INFLUENCE OF ROSE BENGAL ON PLATELET AGGREGATION ACTIVITY

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Abstract

The goal of the study is to comparatively examine the effect of photoactivated rose bengal on platelet aggregation *in vitro* and in circulating blood of male Wistar rats. Platelet-rich plasma (PRP) was obtained from venous blood. The aggregation activity of platelets was determined by the turbidimetric method, the aggregation inducer was ADP at a final concentration of 1.25 μM. Rose bengal (RB) (Acros Organics, USA) was used as a photosensitizer (PS). PRP samples containing the PS were irradiated using ALOD-Izumrud laser (OOO "Alcom Medica", Russia), λ = 532 nm, power density 0.05 W/cm^2, energy density of 6, 12 and 24 J/cm^2. The effect of photoactivated RB on the aggregation of circulating PLT was studied after laser irradiation of the femoral artery of the rats: 30 mW laser power, 2 mm spot diameter and 30 min exposure. RB at concentrations of 0.5 and 1 μg/ml was found to stimulate, and 5-10 μg/ml—to inhibit platelet aggregation. Photoactivation of RB weakens the stimulating effect of laser irradiation on the aggregation of platelets. Photodynamic modification of blood led to an increase in the intensity of platelet aggregation by 24% in comparison to the control group, and by 39.6% compared to the group without photoactivation of RB ($p<0.01$). The data obtained indicate that under the influence of RB photoactivation, the aggregation activity of platelets changes, the severity and direction of the effect depend on the RB concentration. Change in functional activity of platelets is one of the manifestations of photodynamic modification of blood.

Keywords: rose bengal, photoactivation, photodynamic blood modification, platelet rich plasma, platelet aggregation.

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ВЛИЯНИЕ БЕНГАЛЬСКОГО РОЗОВОГО НА АГРЕГАЦИОННУЮ АКТИВНОСТЬ ТРОМБОЦИТОВ

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Резюме

Проведено сравнительное изучение влияния фотоактивированного бенгальского розового на агрегацию тромбоцитов *in vitro* и в циркулирующей крови крыс-самцов Wistar. Из венозной крови получали плазму, обогащенную тромбоцитами (PRP). Агрегационную активность тромбоцитов определяли турбодиметрическим методом, индуктор агрегации – АДФ в конечной концентрации 1,25 μM. В качестве фотосенсибилизатора (ФС) использовали бенгальский розовый (БР) (Acros Organics, США). Пробы PRP, содержащие ФС, облучали с помощью лазерного аппарата АЛОД-Изумруд (ООО «Алком медика», Россия), λ=532 нм, плотность мощности 0,05 Вт/см^2, плотность энергии 6, 12, 24 Дж/см^2. Влияние фотоактивированного БР на агрегацию циркулирующих тромбоцитов изучали после лазерного облучения бедренной артерии крыс. Параметры облучения: мощность 30 мВт; диаметр пятна 2 мм; экспозиция 30 мин. БР в концентрациях 0,5 и 1 мкг/мл стимулирует, а 5–10 мкг/мл – угнетает агрегацию тромбоцитов. Фотоактивация БР ослабляет стимулирующее действие лазерного облучения на агрегацию тромбоцитов. Фотодинамическая модификация крови приводила к увеличению интенсивности агрегации тромбоцитов на 24% по сравнению с контрольной группой, на 39,6% – по сравнению с группой без фотоактивации БР ($p<0,01$). Полученные данные свидетельствуют о том, что под влиянием фотоактивации БР изменяется агрегационная активность тромбоцитов, степень выраженности и направленность эффекта зависят от концентрации БР. Изменение функциональной активности тромбоцитов является одним из проявлений фотодинамической модификации крови.

Ключевые слова: бенгальский розовый, фотоактивация, фотодинамическая модификация крови, плазма богатая тромбоцитами, агрегация тромбоцитов.

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Introduction

The presence of a halogenated xanthene ring in the structure of rose bengal (RB) determines its properties as a photosensitizer (PS). When irradiated with green light (maximum absorption at a wavelength of 546 nm), the RB goes into an excited state and photochemical reactions develop.

