

Photodamage of the cells in culture sensitized with bilirubin

**O A Kozlenkova, L G Plavskaya, A V Mikulich, I A Leusenko,
A I Tretyakova, V Yu Plavskii.**

Laboratory of organic heterogeneous media, B.I. Stepanov Institute of Physics of NAS of Belarus, Minsk, Republic of Belarus

Abstract. It has been shown that exposure to radiation of LED sources of light with an emission band maximum at about 465 and 520 nm having substantially identical damaging effects on animal cells in culture, that are in a logarithmic growth phase and preincubated with pigment. Photobiological effect is caused by photodynamic processes involving singlet oxygen generated by triplet excited sensitizer. Mono-exponential type dependence of cell survival on the energy dose indicates that it is bilirubin that acts as a sensitizer but not its photoproducts. The inclusion of bilirubin in the cells, where it is primarily localized in the mitochondria cells, it is accompanied by multiple amplification photochemical stability compared to pigment molecules bound with albumin

1. Introduction

The development of syndrome of neonatal hyperbilirubinemia (jaundice) is caused by excessive accumulation in the blood, as well as in subcutaneous fat of gall pigment (breakdown product of normal heme catabolism) – Z, Z-bilirubin IX α , due to its overproduction and / or low excretion rate from the body. Phototherapy - the main and most common method for treating neonatal hyperbilirubinemia, which lies in exposure of body surface to radiation with spectral range, which corresponds to the long-wavelength absorption band of bilirubin. It is believed [7-11] that the determining role in reducing the level of bilirubin in newborns under irradiation play photoisomerization processes of the pigment - the formation of its configurational and structural isomers that are more hydrophilic compounds than the native bilirubin, and quickly eliminated from the body. In addition to photoisomerization self-sensitized photolysis of bilirubin involving singlet oxygen also contributes to the reduction of bilirubin level [12]. At the same time, one cannot exclude that the singlet oxygen generated by triplet excited bilirubin, will cause photodynamic damage to other vital structures of molecular and cell organelles. This indicates the need to investigate sensitizing effect of bilirubin and its photoproducts on important biological structures to develop measures for reducing the possible side effects of the action of light during phototherapy. In recent years, the urgency this problem is even more acute in connection with the application for the treatment of neonatal hyperbilirubinemia of new light sources based on high-brightness LEDs, allowing vary over a wide range both the intensity and the wavelength of incident radiation within the absorption band of the pigment [7 - 11].

The aim of present work – comparable studies of photostability of bilirubin in cells and in a complex with albumin as well as study of mechanism of sensitized by pigment damage of cells in culture upon irradiation with LED sources of light of blue and green spectral range.

2. Materials and methods



Photostability of bilirubin was investigated in MEM medium (with 10% bovine serum), in which bilirubin is in a complex with albumin, as well as in kidney cells of an African green monkey BGM in a logarithmic growth phase. The cells were grown in disposable Petri dishes in a nutrient medium MEM (minimal essential medium) with 10% bovine serum at 37 °C and a 5% CO₂ incubator. The cellular monolayers were preincubated with bilirubin in a concentration of 40 μM for 2 hours, and then exposed to radiation of LED sources with emission band maximum at about 465 nm or 520 nm. Bilirubin photostability was evaluated spectrophotometrically by comparing the absorbance of the bilirubin solution extracted from the cells using DMSO immediately after irradiation of the LED source and unirradiated cells. We use an MTT-test as a criterion for the metabolic cells activity assessment.

