

Bilirubin- and Blue-Green Light-Induced Damage of Human Erythrocytes

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Abstract. Phototherapy is widely used for neonatal hyperbilirubinemia treatment; however, the most important but not completely recognized issue is the determination of the optimal spectral range of light that provides an effective reduction of bilirubin level in the blood of a newborn with minimal adverse side effects. This study demonstrates that the exposure of radiation from LED sources of blue (emission wavelength $\lambda_{\max} = 463$ nm, FWHM 20 nm) and green ($\lambda_{\max} = 517$ nm, FWHM 38 nm) spectral regions to the erythrocyte suspension with the same energy dose ($D = 14.4$ J/cm²) leads to approximately equally effective hemolysis of erythrocytes. It is concluded that the damage of erythrocyte membranes is caused by the excitation of endogenous porphyrin and flavin photosensitizers contained in the cells in low concentrations, but characterized by high efficiency of singlet oxygen generation. When bilirubin is added to the erythrocyte suspension, a spontaneous (dark) erythrocyte hemolysis intensifies, but the light-induced hemolysis slows down (compared to cells containing no bilirubin). Under the same conditions, the leakage of potassium ions from light-exposed erythrocytes is accelerating, with the light of green spectral region has a more pronounced effect, despite more intense absorption of blue light by bilirubin localized in cells. © 2023 Journal of Biomedical Photonics & Engineering.

Keywords: hyperbilirubinemia; phototherapy; blue LED radiation; green LED radiation; photohemolysis; potassium leakage; human erythrocyte membrane; protoporphyrin IX; Zn-protoporphyrin IX.

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1 Introduction

The interest to the investigation of possible photodamage of biological systems of various structural levels treated with bilirubin is due to the widespread use of optical technologies for the treatment of hyperbilirubinemia (jaundice) in newborn children [1–6]. As it is known, this disease is caused by the excessive accumulation in the child's body of the bile pigment – Z,Z-bilirubin IX α , which at its high concentrations can effect negatively the health of the newborn. The main and in fact the only method of reduction of bilirubin levels during the jaundice is phototherapy, based on exposure of child's body surface with light of the blue-green spectral range corresponding to the pigment absorption spectrum [1–6].

On light quanta absorption, Z,Z-bilirubin IX α molecule undergoes a number of configuration and structural transformations into photoisomers, that have greater hydrophilicity compared to native bilirubin, and are characterized by a higher excretion rate.

However, despite more than 60 years of history of the use of light for the treatment of hyperbilirubinemia (despite of the use of light for hyperbilirubinemia treatment for more than 60-years), many issues related to the practical implementation of phototherapy technologies are far from being understood. One of the most important but not fully clarified issues is to determine the optimal wavelength of light for hyperbilirubinemia phototherapy. Another fundamental issue is connected to adverse side effects from

phototherapy and their dependence on the spectral range of the incident radiation. For a long time it was believed that the optimal spectral range for phototherapy should correspond to the maximum of absorption spectrum of bilirubin bound to serum albumin $\lambda_{\max} \approx 460$ nm [5]. However, in recent years, quite a lot of data has appeared [6–12] that the light of green spectral region corresponding to the long-wavelength slope of the absorption band of the bilirubin-albumin complex is no less effective than blue light, or even surpasses it.

The long-time experience in using blue light for the treatment of neonatal hyperbilirubinemia has shown that no serious adverse side effects are mostly observed during phototherapy in clinical conditions. However, there is a strong opinion that phototherapy cannot be completely harmless, and it should be used carefully, not exceeding certain recommended light intensities; minimization of phototherapeutic effects is advised [1, 4]. The need to comply with certain restrictive measures during phototherapy is stipulated by some literature data [13–15] giving information that phototherapy implementation in newborns with extremely low body weight (less than 750 g) aggravates the condition of the infant and may be fatal. In addition, there is data about a higher risk of future development of cancer [16–19] and epilepsy [20] of those children who have previously had neonatal phototherapy. Sometimes there are undesirable processes in the newborn's body (bronze baby syndrome, hypocalcemia, dehydration, DNA damage, allergic reactions in the form of asthma attacks, rashes on the body), manifested directly during or after the phototherapy [1, 3, 4, 21, 22]. It is believed that some of them may be caused by the sensitizing effect of bilirubin and its photoisomers [23–26]. There are numerous data on the photocytotoxicity of bilirubin and its structural photoisomer lumirubin against cells of various body tissues [25–30] and, in particular, blood cells [31–34]. At the same time, it has been repeatedly suggested in the literature [22, 23, 27, 28, 35, 36] that the transition from light sources of the blue spectral range to sources of the green spectral range will help not only effectively reduce the level of bilirubin in the blood of newborns, but also reduce adverse side effects of phototherapy. However, there are practically no comparative studies of the adverse side effects upon exposure of blood cells treated with bilirubin to light of the blue and green spectral regions. Such studies are becoming especially relevant because of switching to LED sources of blue-green or green spectral regions in phototherapy equipment for the treatment of neonatal jaundice [1–4, 36], and also in connection with the revealed dependence of the efficiency of the formation of configuration and structural bilirubin photoisomers on the wavelength of exciting light [37–39]. The reason for the variation in the quantum yield of photoisomers formation with a change of the excitation wavelength is the bichromophoric nature of the absorption and emission of light by bilirubin molecules and the dependence of intramolecular distribution of the

excitation energy between two asymmetric tetrapyrrole chromophores on the quantum energy [40, 41].

The purpose of this work is comparative *in vitro* studies of the effectiveness of human erythrocytes damage upon exposure to radiation of LED sources with the same intensity in blue and green spectral regions (promising for reduction of bilirubin levels in newborns with hyperbilirubinemia syndrome) at a bilirubin concentration suggesting phototherapy in infants. The choice of erythrocytes is specified by the known data of previous studies [31–34, 42–44], which identified the erythrocyte membrane as the main target of photocytotoxicity during sensitization with bilirubin. Moreover, in Ref. [45] we demonstrated the ability of low-intensity optical radiation of visible spectral region to have a modifying effect on erythrocyte membranes even in the absence of exogenous bilirubin.

2 Materials and Methods

Saline and buffered solutions were prepared from sodium-phosphate salts produced by Merck (Merck, extra pure, p.a.) on distilled water. Bilirubin produced by “Reakchim” SRPA Biolar high purity grade (Russia) and bilirubin produced by “Fluka” (Germany) with no additional purification were used in the work. The purity of the substances was $\geq 98.5\%$. The bilirubin solution was prepared according to the standard procedure: 2.5 mg of bilirubin were dissolved in 200 μl of 0.05 M NaOH (due to its very low solubility in water at physiological pH values). At calculation of bilirubin concentrations, the molar extinction coefficient $\varepsilon_{440} = 4.75 \cdot 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [41] was assumed.

For preparation of the bilirubin-albumin solution complex, human serum albumin produced by Sigma, USA, fraction V, degree of purification about 99% was used. At calculation of the components concentrations, the following values of molar extinction coefficients were used: bilirubin within the HSA complex $\varepsilon_{460} = 4.70 \cdot 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$, HSA $\varepsilon_{280} = 3.55 \cdot 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [41].

Flavin mononucleotide phosphate (FMN) (fluorimetric purity 76.5%) by Sigma-Aldrich, protoporphyrin IX (purity > 97%), Zn(II)-protoporphyrin IX (purity > 95%) by Frontier Scientific and dimethylsulfoxide by “KupavnaReactive” (Russia, reagent grade) with no additional purification were used.

Erythrocytes were extracted from adult donors' blood of a random group and Rh factor provided by the Republican Scientific and Practical Center of Transfusiology and Medical Biotechnologies of the Ministry of Health of the Republic of Belarus. Blood was placed into 3.8% sodium citrate. Erythrocytes were isolated from supernatant plasma by centrifugal separation at 3000 g for 15 min. Pelleted cells were resuspended in 0.155 M NaCl, centrifuged as above and a buffy coat was removed. The process was triply repeated to take away white blood cells and platelets. Finally, the washed erythrocytes were resuspended in phosphate buffered saline (PBS: 0.155 M NaCl, 1.9 mM NaH_2PO_4 , 8.1 mM Na_2HPO_4 , pH 7.4) to their initial concentration in the blood (hematocrit $\sim 50\%$).

Bilirubin was added into the erythrocytes suspension in PBS to concentration of 171 μM (through 0.05 M NaOH), and such suspension samples were incubated at 37 °C for an hour in the dark simultaneously with control erythrocyte suspension samples without bilirubin. The indicated concentration of bilirubin corresponds to the level at which phototherapy is recommended for newborns with hyperbilirubinemia syndrome. For irradiation, the erythrocyte suspension was diluted to hematocrit $\sim 13\%$. Aliquots of each suspension were exposed to blue or green light for 20 min. Forty min after irradiation, one part of the irradiated sample, as well as control unirradiated samples of identical composition, were centrifuged at 3000 g for 15 min for sedimentation and then the supernatant was analyzed. The second part of all the samples were kept for 18 h in the fridge at 8 °C, and then erythrocytes were also sedimented in them and supernatant was analyzed. The work was carried out at room temperature of 21 ± 1 °C in low light conditions.

The radiation sources were blue (the wavelength of the emission spectrum maximum $\lambda_{\text{max}} = 463$ nm, the full-width at half-maximum FWHM = 20 nm) and green ($\lambda_{\text{max}} = 517$ nm, FWHM = 38 nm) LED matrices forming a uniform intensity light spot with irradiance at the surface level of the samples $I_{\text{LED}} \approx 12$ mW/cm² (phototherapy device “Romashka”, Institute of Physics of the National Academy of Sciences of Belarus). The light exposure was carried out for 20 min in a 10 mm thick layer with slow stirring of the suspension, excluding mechanical hemolysis of red blood cells. The energy dose during this exposure was $D = 14.4$ J/cm², which is approximately comparable to 2 h of phototherapy in the treatment of neonatal hyperbilirubinemia.

The tests indicating photodamage of the erythrocyte membrane were data on the concentrations of potassium K^+ ions and hemoglobin molecules in the solution surrounding the erythrocytes, which was extracted by centrifugation of the suspension as a supernatant. The magnitude of the photobiological effect was characterized by the ratio of optical densities at the Soret band maximum ($\lambda_{\text{max}} = 416$ nm) in the supernatant of experimental and control samples, as well as by the ratio of K^+ ion concentrations in the supernatant of experimental and control samples.

Since, during the experiments, each new part of donor material differed from the previous one due to the random choice of donors by blood type and Rh factor, as well as due to the possible presence of significantly different concentrations of antioxidants in their blood, a relative assessment of potassium ions' and hemoglobin yield in the PBS was carried out for each part of donor material. The number of measurements within one sample of donor blood was at least 3. The total amount of samples of donor blood was at least 4.