RB as a PS is used in oncology, ophthalmology, and some other areas of clinical medicine, as well as in experimental studies [1, 2]. In 1985, B.D. Watson et al. showed for the first time that the irradiation of vessels with green light against the background of preliminary administration of RB naturally leads to the formation of a thrombus [3]. In subsequent studies, it was found that RB is taken up by the endothelium and activated upon subsequent irradiation. In this case, reactive oxygen species are formed, including singlet oxygen, causing photodynamic damage to the endothelium, which manifests itself in the release of thrombogenic substances from it, the expression of adhesion molecules, which initiates the formation of a thrombus [4-6].

RB circulating in the blood is absorbed not only by the endothelium, but also by other cells, including blood cells. During PDT of tumors, photoactivation of RB fixed in the endothelium and, possibly, in blood cells circulating in the irradiation zone occurs. The question of the role of platelets that have experienced photodynamic effects in the formation of a thrombus remains open. According to J. Inamo et al. (1996) photoactivated RB has a weak activating effect on ADP-induced human thrombocyte aggregation (*in vitro*). Based on these data, the authors concluded that RB photoactivation is not of great importance in the development of photodynamically induced thrombosis [7].

The aim of our study was a comparative study of the effect of photoactivated RB on platelet aggregation *in vitro* and in circulating blood.

Materials and methods

The experiments were performed on male Wistar rats (FSUP "Rappolovo" Nursery of Laboratory Animals", FSBI "National Research Center "Kurchatov Institute"). The animals were kept and cared for in accordance with the rules set forth in the European Convention for the Protection of Vertebrate Animals (Strasbourg, 1986). The study was approved by the commission for the maintenance and use of vertebrate laboratory animals at The Pavlov First Saint Petersburg State Medical University as part of the State R&D assignment No. AAAA-A18-118091790075-0 "Development of the principles of laser photodynamic theranostics" (2015–2020).

Platelet aggregation was studied by the turbodimetric method using an AT-02 aggregometer (Russia).

Blood was taken from the jugular vein of anesthetized rats (20% urethane solution, 5 ml/kg body weight, intraperitoneally). A 3.2% sodium citrate solution in a ratio of 9:1 was used as a blood stabilizer. Blood centrifugation mode: to obtain platelet-rich plasma (PRP) – 200 g, 10 min; platelet-poor plasma (PPP) – 1700 g, 30 min.

Platelet aggregation inducer – ADP (Chrono-log Co, USA) at a final concentration of 1.25 μM. The following aggregation parameters were determined: aggregation intensity – increase in light transmission (MA) %; time to reach MA, (t_1) s; aggregation rate – V_{agr} (MA/t_1); MA reduction time by 2 times (t_2) s; disaggregation rate – V_{desagr} ($½\ MA/t_2$).

In experiments *in vitro*, RB (Acros organics, USA) was added to plasma containing a standard number of platelets ($270–350*10^9$/l) at concentrations from 0.5 to 10 μg/ml in experiments to study the effect of RB on platelet aggregation activity without photoactivation.

In experiments to study the effect of photoactivated RB on platelet aggregation *in vitro*, RB was added at a certain concentration of 5 μg/ml to plasma containing a standard number of platelets. After a 5-minute incubation in the dark, the sample was irradiated and platelet aggregation activity was determined. In the comparison groups, the effect of RB (without irradiation) and laser exposure (without RB) on platelet aggregation was studied.

Irradiation procedure

Samples of PRP with a volume of 370 μl were poured into the cells of a 24-well plate and irradiated in the dark using a semiconductor laser device (ALOD, Russia). The end of the light guide was fixed on a tripod and placed at a distance of 10 mm from the plate surface (see Fig. 1). Irradiation parameters: wavelength 532 nm, laser power 0.5 W, power density 0.05 W/cm^2, energy density 6 J/cm^2 (2 min exposure), energy density 12 J/cm^2 (4 min exposure), energy density 24 J/cm^2 (8 min exposure). The laser radiation power was controlled using a power meter (Advantest Q8230, USA) before each experiment.

