registration and processing of chemiluminescence signals was carried out using specialized software "Power Graph 3.3 Professional".

To study the effect of radiation on cell chemiluminescence, a cell suspension with a concentration of $2\cdot 10^6$ ml^{-1} was used. An amount of 220 μl of suspension was irradiated in glass cylindrical cuvettes with an internal diameter of 10 mm and a flat bottom. The suspension was irradiated through the bottom of the cuvette by lasers with a wavelength of 405 or 445 nm. The irradiation time was $t = 3$ min, irradiance at the surface level of the cuvette bottom was $I = 50$mW/cm^2. After cessation of irradiation, the suspension was immediately transferred to a chemiluminometer cuvette. The time from cessation of irradiation to the start of recording the chemiluminescence signal was strictly controlled and was 24 s. Similar manipulations (except for irradiation) were carried out with control samples. Measurements of chemiluminescence of control (non-irradiated) and experimental samples of cell suspensions was alternated in a random order. The luminometer measured chemiluminescence (in relative units) at 22 °C for 5 min after the start of recording.

To determine the type of reactive oxygen species, involved in photochemical processes, initiated by exposure to blue light, 10 μl of one of the specific quenchers (scavengers) of reactive oxygen species was added to the cell suspension 10 min before irradiation: sodium azide – a quencher of singlet oxygen, sodium pyruvate – a hydrogen peroxide scavenger or D-mannitol – a hydroxyl radical scavenger. The concentration of stock solutions of the indicated quenchers (scavengers) was prepared in such a way that after adding 10 μl of one of them to 220 μl of a cell suspension, the final concentration were as follows: 10 mM for sodium azide and sodium pyruvate, 40 mM for D-mannitol. To account for dilution, 10 μl of saline solution was added to samples of cell suspensions that did not contain ROS quenchers (scavengers).

2.5 Registration of Fluorescence of Endogenous Porphyrins in Cell Extracts

As is known, the fluorescence of endogenous porphyrins in a suspension of somatic cells is very difficult to detect due to the predominant luminescence of flavins and flavoproteins, which is due to their higher concentration (exceeding the concentration of porphyrins by about 2−3 orders of magnitude) and a higher (5−10 times) fluorescence quantum yield [29, 77]. More reliably, the fluorescence of free-base porphyrins and their zinc complexes is recorded after the extraction of tetrapyrroles from cells using 3 M hydrochloric acid. This is due to the good solubility of porphyrins in hydrochloric acid and the multidirectional effect of hydrochloric acid on the fluorescence intensity of flavins (the fluorescence of flavin mononucleotide after treatment with 3 M HCl is quenched by more than 60 times) and porphyrins (the fluorescence of protoporphyrin IX increases by more than 20 times compared to an aqueous solution) [29, 77]. A detailed description of methods for extracting porphyrin fluorophores from the studied cell cultures is presented in Refs. [29, 77]. We only note that, when performing comparative studies of porphyrin fluorescence of acidic extracts of HeLa and BGM cells, their initial concentration in saline solution was controlled either using a hemocytometer or a flow cytometer and adjusted by appropriate dilution with saline solution to the same value of $5\cdot 10^5$ cells/ml for each type cells. Moreover, all subsequent manipulations for the extraction of porphyrins from HeLa and BGM cells according to the method [29, 77] were of the same type.

Absorption spectra were measured on a Cary-500 ScanUV-Vis-NIR spectrophotometer (Varian, USA, Australia), fluorescence emission and fluorescence excitation spectra were measured on a Fluorolog-3 spectrofluorimeter (Horiba Jobin Yvon, Inc., France), using standard quartz cuvettes of 10×10×40 mm, as well as special cuvettes with an optical path length of 4×4 mm. The width of monochromator slits was 5 nm.

2.6 Statistical Analysis

Statistical analysis was performed using Statistical Analysis System software (SAS Enterprise Guide, version 7.1, SAS Inst. Inc.). Data were tested for normal

distribution (Shapiro-Wilk test) followed by pairwise comparisons using Student's *t*-test. All data are presented as mean ± standard error of the mean. A *p*-value < 0.05 was chosen to indicate statistical significance. The results presented were obtained from at least 3 independent experiments.

3 Results

3.1 Effect of Blue Light on Metabolic Activity of Cancer and Normal Cells

Studies have shown that the effect of laser radiation of blue spectral region with λ = 405 nm or 445 nm and irradiance of I = 25 mW/cm^2 in the energy range doses of D = 1–15 J/cm^2 on cells of various types (BGM, HeLa) is inhibition of their metabolic activity, controlled 24 h after cessation of irradiation. The results obtained are presented in Fig. 1(a, b).

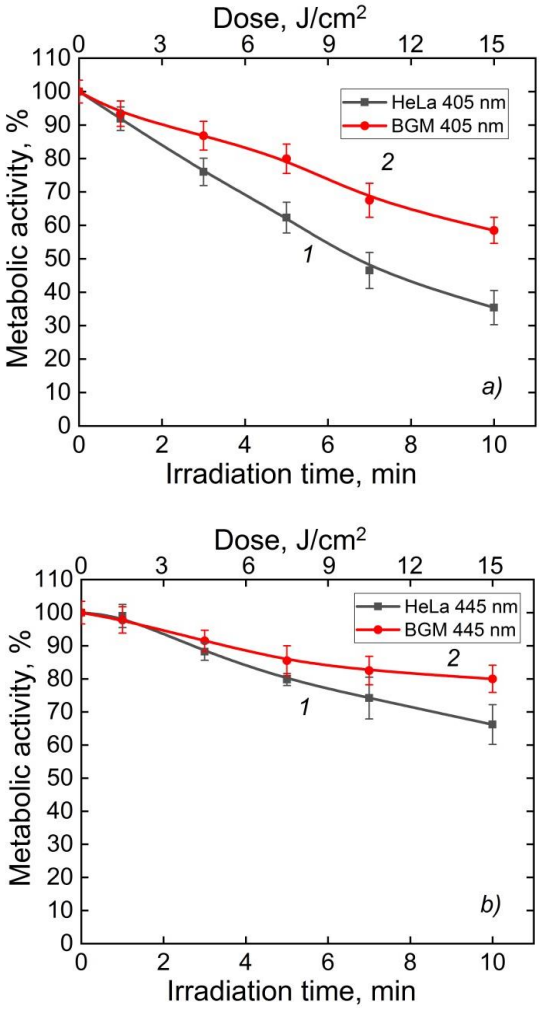

Fig. 1 Dose-dependent metabolic activity of (1) HeLa and (2) BGM cells as a percentage of the control 24 h after exposure to laser radiation with wavelength of λ = 405 nm (a), 445 nm (b) at irradiance of I = 25 mW/cm^2. The lower axis shows the time of irradiation of cell monolayer in minutes, the upper axis shows the dose of radiation in J/cm^2.

It follows from the Fig. 1 that as energy dose of light exposure increases, an increase in the degree of inhibition of the metabolic activity of both HeLa and BGM cells is observed. However, at the same energy dose, the inhibitory effect for radiation with wavelength of λ = 405 nm is significantly higher in relation to HeLa cells (Fig. 1(a), curve 1) than in relation to BGM cells (Fig. 1(a), curve 2). A higher sensitivity of cancer cells (Fig. 1(b), curve 1) compared to normal (Fig. 1(b), curve 2) is also registrated when exposed to radiation with wavelength of λ = 445 nm. It is also characteristic that after exposure to radiation λ = 405 nm, the rate of inhibition of metabolic activity of cells is higher than after exposure to radiation λ = 445 nm. This pattern appears for both cancer and normal cells.

3.2 Effect of Blue Light on Efficacy of Generation of Reactive Oxygen Species in Cancer and Normal Cells

As we have shown previously in experiments with various types of cells [29, 74, 75], important information about photochemical processes occurring in cells can be obtained using chemiluminescence assay, which makes it possible to collectively detect various reactive oxygen species (including superoxide anion radical O_2•-, hydrogen peroxide H_2O_2 and hydroxyl radical OH•), localized both in intracellular and extracellular spaces.

Chemiluminescence intensity kinetic curves for suspension of HeLa (A, B) and BGM (C, D) cells in saline when it is recorded for 5 min in the case of using control (non-irradiated) cell samples (A and C), as well as samples previously exposed to radiation (B, D) λ = 405 nm, irradiance I = 50 mW/cm^2, for t = 180 s (energy dose – D = 9.0 J/cm^2) are presented in Fig. 2. The concentration of HeLa and BGM cells was $2 \cdot 10^6$ ml^{-1}.

