ORIGINAL ARTICLES

THE EFFECT OF CHLORIN E_6 DRUG ON PLATELET AGGREGATION ACTIVITY

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Abstract

The goal of the study is to evaluate the effect of Radachlorin (OOO "RADA-PHARMA", Russia) (RC) on platelet aggregation in ex vivo and in vivo experiments. The experiments were conducted on male Wistar rats. Platelet aggregation activity was determined in platelet-rich plasma (PRP) using a turbidimetric method and the aggregation inducer was ADP at a final concentration of 1.25 μM. PRP samples containing RC were irradiated with ALOD-Granat laser device (OOO "Alkom Medika", Russia) at 662 nm wavelength with 0.05 W/cm^2 power density. After a 5-minute incubation of PRP with RC in the dark, dose-dependent inhibition of platelet aggregation was observed. Laser irradiation (12.5 J/cm^2 and, especially, 25 J/cm^2) increased the inhibitory effect of RC. 3 hours after intravenous administration of RC, the rate and intensity of platelets aggregation did not change, while disaggregation slowed down significantly. Irradiation at a dose of 5 J/cm^2 did not affect the platelets aggregation kinetics, and disaggregation slowed down even more at 10 J/cm^2, and at 20 J/cm^2 the rate and intensity of platelets aggregation decreased, and no disaggregation occurred.

In vitro, RC inhibited the ADP-induced platelet aggregation in rats in a dose-dependent manner; after laser irradiation, this effect was enhanced significantly. The effect of RC on circulating platelets leads to a change in their functional state, which manifests in slowing down the disaggregation after exposure to ADP. After laser irradiation (10 J/cm^2 and, especially, 20 J/cm^2), the severity of the functional changes increases. The role of decreasing the disaggregation activity of platelets in the mechanism of vascular thrombosis in the affected area of photodynamic therapy (PDT) is discussed.

Key words: chlorin e_6, photoactivation, ADP, platelet aggregation.
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ВЛИЯНИЕ ПРЕПАРАТА НА ОСНОВЕ ХЛОРИНА E_6 НА АГРЕГАЦИОННУЮ АКТИВНОСТЬ ТРОМБОЦИТОВ

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Резюме

Цель исследования – изучение влияния радахлорина на агрегационную активность тромбоцитов в опытах *in vitro* и *ex vivo*. Опыты проведены на крысах-самцах линии Wistar. Агрегационную активность тромбоцитов определяли в плазме, обогащенной тромбоцитами (PRP), турбидиметрическим методом, индуктор агрегации – аденозиндифосфат (АДФ) в конечной концентрации 1,25 μM. Пробы PRP, содержащие радахлорин, облучали при плотности мощности 0,05 Вт/см^2. После темновой инкубации в течение 5 мин PRP с радахлорином наблюдали дозозависимое угнетение агрегации тромбоцитов. Лазерное облучение (плотность энергии 12,5 Дж/см^2 и 25 Дж/см^2) усиливало ингибирующее влияние радахлорина. Через 3 ч после внутривенного введения фотосенсибилизатора скорость и интенсивность агрегации тромбоцитов не изменялись, а дезагрегация значимо замедлялась. Облучение при плотности энергии 5 Дж/см^2 не повлияло на кинетику агрегации тромбоцитов, при 10 Дж/см^2 –дезагреция еще больше замедлялась, а при 20 Дж/см^2 – уменьшались скорость и интенсивность агрегации тромбоцитов, а дезагрегация не происходило.

В условиях *in vitro* радахлорин дозозависимо ингибирует АДФ-индуцированную агрегацию тромбоцитов крыс; после лазерного облучения этот эффект значимо усиливается. Воздействие радахлорина на циркулирующие тромбоцитов приводит к изменению их функционального состояния, что проявляется в замедлении дезагрегации после воздействия АДФ. После лазерного облучения (10–20 Дж/см^2) выраженность функциональных изменений увеличивается. Обсуждается вопрос о роли снижения дезагрегационной активности тромбоцитов в механизме тромбоза сосудов в зоне воздействия при фотодинамической терапии.

Ключевые слова: хлорин e_6, фотоактивация, АДФ, агрегация тромбоцитов.

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Introduction

The data on the impact of photodynamic effects on platelet aggregation activity are scarce and highly controversial. *In vivo* experiments with the irradiation of blood vessels of the microcirculatory bloodstream after the administration of various photosensitizers (PS) reveal platelet adhesion and aggregation in the exposure area. It is assumed that their activation occurs due to the influence of biologically active substances (adenosine diphosphate (ADP), thromboxane A2, etc.) coming from damaged endothelium [1], but some authors do not exclude photodynamic activation of platelets circulating in the blood [2]. *In vitro* experiments showed that the combined action of various PS and irradiation leads to the development of structural and functional changes in platelets, including a decrease in their aggregation activity [3–9]. In both experimental and clinical photodynamic therapy of neoplasms, PS is usually administered intravenously 2–3 hours before irradiation, and, therefore, circulating blood cells, including platelets, are exposed to the PS for a long time, which makes it possible to assume that this affects their photosensitivity.

The goal of this work was to study the effect of radachlorin on platelet aggregation activity *in vitro* and *ex vivo* before and after photoactivation.

Materials and methods

The experiments were performed on male Wistar rats weighing 240–280 g (Federal State Unitary Enterprise "Rappolovo Laboratory Animals Farm") in accordance with the "Guidelines for the Use of Laboratory Animals for Scientific and Educational Purposes at the First Pavlov State Medical University of St. Petersburg" [10], compiled on the basis of Directive 2010/63/EC of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes.