The study of the effect of photoactivated RB on circulating platelets was carried out as follows: 1 hour after intravenous administration of RB under conditions of anesthesia in rats, a section of the femoral artery was isolated from the neurovascular bundle (see Fig. 2) and supravascular laser irradiation was performed using a focuser (Alcom Medica LLC, Russia). Irradiation conditions: λ = 532 nm, power 30 mW, spot diameter 2 mm, power density 0.9 W/cm^2, exposure 30 min, energy density 1620 J/cm^2. The parameters of laser radiation were chosen according to the data obtained in previous studies, in which RB photoactivation led to a guaranteed decrease in the blood flow velocity in the vessels and the formation of a thrombus [4]. Irradiation in the same modes of the femoral artery in rats without prior administration of RB did not lead to the formation of a thrombus.

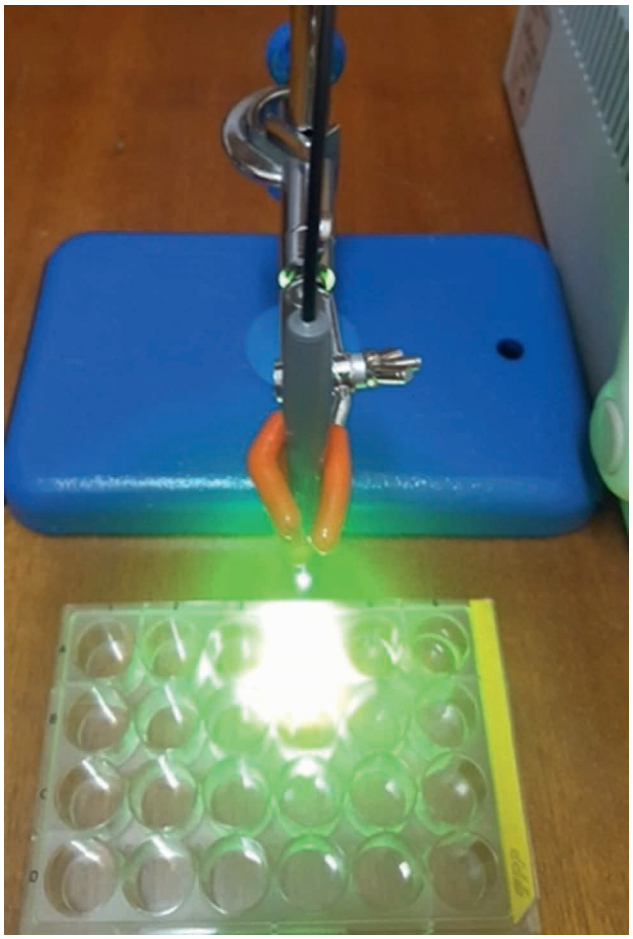

Рис. 1. Процедура облучения *in vitro* PRP в 24-луночном планшете.
Fig. 1. *In vitro* irradiation procedure of platelet-rich plasma in 24 well plate.

After completion of irradiation, blood was taken and platelet aggregation was examined. In the comparison groups, the effect of supravascular irradiation of the femoral artery (without RB) and intravenous administration of RB (without irradiation) on platelet aggregation was evaluated.

Statistical data processing

Data collection was carried out using a spreadsheet Microsoft Excel 2007. Quantitative data were tested for normal distribution using the Shapiro-Wilk W test. We used non-parametric methods of statistical analysis using the Mann-Whitney test. Numerical data are presented as median (lower quartile/upper quartile). The significance of the established differences was judged by the level of values $p<0.05$.

Results

In experiments *in vitro*, RB was added to PRP (final concentration from 0.5 to 10 μg/ml) and platelet aggregation was examined after a 5-minute incubation in the dark. As can be seen from the data in Fig. 3, at a concentration of 0.5 and 1 μg/ml, MA increased by 52.3 and 34.6%, respectively, compared with the control ($p<0.01$), while the rate of aggregation and disaggregation did not change significantly. The intensity of platelet aggregation at a RB concentration of 2.5 μg/ml did not differ from the control, however, the rate of aggregation and disaggregation slowed down significantly by 22.2 and 26%, respectively. An increase in the concentration of RB to 5 and 10 μg/ml led to a decrease in the intensity of platelet aggregation by 43.9 and 53.3%, respectively. The rates of aggregation and disaggregation decreased significantly ($p<0.01$).