Analysis of the data presented in Fig. 2 shows the presence of significant differences in the course of kinetic curves of chemiluminescence intensity of irradiated and non-irradiated cell suspensions. These differences are manifested primarily in the fact that the kinetic curves of changes in chemiluminescence intensity for irradiated cell suspensions (B, D) are descending curves, while the average chemiluminescence intensity for control (intact) cell suspensions (A and C) remains unchanged throughout the entire signal recording period. In addition, the amplitude of signal fluctuations relative to the average curve approximating the kinetics of signal changes is significantly higher for irradiated cells.

However, the main conclusion that follows from the kinetic curves of chemiluminescence intensity of irradiated samples of cell suspensions is that, at the same concentration in the suspension of HeLa (B) and BGM cells (D), the integrated signal intensity (chemiluminescence light sum) is reliably higher in case of preliminary exposure of cancer cells to light.

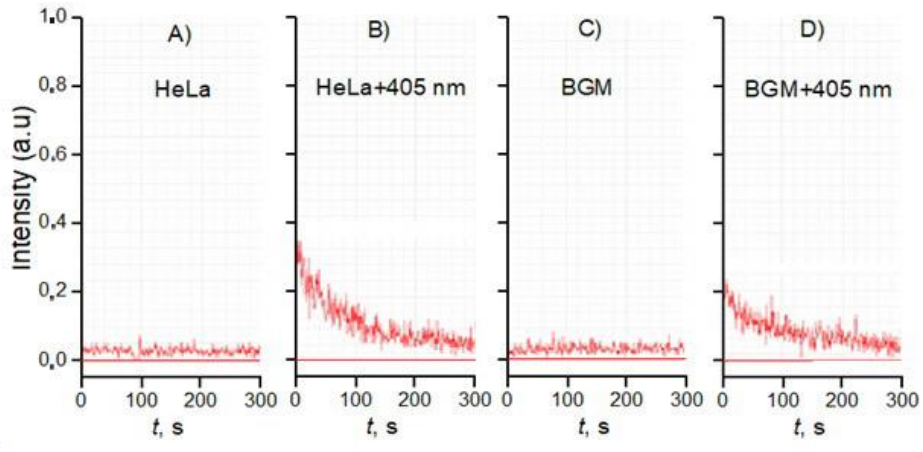

Fig. 2 Chemiluminescence intensity kinetic curves for suspension of (A, B) HeLa and (C, D) BGM cells with concentration of $2 \cdot 10^6$ ml^{-1} in saline solution: (A) and (C) control cell suspensions not exposed to optical radiation; (B) and (D) cell suspensions after exposure to laser radiation with wavelength of $\lambda = 405$ nm, irradiance I = 50 mW/cm^2, for t = 180 s (dose D = 9.0 J/cm^2).

Since the presence of chemiluminescence signal as a result of exposure to light is explained by the occurrence of photochemical reactions involving various types of ROS, the results obtained indicate higher levels of light-induced ROS formation in HeLa cells compared to normal BGM cells.

3.3 Chemiluminescence Assay to Study the Mechanism of Light-Induced Damage of HeLa Cells

Using chemiluminescence assay, it was possible to show that the main type of reactive oxygen species that initiates damage to cancer cells when exposed to blue light is singlet oxygen. This conclusion follows from the results of studying the effect of specific ROS quenchers (scavengers) (sodium azide − a quencher of singlet oxygen, sodium pyruvate − a scavenger of hydrogen peroxide, D-mannitol − a scavenger of hydroxyl radicals), which were added to the cell suspension 10 min before its irradiation, on the chemiluminescence light sum of cell suspension. It is important to note that all the quenchers (scavengers) of reactive oxygen species used in this work do not absorb at wavelengths of the acting radiation $\lambda = 405$ and 445 nm. Therefore, the change in the magnitude of the light-induced effect in cells cannot be associated with screening of the acting radiation by quenchers (scavengers). The results obtained are presented in Fig. 3, in which the integral, the area (S) under the chemiluminescence curve from the beginning of recording ($t = 0$ s) to $t = 300$ s, is taken as a parameter characterizing the integral intensity of chemiluminescence (see Fig. 2).

As follows from the Fig. 3, for all non-irradiated samples of cell suspensions, not containing (Contr) and containing (NaN_3, SP, Man) quenchers of ROS, the integral intensity of chemiluminescence is recorded at approximately the same low level ($S_{contr} \rightarrow S_{NaN3} \rightarrow S_{SP} \rightarrow S_{Man}$). Consequently, the addition of ROS quenchers (scavengers) has no effect on the spontaneous (not initiated by light) chemiluminescence of HeLa cell suspension. However, after 180 s exposure of cell suspension, not containing quenchers, to laser radiation with wavelength of $\lambda = 405$ nm, $I = 50$ mW/cm^2, $D = 9.0$ J/cm^2 (Ir), the integral signal intensity increases significantly, reaching the value $S_{Ir} = 1.0$. The addition of singlet oxygen quencher, sodium azide, to the cell suspension before irradiation followed by exposure to blue light of the same parameters (NaN_3 + Ir) leads to a significant increase in the signal compared to the non-irradiated control, containing the specified quencher (NaN_3).

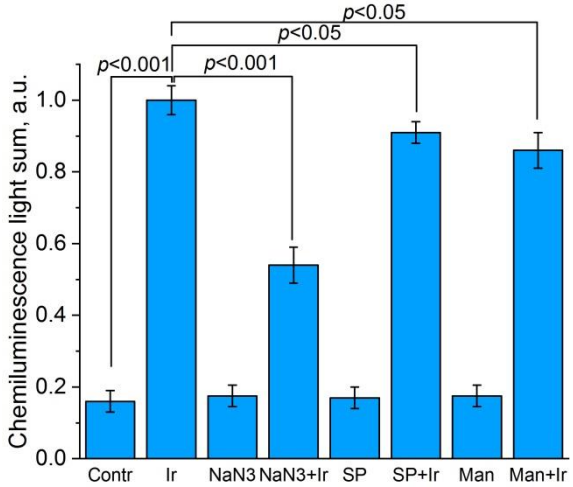

Fig. 3 Integral chemiluminescence of HeLa cell suspension without irradiation and without external additives (Contr); without irradiation in the presence of 10 mM sodium azide (NaN_3), 10 mM sodium pyruvate (SP), 40 mM D-mannitol (Man); irradiated suspension in the absence of external additives (Ir), in the presence of 10 mM sodium azide (NaN_3 + Ir), 10 mM sodium pyruvate (SP + Ir) or 40 mM D-mannitol (Man + Ir). Parameters of optical radiation: $\lambda = 405$ nm, $I = 50$ mW/cm^2, duration $t = 180$ s, $D = 9.0$ J/cm^2.

However, compared to the irradiation without additives (Ir), the integral intensity of chemiluminescence of the cell suspension, irradiated in the presence of NaN_3 (NaN_3 + Ir) is lower by 46%, $S_{NaN3+Ir} = 0.54$. A decrease in the magnitude of the photobiological effect in the presence of singlet oxygen quencher indicates the participation of this form of oxygen in photochemical processes in HeLa cells.

Unlike sodium azide, the addition of hydrogen peroxide scavenger, sodium pyruvate, followed by exposure to blue light has a significantly less effect on the integral intensity of the chemiluminescent signal ($S_{SP+Ir} = 0.91$) compared to the irradiation in the absence of quenchers ($S_{Ir} = 1.0$). Moreover, despite the slight differences in the indicated variants for irradiation of cell suspension, the participation of hydrogen peroxide in the photochemical processes initiated by blue light in cells is reliably recorded ($p < 0.05$).

As for the effect of hydroxyl radical scavenger – D-mannitol, as it follows from Fig. 3 that its addition to the cell suspension followed by exposure to light (Man + Ir) also leads to the registration of chemiluminescent signal that practically corresponds to the variant of cell irradiation without additives (Ir): $S_{Man+Ir} = 0.86$. Therefore, it can be assumed that when monitoring the participation of ROS in photodamage to HeLa cells immediately after the cessation of light exposure, hydroxyl radicals also play a certain role in the photochemical processes under study.