To reveal the possible contribution of singlet oxygen to photodynamic processes, sodium azide (NaN_3), a well-known quencher of this active oxygen form, in final concentration of 50 mM, was added into some samples with bilirubin before irradiation.

The analysis of the K^+ ions in the supernatant solution was carried out with an inductively coupled plasma atomic emission spectrometer (IRIS Intrepid II XDL DUO, Thermo Electron Corp., USA) with calibration by the standard sample of K^+ aqueous solution 8092-97(18K-1). The presence of hemoglobin in the solution was determined spectrophotometrically. Solutions absorption spectra were measured by Cary 500 Scan UV-Vis-NIR spectrophotometer (Varian, USA) in 1-cm quartz cuvettes.

3 Statistical Analysis

Data are expressed as mean $\pm$ error of mean. Statistical analysis was performed using Microsoft Excel. Differences between means were assessed by the Kramer-Welch criterion (applied to small independent samples) [46], based on statistics $T_{\text{emp}} = |\bar{x} - \bar{y}| / (D_x/n_x + D_y/n_y)^{0.5}$, where $\bar{x}$ and $\bar{y}$ are comparable means, D is the sample variance, $n = 4$. A value of $\alpha = 0.05$ was chosen as the level of statistical significance, unless otherwise specified.

4 Results

4.1 Spectral Characteristics of Bilirubin, Erythrocyte Suspension and Optical Radiation Sources

It is well known that bilirubin is a hydrophobic compound, and, in human blood, it is mainly in complex with serum albumin, which is able to bind two pigment molecules in different parts of the protein globule with association constants $k_{\text{as}} = 5.5 \cdot 10^7 \text{ M}^{-1}$ and $k_{\text{as}} = 4.4 \cdot 10^6 \text{ M}^{-1}$ [40]. Less than 10–15% of the blood total bilirubin is in a water-soluble form, being conjugated with glucuronic acid. When bilirubin concentrations in the blood exceed the albumin binding capacity, erythrocytes along with lipoproteins become the closest target for it. Human erythrocytes are capable to bind bilirubin in concentrations up to 0.93 nM/mg of protein [43] or about 1 mg per 10 ml of cells [47]. It is believed that bilirubin binding sites can be located both outside and inside of erythrocyte membrane and these sites are not formed by proteins or, at least, membrane proteins do not play a significant role in bilirubin binding [42, 43].

The absorption spectra of bilirubin in PBS (curve 1), as well as in complex with human serum albumin at 1/1 molar ratio (curve 2) are shown in Fig. 1. The absorption spectrum of bilirubin bound to erythrocyte ghosts (0.1 ml; 5 mg of ghost protein/ml, 20 μM bilirubin) is also shown here (curve 3), reproduced from Ref. [42]. A distinctive feature of bilirubin absorption spectrum in an aqueous medium is its rapid variability over time caused by the formation of aggregated forms and oxidation by air oxygen. Bilirubin complexes with albumin and erythrocyte membranes (ghosts) are significantly more stable. Note that the incorporation of bilirubin into the albumin protein globule is accompanied by a long-wave

shift of the pigment absorption spectrum from $\lambda_{\max} = 437$ nm for bilirubin in the buffer solution to $\lambda_{\max} = 460$ nm.

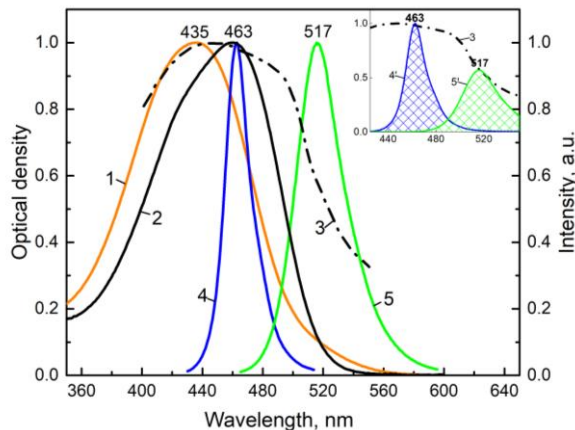


Fig. 1 The normalized absorption spectra of bilirubin in PBS (1), in complex with human serum albumin at 1/1 molar ratio (2), in erythrocyte ghosts (0.1 ml; 5 mg of ghost protein/ml, 20 μ M bilirubin) from Ref. [42] (3), and normalized emission spectra of blue (4), and green (5) LEDs. The emission spectra of blue (4') and green (5') LEDs normalized to equal integral intensity are shown in the inset together with the spectrum of bilirubin in erythrocyte ghosts (3).

In contrast to the absorption spectrum of the bilirubin-albumin complex, the absorption spectrum of bilirubin bound to erythrocyte membranes is significantly broadened, its maximum is in the region of $\lambda_{\max} = 450$ nm; in addition, a shoulder appears in the spectrum in the region of 490 nm. It is characteristic that those similar broadened bilirubin absorption spectra with a pronounced shoulder in the region of 500 nm are also observed when bilirubin is embedded in the synaptosomal plasma membrane vesicles isolated from rat brain [48].

Normalized emission spectra of blue (curve 4) and green (curve 5) LEDs used to irradiate the erythrocytes suspension are also shown in Fig. 1. Since the emission spectra of LEDs are characterized by quite different FWHM values, for clarity, the inset in Fig. 1 shows the emission spectra of blue (curve 4') and green (green 5') LEDs, normalized to the same integral intensities (which under identical experimental conditions means the same irradiance of blue and green light at the surface level of the samples). A fragment of the absorption spectrum of bilirubin in erythrocyte ghosts is also shown here (curve 3). It can be seen that the radiation with $\lambda_{\max} = 463$ nm almost corresponds to the maximum of the absorption spectrum of free bilirubin, as well as bilirubin bound both to albumin and erythrocyte membranes (ghosts). But the radiation with $\lambda_{\max} = 517$ nm corresponds to the long-wavelength slope of the pigment absorption band, where the absorption of light by bilirubin is less than in the region of the maximum of the absorption spectrum. Therefore, at the same irradiance (12 mW/cm²) of the incident radiation of

blue and green LEDs, the absorption of blue light by bilirubin bound to erythrocytes in suspensions will be greater than the absorption of green light.

Studies have shown that the radiation of LED sources in blue ($\lambda_{\max} = 463$ nm) and green ($\lambda_{\max} = 517$ nm) spectral regions, upon exposure of cell suspension treated with bilirubin, is to some extent shielded by hemoglobin contained in erythrocytes. At the same time, the shielding effect of hemoglobin is approximately the same for the LED sources used. This conclusion follows from the data in Fig. 2, which shows the absorption spectra of the erythrocyte suspension and the emission spectra of blue and green LEDs.

It can be seen that the absorption spectrum of the erythrocyte suspension with maxima in the region of $\lambda_{\max} = 418$ nm (Soret band) and $\lambda_{\max} = 543$ and 578 nm (Q-bands) corresponds to the absorption spectrum of hemoglobin. At the same time, the areas of overlap of the suspension absorption spectrum with the emission spectra of blue and green LEDs (shaded areas in Fig. 2) are approximately equal. In addition, mixing of suspensions during their irradiation almost eliminates the possible role of incident light shielding in the observed differences (see below) of the damaging effects of blue and green light on the erythrocyte membranes.

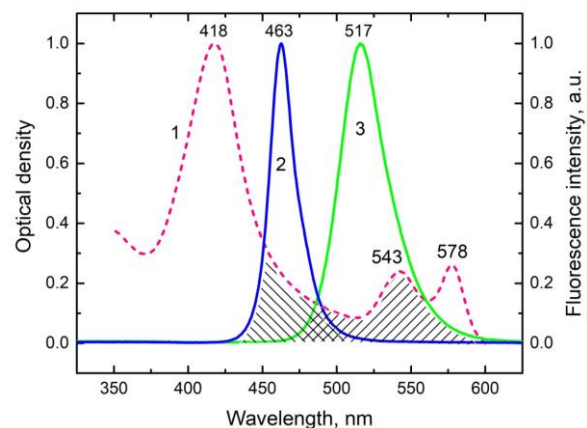


Fig. 2 The normalized absorption spectrum of the erythrocyte suspension (1) and the emission spectra of blue LED (2) and green LED (3).

4.2 The Effect of LED Sources Radiation of Blue and Green Spectral Regions to the Permeability of Erythrocyte Membranes for Potassium Ions

To quantify the change in the permeability of erythrocyte membranes for potassium K^+ ions under the influence of radiation, the ratio $[K^+]_{ir}/[K^+]_{un}$ was used, where $[K^+]_{ir}$ and $[K^+]_{un}$ are the concentrations of K^+ ions in the supernatant from irradiated and unirradiated control erythrocyte suspensions, accordingly.

The performed studies have shown that LED sources radiation of blue ($\lambda_{\max} = 463$ nm) or green ($\lambda_{\max} = 517$ nm) spectral regions at irradiance of $I_{LED} \approx 12$ mW/cm² applied to the suspension of human erythrocytes for 20 min (energy dose $D = 14.4$ J/cm²)

without adding the bilirubin almost does not affect the permeability of cell membranes for potassium ions when monitoring this process both 40 min after the termination of irradiation and 18 h later. This conclusion follows from the presented in Fig. 3 arithmetic mean values of $[K^+]_{ir}/[K^+]_{un}$, determined by four independent experiments (with erythrocytes of four donors).

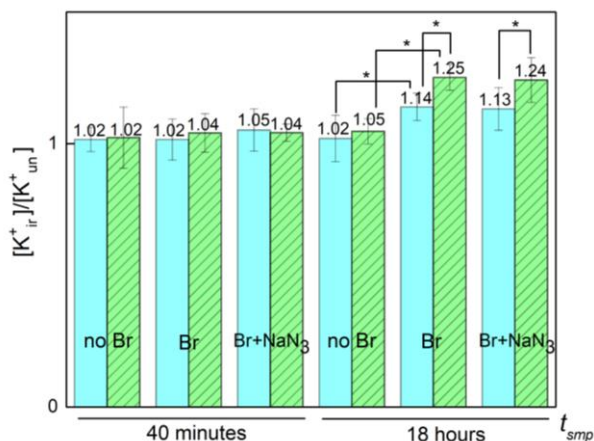


Fig. 3 The ratio of the concentration of potassium ions $[K^+]_{ir}/[K^+]_{un}$ in supernatants (irradiated relative to unirradiated suspensions) after exposure to blue LEDs radiation ($\lambda_{max} = 463$ nm, blue bars) or green LEDs radiation ($\lambda_{max} = 517$ nm, green bars) with irradiance of $I_{LED} = 12$ mW/cm² for 20 min on the suspension of human erythrocytes: without bilirubin (no Br), with 171 μ M Br (Br), with 171 μ M Br and 50 mM NaN₃ (Br + NaN₃), sampling time were 40 min and 18 h after the exposure. Error bars are refer to errors of $[K^+]_{ir}/[K^+]_{un}$ means. An asterisk * indicates differences in values at the significance level of 0.05.