3. Results and discussion

Studies have shown that bilirubin can have a sensitizing effect on the cells (as manifested in the reduction of viability) when excited by optical radiation with wavelength $\lambda = 465$ nm and $\lambda = 520$ nm. It is found that the biological effect induced by the light in the presence of bilirubin depends on the physiological state of the cells, the dose of incident radiation and the concentration of the photosensitizer. Changing the power density of light in 3-4 times with appropriate compensation of dose due to the irradiation time has practically no influence on the photobiological effect (table 1). Adding in cell culture together with the sensitizer a quencher of singlet oxygen - sodium azide - significantly reduces the damaging effect of light towards the cells. This is indicated by the MTT-test data presented in Table 1. One can see that fraction of viable cells (relative to control) in the absence of other additives upon irradiation of the cells ($P = 20$ mW / cm², $t = 5$ min) sensitized with bilirubin is $\gamma = (45,5 \pm 1,6)\%$. Under the same conditions of irradiation for cells in the presence of 5 mM of sodium azide fraction of viable cells is significantly increased and $\gamma = (97,9 \pm 5,7)\%$. Thus, sodium azide actually blocks the destruction of the cells sensitized with bilirubin. This fact is evidence of the decisive role of singlet oxygen in the mechanism of photodegradation of cells sensitized with bilirubin. Also the addition of sodium azide in the culture medium prior to irradiation reduces in some degree bilirubin discoloration. Thus, singlet oxygen plays a determining role in the study of photochemical processes. In the absence of bilirubin, as well as in case of incubation of the cells with bilirubin ($C_{BR} = 40$ μM) without light exposure, the effect is extremely low.

Table 1. Effect of sodium azide on photosensitized bilirubin cell damage in the conditions of constant light exposure dose of energy ($\lambda = 465$ nm) by varying the time and power density of incident radiation.

Parameters	Control group	Experimental group		
The power density of incident radiation, mW/sm ²	0	20	10	5
Exposure time, min	0	5	10	20
Energy dose, J / cm ²	0	6	6	6
Percentage of viable cells, incubated for 2 hours with 40 μM bilirubin,%	100,0±7,5	45,5±1,6	50,0±6,4	48,7±12,5
Percentage of viable cells, incubated for 2 hours with 40 μM bilirubin and 5 mM sodium azide,%	100,0±13,5	97,9±5,7	91,2±10,4	90,2±1,0

It is well known [13 - 15] that the lipophilic bilirubin being readily soluble in lipids of cell membranes, can penetrate and accumulates in the mitochondria. For this reason, the high concentration of bilirubin in the mitochondria, even in dark conditions may alienate respiration and oxidative phosphorylation in them, induce apoptosis, disrupt protein synthesis, and have other toxic

effects. As already mentioned, at the chosen concentrations (40 μM in growth medium) bilirubin in dark conditions has no appreciable effect on the survival of kidney cells of African green monkey BGM. Nevertheless, these results indirectly confirm the localization of bilirubin in mitochondrial structures, since preincubation of cells with bilirubin greatly affects the structure of crystals of formazan formed in the mitochondria of living cells for the MTT-test. Pictures (figure1) obtained at the same magnification ($40\times$) clearly show that after incubation of cells with bilirubin formazan crystals become significantly larger (figure 1B) compared with the control intact cells (figure 1A). Irradiation of cells in the presence of bilirubin significantly affect cell viability, as evidenced by low packing density of crystals of formazan formed in the mitochondria (figure 1D). Thus in the absence of irradiation bilirubin cells (figure 1C) has practically no effect on the arrangement density of mentioned crystals.