Thus, we can conclude that chemiluminescence initiated by exposure of cell suspension to blue light is mainly caused by generation of singlet oxygen in cells due to the excitation of endogenous photosensitizers and, to a lesser extent, due to processes involving hydrogen peroxide and hydroxyl radicals.

3.4 Determination of Type of Molecules Responsible for Formation of Chemiluminescence Signal upon Exposure of Cell Suspension to Laser Radiation with Wavelengths of λ = 405 nm and λ = 445 nm

The next stage of our research was aimed at elucidating the type of molecules responsible for formation of chemiluminescence signal induced by exposure of cell suspension to blue light with wavelengths of λ = 405 nm and λ = 445 nm. As already mentioned, there is reason to believe [29, 77] that this signal may be due to the participation of ROS in photochemical reactions in cells formed as a result of the excitation of free-base porphyrins (protoporphyrin IX, coproporphyrin III, uroporphyrin III) and their zinc complexes by blue light, as well as flavin molecules (riboflavin, RF, flavin mononucleotide, FMN, flavin adenine dinucleotide, FAD). As is known [86–88], these compounds in monomeric form are characterized by high efficacy of singlet oxygen generation and are also capable (albeit with much less efficacy) of generating other types of ROS [89]. The absorption spectra of one of the flavins (FMN), protoporphyrin IX as well as Zn-protoporphyrin IX in monomeric form in DMSO are shown in Fig. 4. Note that the absorption spectra of RF, FMN and FAD are practically the same. For this reason, the presented data allow us to draw a conclusion about relationship between maxima in absorption spectra of above sensitizers of flavin and porphyrin nature.

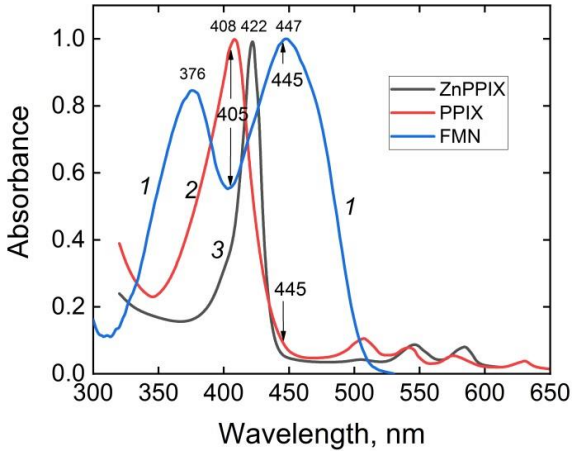

Fig. 4 Absorption spectra of endogenous photosensitizers: FMN in (1) buffer, (2) protoporphyrin IX and (3) Zn-protoporphyrin IX in DMSO. Arrows are wavelengths of radiation acting on cells

From Fig. 4 it follows that the absorption spectra of flavins in aqueous environment are characterized by two maxima: short-wavelength maximum in the region of 376 nm and long-wavelength maximum in the region of 447 nm, as well as a local minimum in the region of 405 nm. Protoporphyrin IX is characterized by the presence of an intense absorption band (Soret band, $S_2 \leftarrow S_0$ – transition) in the region of $\lambda_{max} = 408$ nm (B(0,0)) and four significantly weaker Q-bands ($S_1 \leftarrow S_0$ – transition) in the green, yellow and red spectral regions with maxima at $\lambda_{max} = 507$ nm ($Q_y(1,0)$), $\lambda_{max} = 543$ nm ($Q_y(0,0)$), $\lambda_{max} = 575$ nm ($Q_x(1,0)$), $\lambda_{max} = 630$ nm ($Q_x(0,0)$). In other words, laser radiation with wavelength of λ = 405 nm corresponds to the maximum of absorption band of protoporphyrin IX and to the region of the local minimum of absorption spectrum of flavins. At the same time, laser radiation with wavelength of λ = 445 nm corresponds to the maximum absorption of flavins and to the region of local minimum of the absorption spectrum of protoporphyrin IX. Moreover, the absorption coefficient of porphyrin in the region of 445 nm is approximately 20 times lower than its value at 405 nm.

The absorption spectrum of Zn-protopophyrin IX in DMSO is characterized by the presence of intense Soret band ($S_2 \leftarrow S_0$ – transition) with maximum at $\lambda_{max} = 422$ nm (B(0,0)) and two weaker Q-bands ($S_1 \leftarrow S_0$ – transition) with maxima at $\lambda_{max} = 547$ nm (Q(1,0)), $\lambda_{max} = 584$ nm (Q(0,0)). From Fig. 4 it follows that Q-absorption bands of Zn-protopophyrin IX are located outside the absorption spectrum of flavins, and the Soret band falls on the short-wavelength slope of their long-wavelength absorption band.

Naturally, the contribution of one or another endogenous acceptor to photosensitized damage of cellular structures depends on a number of factors: (a) the ratio of absorption coefficients of acceptors at the wavelength of the influencing radiation; (b) ratio of acceptor concentrations; (c) their photosensitizing properties (for example, the efficacy of ROS generation); (d) the presence near the sensitizer of biologically important molecules capable of interacting with ROS or triplet-excited sensitizer (in photochemical processes by a radical mechanism - type I mechanism); (e) the presence in the immediate vicinity of the sensitizer of ROS quenchers (scavengers) or quenchers of the triplet-excited state of the photosensitizer.

From the data presented in Fig. 4, it is clear that in the blue spectral region, in the case of a noticeable role of porphyrins in the effects of sensitization, one should expect that their contribution will be most pronounced in the region of the local minimum of absorption band of flavins (λ_{min} = 405 nm), since in this region one of the porphyrin photosensitizers (protoporphyrin IX) is characterized by an intense band with a maximum at λ_{max} = 408 nm. In this regard, it was of interest to compare the efficacy of ROS formation when exposed to laser radiation with wavelength of λ = 405 nm, corresponding to the long-wave absorption maximum of protoporphyrin IX, and radiation with a wavelength of λ = 445 nm, corresponding to the maximum in the absorption spectrum of flavins.

Results of comparative studies of chemiluminescence light sum (in relative units) induced by exposure of suspension of HeLa cells to laser radiation with wavelength of λ = 405 nm and λ = 445 nm for t = 180 s, irradiance I = 50 mW/cm^2 (D = 9 J/cm^2 energy dose) are presented in Fig. 5.

It follows from the Fig. 5 that the value of the light sum of chemiluminescence induced by exposure to radiation with λ = 405 nm significantly exceeds the corresponding indicator when exposed to radiation with λ = 445 nm. This confirms our assumption about the ability of endogenous porphyrins to contribute to photochemical processes in cells when exposed to blue light.

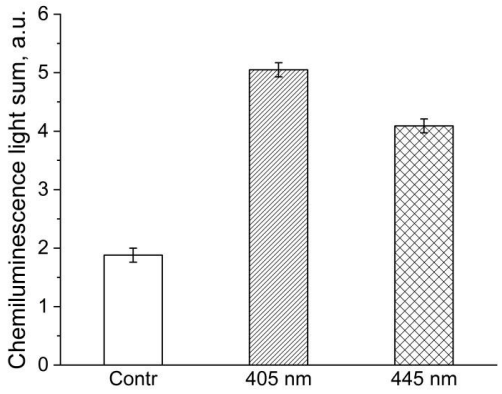

Fig. 5 Chemiluminescence light sum (a.u.) upon exposure of HeLa cell suspension to laser radiation λ = 405 nm and λ = 445 nm, irradiance I = 50 mW/cm^2 (dose D = 9 J/cm^2) during t = 180 s.

Thus, the results obtained indicate that significant contribution to the processes affecting the metabolic activity of cells when exposed to laser radiation with λ = 405 nm (Fig. 1) can be made by endogenous porphyrins due to the sensitized formation of ROS, and, above all, singlet oxygen (Fig. 3). At the same time, the levels of ROS formation, controlled by chemiluminescence, are higher when HeLa cells are exposed to blue light compared to non-transformed BGM cells (Fig. 2), which are also characterized by lower photosensitivity to the action of this radiation compared to HeLa cells (Fig. 1).