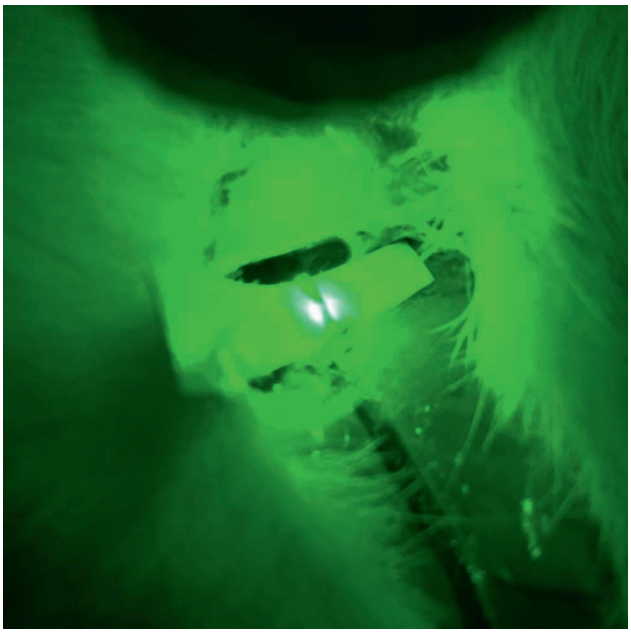

Рис. 2. Надсосудистое лазерное облучение бедренной артерии у крыс.
Fig. 2. Supravascular laser irradiation of the femoral artery in rats.

Thus, the direction and severity of the effect of RB on platelet aggregation *in vitro* depended on the concentration: at low concentrations (0.5 and 1 μg/ml), stimulation was observed, and at higher concentrations, inhibition.

PRP irradiation (without RB) significantly increased the intensity of platelet aggregation compared to the control: at 6 J/cm^2, MA increased by 55.1%; at 12 J/cm^2 – by 65.4%; at 24 J/cm^2 – by 90.7% ($p<0.01$). The rates of aggregation and disaggregation did not change significantly.

In the next group of experiments, after a 5-minute incubation of PRP with RB at a concentration of 5 μg/ml, the samples in the dark were subjected to laser irradiation with an energy density of 12 J/cm^2.

As can be seen from Fig. 4, laser irradiation (532 nm) of PRP after incubation with RB (5 μg/ml) led to a decrease in MA, while irradiation without RB increased the intensity of aggregation ($p<0.01$). Thus, RB photoactiva-

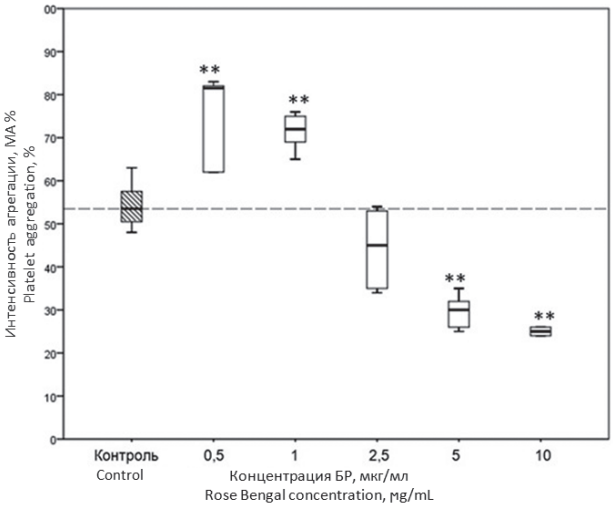

Рис. 3. Влияние бенгальского розового на интенсивность агрегации тромбоцитов.
Примечание: ** – p<0,01 по сравнению с контролем.
Fig. 3. Influence of rose bengal on the intensity of platelet aggregation.
Note:** – p <0.01 compared to control.