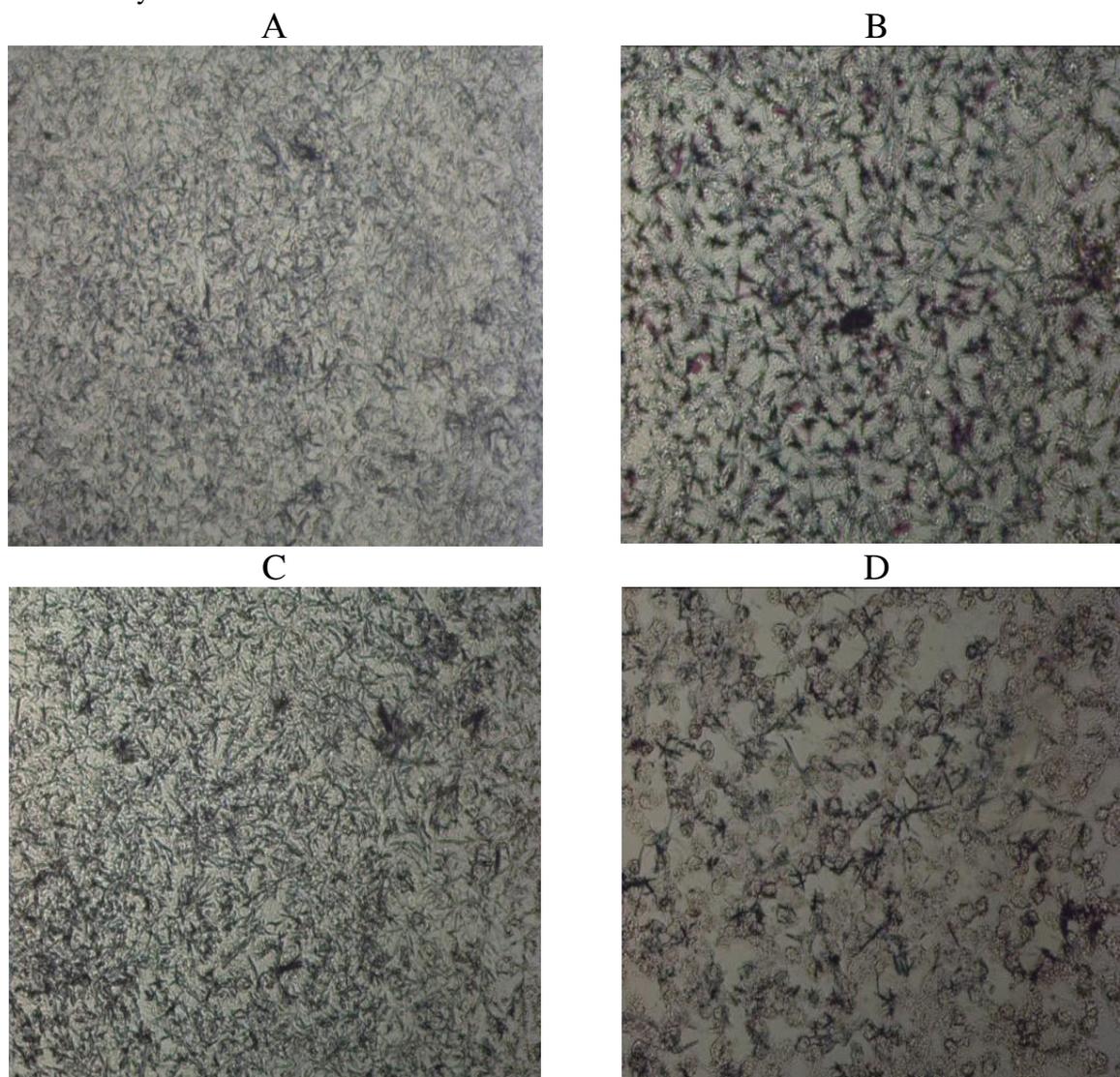


Figure 1. Effect of bilirubin and light exposure ($\lambda_{\text{ex}} = 465 \text{ nm}$, $P = 10 \text{ mW} / \text{cm}^2$, $t = 10 \text{ min}$) on the structure of crystals of formazan formed in the mitochondria of metabolically active cells A - cells in the absence of bilirubin and without irradiation (control); B - cells with bilirubin without irradiation; C - irradiated cells in the absence of bilirubin; T - cells are irradiated with bilirubin.

Dependence of cell survival on energy dose upon irradiation of cells in the presence of bilirubin with LED sources of light with an emission band maximum at about 465 and 520 nm is mono-

exponential function (figure 2). This indicates that it is bilirubin that acts as a sensitizer but not its photoproducts. Another unique feature of these curves - practically identical photobiological effect for radiation with $\lambda = 465$ nm, corresponding to the maximum of the absorption spectrum of bilirubin-albumin complex, and for radiation with $\lambda = 520$ nm corresponding to the long-wavelength slope of the specified spectrum.