3.5 Assessment of Relative Concentration of Endogenous Porphyrins in Cancer and Normal Cells Using Fluorescence Spectroscopy

To clarify the reasons for the increased photosensitivity of cancer cells to blue light, we performed comparative assessments of the relative concentrations of endogenous porphyrins in cancer and normal cells using fluorescent analysis. As already noted, due to the low concentration of endogenous porphyrins in cells (at the level of ~1 nM), the low quantum yield of their fluorescence (φ_{fl} = 0.01–0.08 [77]), high concentration of flavins in cells (0.3–1 μM) [90, 91], higher quantum yield of their fluorescence (φ_{fl} = 0.08–0.26 [77]), as well as due to the overlap of absorption spectra of porphyrin and flavin photosensitizers, detection of porphyrin fluorescence in living cells is associated with significant difficulties. Fluorescence of endogenous porphyrins is more reliably recorded in acidic cell extracts [29, 77]. As was shown earlier [77], treatment of protoporphyrin IX with 3 M hydrochloric acid leads to an increase in the quantum yield of its fluorescence compared to aqueous solutions by approximately 20 times, while acid treatment of FMN is accompanied by a decrease in the quantum yield of its fluorescence by approximately 60 times. Naturally, such a multidirectional effect of HCl on the fluorescent characteristics of porphyrins and flavins improves the possibility of recording the luminescence of endogenous porphyrins in acidic cell extracts with an appropriate choice of the fluorescence excitation wavelength. The reasons for the multidirectional influence of hydrochloric acid on the spectral and fluorescent characteristics of flavins and free-base porphyrins are analyzed in detail in Ref. [77].

As an example, in Fig. 6, fluorescence emission spectra (curves 1 and 2) at excitation wavelength of λ_{ex} = 405 nm and fluorescence excitation spectra (curves 4 and 5) at registration wavelength of λ_{em} = 656 nm of acidic extracts of HeLa (curves 1 and 4) and BGM cells (curves 2 and 5) are shown. The fluorescence emission spectra (curve 3) and fluorescence excitation spectra (curve 6) of the extractant (3 M hydrochloric acid) are also shown.

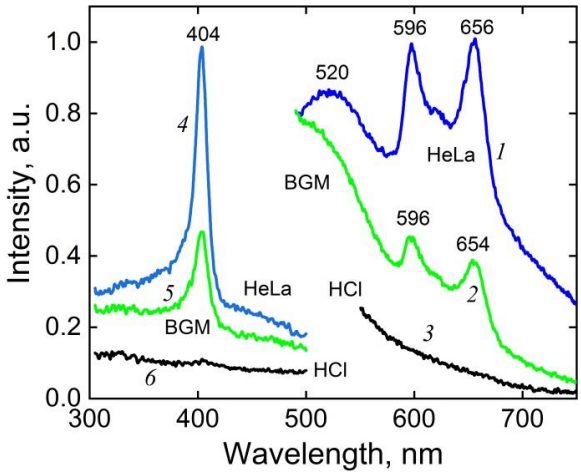

Fig. 6 Fluorescence emission (1–3) and fluorescence excitation (4–6) spectra of HeLa (1, 4), BGM (2, 5) cell extracts and extractant – 3 M hydrochloric acid (3, 6) at excitation wavelength of λ_{ex} = 405 nm (1–3) and registration wavelength of λ_{em} = 656 nm (4–6). Cell concentration for extraction was $5 \cdot 10^5$ ml^{-1}.

From the data presented it is clear that when fluorescence is excited by radiation with λ_{ex} = 405 nm, the emission spectrum of acidic cell extracts is characterized by a band with a pronounced maximum in the region of λ_{max} = 520 nm, caused by the fluorescence of flavin compounds present in acidic extracts and, above all, riboflavin [77]. On the long-wavelength slope of the flavin fluorescence band, a double-humped band with maxima at λ_{max} = 596 and 656 nm, characteristic for fluorescence of porphyrin compounds, is clearly detected. As was shown earlier [77], for extracts of HeLa cells in 3M HCl, the indicated fluorescence is closest to the fluorescence of chemically pure protoporphyrin IX in the indicated solvent.

In fluorescence excitation spectrum of cell extracts when using 3M HCl as an extractant and registration wavelength λ_{em} = 656 nm, a narrow band with a maximum at λ_{max} = 404 nm, characteristic of chemically pure protoporphyrin IX in hydrochloric acid, is clearly recorded [77].

As expected, there are no extreme points when recording the fluorescence emission spectra (curve 3) and fluorescence excitation spectra (curve 6) of a chemically pure extractant – 3M HCl (Fig. 6).

A comparison of fluorescence emission and fluorescence excitation spectra of HeLa (curves 1 and 4) and BGM (curves 2 and 5) cell extracts shows that they are qualitatively very similar. However, the intensity of the porphyrin fluorescence signal for HeLa cells is approximately 2.5 times higher than for normal BGM cells. The same quantitative pattern can be traced when analyzing the fluorescence excitation spectra of these types of cells. Since the fluorescence intensity is directly proportional to the concentration of fluorescent molecules, it can be concluded that the concentration of endogenous porphyrins in HeLa cells is approximately 2.5 times higher than in normal, non-transformed BGM cells. Taking into account the fundamental nature of the conclusion about the higher level of endogenous porphyrin concentrations in cancer cells, comparative studies of the intensity of porphyrin fluorescence for cancer and non-cancerous cells treated with hydrochloric acid were carried out in 5 pairs of independent experiments, which confirmed the above. Moreover, this conclusion was also confirmed by extracting endogenous porphyrins from cells using acetone, which, according to Ref. [77], allows identifying porphyrins in cancer cells.

4 Discussion

As already noted, in the overwhelming number of experimental studies that compared the effect of blue light from laser, LED or lamp sources on cancer and normal cells, it was found that at equal spectral and energy parameters of specified physical factor, a higher degree of photoinhibition of metabolic activity of cancer cells is noted [4, 8, 23, 31, 36, 39, 46, 48, 49, 57, 59, 60], as well as higher levels of light-induced ROS production [23, 28, 60].

The results of comparative studies of the effect of blue light on cancer and normal cells available in the literature are summarized in Table 1.

Table 1 Influence of blue light on rate of cell proliferation, ROS level and apoptosis of cancer and normal cells.

Type of cancer cells	Type of normal cells	Source of light, wave-length	Irradiance, mW/cm^2, Fluence, J/cm^2	Rate of cell proliferation as percentage to control, %			Light-induced ROS formation (a.u.)		Apoptosis, %		Ref.
				Cancer cells		Normal cells	Cancer cells	Normal cells	Cancer cells	Normal cells	
HT29 and HCT116 human colon cancer cells	CSC-2FO human fibroblast cells	LED, 465 nm	30 mW, 10 min/day during 5 days	HT29 24.8	HCT116 37.1	CSC-2FO ~100					[4]
HCT116 and HT29 colorectal cancer cells	MRC-5 human embryonic lung fibroblast	LED, 465 nm	3×10^4 lux, 2 h	HT29 ~50	HCT116 ~50	MRC-5 ~100					[8]
OSC2 gingival (oral) squamous carcinoma cells	NHEK normal human pooled primary epidermal keratinocytes	QTH#, 380–50 0 nm	5 J/cm^2	OSC2 90	– –	NHEK 130	OSC2 –	NHEK –	OSC2 detected	NHEK Not detected	[23]
			15 J/cm^2		–		125	100	–	–	
			60 J/cm^2	20	–	120	–	–	–	–	

Table 1 Cont.