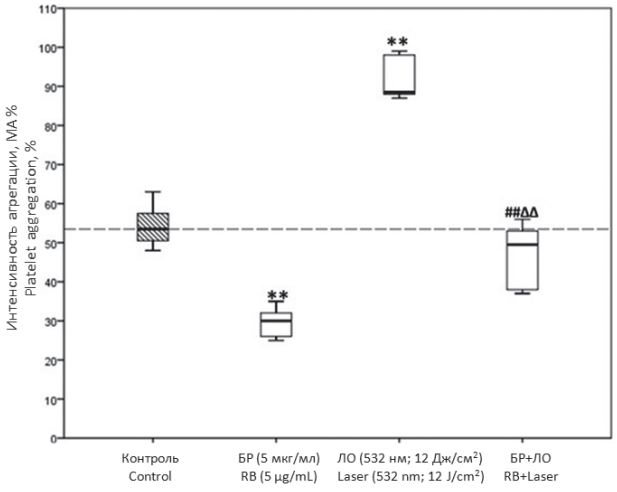

Рис. 4. Влияние БР, лазерного облучения, лазерного облучения на фоне предварительного добавления в PRP БР на интенсивность агрегации тромбоцитов.
Примечание: ** – p<0,01 по сравнению с контролем; ## – p<0,01 по сравнению с БР; ΔΔ – p<0,01 по сравнению с группой облучения.
Fig. 4. Influence of the RB, laser irradiation, laser irradiation with addition of RB to platelet-rich plasma on the intensity of platelet aggregation.
Note: ** – p <0.01 compared to control; ## – p <0.01 compared to RB; ΔΔ – p <0.01 in comparison with the irradiation group.

tion weakens the stimulating effect of laser irradiation on platelet aggregation (Fig. 4).

Photodynamic modification of circulating blood in our studies was carried out by irradiating the femoral artery in rats against the background of preliminary administration of RB.

As can be seen from the table, one hour after the intravenous administration of RB, the intensity of platelet aggregation did not change significantly, however, the time to reach MA was 20.7% less versus control (p<0.01).

Thus, prolonged contact of circulating platelets with RB affected their functional activity, but to a lesser extent than in *in vitro* experiments.

Irradiation of the femoral artery in rats without prior administration of RB (blood photomodification) did not lead to a significant change in platelet aggregation activity.

Photodynamic modification of blood (irradiation of the artery) led to an increase in the intensity of platelet aggregation by 24% compared with the control group, and by 39.6% compared with the group without RB photoactivation (p<0.01). The aggregation rate increased by 36.6% compared to the control group and by 27.3% compared to the group without RB photoactivation (p<0.05) (see Table). The disaggregation rate did not differ from the data in other groups.

Discussion

Weak lipophilicity, the presence of a double negative charge at physiological pH limits the penetration of RB into cells at low concentrations and in the absence of carriers, such as albumin. This explains the fact that in an aqueous solution RB has practically no effect on normal cells, while at the same concentrations it has a cytotoxic effect on the cultured cells of some tumors [1, 2, 8, 9].

In our experiments, it was found that RB has an effect on platelet aggregation, while the degree of severity and direction of the effect depend on the concentration of RB. The penetration of RB into intact platelets most likely occurs by endocytosis of the RB complex with albumin. There is evidence in the literature that some PS have a direct effect on platelets, but most authors observed an inhibitory effect only after PS photoactivation [10-13].

According to J. Inamo et al. RB at a final concentration of 5 μg/ml did not affect ADP-induced aggregation of human platelets. According to our data, RB at concentrations of 5 and 10 μg/ml naturally inhibited rat platelet aggregation, and at lower concentrations (0.5–1.0 μg/ml) stimulated it. Perhaps these differences are associated with the specific features of human platelets. J. Inamo et al. noted some stimulating effect of photoactivated RB on platelet aggregation (532 nm, dose not specified), but this effect did not differ from the effect of irradiation itself on platelets.