As it follows from Fig. 3 (bars “no Br”), 40 min after irradiation, K⁺ ions yield differs from the control level, taken as 100%, by no more than 2%. Eighteen hours after the light exposure to erythrocytes, the K⁺ ions yield almost did not change compared to control samples, exceeding their value only by 2–5%. Note that the control unirradiated samples were incubated under the same conditions for 18 h as the irradiated ones. In this case, there were also no significant differences in the effect of green and blue LEDs.

It was found that the effect of light both from blue and green spectrum regions on the erythrocyte suspension in the presence of bilirubin also practically does not lead to an increase in the concentration of K⁺ ions in the supernatant (compared to unirradiated samples) when monitoring concentrations 40 min after the termination of irradiation (Fig. 3, bars “Br”, $t_{smp} = 40$ min).

A different situation is realized 18 h after the termination of light exposure to erythrocytes treated with bilirubin. In these conditions, potassium leakage from the erythrocytes increased on average (compared to control samples for this time period) by 14% when exposed to blue LED light and by 25% when exposed to green LED light (Fig. 3, bars “Br”, $t_{smp} = 18$ h). In this case, there are significant differences ($\alpha < 0.05$, marked with * in Fig. 3)

in K⁺ ions leakage between control and irradiated suspensions, as well as between suspensions irradiated by blue and green LEDs. Moreover, the radiation of green spectrum region has a more pronounced (by about 10%) damaging effect on erythrocytes, despite of weaker absorption of light in this region by bilirubin localized in the erythrocytes (Fig. 1, curve 3).

As it is known, bilirubin is a rather weak photosensitizer: the quantum yield of singlet oxygen generation sensitized by it is $\phi_{\Delta} \approx 0.01$ [3]. Nevertheless, according to literature data [44], photodamage of erythrocyte membranes sensitized by bilirubin can be determined by the participation of this active oxygen form. In this regard, we investigated the possible participation of singlet oxygen in the photodamage of erythrocyte membranes by controlling the effect of sodium azide, a well-known singlet oxygen quencher, on the magnitude of the photobiological effect, estimated by the K⁺ ions yield from the cells. The performed studies have shown (Fig. 3, bars “Br+NaN₃” versus “Br”, $t_{smp} = 18$ h) that the addition of sodium azide to the bilirubin-sensitized erythrocyte suspension before irradiation reduces the value of the effect $[K^+]_{ir}/[K^+]_{un}$ (18 h after the irradiation) on average by only 1% when exposed to both blue and green light. This value is not statistically significant according to the assessment. Thus, it can be concluded that sodium azide does not actually block the light-induced yield of potassium ions, and, accordingly, singlet oxygen almost does not participate in the photochemical processes responsible for changing the permeability of cell membranes to potassium ions.

4.3 The Effect of Radiation from LED Sources in the Blue and Green Spectral Regions on the Release of Hemoglobin from Erythrocytes

The release of hemoglobin from the erythrocytes is the most frequently studied parameter characterizing the breakage of the integrity of the erythrocyte cell membrane under the influence of physical factors [31, 32, 44, 47]. It should be noted that when the erythrocyte cell membrane is damaged, along with hemoglobin, other heme derivatives (hemin, cytochromes, catalase and peroxidase enzymes, protoporphyrin, etc.) may appear in the extracellular space. However, the contribution to the recorded absorption spectrum of the supernatant, collected during the sedimentation of erythrocytes and diluted multiply with NaCl-isotonic solution is actually determined by hemoglobin. Fig. 4 shows the absorption spectra of supernatants from erythrocyte suspensions with bilirubin (curves 1, 2, 3), with bilirubin and azide (curves 4, 5, 6) in NaCl-isotonic solution, which were irradiated with blue (curves 2, 6) and green (curves 3, 5) LEDs with irradiance of $I_{LED} \approx 12$ mW/cm² for 20 min, as well as from their unirradiated analogues (curves 1, 4). The supernatants were extracted from erythrocyte suspensions $t_{smp} = 40$ min after the light exposure.

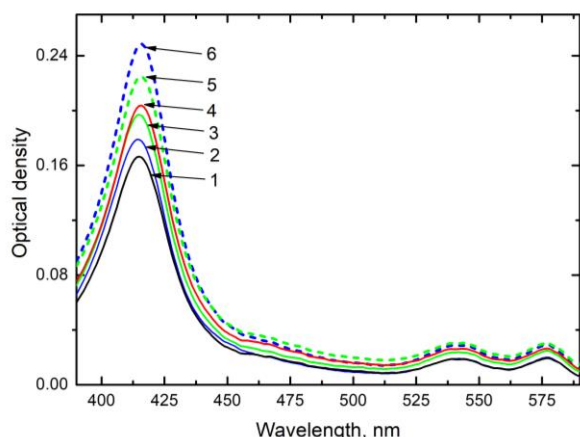


Fig. 4 Absorption spectra of supernatants from erythrocyte suspensions with bilirubin (1, 2, 3), with bilirubin and 50 mM NaN_3 (4, 5, 6) in NaCl-isotonic solution, irradiated with blue LED (2, 6), green LED (3, 5) and unirradiated (1, 4). $I_{\text{LED}} \approx 12 \text{ mW/cm}^2$, exposure time is 20 min, sampling time is 40 min.

The absorption spectra of supernatants in NaCl-isotonic solution for irradiated and unirradiated erythrocyte suspensions look like typical hemoglobin absorption spectra with maxima in the region of 416 nm (Soret band), as well as in the region of 540 nm and 575 nm.

As follows from Fig. 4, the greatest dynamic range of changes upon exposure of the erythrocytes to light is observed at the maximum of the Soret band. For this reason, further, the $OD_{\text{ir}}/OD_{\text{un}}$ value was chosen as a quantitative measure to characterize the erythrocyte hemolysis, where OD_{ir} and OD_{un} are optical densities at the maximum of the Soret band (around 416 nm) of the supernatants diluted multiply in NaCl-isotonic solution in the same proportions for irradiated and unirradiated control erythrocyte suspensions, respectively.

The performed studies showed the presence of erythrocyte hemolysis in unirradiated control samples, which is manifested in an increase in the optical density of the Soret band maximum of hemoglobin in the supernatant (Table 1) from $OD_{\text{un}} = 0.129 \pm 0.045$ when analyzed after 40 min of incubation at a room temperature to $OD_{\text{un}} = 0.147 \pm 0.049$ after 18 h of dark storage at 8 °C. The addition of bilirubin at a concentration of 171 μM to the erythrocytes suspension (without exposure to light) increases the rate of hemolysis. This is confirmed by an increase in optical density at the maximum of the Soret band of hemoglobin in the supernatant already 40 min after incubation of cells with bilirubin ($OD_{\text{un}} = 0.152 \pm 0.019$) compared to cells without bilirubin additives ($OD_{\text{un}} = 0.129 \pm 0.045$). This tendency remains when analyzing the release of hemoglobin from erythrocytes after 18 h of dark storage: for cells with bilirubin $OD_{\text{un}} = 0.187 \pm 0.026$; for cells without addition of bilirubin $OD_{\text{un}} = 0.147 \pm 0.049$.

The results of studies of the effect of light from blue ($\lambda_{\text{max}} = 463 \text{ nm}$) and green ($\lambda_{\text{max}} = 517 \text{ nm}$) spectral

regions on erythrocyte hemolysis during irradiation (irradiance $I_{\text{LED}} \approx 12 \text{ mW/cm}^2$, exposure time 20 min) without bilirubin, as well as upon sensitization of cells with 171 μM of bilirubin, are shown in Fig. 5. The results are presented in the form of histograms reflecting the change of the optical density of $OD_{\text{ir}}/OD_{\text{un}}$ in the supernatant of erythrocytes suspensions irradiated against unirradiated ones. Data on the effect of sodium azide, a singlet oxygen quencher, on the hemolysis process sensitized by bilirubin are also shown in Fig. 5. The data are presented as an arithmetic mean for four independent experiments.

From the data presented in Table 1 and Fig. 5, it follows that the most pronounced photodamage of erythrocytes, controlled by the release of hemoglobin, is observed upon exposure to light in the blue and green spectral regions without adding bilirubin (bars “no Br”). So, already 40 min after irradiation of erythrocyte suspensions without bilirubin with blue light, $OD_{\text{ir}}/OD_{\text{un}} = 1.41 \pm 0.30$, and when exposed to green radiation, $OD_{\text{ir}}/OD_{\text{un}} = 1.57 \pm 0.49$. 18 h after the termination of irradiation and dark storage of erythrocyte suspensions, the corresponding $OD_{\text{ir}}/OD_{\text{un}}$ values are 1.48 ± 0.42 for blue light and 1.34 ± 0.24 for green one. Thus, irradiation of erythrocytes without bilirubin accelerates the release of hemoglobin from erythrocytes by an average of 40–50% relative to the corresponding unirradiated suspensions.

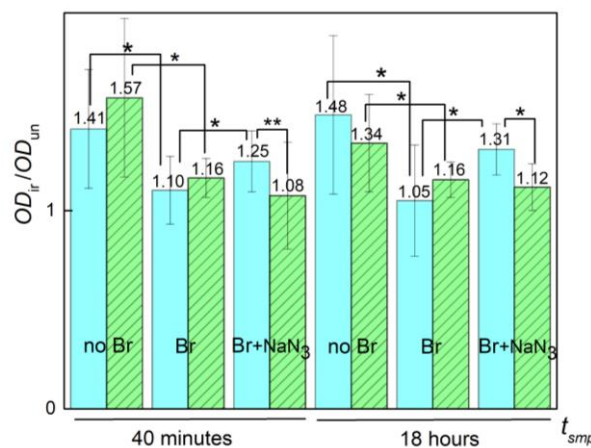


Fig. 5 Change of the optical density $OD_{\text{ir}}/OD_{\text{un}}$ in supernatant (of the irradiated and unirradiated suspensions) after exposure to blue LED ($\lambda_{\text{max}} = 463 \text{ nm}$, blue bars) or green LED ($\lambda_{\text{max}} = 517 \text{ nm}$, green bars) at irradiance $I_{\text{LED}} \approx 12 \text{ mW/cm}^2$ for 20 min to an erythrocytes suspension: without bilirubin (no Br), with 171 μM of bilirubin (BR), with 171 μM of bilirubin (BR) and 50 mM of sodium azide (Br + NaN_3), $t_{\text{smp}} = 40 \text{ min}$ and 18 h after the light exposure. Error bars refer to errors of $OD_{\text{ir}}/OD_{\text{un}}$ means. An asterisk * indicates differences in values at the significance level of 0.05; ** – at the significance level of 0.2.