OSC2 gingival (oral) squamous carcinoma cells	NHEK normal human epidermal keratinocytes	QTH#, 380−500 nm	550 mW/cm^2 15 J/cm^2 30 J/cm^2				OSC2 12 14	28 3 3			[28]
HeLa human cervical cancer cells	hDF human dermal fibroblasts	LED, 450 nm	25700 cd/m^2, 30 min after 24 h	HeLa 62		hDF 110					[31]
SYO-1, HS-SY-II, Saka-SS, Yamato-SS synovial sarcoma cells	HEK293 human embryonic kidney cell line	LED, 465 nm	48 h 0.6 mW/cm^2 48 h 0.6 mW/cm^2	SYO-1 45 Saka-SS 33	HS-SY-II 70 Yamato-SS 58	HEK293 80 – –					[36]
U937 tumor cells with diffuse histiocytic lymphoma	PBMC peripheral blood mononuclear cells	LED, 456 nm	0.25 mW/cm^2 2 h	U937 38	–	PBMC 90					[39]
PaCa-2, PANC-1, BxPC-3 human pancreatic cancer cells	H6c7 human pancreatic duct epithelial cell line; HUVEC human umbilical vein endothelial cells	LED, 460 nm	5 h/day for 5 days 5 mW/cm^2 10 mW/cm^2 5 h/day for 5 days 5 mW/cm^2 10 mW/cm^2	PaCa-2 52 30 – – –	PANC-1 41 42 BxPC-3 54 55	H6c7 83 78 HUVEC – 80					[49]
SRSY5Y human neuroblastoma cancer	HDF human dermal fibroblasts	LED, 460–470 nm	10,540 lux 6 h irr. + 12 h dark+6 h irr. after 24 h after 48 h after 72 h	– –	SRSY5Y 83 91 91	HDF 83 96 97		SRSY5Y 2.70	HDF 26.60		[57]
PC-3 prostate cancer cells, A2780 ovarian carcinoma cells, G361 malignant melanoma cells	PNT1A prostatic epithelial tissue cells	plasma light source, 485 nm, bandwidth 25 nm	208 mJ/cm^2 208 mW/cm^2·1s	PC-3 $k^{\#}$ = –0.53 G361 $k^{\#} = 0^{\#}$	A2780 $k^{\#}$ = –2.12	PNT1A $k^{\#}$ = 3.38					[59]
A549 human lung carcinoma cells	MRC-5 human lung fibroblast	diode-pumped solid state laser, 473 nm	750 mW/cm^2 5 min				A549 44	MRC-5 15			[60]
erythroblastic leukemic cells (EBL)	peripheral blood lymphocytes (PBL)	LED, 470 nm	5.7 mW/cm^2, 3 h Day 0 Day 4 Day 7	EBL 100 19.0 18.3		PBL 100 67.5 79.3					[46]
MCF-7 human epithelial breast carcinoma, OSC-2 human oral squamous cell carcinoma keratinocytes	NHEK normal human foreskin keratinocytes, WI-38 human lung fibroblasts, Balb/c 3T3 mouse lung fibroblasts, HGF human gingival fibroblasts	Ar-laser 400−500 nm	202 mW/cm^2 60 J/cm^2	MCF-7 86	OSC-2 92	NHEK 130 Balb/c 3T3 5 WI-38 145 HGF 150					[33]

Table 1 Cont.

			MCF-7	OSC-2	NHEK	
			79	14	128	
					Balb/c 3T3	
		PAC#, 400−500 nm	1690 mW/cm^2 60 J/cm^2		0	
					WI-38	
					87	
					HGF	
					83	
			MCF-7	OSC-2	NHEK	
			18	5	104	
					Balb/c 3T3	
		QTH#, 400−500 nm	556 mW/cm^2, 5 J/cm^2		0	
					WI-38	
					122	
					HGF	
					82	
T24 and EJ human bladder cancer cells	SV-HUC-1 human uroepithelial cells	diode laser, 450 nm	100 mW/cm^2 20–240 s 2, 4, 8, 12, 16, 20, 24 J/cm^2	T24	EJ	SV-HUC-1	[48]
			IC_{50}# after 24 h, J/cm^2	9.8	14.6	20.0	
			IC_{50}# after 48 h, J/cm^2	8.0	8.9	17.9	

QTH# – dental quartz-tungsten-halogen lamp; 94% power of radiation in the 400−500 nm range [58];

PAC# – plasma arc curing lamp, 81 % power of radiation in the 400−500 nm range [58];

k# – the slope of the approximating line, reflecting the change in the number of cells during 24 h of the experiment (increase in value per hour);

IC_{50}# – half maximal inhibitory energy dose (an energy dose that reduces the proliferative activity of cells by half.

From the analysis of the literature data, it follows that most often comparison of the effect of blue light on cancer and normal cells was carried out by monitoring the light-induced change in metabolic activity (viability) of the cells [4, 8, 23, 31, 36, 48], their proliferation [4, 31, 39, 46, 49, 57, 59] and migration [36, 48, 57, 59, 60], as well as changes in cell cycle parameters [4, 49, 57].

Most of the studies presented in Table 1, and confirming the higher sensitivity of cancer cells to blue light, were performed using LED sources [4, 8, 29, 31, 36, 39, 46, 49, 57]. However, this conclusion is also true when cells are exposed to laser radiation, which follows from the data presented in the present work and in Refs. [33, 48, 60], as well as a quartz-tungsten-halogen lamp [23, 28, 33] and a plasma arc curing lamp [59]. Thus, the more pronounced effect of blue light on cancer cells compared to non-cancerous cells is not associated with the laser specificity of the characteristics of optical radiation. Moreover, as shown in Ref. [29], there are no fundamental differences in the effect of laser radiation and radiation from LED sources emitting in the blue spectral region on cells. For example, according to Ref. [29], when exposed to laser radiation with λ = 405 nm and an LED source with λ_{max} = 405 nm, for which full width at half maximum FWHM = 17 nm, the changes in the metabolic activity of cells are equivalent. The same conclusion follows when comparing the photobiological effects initiated by laser radiation with λ = 445 nm and radiation from an LED source with λ_{max} = 445 nm, FWHM = 18 nm [29].

Some of the above studies note that while there is a significant inhibitory effect of blue light with wavelengths of 450, 456, 460, 465, 470, 473, 485, 380–500, 450–500 nm on the proliferation and migration of cancer cells, its effect on normal cells are practically absent [4, 23, 31, 46, 49, 59, 60]. Other studies [8, 33, 39, 48, 57] show the presence of photobiological effect when exposed to blue light with wavelengths of 450, 465, 460–470, 400–500, 450–500 nm on both cancer and normal, non-transformed cells. Moreover, according to Refs. [8, 33, 39, 48, 57], the inactivating effect of blue light is more pronounced against cancer cells. For example, according to Ref. [39], irradiation of human leukemic monocytic lymphoma cells U937 with LED source peaking at λ_{max} = 56 nm for t = 2 h at irradiance of I = 0.25 mW/cm^2 (energy dose was D = 1.8 J/cm^2) leads

to a decrease in proliferation by 75%, while the same effect on normal non-transformed peripheral blood mononuclear cells is accompanied by a decrease in the corresponding proliferation rates by only 10%.

In our opinion, the absence of effect of blue light on normal cells in studies [4, 23, 31, 46, 49, 59, 60] is due to the insufficiently high energy dose of light exposure used. This is indicated by numerous studies of effect of blue light of the above wavelengths on non-tumor cells, which leave no doubt about its ability to influence the functional characteristics of this type of cell [1, 92]. In addition, the authors [48] showed that in two bladder cancer cell lines upon exposure to laser radiation with wavelength of $\lambda = 450$ nm, a decrease in their viability begins to be recorded at an energy dose of $D > 4$ J/cm^2, while for non-cancer uroepithelial cell viability begins to decrease at $D > 16$ J/cm^2. In other words, the photobiological effect, which was absent in relation to normal cells at low energy doses, begins to appear at higher doses.

At the same time, it should be noted that some reports [27] indicate the absence of significant differences in the effect of blue light ($\lambda = 465$ nm, energy dose was $D = 123$ J/cm^2 per day, total dose for three days of cell irradiation was $D = 369$ J/cm^2) on cancer and normal cells, and according to certain indicators (decrease in viability, percentage of cells in the apoptosis stage), the photobiological effect on normal cells according to Ref. [27] is higher than the corresponding value for cancer cells. In our opinion, one of the reasons for the fundamental differences between these results and the data of other authors may be the excessively high dose on the cells, which is almost 50 times higher than the doses usually used in other literature sources. However, despite this work, the totality of data contained in Table 1 strongly suggests a higher sensitivity of cancer cells to blue light compared to normal, non-transformed cells.

In this regard, it is noteworthy that the higher sensitivity of cancer cells compared to normal cells has also been confirmed with cells obtained from the same organ [33, 48, 59]. Thus, in studies [33], performed using a lamp source emitting in the range of 400–500 nm, or an argon laser generating radiation in the range of 450–500 nm, it was shown that the decrease in survival (energy dose was $D = 60$ J/cm^2) is strongly pronounced against cancer cells (carcinoma) of the oral cavity, while the inhibitory effect is barely registered against normal cells of the same location.

The same results were obtained by the authors [59] when monitoring, using a multimodal holographic microscope, the migration of cancer and normal prostate cells, previously exposed to lamp source (halogen lamp) with wavelength of $\lambda = 485 \pm 12.5$ nm at an energy dose of $D = 0.208$ mJ/cm^2.