Changes in the functional state of circulating platelets during irradiation of the femoral artery of rats against the background of preliminary administration of RB can be the result of both direct and indirect photodynamic effects. In the 90s of the last century, V.H. Fingar et al. showed that during PDT of experi-

Таблица
Влияние фотодинамической модификации крови на показатели АДФ-индуцированной агрегации тромбоцитов
Table
Influence of photodynamic blood modification on indicators of ADP-induced platelet aggregation

Группа Group	Показатели агрегации Aggregation parameters				
	Максимальная амплитуда агрегации (МА), % Maximum aggregation amplitude (MA), %	Время достижения МА, с Time to reach MA, s	Время уменьшения МА в 2 раза, с Time to decrease MA by 2 times, s	Скорость агрегации, %/с Aggregation rate, %/s	Скорость дезагрегации, %/с Disaggregation rate, %/s
Контроль Control (n=15)	54 (52–58)	130 (113–143)	219 (192–251)	0,41 (0,38–0,48)	0,31 (0,25–0,35)
БР (17 мг/кг) RB (17 mg/kg) (n=6)	48 (33–54)	103 (97–112)**	173 (153–186)**	0,44 (0,34–0,47)	0,32 (0,31–0,32)
ЛО 532 нм LI 532 nm (n=6)	57,5 (52–60)	126,5 (122–141)	222,5 (202–249)	0,45 (0,42–0,47)	0,31 (0,3–0,32)
БР+ЛО RB+LI (n=8)	67 (61–77)**## Δ	126 (103–146)	226 (179–264)	0,56 (0,48–0,61)* # Δ	0,37 (0,31–0,44)

Примечание: n — число животных; * – p<0,05 по сравнению с контролем; ** – p<0,01 по сравнению с контролем; # – p<0,05 по сравнению с группой ФС; ## – p<0,01 по сравнению с группой ФС; Δ – p<0,05 по сравнению с группой облучения. БР – бенгальский розовый; ЛО – лазерное облучение; РБ+ЛО – лазерное облучение после предварительного введения БР.
Note: n — number of animals; * – p<0.05 compared to control; ** – p<0.01 compared to control; # – p<0.05 compared to the RB group; ## – p<0.01 compared to the RB group; Δ – p<0.05 compared to the irradiation group. RB - rose bengal; LI – laser irradiation; RB+LI - laser irradiation after preliminary administration of RB.

mental tumors, the content of thromboxane A2 (TxA2) in the blood increases. The authors explain this by the release of TxA2 from the endothelium of tumor vessels in the zone of photodynamic exposure [14]. TxA2 is known to be a platelet activator, which, according to the authors, promotes thrombus formation in tumor vessels during PDT. The described mechanism of changes in platelet activity during PDT can be considered as indirect, that is, not associated with direct photodynamic damage to circulating platelets. In our experiments, the femoral artery was irradiated against the background of preliminary RB injection. In previous studies, it was shown that with the experimental design used, photodynamically induced thrombi are formed in all cases, that is, there was damage to the endothelium in the irradiation zone [4, 18]. However, the damage area (3.14 mm^2) was incommensurably smaller than in the experiments of V.H. Fingar et al. during irradiation of tumor vessels.

There is evidence in the literature that intravenous and supravascular laser irradiation of blood after the preliminary administration of PS has a cytotoxic effect on circulating tumor cells. This effect is considered as a result of photodynamic modification of blood [15-18]. It can be assumed that platelets circulating in the blood in the area of femoral artery irradiation also develop photochemical processes that affect their functional state, that is, there is a direct photodynamic effect.

Conclusion

In our study, RB, like some other PS in our previous experiments, had a direct effect on platelets and their functional activity *in vitro* and *in vivo* [19]. The severity of the effect *in vitro* depends on the concentration of the drug. At low concentrations of RB, stimulation of the aggregation activity of platelets is observed, at high concentrations – inhibition. Unlike PS of the chlorine series, photoactivation of RB at a concentration of 5 μg/ml did not increase the inhibitory effect of platelet aggregation under *in vitro* conditions [19].

It has been shown for the first time that photomodification of blood against the background of preliminary administration of RB leads to moderate activation of the functional activity of platelets. A change in the functional activity of platelets is one of the manifestations of photodynamic modification of blood. Considering that the photodynamic model of thrombosis using RB is widely used in preclinical studies, these data should be taken into account when studying the effectiveness of antithrombotic agents.

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