Table 1 Optical density at the maximum of the absorption spectrum of supernatant from erythrocyte suspension.

Supernatant from suspensions unirradiated or irradiated by	OD in “no Br” sample		OD in “Br” sample		OD in “Br + NaN ₃ ” sample	
	$t_{\text{smp}} = 40 \text{ min}$	$t_{\text{smp}} = 18 \text{ h}$	$t_{\text{smp}} = 40 \text{ min}$	$t_{\text{smp}} = 18 \text{ h}$	$t_{\text{smp}} = 40 \text{ min}$	$t_{\text{smp}} = 18 \text{ h}$
Unirradiated	0.129 ± 0.045	0.147 ± 0.049	0.152 ± 0.019	0.187 ± 0.026	0.177 ± 0.025	0.229 ± 0.037
Blue LED	0.171 ± 0.046	0.202 ± 0.043	0.168 ± 0.021	0.197 ± 0.033	0.217 ± 0.025	0.298 ± 0.043
Green LED	0.185 ± 0.046	0.187 ± 0.026	0.178 ± 0.024	0.216 ± 0.030	0.185 ± 0.024	0.260 ± 0.051

* OD (Optical density) values are presented as an arithmetic mean $\pm$ error of mean for four independent experiments (with erythrocytes of four donors), t_{smp} (sampling time) is time interval between stopping irradiation and starting to monitor changes in erythrocytes.

During the experiments, it was found out that irradiation of the erythrocyte suspension in the presence of bilirubin not only does not enhance hemolysis, but even slightly reduces it compared to the data of irradiation without bilirubin. Thus, 40 min after the irradiation of erythrocytes sensitized by bilirubin with blue light, the average value of $OD_{\text{ir}}/OD_{\text{un}} = 1.10 \pm 0.17$, and with green light – the average value of $OD_{\text{ir}}/OD_{\text{un}} = 1.16 \pm 0.10$. In 18 h, the value of the blue light-photoinduced effect decreases slightly to $OD_{\text{ir}}/OD_{\text{un}} = 1.05 \pm 0.28$. That is, the hemoglobin release from the erythrocytes irradiated in the presence of bilirubin, exceeds the corresponding value for unirradiated cells on average by 5–15%, which is significantly lower than for irradiation of erythrocytes without bilirubin (see above). Thus, the presence of 171 μM of bilirubin in the suspension and its subsequent binding to erythrocytes significantly (by ~ 20 –40%) increases the resistance of cells to hemolysis initiated *in vitro* by the light exposure of blue-green spectral region. It should be noted that the mean $OD_{\text{ir}}/OD_{\text{un}}$ values of the samples without bilirubin and those with bilirubin differ statistically significantly (according to the Kramer-Welch test applied to small independent samples) both under blue and green LEDs irradiation.

The performed studies also demonstrated (Table 1 and Fig. 5) that the addition of sodium azide to the erythrocyte suspension, at least does not block the photodamage of erythrocyte membranes initiated by exposure to light of the blue and green spectral regions in the presence of bilirubin. Moreover, when exposed to blue light in the presence of bilirubin and azide, the damaging effect exceeds the effect observed in the absence of azide (Fig. 5, blue bars “Br + NaN₃” versus “Br”, $t_{\text{smp}} = 40 \text{ min}$, 18 h): when analyzed 40 min after irradiation, the corresponding values of $OD_{\text{ir}}/OD_{\text{un}}$ are 1.25 and 1.10, and in 18 h of incubation $OD_{\text{ir}}/OD_{\text{un}} = 1.31$ and 1.05, respectively. The given measures are statistically significantly different (with the significance level of 0.05).

In contrast to azide influence to the effects induced with blue light, green light irradiation of the erythrocytes suspension with bilirubin in the presence of azide leads to the decrease in hemoglobin release compared to similar samples without azide (Fig. 5, green bars “Br” versus “Br + NaN₃”, $t_{\text{smp}} = 40 \text{ min}$, 18 h). In this case, when analyzed 40 min after irradiation, the corresponding average values are: for samples with bilirubin $OD_{\text{ir}}/OD_{\text{un}} = 1.16$ and for samples with bilirubin and azide it is 1.08, and in 18 h $OD_{\text{ir}}/OD_{\text{un}} = 1.16$ and 1.12. Thus, the addition of NaN₃ before irradiation of bilirubin-containing erythrocytes with green light (contrary to exposure with blue light) somewhat reduces the release of hemoglobin through the cell membrane, which may serve as confirmation of the partial participation of singlet oxygen in the photochemical process.

5 Discussion

5.1 Effect of LEDs Radiation on Erythrocytes in the Absence of Exogenous Bilirubin

The performed study has shown that the effect of radiation from LED sources of blue ($\lambda_{\text{max}} = 463 \text{ nm}$) or green ($\lambda_{\text{max}} = 517 \text{ nm}$) spectral regions at irradiance of $I_{\text{LED}} \approx 12 \text{ mW/cm}^2$ for 20 min (energy dose $D = 14.4 \text{ J/cm}^2$) on the suspension of human erythrocytes without the addition of bilirubin practically does not affect the permeability of cell membranes for potassium ions (Fig. 3). The photoinduced effect assessed by this test, is practically absent when it is monitored both 40 min and 18 h after the irradiation. The results obtained correspond to the literature data, which studied the effect of blue light from special fluorescent lamps (with a maximum in the emission spectrum at 450–460 nm) on the potassium ions leakage from erythrocytes [31], and Na⁺ ions leakage from the sealed erythrocyte ghosts [32]. Therefore, it can be stated that upon exposure to blue light, no endogenous compounds sensitize damage to the molecules that control the release of potassium ions from

erythrocytes (for example, the enzyme Na/K-ATPase, providing an asymmetric distribution of sodium and potassium ions on both sides of the erythrocyte membrane). Another possible explanation for this result is that the antioxidant system of the erythrocyte prevents possible damage to the molecular structures of membranes responsible for permeability to potassium ions [49]. According to Ref. [49], “Under intensive photodynamic action resulting in cell lysis directly on lighting main defence role plays membrane bound α -tocopherol; under erythrocyte hemolysis in dark after shot photodynamic action – decisive meaning has cytoplasm glutathione”; and in general, the nature and mechanisms of the mutually coordinated functioning of these and other antioxidants present in the cell depend on the intensity and site of application of the oxidative action.

A different situation is realized when analyzing the hemoglobin molecules release from erythrocytes under irradiation with the same parameters. According to the data obtained (Fig. 5), irradiation of the erythrocyte suspension without bilirubin accelerates the release of hemoglobin by an average of 45% relative to the corresponding unirradiated suspensions. At the same time, a significant excess of the optical density of the supernatant from the irradiated samples over the control variant is observed both when assessing hemoglobin concentrations 40 min and 18 h after the termination of irradiation. It is characteristic that, 40 min after the termination of the irradiation, a more pronounced effect is observed upon exposure to light in green spectral region: mean value $OD_{ir}/OD_{un} = 1.57$, while after irradiation with blue light, mean value $OD_{ir}/OD_{un} = 1.41$. However, 18 h after the irradiation and dark storage of erythrocyte suspensions, the corresponding mean values OD_{ir}/OD_{un} are 1.48 for blue light and 1.34 for green one. That is, the magnitude of the effect (in relation to the control unirradiated samples) upon exposure to blue light on the second day, at least, does not decrease, and, upon exposure to green light, it decreases by $\sim 20\%$.

Based on the data obtained, it can be assumed that damage to erythrocyte membranes upon exposure to blue-green light in the absence of exogenous bilirubin is due to photochemical reactions sensitized by endogenous compounds. At the same time, it is well known that hemoglobin is not a photosensitizer and does not generate singlet oxygen upon exposure to light [50]. In our opinion, flavin compounds (riboflavin, flavin mononucleotide, FMN, and flavin adenine dinucleotide, FAD) and porphyrins (protoporphyrin IX, PPIX and zinc-protoporphyrin IX, ZnPP) can primarily be such endogenous photosensitizers that absorb visible radiation and generate reactive oxygen species (ROS) with high efficiency. The participation of endogenous flavin and porphyrin photosensitizers in regulatory and lethal effects of optical radiation in the visible spectral region was previously established in our laboratory in relation to microbial cells and spermatozoa [51–53]. It is known that flavin and porphyrin photosensitizers are capable of generating singlet oxygen 1O_2 quite efficiently [53, 54].

The quantum yield of 1O_2 generation in an aqueous medium is $\phi_\Delta = 0.54$ for riboflavin, $\phi_\Delta = 0.51$ for FMN, $\phi_\Delta = 0.07$ for FAD, $\phi_\Delta = 0.77$ for the monomeric form of PPIX and $\phi_\Delta = 0.91$ for the monomeric form of ZnPP (in the presence of Triton X-100 in water). Along with singlet oxygen, riboflavin and protoporphyrin IX also sensitizes the formation of other ROS: superoxide radicals, hydrogen peroxide. At the same time, flavin photosensitizers (riboflavin, FMN and FAD) have similar absorption characteristics: their long-wave maximum in the absorption spectrum is located in the region of 445 nm. PPIX and ZnPP in aqueous media are prone to aggregation and the position of the most intense band (Soret band) in their absorption spectrum strongly depends on the pH of the medium, the formation of complexes with macromolecules, etc. As a rule, the Soret band maximum for PPIX and ZnPP is in the region of 380–410 nm. Currently, the presence of porphyrin and flavin photosensitizers in human erythrocytes is beyond doubt: they are recorded by fluorescence, acetone extraction followed by fluorescence analysis, chromatographic method, etc. According to Ref. [55], the concentration of FMN and FAD in adult human erythrocytes is 44 and 469 nM, respectively; riboflavin is present only in trace amounts. As for PPIX, according to Ref. [56], its concentration in the adult healthy human erythrocytes is 0.60–0.75 $\mu\text{g/ml}$ of erythrocytes and can vary significantly at various diseases. It is also known that non-heme protoporphyrins present in a healthy body in the form of approximately 85–95% ZnPP and 5–15% PPIX [57].

We should note that in the presence of several photosensitizers in the system, their relative contribution to the realization of photobiological effect depends on a number of factors: the generation efficiency of ROS, concentration, molar extinction coefficient, localization near the sensitive biologically important biosubstrate, the presence of closely located ROS quenchers, etc. Therefore, it is quite a difficult task to assess the contribution of a particular photoacceptor to the total photobiological effect, taking into account the overlap of the absorption spectra of potential endogenous photosensitizers, as well as the multiplicity of influencing factors. At the same time, certain conclusions can be made from the analysis of the absorption spectra of photosensitizers. As an example, Fig. 6 shows the absorption spectra of monomeric forms of potential endogenous photosensitizers: FMN in the PBS (curve 1), PPIX (curve 2) and ZnPP (curve 3) in dimethylsulfoxide. The arrows in Fig. 6 indicate the wavelengths of radiation from LED sources affecting the erythrocyte suspension.