As already noted, a higher sensitivity of bladder cancer cells compared to normal uroepithelial cells was established in Ref. [48] when exposed to semiconductor laser radiation with wavelength of $\lambda = 450$ nm in the energy dose range of $D = 4$–24 J/cm^2.

The results of our research, presented in Fig. 1, are in full accordance with most of the above-cited works. From these data it follows that the rate of light-induced decrease in the metabolic activity of cancer cells is higher than that of normal cells both when exposed to radiation $\lambda = 405$ nm and $\lambda = 445$ nm. Moreover, the differences in the magnitude of the inhibitory effect of light between these types of cells increase as the energy dose increases. However, at initial stage of dose dependence, these differences are minimal, which is consistent with the results of Ref. [48].

And the second important conclusion, following from the results of Fig. 1, is that the rate of decrease in metabolic activity of both cancer and normal cells is significantly higher when exposed to radiation with a wavelength of $\lambda = 405$ nm compared with $\lambda = 445$ nm.

At the same time, our studies using chemiluminescence assay (Fig. 2 and Fig. 3) showed that reactive oxygen species, and, above all, singlet oxygen, play an important role in reducing the metabolic activity of cells when exposed to blue light. This is indicated by a significant decrease in the chemiluminescence signal when a specific singlet oxygen quencher, sodium azide, is added to the cell suspension before irradiation. In addition to singlet oxygen, hydrogen peroxide and hydroxyl radicals also play a certain role in photochemical processes in cells when exposed to blue light, since the addition of a specific hydrogen peroxide scavenger, sodium pyruvate, or hydroxyl radicals scavenger, D-mannitol, to the cell suspension before irradiation leads to reliable decrease in the chemiluminescence signal (Fig. 3).

The results obtained confirming the participation of ROS in photochemical processes in cells exposed to blue light are in good agreement with numerous literature reports [7, 8, 16, 19–21, 23, 28, 30, 35, 36, 38, 41, 60], in which the formation of ROS was detected using specific fluorescent probes. At the same time, our studies have shown (Fig. 2) that with the same spectral and energy characteristics of laser radiation ($\lambda = 405$ nm, $I = 50$ mW/cm^2, $t = 180$ s, $D = 9$ J/cm^2) the light sum of chemiluminescent signal is significantly higher for cancer cells than for normal cells. This result is in accordance with the data of other authors [23, 28, 60]. Thus, studies [23] show that the level of ROS formation, assessed using a fluorescent probe, in epithelial carcinoma cells upon exposure to broadband blue light ($\lambda = 380$–500 nm, $D = 60$ J/cm^2) from lamp source at 30% higher than when irradiating normal epithelial keratinocytes (see Table 1).

Even more significant differences in level of light-induced ROS formation in the same types of cancer and normal cells, studied in Ref. [23], are reported by the authors [28] of the same research group when exposed to radiation $\lambda = 380$–500 nm, but at energy doses $D = 15$ and 30 J/cm^2.

Thus, the results of our studies, obtained using chemiluminescence assay, indicating a higher level of light-induced ROS formation in cancer cells compared to normal cells, are fully consistent with literature data

based on recording the fluorescence of specific probes in cancer and normal cells.

In addition to studies confirming higher levels of light-induced ROS formation in cancer cells, we also performed comparative studies of ROS levels in HeLa cells exposed to laser radiation with wavelength of $\lambda = 405$ nm, located in the region of the maximum of the Soret band of protoporphyrin IX, and laser radiation with wavelength of $\lambda = 445$ nm, corresponding to the long-wave maximum of absorption spectrum of flavins (Fig. 4). In this case, radiation with wavelength of $\lambda = 405$ nm corresponds to the local minimum in absorption spectrum of flavins, and radiation with $\lambda = 445$ nm corresponds to the region of local minimum in absorption spectrum of porphyrins.

As follows from the results presented in Fig. 5, the level of ROS formation induced by exposure of suspension of HeLa cells to radiation with $\lambda = 405$ nm is significantly higher than when exposed to radiation with $\lambda = 445$ nm at equal energy parameters ($I = 50$ mW/cm^2, $t = 180$ s, $D = 9$ J/cm^2). In our opinion, the result obtained is a strong argument indicating the contribution of porphyrins to the effects of sensitized ROS formation when cells are exposed to blue light. Since, in the case of the predominant role of flavins in the effects of sensitization, one would expect a higher level of ROS formation when cell suspension is exposed to radiation $\lambda = 445$ nm, corresponding to the long-wave maximum of absorption spectrum of flavins and the region of local minimum in absorption spectrum of porphyrins (Fig. 4). In addition, in the case of the predominant role of flavins in sensitized (due to endogenous compounds) reactions in cells, one would expect a higher rate of decrease in the metabolic activity of cells when exposed to radiation $\lambda = 445$ nm compared to radiation $\lambda = 405$ nm. However, as follows from Fig. 1, the rate of decrease in metabolic activity of both cancer and normal cells when exposed to radiation with $\lambda = 405$ nm significantly exceeds this indicator when cell suspension is exposed to radiation with $\lambda = 445$ nm.

It should be noted that the results of our studies, indicating a greater sensitivity of HeLa cells to radiation with $\lambda = 405$ nm compared to longer wavelength light with $\lambda = 445$ nm, are in good agreement with the results of the authors [93], who studied effect of low-intensity blue light on DNA synthesis in HeLa cells. These studies have shown that of the four wavelengths of a mercury-quartz lamp $\lambda_{max} = 313$, 365, 404 and 434 nm (selected from its emission spectrum using a monochromator) with an energy dose of $D = 1$ mJ/cm^2, the maximum stimulating effect on DNA synthesis in cells is observed at wavelength of 404 nm. In other words, studies [93] have shown that light with a wavelength corresponding to the absorption maximum of protoporphyrin IX and the local minimum in the absorption spectrum of flavins is characterized by the greatest biological activity. This confirms our hypothesis about the ability of endogenous porphyrins to act as one of the acceptors that, after absorbing light, can initiate a cascade of photochemical reactions affecting the course of metabolic processes in the cell. Note that the same results were obtained when studying the spectral dependence of the inactivation effect on normal non-transformed somatic cells [94, 95]. Thus, according to data [94], which studied the effect of LED radiation with $\lambda_{max} = 410$, 420, 453 and 480 nm on fibroblasts, the greatest effect at energy doses $D = 15$, 30, 60, 90 J/cm^2 on cell viability and proliferation, as well as the generation of reactive oxygen species was caused by radiation with $\lambda_{max} = 410$ nm. Cytotoxicity was not observed when exposed to light with $\lambda_{max} = 453$ and 480 nm. And, in studies [95] of the cytotoxicity of blue light with $\lambda_{max} = 412$, 419, 426 and 453 nm at energy doses $D = 33$, 66, 100 J/cm^2, as well as its effect on the proliferation of human keratinocytes and skin endothelial cells, the most effective was radiation with $\lambda_{max} = 412$ nm; the photobiological effect decreased with increasing radiation wavelength. Moreover, despite the fact that radiation with $\lambda_{max} = 453$ nm practically corresponds to the absorption maximum of flavins ($\lambda_{max} = 447$ nm), it (in contrast to radiation with $\lambda_{max} = 412$, 419, 426 nm) had practically no toxic effect on cells even with increasing energy dose to D = 500 J/cm^2. At the same time, as can be seen from Fig. 4, radiation with wavelengths of 410, 412, 419 and 420 nm corresponds to the region of the local minimum in the absorption spectrum of flavins and the region of maximum of the Soret band in the absorption spectrum of endogenous porphyrins, and radiation $\lambda_{max} = 453$ nm practically corresponds to the region of the maximum in the absorption spectrum of flavins and the local minimum in the absorption spectrum of porphyrins. Thus, when studying the effect of blue light on non-cancer cells, the contribution of endogenous porphyrins to the effects of photoinactivation is also beyond doubt.

Finally, comparative studies on fluorescence (Fig. 6) performed with acidic extracts of cancer and normal cells revealed that the concentration of protoporphyrin IX in cancer cells is approximately 2.5 times higher than its concentration in normal cells. In our opinion, the higher concentration of endogenous porphyrins in cancer cells is the reason for their higher sensitivity to blue light compared to normal cells.