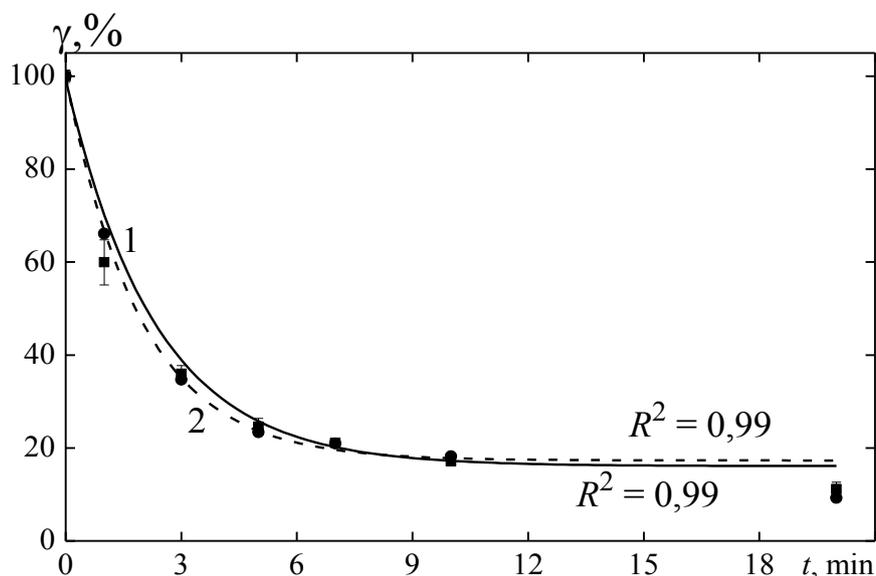


Figure 2. The dependence of viable cells percentage sensitized with bilirubin on the irradiation time by LED sources of light with emission band maximum at $\lambda_{em} = 465$ nm (curve 1) and $\lambda = 520$ nm (curve 2), power density $P = 20$ mW / cm².

Apparently, it should be stated about sharp changes in the spectral characteristics of bilirubin upon its binding to cellular organelles because of structural rearrangements of tetrapyrrole due to changing microenvironment.

It was shown that exposure to radiation of LED sources of light with emission band (maximum at about 465 nm), corresponding to the absorption spectrum of bilirubin, in the conditions causing almost complete bilirubin photodestruction in the nutrient media practically does not cause photolysis of bilirubin localized in the mitochondria (Figure 3). The findings suggest that bilirubin has a high photochemical stability in the lipid environment. Among the possible causes of increased bilirubin photostability at its intracellular localization are considered: a) the formation, along with the monomers, of tetrapyrrole dimeric forms; b) the presence of closely located antioxidants; c) slow diffusion of oxygen involved in the reaction self-sensitized discoloration of the pigment; g) quenching of the triplet state of bilirubin by other biomolecules.