As it follows from Fig. 6, LED sources of radiation both with $\lambda_{\text{max}} = 463$ nm and $\lambda_{\text{max}} = 517$ nm is able to excite each of mentioned above endogenous photosensitizers, since it is absorbed by these compounds to one degree or another. Moreover, the radiation of blue spectral region is near the maximum of the long-wave absorption band FMN ($\lambda_{\text{max}} = 447$ nm), while the radiation of green spectral region falls on the long-wavelength slope of the absorption spectrum of the specified flavin.

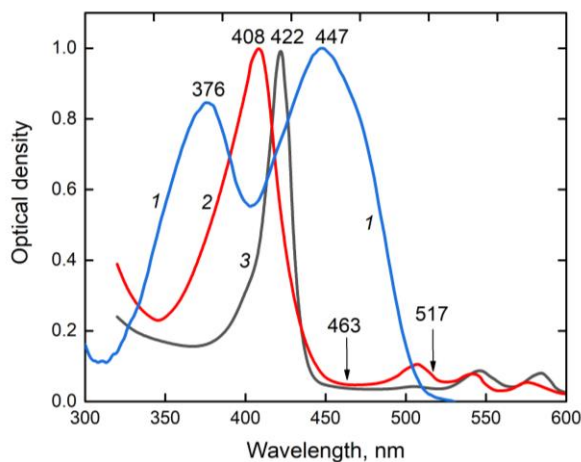


Fig. 6 Normalized absorption spectra of endogenous photosensitizers: 1 – FMN in PBS, 2 and 3 – PPIX and Zn-PPIX in dimethylsulfoxide, respectively. The concentration in solutions was 1 μ M. Arrows indicate the radiation wavelengths of LED sources.

As for porphyrin photosensitizers, the radiation with both $\lambda_{\text{max}} = 463$ nm and $\lambda_{\text{max}} = 517$ nm corresponds to the minimum absorption of ZnPP, characterized by an intense Soret band ($S_2 \leftarrow S_0$ – transition) with maximum for the monomeric form in the region $\lambda_{\text{max}} = 422$ nm ($B(0,0)$) and two Q-bands ($S_1 \leftarrow S_0$ – transition) with maxima at $\lambda_{\text{max}} = 547$ nm ($Q(1,0)$) and $\lambda_{\text{max}} = 584$ nm ($Q(0,0)$). Light absorption by PPIX is due to the presence of an intense Soret band ($S_2 \leftarrow S_0$ – transition) in the spectrum with maximum for the monomeric form in the region of $\lambda_{\text{max}} = 408$ nm ($B(0,0)$) and four Q-bands ($S_1 \leftarrow S_0$ – transition) with maxima at $\lambda_{\text{max}} = 507$ nm ($Q_y(1,0)$), $\lambda_{\text{max}} = 543$ nm ($Q_y(0,0)$), $\lambda_{\text{max}} = 575$ nm ($Q_x(1,0)$) $\lambda_{\text{max}} = 630$ nm ($Q_x(0,0)$). As it can be seen from Fig. 6, the radiation of green spectral region with $\lambda_{\text{max}} = 517$ nm is absorbed by protoporphyrin IX somewhat more efficiently than the radiation of blue region with $\lambda_{\text{max}} = 463$ nm.

Analysis of the abovementioned spectral characteristics of endogenous photosensitizers allows us to conclude that if the light-induced release of hemoglobin molecules from the erythrocytes was stipulated by sensitizing effect of flavins, then the observed photobiological effect in the absence of exogenous bilirubin (Fig. 5) should be much more pronounced when the erythrocyte suspension is exposed to light of blue spectral region, since radiation with a wavelength of $\lambda_{\text{max}} = 463$ nm is absorbed approximately 50 times more efficiently than radiation with $\lambda_{\text{max}} = 517$ nm. At the same time, according to Fig. 5, when controlling the hemoglobin molecules release 40 min after the erythrocyte suspension irradiation in the absence of bilirubin, a more pronounced effect is observed when exposed to green region radiation. And although the situation slightly changes when monitoring the process 18 h after the irradiation (Fig. 5), the data presented cast doubt on the leading role of flavins in sensitizing photodamage to the erythrocyte membrane

when exposed to radiation in blue-green spectrum region. At the same time, it is not possible to make a choice in favor of ZnPP or PPIX due to their different concentrations in the erythrocytes [56, 57], as well as overlapping absorption spectra.

Thus, we have reasons to believe that the excitation of endogenous porphyrin and flavin photosensitizers localized in erythrocytes, when exposed to radiation from blue-green spectral region and the subsequent generation of ROS (primarily singlet oxygen) by them can cause photodamage of the cell membrane, initiating the release of hemoglobin molecules – photohemolysis. The ability of optical radiation of blue spectral region ($\lambda_{\text{max}} = 405$ nm, $I = 30$ mW/cm², energy dose – 72 J/cm²) to affect the erythrocyte suspension in the absence of exogenous photosensitizers is also evidenced by the results of studies [58], in which a significant decrease (by 0.44%) in erythrocytes volume as a result of their irradiation has been determined.

It should be noted that the noticeable effect of blue light from LED sources on the release of hemoglobin from erythrocytes during their irradiation of suspension without bilirubin does not agree with data [31, 34], according to which, irradiation of the erythrocytes suspension with the light of blue fluorescent lamp almost does not lead to an acceleration of hemolysis compared to control unirradiated samples (in the stated studies, control of hemoglobin concentrations in the supernatant was also carried out 18 h after the irradiation). At the same time, the energy parameters of the exposed radiation in our studies and in Ref. [34] ($\lambda_{\text{max}} = 450$ nm, $I \approx 8$ mW/cm², dose – 14 J/cm²) were also quite close. In our opinion, a possible reason for the observed differences in the results is the different methods of hemolysis control: in studies [34], it was initiated osmotically by adding a suspension of irradiated or non-irradiated erythrocytes into NaCl solution of low (0.35–0.45%) concentration (hypotonic solution). Besides, in study [34], not free bilirubin but its complex with albumin (ratio bilirubin/albumin = 0.8) was added into the erythrocyte suspension. The absence of data about the light energy parameters in Ref. [31] does not allow us to determine the reason for the observed differences between our results and data [31] at irradiation of the erythrocytes suspension without bilirubin.

As for green spectrum region radiation, we failed to find in the literature studies on its effect on hemolysis of human erythrocytes *in vitro*. However, in Ref. [59] it was found that against the background of experimental endotoxic shock in the membranes of rat erythrocytes, a more effective increase in lipid oxidation occurs at irradiation *in vivo* with green laser radiation of 532.5 nm (dose – 0.75 J/cm²), than at irradiation with blue of 441.2 nm or red of 632.8 nm laser radiation at the same dose. The authors [59] also believe that this may be an indirect confirmation of the hypothesis about porphyrins as primary photoacceptors in erythrocytes.

5.2 Effect of LEDs Radiation on Erythrocytes in the Presence of Exogenous Bilirubin

As it has been already noted, the study of the sensitizing effect of bilirubin on erythrocyte membranes is of particular interest due to the importance of reducing the effectiveness of this process as a side adverse effect during phototherapy of hyperbilirubinemia in newborns. The performed studies have shown (Table 1) that the interaction of bilirubin at concentration of 171 μM (at which phototherapy of newborns with hyperbilirubinemia syndrome is prescribed) leads to molecular structural changes of the erythrocyte membrane, which is confirmed by the intensification of hemoglobin release through the membrane in dark conditions (without suspension irradiation). Evidence of this is an increase of the optical density in the Soret band maximum of hemoglobin in the supernatant both 40 min and 18 h after incubation of erythrocytes with bilirubin in comparison with cells that do not contain pigment. The effects registered by us when bilirubin binds to the erythrocyte membrane are in accordance with studies [32, 44]. According to these results, the damage of erythrocyte membranes resulting from cross-linking of membrane proteins plays a critical role in cell lysis. At the same time, peroxidation of unsaturated membrane lipids, which occurs during irradiation, also disturbs the membrane structure. However, protein modification, rather than peroxidation of unsaturated membrane lipids, is the determining factor in the initiation of cell lysis (in the photohemolytic mechanism) [44].

It could be expected that the effect of blue light corresponding to the absorption band of bilirubin on cells loaded with this pigment would contribute to their accelerated destruction due to the photodynamic effect. Moreover, the ability of bilirubin and its photoproducts to sensitize photodamage of erythrocyte membranes has been repeatedly noted in the literature [31, 32, 34, 44]. However, our studies have shown that irradiation of the erythrocyte suspension in the presence of 171 μM of bilirubin does not enhance hemolysis, moreover slightly reduces it compared to the data of irradiation without bilirubin.

The analysis 40 min after irradiation with blue light has shown that the excess of the optical density of the supernatant over the control samples was, on average, 40% in the absence of bilirubin and 10% in the case of sensitization with bilirubin; for green light the corresponding values were 57% and 16% (Fig. 5). The analysis of hemolysis 18 h after the irradiation with blue light has shown that the magnitudes of the photohemolytic effect for the erythrocyte suspensions not containing and containing bilirubin are 48 and 5%, respectively, and when exposed to green light – 34% and 16%. Thus, the binding of bilirubin to erythrocytes significantly (by ~ 20 –40%) increases the resistance of cells to hemolysis initiated *in vitro* by exposure to light in blue and green spectral regions compared to the erythrocytes without bilirubin. In our opinion, the protective effect of bilirubin is connected with the

antioxidant properties of bilirubin (and its photoproducts), which is one of the most powerful endogenous antioxidants [60, 61]. Thus, according to Ref. [60], 10 nM of bilirubin is enough to protect cultured cells from the destructive effects of 10,000-fold molar excess of hydrogen peroxide. Since bilirubin is a very weak photosensitizer (the quantum yield of the singlet oxygen formation sensitized by it is 0.01 [3]), then, likely, its antioxidant effect prevails over the sensitizing one in the processes controlling the release of hemoglobin through the erythrocyte membrane.

One of the reasons for the difference between our data and the results of [31, 34], having observed the acceleration of hemolysis due to the sensitizing effect of bilirubin, are different conditions for addition of the pigment to erythrocyte suspension. In studies [31, 34], bilirubin was added as part of a complex with albumin, while in our studies – in phosphate buffered saline. At the same time, according to Ref. [48, 62], the incorporation of bilirubin into biomembranes can be carried out both in the form of a monoanion and in the form of aggregates of molecules, which can affect the sensitizing and antioxidant properties of tetrapyrrol.