Taking together, the results obtained in this work, show: (a) a higher rate of inhibition of the metabolic activity of cells by radiation with wavelength $\lambda = 405$ nm, corresponding to the maximum in the absorption spectrum of protoporphyrin IX, compared to the action of light with $\lambda = 445$ nm, corresponding to the maximum in the absorption spectrum of flavins; (b) a higher rate of inhibition of metabolic activity of HeLa cancer cells by blue light compared to normal BGM cells; (c) higher levels of ROS formation, recorded using the chemiluminescence assay, when a cell suspension is exposed to radiation with $\lambda = 405$ nm compared with $\lambda = 445$ nm; (d) higher levels of ROS formation when exposed suspension of HeLa cells to blue light compared to normal BGM cells; (e) the higher concentration of endogenous porphyrins recorded by the fluorescent method in HeLa cells compared to normal BGM cells allows us to conclude that endogenous porphyrins

localized in cells are capable of playing a leading role in the sensitized formation of ROS when exposed to blue light, affecting metabolic processes in cells, and the higher concentration of endogenous porphyrins in cancer cells is one of the reasons for their higher sensitivity compared to normal cells to blue light.

In connection with the above, a reasonable question arises: why does the contribution of endogenous porphyrins to the effects of sensitized processes in cells become so significant, despite their very low concentration. Indeed, according to Refs. [29, 77], in HeLa cells, the concentration of endogenous porphyrins is ~ 1 nM, while the concentration of flavins in the same cells, according to Refs. [90, 91], is 0.3–1 μM. Moreover, the ratio of the concentrations of riboflavin, FMN and FAD varies depending on the method of their extraction from cells from 0.40/0.17/0.42 to 0.16/0.096/0.75 [90]. And the quantum yield for the formation of singlet oxygen in aqueous environment at neutral pH value is $\varphi_\Delta = 0.54$ for riboflavin, $\varphi_\Delta = 0.51$ for FMN, and $\varphi_\Delta = 0.07$ for FAD [88]. In addition to singlet oxygen, flavins are also capable of generating superoxide radicals [96] and hydroperoxides [97] upon photoexcitation, although with much less efficacy. As for endogenous porphyrins, the efficacy of singlet oxygen generation upon photoexcitation strongly depends on the state of aggregation and is most significant for the monomeric form. Thus, for monomeric form of protoporphyrin IX $\varphi_\Delta = 0.77$ [87], uroporphyrin $\varphi_\Delta = 0.80$ [98], coproporphyrin $\varphi_\Delta = 0.58$ [99], zinc protoporphyrin IX $\varphi_\Delta = 0.91$ [100], zinc-coproporphyrin $\varphi_\Delta = 0.54$ [101]. In other words, upon photoexcitation of protoporphyrin, uroporphyrin and zinc protoporphyrin, the efficacy of singlet oxygen generation is approximately 1.5 times higher than the corresponding indicators for riboflavin and FMN, and an order of magnitude higher than for FAD. Since FAD makes up 42–75% of the total flavin content in the cell [94], we can conclude that the quantum yield of singlet oxygen generation is significantly higher when this process is sensitized by porphyrins compared to flavins.

As already noted, in addition to singlet oxygen, the free-base of porphyrins are capable of generating superoxide radicals [102] and hydroperoxides [89]. However, the efficacy of such generation is significantly lower than the efficacy of sensitized formation of singlet oxygen. Consequently, for both porphyrins and flavins, the main intermediate that determines their sensitizing effect is singlet oxygen.

In addition to high efficacy of singlet oxygen formation, another reason for significant contribution of endogenous porphyrins to sensitized damage of cellular structures may be the higher values of the molar extinction coefficient of tetrapyrroles compared to flavins. It is known that, at the maximum of the long-wave absorption band, the molar extinction coefficients are $\varepsilon_{447} = 12.5$ mM·cm^{-1} for riboflavin and FMN, and $\varepsilon_{447} = 11.3$ mM·cm^{-1} for FAD [103]. For endogenous porphyrins in monomeric form (in chloroform), the corresponding values of the molar extinction coefficients at the maximum of the Soret band are at least an order of magnitude higher: for protoporphyrin IX – $\varepsilon_{408} = 171$ mM·cm^{-1}; for coproporphyrin – $\varepsilon_{400} = 180$ mM·cm^{-1}; for uroporphyrin – $\varepsilon_{406} = 217$ mM·cm^{-1} [104]. Naturally, such significant differences in the molar extinction coefficients of porphyrins and flavins also contribute to the manifestation of the sensitizing properties of endogenous tetrapyrroles when exposed to blue light.

In addition to above factors that contribute to significant role of endogenous porphyrins in photobiological processes in cells when exposed to blue light, differences in intracellular localization of porphyrins and flavins may play a certain role. As for porphyrins, according to fluorescence microscopy, endogenous protoporphyrin IX, the formation of which in cells is induced by 5-aminolevulinic acid (ALA), is initially localized in mitochondria, and, in this case, it has the most pronounced photodynamic effect [105]. Unlike endogenous protoporphyrin IX, chemically pure exogenous protoporphyrin IX is predominantly distributed in cell membranes. At the same concentrations of endogenous porphyrin in cells and its exogenous analogue, photodynamic damage to cells (both cancer and normal) was significantly higher for the endogenous porphyrin [105]. This result indicates the importance of the region of localization of the sensitizer in the cell for the manifestation of its photodynamic activity.

Regarding the localization of flavins in cells, according to confocal and fluorescence lifetime imaging microscopy [106], endogenous flavins are predominantly localized outside the cell nucleus in mitochondria. Their fluorescence is characterized by a maximum in the region of $\lambda_{max} < 500$ nm, and the fluorescence lifetime is $\tau < 1.4$ ns, which indicates that flavins are predominantly in protein-bound form. In some part of the cells, the fluorescence came from flavins localized inside the cell nuclei, in nucleoli, demonstrating a longer fluorescence lifetime and a red-shifted spectral maximum, indicating the presence of flavins in free form [106]. Somewhat different data from the above were obtained by the authors of Ref. [78]. According to their results, based on the use of electron paramagnetic resonance methods to detect oxyradicals formed in cells upon exposure to blue light, flavin photosensitizers are found predominantly in the cytosol of cells and have a mass of less than 12 kDa. This also indicates a bound form of flavins, probably flavin-protein complexes.

In this regard, it should be noted that when flavins form complexes with proteins, there is reason to believe that the sensitizing effect of flavins will be primarily directed at the protein with which they are in complex [107, 108]. This is due to the short lifetime of singlet oxygen in aquatic biological environments, and, accordingly, the short length of its path from the place of formation, that is, from the sensitizer. In fact, the carrier protein protects cellular structures from sensitized photodamage, which may also be responsible for the reduction in contribution of flavins to cell photodamage upon exposure to blue light. And finally, it is well

known [109] that riboflavin, along with a sensitizing pro-oxidant effect, also has pronounced antioxidant properties. Consequently, upon photoexcitation of flavins with subsequent formation of ROS, flavin molecules can act as ROS quenchers through the mechanism of physical or chemical quenching, including the conversion of reduced riboflavin to the oxidized form [109].

Thus, (a) higher efficacy of singlet oxygen formation sensitized by porphyrins and higher values of their molar extinction coefficients compared to flavins; (b) localization of porphyrins in mitochondria, while a significant proportion of flavins are localized in the cytosol; (c) binding of flavins to proteins that perform a protective function against flavin-sensitized damage to cellular structures; (d) the pronounced antioxidant properties of flavins promote the significant contribution of endogenous porphyrins to the regulatory effects, initiated in cells by exposure to blue light, despite their lower (compared to endogenous flavins) concentration.

It should be noted that the possible role of endogenous porphyrins as acceptors of radiation in the blue spectral region in cells of various types was indicated in a number of works [1, 2, 18, 29, 64, 65, 73–77, 110, 111]. At the same time, in an interesting and informative review [2], it is stated without evidence that the concentration of flavins and porphyrins in cancer cells is low compared to normal cells, and it remains controversial whether they are precisely those photoreceptors that, upon exposure to blue light, suppress cell viability. In addition, according to Ref. [2], due to the presence of various components in cell culture media, it is difficult to determine which compound causes blue light-induced cell death.