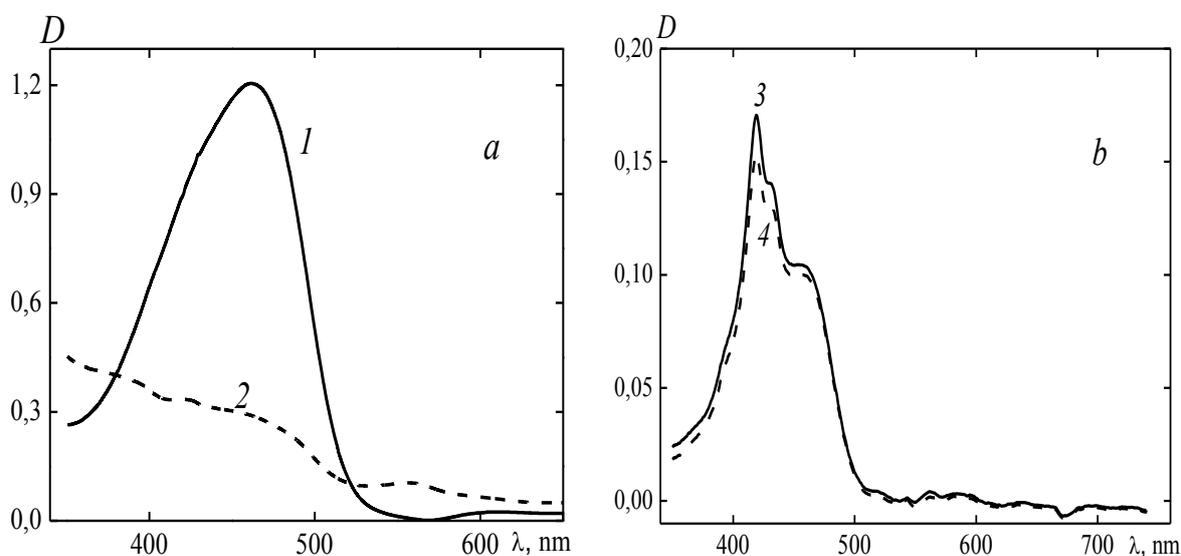


Figure 3. Absorption spectra of bilirubin in medium (a) and a DMSO solution (b) after extraction from the cells without prior exposure (curves 1 and 3) and after exposure to radiation $\lambda = 465$ nm, power density $P = 20$ mW / cm² for $t = 5$ min (curves 2 and 4).

4. Conclusions

It is shown that bilirubin located within cells is characterized by high photochemical stability and can act as a photosensitizer, causing photodamaging lethal effect on animal cells in culture. The exposure to radiation of LED sources of light with an emission band maximum at about 465 and 520 nm causes substantially identical damaging effects on animal cells in culture that may be due to by a significant change in the spectral characteristics of bilirubin upon entering into the cells. The addition of sodium azide to the system being irradiated, the quencher of singlet oxygen, reduces the biological effect that points to the decisive role of singlet oxygen in the mechanism of photodynamic action sensitized with bilirubin.

References

- [1] Xiong T, Qu Y, Cambier S, Mu D 2011. *Eur J Pediatr.* **170** p 1247–1255.
- [2] Плавский В Ю, Мостовников В А, Третьякова А И, Мостовникова Г Р 2008. *Журн. прикл. спект.* **75** с 383–394.
- [3] Böhm F, Drygalla F, Charlesworth P, Böhm K, Truscott T G, Jokiel K 1995. *Photochem. Photobiol.* **62** p 980–983.
- [4] Oláh J, Tóth-Molnár E, Kemény L, Csoma Z 2013. *Brit J Dermatol.* **169** p 243–249.
- [5] Tanaka K, Hashimoto H, Tachibana T, Ishikawa H, Ohki T 2008 *Pediatr Surg Int.* **24** p 837–842.
- [6] Liu G S, Wu H, Wu B Q, Huang R Z, Zhao L H, Wen Y 2008. *World J Pediatr.* **4** p 31–35.
- [7] Plavskii V Yu 2012 *Nova Science Publishers Inc* (New York). ed. J F Novotny and F Sedlacek p 1–65.
- [8] Maisels M J, McDonagh A F 2008. *N. Engl. J. Med.* **358** p 920–928.
- [9] Stokowski L A 2006. *Adv. Neonatal Care.* p 303–312.
- [10] Плавский В Ю, Третьякова А И, Мостовникова Г Р 2014. *Опт. журн.* **81** с 51–62.
- [11] Плавский В Ю, Третьякова А И, Микулич А В, Плавская Л Г 2014. *Инновационные технологии в медицине* **2** с 84–98.
- [12] Landen G L, Park Y T, Lightner D A 1983 *Tetrahedron* **39** p 1893–19071.

- [13] Chuniaud L, Dessante M, Chantoux F, Blondeau J P , Francon J, Trivin F 1996. *Clin Chim Acta.* **256** p 103–114.
- [14] Akin E, Clower B, Tibbs R, Tang J, Zhang J 2002. *Brain Res* **931** p 168–175.
- [15] Keshavan P, Schwemberger S. J, Smith D L, Babcock G F, Zucker S D 2004 *Int J Cancer.* **112** p 433–445.