Studies have shown that, unlike hemolysis processes, where the antioxidant properties of bilirubin are primarily manifested, when studying the effect of light exposure on the leakage of potassium ions, the sensitizing properties of this pigment are more pronounced (Fig. 3). Moreover, the most significant light-induced structural changes of membranes (estimated by the potassium leakage) in the presence of exogenous bilirubin are observed not immediately after the light exposure of blue or green spectral regions, but 18 h after irradiation. Consequently, for the implementation of photodynamic damage to membranes when they are sensitized by bilirubin, a certain time is required. This conclusion is supported by the data of the authors [63] that the processes of peroxidation of unsaturated membrane lipids of the erythrocyte membranes during their sensitization with tetrapyrroles continue after the irradiation procedure. The authors [64] also point to the reliably registered secondary production of ROS and, above all, hydrogen peroxide hundreds of minutes after the irradiation of various cell types in the presence of a photosensitizer.

Another discovered characteristic feature of the bilirubin-sensitized leakage of potassium ions from erythrocytes is an “abnormal” dependence on the wavelength of the incident radiation. Thus, at monitoring of this process 18 h after the light exposure, potassium leakage from erythrocytes increased on average (compared to control samples for this time period) by 14% when exposed to blue LED light and by 25% when exposed to green LED light. Thus, upon sensitization of erythrocytes with bilirubin, the radiation of green spectral region has a more pronounced effect on the potassium ions leakage, despite the weaker absorption of light in this region by bilirubin localized in erythrocytes (Fig. 1, curve 3). At the same time (as it was already noted), in the absence of bilirubin, the exposure of the erythrocyte

suspension to light of blue or green spectral regions almost does not affect the potassium ions leakage, exceeding the values for control (unirradiated) samples by 2–5% ($[K^+]_{ir}/[K^+]_{un} \approx 1.02\text{--}1.05$). Consequently, the difference in the effect of blue and green light is stipulated by the different efficiency of photochemical processes affecting the potassium ions leakage during photoexcitation of bilirubin or its photoproducts with radiation wavelength of $\lambda_{max} = 463$ nm and $\lambda_{max} = 517$ nm without a noticeable contribution of endogenous photosensitizers. The reasons for observed higher photobiological effect of green light compared to blue one in the case of bilirubin-sensitized erythrocytes require a separate discussion. Literature studies of erythrocytes on this issue are unknown to us. At the same time, there are comparative data in the literature [65] on the survival of bilirubin-sensitized cultured animal cells when exposed to radiation of blue ($\lambda = 457.9$ nm) and green ($\lambda = 514.5$ nm) spectral regions. As in our studies with the potassium ions leakage from erythrocytes, the photobiological effect due to the sensitizing action of bilirubin, estimated with colorimetric MTT assay, in cultured somatic cells is more pronounced when exposed to radiation of green spectral region than blue one. Besides, our data on the photosensitizing effect of bilirubin to the potassium ions leakage from the erythrocytes under the blue radiation are in agreement with the results of studies [31].

In our opinion, the abnormal (based on the absorption spectrum of bilirubin bound to erythrocyte membranes) dependence of the light-induced potassium ions leakage from the erythrocytes on the wavelength of the incident radiation can be explained by several reasons. One of them is that along with bilirubin, its configurational and structural photoisomers can contribute to the sensitization effects, since the ability of bilirubin photoproducts to sensitize (with greater efficiency compared to bilirubin) the damage of biomolecules in solution is well known [23, 26]. At the same time, the quantum yield of photoproducts, as well as the ratio between the concentrations of individual photoproducts, strongly depend on the wavelength of the radiation due to a) bichromophoric nature of the absorption and emission of light by bilirubin molecules; b) presence of intramolecular energy transfer between two unequal chromophores forming a pigment molecule; c) dependence of the efficiency of intramolecular energy transfer of electronic excitation on the radiation wavelength [37–41, 66, 67].

Another reason for the increase in the bilirubin-sensitized potassium ions leakage during the transition from blue irradiation with $\lambda_{max} = 463$ nm to green irradiation with $\lambda_{max} = 517$ nm may be the heterogeneity (heterogeneous nature) of the microenvironment of sensitizer molecules in the erythrocyte membrane. This is indicated by the broadened absorption spectrum of bilirubin embedded in the erythrocyte membrane (Fig. 1, curve 3), as well as a strong dependence of the position of the fluorescence excitation spectrum of bilirubin bound to human erythrocyte ghosts on the registration

wavelength, found in Ref. [68]. In this regard, there are reasons to believe that radiation of different wavelengths can excite bilirubin localized in different sites/regions of the erythrocyte membrane and initiate various photochemical processes (lipid peroxidation, cross-linking of proteins and damage to protein-lipid contacts in the membrane, inactivation of the Na/K-ATPase enzyme, etc.).

5.3 Involvement of ROS in Bilirubin-Sensitized Photodamage of Erythrocyte Membranes

Another characteristic feature of photochemical processes in the erythrocyte membranes initiated by exposure to light in the presence of exogenous bilirubin should be noted. It consists in a weakly pronounced effect of sodium azide, a well-known singlet oxygen quencher, on the light-induced leakage of both potassium ions (Fig. 3) and hemoglobin molecules (Fig. 5).

In our opinion, the reasons for a weak effect of this singlet oxygen quencher on photochemical processes in erythrocytes during their bilirubin – sensitization may be the following: a) low availability of singlet oxygen generation sites in the membrane for sodium azide; b) participation in damage to erythrocyte membranes of other ROS and free radicals, for which sodium azide does not act as a quencher. Moreover, unlike singlet oxygen, other reactive oxygen species (primarily hydrogen peroxide) can play a main role in the membrane damage already after the light exposure [64]. Since the lifetime of singlet oxygen in an aqueous medium is about 3 μ s, its damaging effect on the erythrocyte membrane actually stops immediately after the irradiation stops, while chemical reactions mediated by hydrogen peroxide continue for a long time [63, 64]. Another reason for the weakly expressed effect of azide on the photobiological processes, estimated by the potassium ions or hemoglobin molecules leakage, may be the ability of azide ions to interact with heme iron [69], which actually reduces the concentration of free (unbound) intracellular azide capable to quench singlet oxygen generated by a photosensitizer. The spectral data obtained (Fig. 4, Table 1) also allow us to confirm the existence of such an interaction. This is manifested in a higher optical density of the supernatants (in NaCl-isotonic solution) from unirradiated erythrocytes suspensions with bilirubin and azide (curve 4 in Fig. 4) compared to the corresponding data for the erythrocyte suspension with bilirubin without NaN_3 (curve 1 in Fig. 4). Thus, for the studied biological system, there is a pronounced interaction of sodium azide with the erythrocyte membrane and erythrocyte hemoglobin under dark conditions, which can affect the photochemical and subsequent chemical reactions in cells [70].

Attention is also drawn to the seemingly abnormal effect of sodium azide on blue-light-induced release of hemoglobin molecules from the bilirubin-sensitized erythrocytes (Table 1 and Fig. 5). According to the experimental data obtained, when exposed to blue light in the presence of bilirubin and azide, the damaging

effect on membranes, estimated by the release of hemoglobin molecules from cells, exceeds the effect observed in the absence of azide (Fig. 5, blue bars “Br+NaN₃” versus “Br”). That is, the addition of the known singlet oxygen quencher sodium azide to the suspension of cells not only does not reduce the magnitude of the photobiological effect, but also increases it. In this regard, it should be noted that an increase in the photobiological effect due to the addition of sodium azide for the first time was noted in Refs. [71, 72] during the sensitization of microbial cells with methylene blue. The authors [71, 72] have shown that this effect is stipulated by the formation of azide radicals (having a high reactivity) due to electron transfer from the excited form of the sensitizer. According to Refs. [71, 72], the decrease or increase in the efficiency of photochemical reactions due to the addition of azide largely depends on the contribution to the total process of reactions of type II (occurring with the participation of singlet oxygen) or type I (due to radical processes).

Typically, that the addition of NaN₃ before irradiation of bilirubin-containing erythrocytes with green light (in contrast to exposure to blue light) slightly reduces the release of hemoglobin through the cell membrane, which may be a confirmation of the partial participation of singlet oxygen in the photochemical process. On the other hand, the multidirectional action of azide on hemolytic effects initiated in erythrocyte membranes by light of the same intensity in blue and green spectral regions indicates the different nature of the photochemical processes underlying them. In this regard, we can expect a significant role of bilirubin photoisomers in the effects of sensitized damage of erythrocyte membranes. Moreover, the contribution of various configurational and structural isomers of bilirubin to the total light absorption may differ markedly when exposed to light with wavelengths of $\lambda_{\max} = 463$ nm and $\lambda_{\max} = 517$ nm. The reasons for this are the different absorption spectra of bilirubin photoisomers [23], as well as the dependence of the quantum yield of the bilirubin photoisomer formation on the wavelength of the incident radiation [37–41, 65, 66].

6 Conclusions

The effect of radiation from LED sources of blue ($\lambda_{\max} = 463$ nm) or green ($\lambda_{\max} = 517$ nm) spectral regions at irradiance of $I = 12$ mW/cm² for 20 min (energy dose $D = 14.4$ J / cm²) on the human erythrocyte suspension in the phosphate buffered saline leads to erythrocyte membrane damages, which is confirmed by an increase in the optical density of supernatant in the Soret band of hemoglobin. The values of photohemolytic effects are approximately equal when exposed to radiation from blue and green spectral regions.

Molecular-structural changes in erythrocyte membranes when exposed to light of the mentioned above parameters are most likely stipulated by the excitation of endogenous porphyrin (protoporphyrin IX, zinc-protoporphyrin) and flavin (flavin mononucleotide,

flavinadenin dinucleotide) photosensitizers contained in the erythrocytes in low concentrations, but characterized by high efficiency of generation of reactive oxygen species. The leading role of porphyrin compounds in the effects of sensitization is substantiated.

The effect of radiation of the same parameters on the erythrocyte suspension almost does not influence the permeability of cell membranes for potassium ions. The difference in the effect of blue-green radiation on the release of hemoglobin molecules from erythrocytes and ion transport through the membrane is consistent with the known information about the spatial separation of these processes in the membrane and the difference in the mechanisms mediating them.

Addition of exogenous bilirubin to the erythrocyte suspension at concentration of 171 μ M, at which phototherapy is prescribed for newborns with hyperbilirubinemia syndrome, accelerates spontaneous (dark) hemolysis, but reduces the light-induced hemoglobin release relative to cells that do not contain bilirubin.

Bilirubin, along with weak sensitizing properties, has antioxidant functions, reducing the release of hemoglobin molecules from the erythrocytes initiated by photochemical reactions in the membrane due to the excitation of endogenous porphyrin and flavin photosensitizers localized in these cells.