A point of view close to the conclusions of Ref. [2] is also shared by the authors of Ref. [18], according to whom the concentration of flavins and porphyrins in cancer cells compared to normal cells is controversial and very few direct or significant comparisons are available. Additionally, due to the variety of cell culture media used, determination of specific chromophore from a cocktail makes it difficult to identify the compound responsible for blue light-specific cell death [18].

We believe that the results obtained in this work convincingly prove the important role of endogenous porphyrins in the implementation of photobiological processes in cells upon exposure to blue light, as well as the importance of higher concentrations of endogenous porphyrins in cancer cells compared to normal ones in initiating higher levels of formation ROS, and as a result - in ensuring a higher sensitivity of cancer cells to the blue light.

At the same time, the results of comparative studies of the effect of optical radiation with wavelengths of $\lambda = 405$ nm and $\lambda = 445$ nm on the metabolic activity of cells (see Fig. 1) and on the integral intensity of chemiluminescence of cell suspension (see Fig. 5), which revealed the participation endogenous porphyrins in sensitized processes in cells, do not at all deny the participation of endogenous flavins in these processes. As already noted, the absorption coefficient of protoporphyrin IX upon transition from wavelength $\lambda = 405$ nm to $\lambda = 445$ nm decreases by more than 10 times, while the integral intensity of chemiluminescence of cell suspension according to the data in Fig. 5 is reduced by only 19%. The rate of light-induced decrease in the metabolic activity of cells also changes less significantly (as the absorption coefficient) when moving from wavelength of $\lambda = 405$ nm to $\lambda = 445$ nm (Fig. 1). All this indicates the contribution of not only endogenous porphyrins, but also endogenous flavins in the sensitization of cells when exposed to blue light. In this case, the contribution of flavins is most pronounced in the region of maximum of their absorption spectrum, where porphyrins are characterized by minimal absorption. Conversely, the contribution of porphyrins is most pronounced in the region of the maximum absorption band in the Soret band, corresponding to the local minimum of the absorption spectrum of flavins (Fig. 4).

In our opinion, the participation of ROS (singlet oxygen, hydroxyl radicals, hydrogen peroxide, superoxide anion radical, etc.) formed during exposure of cells to blue light is not limited to lipid peroxidation, oxidation of proteins and nucleic acids, inhibition of enzymes and activation of apoptosis, which in ultimately leads to cell death [112]. Indeed, at high energy doses of light exposure, the contribution of these destructive processes initiated by ROS to the decrease in the metabolic activity of cells is significant, which is confirmed by the registration of a large proportion of dead cells [7, 17, 19, 22, 35, 43, 47, 49, 52]. At the same time, it is well known that low concentrations of ROS can act as signaling molecules that can have a regulatory effect on metabolic processes in cells [1, 64–66]. A change in the intracellular concentration of ROS under light can change the redox status of the cell and can be a direct cause of both the activation of proliferative processes in cells (at a relatively low level of ROS) and the inhibition of their proliferation (at an increased concentration of ROS).

Indeed, studies [4, 12, 27, 43, 49, 57], using flow cytometry, have shown that at low energy doses of blue light, a decrease in the metabolic activity of cells is not realized due to their death, since the decrease in metabolic activity is practically not accompanied an increase in the number of necrotic and apoptotic cells. It is believed [4, 12, 27, 43, 49, 57] that the inhibitory effect of light in this case is the result of its influence on the cell cycle, and is explained by the inhibition of the transition from G1 to S-phase and an increase in the duration of the M-phase. Note that, when monitoring cell cycle parameters, the specificity of the response of cancer and normal cells to the blue light is also noted. Inhibition of cell growth not as a result of the death of some cells, but due to prolongation of the stages of the cell cycle, also indicates the regulatory (non-destructive) nature of the action of blue light at relatively low energy doses. At the same time, as the energy dose of light exposure increases, along with a decrease in the metabolic and proliferative

activity of cells, an increase in the number of dead cells is noted, and cell cycle arrest is also initiated.

Another confirmation of the mild regulatory nature of the action of blue light on animal somatic cells (including cancer cells) is the registration of its stimulating effect in a certain dose range: stimulation of DNA and RNA synthesis when exposed to narrow-band blue light with a wavelength of λ_{max} = 404 and 434 nm at energy dose D = 1 mJ/cm^2 [93], stimulation of cell proliferation by laser radiation with a wavelength λ = 405 nm at energy dose of D = 30 J/cm^2 [11], stimulation of metabolic activity of cells by laser radiation or radiation from LED source with λ_{max} = 405 nm, at D = 0.45 J/cm^2 [29]. At the same time, the dose dependence of the biological effect of blue light, according to Refs. [29, 93], is biphasic in nature, which indicates its compliance with the well-known Arndt–Schultz rule [113], according to which, at low doses of exposed radiation, a slight stimulating effect is observed, which increases with increasing energy dose until the maximum is reached; further increase in the dose leads first to a decrease in the stimulating effect to the control level, and then to a inhibitory effect compared to non-irradiated samples, and finally to the death of the biological object. As noted earlier [74, 75], compliance of the dose dependence with the Arndt–Schultz rule indicates the regulatory nature of the action of a physical factor on a biological system, that is, when blue light is exposed to cancer and normal cells, all the signs inherent in the effects of photobiomodulation appear [113].

However, it should be noted that higher levels of ROS production in cancer cells are not the only reason for their greater sensitivity to blue light. Thus, some studies note that the causes of increased sensitivity of cancer cells are excessive accumulation of ROS, as well as deficiency of antioxidants, which leads to oxidative damage to mitochondria [23]. A similar point of view is shared by the authors of Ref. [36], according to whom cancer and normal cells differ in their cellular response to ROS, and cancer cells may be more prone to apoptosis as a result of an imbalance of the intracellular antioxidant system caused by excessive production of ROS.

Thus, the results presented in this paper, compared with the analysis of literature data, provide an answer to the reasons for the higher sensitivity of cancer cells compared to non-cancerous cells to the action of optical radiation in the blue spectral region (both laser and non-laser sources).

5 Conclusions

The studies carried out in this work showed the important role of endogenous porphyrins (free-base and their zinc complexes) in the generation of reactive oxygen species in cells (and, above all, singlet oxygen), which are capable of influencing, by changing the redox state of cells upon absorption of blue light, the metabolic processes occurring in them. It has been shown that the decisive role of porphyrins in the effects of cell sensitization occurs despite higher, at least two orders of magnitude, concentrations of flavins (riboflavin, FMN, FAD) in cells.

The manifestation of the sensitizing properties of porphyrins in cells is facilitated by: (a) higher efficacy of formation of singlet oxygen sensitized by them and higher values of their molar extinction coefficients compared to flavins; (b) localization of porphyrins in mitochondria, while a significant proportion of flavins are localized in the cytosol; (c) binding of flavins to proteins that perform a protective function against flavin-sensitized damage to cellular structures; (d) pronounced antioxidant properties of flavins, which contributes to the quenching of ROS generated by them during photoexcitation.

The leading role of porphyrins, and not flavins, in the photobiological processes that determine cell metabolism when exposed to blue light is confirmed by a higher rate of inhibition of cell metabolic activity and higher levels of ROS formation, recorded using the chemiluminescence assay, when a cell suspension is exposed to radiation with λ = 405 nm compared to λ = 445 nm. In this case, radiation with a wavelength of λ = 405 nm corresponds to the maximum of the absorption spectrum of protoporphyrin IX and the local minimum of the absorption spectrum of flavins, and radiation with λ = 445 nm corresponds to the maximum of the absorption spectrum of flavins and the region of local minimum of the absorption spectrum of porphyrins.

For the first time, it has been shown that one of the reasons for the increased sensitivity of cancer cells compared to normal cells to the blue light is the higher concentration of endogenous porphyrin sensitizers, which is confirmed by fluorescent analysis methods. The determining role of porphyrins in the difference in the reactions of cancer and normal cells to the blue light on their suspension is also evidenced by higher levels of ROS formation and a higher rate of light-induced inhibition of the metabolic activity of HeLa cancer cells compared to normal BGM cells.

In addition to differences in the concentrations of endogenous porphyrin sensitizers in cancer and normal cells, the reason for the specificity of their reactions to exposure to blue light may lie in the fact that cancer and normal cells differ in their cellular response to the effects of ROS; and cancer cells may be more prone to apoptosis as a result of an imbalance in the intracellular antioxidant system caused by excess ROS production.

Declarations of Interests

The authors declare no conflict of interest.

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