Bilirubin-sensitized leakage of potassium ions is more pronounced upon exposure to radiation of green spectral region, despite the weaker absorption of light in this region by bilirubin localized in erythrocytes. The reason for the abnormal dependence of the light-induced leakage of potassium ions from the erythrocytes on the wavelength of the incident radiation may be the participation of bilirubin photoisomers in sensitization processes, the quantum yield of which depends on the exciting radiation wavelength. Another reason for the increase in the potassium ions leakage during the transition from blue to green radiation may be the heterogeneity of the microenvironment of bilirubin molecules in the erythrocyte membrane. As a result, radiation of different wavelengths can excite bilirubin localized in different membrane sites and initiate different destructive processes in the membrane.

Photochemical destructive processes in the erythrocyte membrane, initiated due to the sensitizing effect of bilirubin and its photoisomers, proceed with insignificant participation of singlet oxygen, which is confirmed by a slight decrease in the leakage of potassium ions and hemoglobin molecules when the ¹O₂ quencher, sodium azide, is added to the suspension before irradiation.

Reliably recorded damages of the bilirubin-sensitized erythrocyte membranes upon exposure to radiation of blue-green spectral region indicates the importance of optimizing the parameters of phototherapy of hyperbilirubinemia of newborns in order to reduce effectively the level of bilirubin and ensure minimal possible adverse side effects. The presence of the damaging effect of blue-green light on erythrocyte

membranes should also be taken into account at the development of photobiomodulation methods (including those based on intravenous exposure to blue light) and photodecontamination based on the inactivation of microorganisms due to excitation of endogenous photosensitizers.

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References

1. T. Hansen, M. Maisels, F. Ebbesen, H. Vreman, D. Stevenson, R. Wong, and V. Bhutani, “Sixty years of phototherapy for neonatal jaundice—from serendipitous observation to standardized treatment and rescue for millions,” *Journal of Perinatology* 40(2), 180–193 (2020).
2. A. Lamola, “A pharmacologic view of phototherapy,” *Clinics in Perinatology* 43(2), 259–276 (2016).
3. V. Plavskii, A. Tret'yakova, and G. Mostovnikova, “Phototherapeutic systems for the treatment of hyperbilirubinemia of newborns,” *Journal of Optical Technology* 81(6), 341–348 (2014).
4. S. Itoh, H. Okada, T. Kuboi, and T. Kusaka, “Phototherapy for neonatal hyperbilirubinemia,” *Pediatrics International* 59(9), 959–966 (2017).
5. V. Bhutani, Committee on Fetus and Newborn, “Phototherapy to prevent severe neonatal hyperbilirubinemia in the newborn infant 35 or more weeks of gestation,” *Pediatrics* 128(4), 1046–1052 (2011).
6. D. Seidman, J. Moise, Z. Ergaz, A. Laor, H. Vreman, D. Stevenson, and R. Gale, “A prospective randomized controlled study of phototherapy using blue and blue-green light-emitting devices, and conventional halogen-quartz phototherapy,” *Journal of Perinatology* 23(2), 123–127 (2003).
7. F. Ebbesen, P. Vandborg, P. Madsen, T. Trydal, L. Jakobsen, and H. Vreman, “Effect of phototherapy with turquoise vs. blue LED light of equal irradiance in jaundiced neonates,” *Pediatric Research* 79(2), 308–312 (2016).
8. V. Plavskii, A. Mikulich, I. Leusenko, A. Tret'yakova, L. Plavskaya, N. Serdyuchenko, J. Gao, D. Xiong, and X. Wu, “Spectral range optimization to enhance the effectiveness of phototherapy for neonatal hyperbilirubinemia,” *Journal of Applied Spectroscopy* 84(1), 92–102 (2017).
9. T. Kuboi, T. Kusaka, H. Okada, M. Arioka, K. Nii, M. Takahashi, S. Yamato, T. Sadamura, A. Nakano, and S. Itoh, “Green light-emitting diode phototherapy for neonatal hyperbilirubinemia: Randomized controlled trial,” *Pediatrics International* 61(5), 465–470 (2019).
10. S. Kato, O. Iwata, Y. Yamada, H. Kakita, T. Yamada, H. Nakashima, T. Sugiura, S. Suzuki, and H. Togari, “Standardization of phototherapy for neonatal hyperbilirubinemia using multiple-wavelength irradiance integration,” *Pediatrics & Neonatology* 61(1), 100–105 (2020).
11. F. Ebbesen, P. Vandborg, and M. Donneborg, “The effectiveness of phototherapy using blue-green light for neonatal hyperbilirubinemia – Danish clinical trials,” *Seminars in Perinatology* 45(1), 151358 (2021).
12. F. Ebbesen, M. Rodrigo-Domingo, A. Moeller, H. Vreman, and M. Donneborg, “Effect of blue LED phototherapy centered at 478 nm versus 459 nm in hyperbilirubinemic neonates: a randomized study,” *Pediatric Research* 89(3), 598–603 (2021).
13. B. Morris, W. Oh, J. Tyson, D. Stevenson, D. Phelps, and T. O'Shea, “Aggressive vs. conservative phototherapy for infants with extremely low birth weight,” *New England Journal of Medicine* 359(18), 1885–1896 (2008).
14. J. Tyson, C. Pedroza, J. Langer, C. Green, B. Morris, and D. Stevenson, “Does aggressive phototherapy increase mortality while decreasing profound impairment among the smallest and sickest newborns?” *Journal of Perinatology* 32(9), 677–684 (2012).
15. C. Arnold, C. Pedroza, and J. E. Tyson, “Phototherapy in ELBW newborns: does it work? Is it safe? The evidence from randomized clinical trials,” *Seminars in Perinatology* 38(7), 452–464 (2014).
16. S. Yahia, A. E. Shabaan, M. Gouida, D. El-Ghanam, H. Eldegl, A. El-Bakary, and H. Abdel-Hady, “Influence of hyperbilirubinemia and phototherapy on markers of genotoxicity and apoptosis in full-term infants,” *European Journal of Pediatrics* 174(4), 459–464 (2015).
17. N. Auger, C. Laverdière, A. Ayoub, E. Lo, and T. M. Luu, “Neonatal phototherapy and future risk of childhood cancer,” *International Journal of Cancer* 145(8), 2061–2069 (2019).
18. J. Oláh, E. Tóth-Molnár, L. Kemény, and Z. Csoma, “Long-term hazards of neonatal blue-light phototherapy,” *British Journal of Dermatology* 169(2), 243–249 (2013).
19. N. Ramy, E. A. Ghany, W. Alsharany, A. Nada, R. K. Darwish, W. A. Rabie, and H. Aly, “Jaundice, phototherapy and DNA damage in full-term neonates,” *Journal of Perinatology* 36(2), 132–136 (2016).

20. T. Newman, Y. Wu, M. Kuzniewicz, B. Grimes, and C. McCulloch, “[Childhood seizures after phototherapy](#),” *Pediatrics* 142(4), e20180648 (2018).
21. T. Xiong, Y. Qu, S. Cambier, and D. Mu, “[The side effects of phototherapy for neonatal jaundice: what do we know? What should we do?](#)” *European Journal of Pediatrics* 170(10), 1247–1255 (2011).
22. J. Wang, G. Guo, A. Li, W. Q. Cai, and X. Wang, “[Challenges of phototherapy for neonatal hyperbilirubinemia](#),” *Experimental and Therapeutic Medicine* 21(3), 231 (2021).
23. V. Plavskii, V. Mostovnikov, A. Tret'yakova, and G. Mostovnikova, “[Sensitizing effect of Z, Z-bilirubin IX \$\alpha\$ and its photoproducts on enzymes in model solutions](#),” *Journal of Applied Spectroscopy* 75(3), 407–419 (2008).
24. G. Wondrak, M. Jacobson, and E. Jacobson, “[Endogenous UVA-photosensitizers: mediators of skin photodamage and novel targets for skin photoprotection](#),” *Photochemical & Photobiological Sciences* 5, 215–237 (2006).
25. B. Rosenstein, J. Ducore, and S. Cummings, “[The mechanism of bilirubin-photosensitized DNA strand breakage in human cells exposed to phototherapy light](#),” *Mutation Research/DNA Repair Reports* 112(6), 397–406 (1983).
26. F. Bohm, F. Drygalla, P. Charlesworth, K. Bohm, T. Truscott, and K. Jokiel, “[Bilirubin phototoxicity to human cells by green light phototherapy in vitro](#),” *Photochemistry and Photobiology* 62(6), 980–983 (1995).
27. O. Kozlenkova, L. Plavskaya, A. Mikulich, I. Leusenko, A. Tret'yakova, and V. Plavskii, “[Photodamage of the cells in culture sensitized with bilirubin](#),” *Journal of Physics: Conference Series* 741(1), 012063 (2016).
28. E. Bruzell Roll, T. Christensen, “[Formation of photoproducts and cytotoxicity of bilirubin irradiated with turquoise and blue phototherapy light](#),” *Acta Paediatrica* 94(10), 1448–1454 (2005).
29. T. Christensen, G. Kinn, T. Granli, and I. Amundsen, “[Cells, bilirubin and light: formation of bilirubin photoproducts and cellular damage at defined wavelengths](#),” *Acta Paediatrica* 83(1), 7–12 (1994).
30. T. Christensen, E. B. Roll, A. Jaworska, and G. Kinn, “[Bilirubin and light induced cell death in a murine lymphoma cell line](#),” *Journal of Photochemistry and Photobiology B: Biology* 58(2–3), 170–174 (2000).
31. G. Odell, R. Brown, and A. Kopelman, “[The photodynamic action of bilirubin on erythrocytes](#),” *The Journal of Pediatrics* 81(3), 473–483 (1972).
32. M. Deziel, A. Girotti, “[Bilirubin photosensitized lysis of resealed erythrocyte membranes](#),” *Photochemistry and Photobiology* 31(6), 593–596 (1980).
33. M. Tozzi-Ciancarelli, G. Amicosante, A. Menichelli, S. Di Giulio, and D. Del Principe, “[Photodynamic damage induced by bilirubin on human platelets: possible relevance to newborn pathology](#),” *Neonatology* 48(6), 336–340 (1985).
34. E. Bruzell Roll, T. Christensen, and O. Gederaas, “[Effects of bilirubin and phototherapy on osmotic fragility and haematoporphyrin-induced photohaemolysis of normal erythrocytes and spherocytes](#),” *Acta Paediatrica* 94(10), 1443–1447 (2005).
35. K. Nii, H. Okada, S. Itoh, and T. Kusaka, “[Characteristics of bilirubin photochemical changes under green light-emitting diodes in humans compared with animal species](#),” *Scientific Reports* 11(1), 6391 (2021).
36. V. Plavskii, “[Current State and Prospects for Development of Systems for Photodynamic Therapy of Neonatal Hyperbilirubinemia](#),” *Biomedical Engineering* 47, 91–95 (2013).
37. A. McDonagh, G. Agati, F. Fusi, and R. Pratesi, “[Quantum yields for laser photocyclization of bilirubin in the presence of human serum albumin. Dependence of quantum yield on excitation wavelength](#),” *Photochemistry and Photobiology* 50(3), 305–319 (1989).
38. J. Ennever, T. Dresing, “[Quantum yields for the cyclization and configurational isomerization of 4E,15Z-bilirubin](#),” *Photochemistry and Photobiology* 53(1), 25–32 (1991).
39. S. Onishi, S. Itoh, and K. Isobe, “[Wavelength-dependence of the relative rate constants for the main geometric and structural photoisomerization of bilirubin IX \$\alpha\$ bound to human serum albumin: demonstration of green light at 510 nm as the most effective wavelength in photochemical changes from \(ZZ\)-bilirubin IX \$\alpha\$ to \(EZ\)-cyclobilirubin IX \$\alpha\$ via \(EZ\)-bilirubin](#),” *Biochemical Journal* 236(1), 23–29 (1986).
40. V. Plavskii, V. Mostovnikov, G. Mostovnikova, and A. Tret'yakova, “[Spectral fluorescence and polarization characteristics of Z, Z-bilirubin IX \$\alpha\$](#) ,” *Journal of Applied Spectroscopy* 74(1), 120–132 (2007).
41. V. Plavskii, V. Mostovnikov, A. Tret'yakova, and G. Mostovnikova, “[Photophysical processes that determine the photoisomerization selectivity of Z, Z-bilirubin IX \$\alpha\$ in complexes with albumins](#),” *Journal of Optical Technology* 74(7), 446–454 (2007).
42. H. Sato, S. Aono, R. Semba, and S. Kashiwamata, “[Interaction of bilirubin with human erythrocyte membranes. Bilirubin binding to neuraminidase-and phospholipase-treated membranes](#),” *Biochemical Journal* 248(1), 21–26 (1987).
43. H. Sato, S. Kashiwamata, “[Interaction of bilirubin with human erythrocyte membranes](#),” *Biochemical Journal* 210(2), 489–496 (1983).
44. A. Girotti, “[Bilirubin-photosensitized crosslinking of polypeptides in the isolated membrane of the human erythrocyte](#),” *Journal of Biological Chemistry* 253(20), 7186–7193 (1978).
45. J. Kruchenok, A. Sobchuk, G. Kurilo, N. Nemkovich, and A. Rubinov, “[Study of interference laser field action on erythrocyte membranes by fluorescent probe with intramolecular proton transfer](#),” *Proceedings of SPIE* 6257, 62570W (2006).

46. S. Bondarchuk, I. Bondarchuk, [Statistical processing of experimental data in MS Excel: tutorial](#), Tomsk State Pedagogical University Publishing, Tomsk (2018). [in Russian]
47. A. Lamola, J. Esinger, W. Blumberg, S. Patel, and J. Flore, [“Fluorometric study of the partition of bilirubin among blood components: basis for rapid microassays of bilirubin and bilirubin binding capacity in whole blood,”](#) *Analytical Biochemistry* 100(1), 25–42 (1979).
48. J. Vazquez, M. Garcia-Calvo, F. Valdivieso, F. Mayor, and Jr. F. Mayor, [“Interaction of bilirubin with the synaptosomal plasma membrane,”](#) *Journal of Biological Chemistry* 263(3), 1255–1265 (1988).
49. T. Vadetskaya, N. Shukanova, and A. Vorobej, [“Role of \$\alpha\$ -tocopherol and glutathione in membrane defense under photodynamic action on erythrocytes,”](#) *Doklady Nacional’noj akademii nauk Belarusi* 42, 101–103 (1998). [in Russian]
50. S. V. Lepeshkevich, A. S. Stasheuski, M. V. Parkhats, V. A. Galievsky, and B. M. Dzhagarov, [“Does photodissociation of molecular oxygen from myoglobin and hemoglobin yield singlet oxygen?”](#) *Journal of Photochemistry and Photobiology B: Biology* 120, 130–141 (2013).
51. V. Plavskii, A. Mikulich, A. Tretyakova, I. Leusenka, L. Plavskaya, O. Kazyuchits, I. Dobysh, and T. Krasnenkova, [“Porphyrins and flavins as endogenous acceptors of optical radiation of blue spectral region determining photoinactivation of microbial cells,”](#) *Journal of Photochemistry and Photobiology B: Biology* 183, 172–183 (2018).
52. V. Plavskii, A. Mikulich, N. Barulin, T. Ananich, L. Plavskaya, A. Tretyakova, and I. Leusenka, [“Comparative effect of low-intensity laser radiation in green and red spectral regions on functional characteristics of sturgeon sperm,”](#) *Photochemistry and Photobiology* 96(6), 1294–1313 (2020).
53. V. Plavskii, N. Barulin, A. Mikulich, A. Tretyakova, T. Ananich, L. Plavskaya, I. Leusenka, A. Sobchuk, V. Sysov, O. Dudinova, A. Vodchits, I. Khodasevich, and V. Orlovich, [“Effect of continuous wave, quasi-continuous wave and pulsed laser radiation on functional characteristics of fish spermatozoa,”](#) *Journal of Photochemistry and Photobiology B: Biology* 216, 112112 (2021).
54. J. Fernandez, M. Bilgin, and L. Grossweiner, [“Singlet oxygen generation by photodynamic agents,”](#) *Journal of Photochemistry and Photobiology B: Biology* 37(1–2), 131–140 (1997).
55. S. Hustad, M. McKinley, H. McNulty, J. Schneede, J. Strain, J. Scott, and P. Ueland, [“Riboflavin, flavin mononucleotide, and flavin adenine dinucleotide in human plasma and erythrocytes at baseline and after low-dose riboflavin supplementation,”](#) *Clinical Chemistry* 48(9), 1571–1577 (2002).
56. W. Marsh Jr, D. Nelson, and H. Koenig, [“Free erythrocyte protoporphyrin \(FEP\) I. Normal values for adults and evaluation of the hematofluorometer,”](#) *American Journal of Clinical Pathology* 79(6), 655–660 (1983).
57. G. Hennig, C. Gruber, M. Vogeser, H. Stepp, S. Dittmar, R. Sroka, and G. Brittenham, [“Dual-wavelength excitation for fluorescence-based quantification of zinc protoporphyrin IX and protoporphyrin IX in whole blood,”](#) *Journal of Biophotonics* 7(7), 514–524 (2014).
58. M. Musawi, M. Jafar, B. Al-Gailani, N. Ahmed, F. Suhaimi, and N. Suardi, [“In vitro mean red blood cell volume change induced by diode pump solid state low-level laser of 405 nm,”](#) *Photomedicine and Laser Surgery* 34(5), 211–214 (2016).
59. T. Machneva, N. Kosmacheva, Yu. Vladimirov, and A. Osipov, [“The effects of low power laser radiation on blue, green and red ranges on free radical processes in rat blood in endotoxic shock,”](#) *Biochemistry (Moscow), Supplement Series B: Biomedical Chemistry* 6(3), 237–246 (2012).
60. A. McDonagh, [“The biliverdin–bilirubin antioxidant cycle of cellular protection: Missing a wheel?”](#) *Free Radical Biology and Medicine* 49(5), 814–820 (2010).
61. A. Dvořák, K. Pospisilova, N. Capkova, L. Muchova, M. Vecka, N. Vrzackova, J. Krizová, J. Zelenka, and L. Vítek, [“The effects of bilirubin and lumirubin on metabolic and oxidative stress markers,”](#) *Frontiers in Pharmacology* 12, 567001 (2021).
62. M. A. Brito, R. F. Silva, and D. Brites, [“Bilirubin toxicity to human erythrocytes: a review,”](#) *Clinica Chimica Acta* 374(1–2), 46–56 (2006).
63. A. Frolov, G. Gurinovich, [“The laws of delayed photohaemolysis sensitized by chlorin e6,”](#) *Journal of Photochemistry and Photobiology B: Biology* 13(1), 39–50 (1992).
64. N. Peskova, A. Brilkina, A. Gorokhova, N. Shilyagina, O. Kutova, A. Nerush, A. Orlova, L. Klapshina, V. Vodeneev, and I. Balalaeva, [“The localization of the photosensitizer determines the dynamics of the secondary production of hydrogen peroxide in cell cytoplasm and mitochondria,”](#) *Journal of Photochemistry and Photobiology B: Biology* 219, 112208 (2021).
65. V. Plavskii, L. Plavskaya, T. Ananich, V. Katarkevich, A. Mikulich, I. Leusenko, A. Tretyakova, O. Dudinova, P. Mazmanyany, V. Karapyan, and G. Margaryan, [“New approaches to improve efficacy and reduce side effects of phototherapy for neonatal hyperbilirubinemia using led and laser sources,”](#) *Photobiology and Photomedicine* 26, 63–72 (2019).
66. H. Vreman, S. Kourula, J. Jašprová, L. Ludvíková, P. Klán, L. Muchova, L. Vítek, B. Cline, R. Wong, and D. Stevenson, [“The effect of light wavelength on in vitro bilirubin photodegradation and photoisomer production,”](#) *Pediatric Research* 85(6), 865–873 (2019).

67. G. Agati, F. Fusi, G. Donzelli, and R. Pratesi, “[Quantum yield and skin filtering effects on the formation rate of lumirubin](#),” *Journal of Photochemistry and Photobiology B: Biology* 18(2–3), 197–203 (1993).
68. V. Glushko, M. Thaler, and M. Ros, “[The fluorescence of bilirubin upon interaction with human erythrocyte ghosts](#),” *Biochimica et Biophysica Acta (BBA)-General Subjects* 719, 65–73 (1982).
69. R. Bormett, S. Asher, P. Larkin, W. G. Gustafson, N. Ragunathan, T. Freedman, L. Nafie, S. Balasubramanian, S. Boxer, N. Yu, K. Gersonde, R. Noble, B. Springer, and S. Sligar, “[Selective examination of heme protein azide ligand-distal globin interactions by vibrational circular dichroism](#),” *Journal of the American Chemical Society* 114(17), 6864–6867 (1992).
70. E. Nagababu, J. Mohanty, S. Bhamidipaty, G. Ostera, and J. Rifkind, “[Role of the membrane in the formation of heme degradation products in red blood cells](#),” *Life Sciences* 86(3–4), 133–138 (2010).
71. L. Huang, T. G. S. Denis, Y. Xuan, Y. Huang, M. Tanaka, A. Zadlo, T. Sarna, and M. Hamblin, “[Paradoxical potentiation of methylene blue-mediated antimicrobial photodynamic inactivation by sodium azide: Role of ambient oxygen and azide radicals](#),” *Free Radical Biology and Medicine* 53(11), 2062–2071 (2012).
72. M. Hamblin, H. Abrahamse, “[Inorganic salts and antimicrobial photodynamic therapy: mechanistic conundrums?](#)” *Molecules* 23(12), 3190 (2018).