The animals were fed an unlimited diet of standard K-120 food (Manufacturer: Inform-Korm, Russia) and given unlimited quantity of water, and had a specific light regime of 12 hours to 12 hours (light: darkness ratio). The temperature was maintained within the range of 22–25°C, and the relative humidity was 50 to 70%. The quarantine lasted for 14 days.

Blood sampling for platelet aggregation was performed from the jugular vein in anesthetized rats (20% urethane solution, 5 ml/kg intraperitoneally). Sodium citrate (3.2%) in a 9:1 ratio was used as a blood stabilizer. To obtain platelet-rich plasma (PRP), blood was centrifuged for 10 min (200 g) at room temperature. Part of the PRP was collected in a plastic tube, and platelet-poor plasma (PPP) was obtained from the remaining blood by centrifugation for 30 min (1700 g), which was used to calibrate the optical density scale of the aggregometer and dilute PRP to a platelet concentration of $200–300 \cdot 10^9$/l. A platelet aggregation study was performed within 2 hours after PRP was produced.

Platelet aggregation activity was determined by a turbidimetric method with an AT-01 aggregometer (Manufacturer: NPF Medtech, Russia); ADP (Manufacturer: CHRONO-LOG Corporation, USA) at a final concentration of 1.25 μM was used as an aggregation inducer.

During the study, the following aggregogram indicators were recorded:

– the maximum amplitude of aggregation (MA) refers to the maximum increase in the optical transmission coefficient from the moment of ADP introduction, as a % to the optical transmission of platelet-free plasma;

– t_1 is time needed to reach MA, s;

– V_{agr} is the aggregation rate, which is MA/t_1, $\%\times s^{-1}$;

– t_2 is the time needed to halve MA, s;

– V_{disagr} is the disaggregation rate, $½\ MA/t_2$, $\%\times s^{-1}$.

PRP samples were irradiated in a polypropylene cuvette (d = 7 mm, h = 45 mm) with ALOD-Granat semiconductor laser apparatus (manufacturer: OOO Alkom Medica, Russia), wavelength: 662 nm. A fiber with a lens for external irradiation was used (OOO Polironik, Russia), fixed in a clamp stand. The fiber end face was positioned at a distance of 40 mm from the sample surface.

The photosensitizer used was a 0.35% solution of radachlorin (OOO RADAPHARMA, Russia, registration certificate No. ЛС-001868 dated December 16, 2011). After intravenous administration, the concentration of radachlorin in rat plasma was determined by spectrophotometry. At the time of administration, the calculated concentration of radachlorin, taking the hematocrit into account, was 160 μg per 1 ml of plasma. PPP was diluted to half the concentration with PBS (phosphate-buffered saline, pH 7.4) and the optical density was determined at 662 nm wth an SF-2000 spectrophotometer (AO LOMO, Russia). 800 μl of the drug was introduced into a quartz cuvette (l = 0.5 cm), the measurement was carried out in comparison with the plasma of control rats diluted to a half of the concentration with PBS. The concentration of radachlorin in the plasma was determined on the basis of a calibration graph plotted for radachlorin diluted in the

control plasma in PBS, to the concentration in range from 2.5 to 30 μg/ml.

In the first group of experiments, the effect of radachlorin on platelet aggregation *in vitro* was investigated. Radachlorin at a final concentration of 10, 20 and 40 μg/ml was added to the standard platelet content plasma, and after a 5-minute incubation in the dark, platelet aggregation activity was determined.

In the group of experiments that followed, the effect of photoactivated radachlorin on platelet aggregation *in vitro* was investigated. Radachlorin (14 μg/ml) was introduced into the plasma with the standard platelet count, and, after a 5-minute incubation in the dark, the sample was irradiated and platelet aggregation activity was determined. In the comparison group, the effect on platelet aggregation of irradiation with the same dose was investigated. Radiation exposure modes: the power density on the sample surface was 0.05 W/cm^2, and the energy density was 12.5 and 25 J/cm^2.

In the third group of experiments, an intravenous bolus injection of radachlorin (5 mg/kg) was administered into the tail vein of the animals. Before blood sampling, the animals were kept in the dark. After 3 hours, a blood sample was taken from the jugular vein, platelet-standard plasma was produced and then irradiated, and platelet aggregation activity was determined. Radiation exposure modes: the power density on the sample surface was 0.05 W/cm^2, and the energy density was 5, 10, and 20 J/cm^2.

The statistical analysis of the results was performed with IBM SPSS Statistics Version 20.0 software package. The significance of differences between the measured parameters was evaluated with Mann-Whitney U test. The differences were considered statistically significant at p values under 0.05. The results are presented as median (lower/upper quartile). A correlation analysis was performed with the use of the Spearman test.

Results and discussion

In our experiments, adenosine diphosphate (ADP) at a concentration of 1.25 μM was used to induce platelet aggregation in rat blood; the aggregation was reversible. The data on the effect of radachlorin on platelet aggregation *in vitro* are given in Table 1. After a 5-minute incubation of PRP with radachlorin, the kinetics of the process changed: the aggregation intensity decreased, aggregation and disaggregation slowed down. The severity of these effects directly depended on the concentration of radachlorin. As can be seen from Table 1, the decrease in platelet aggregation activity during incubation with radachlorin was dose-dependent (Spearman's rank correlation coefficient r = –0.915; p <0.001).

In the next group of experiments, after a 5-minute dark incubation of PRP with radachlorin, the samples were irradiated (12.5 and 25 J/cm^2). As an additional control, platelet aggregation immediately after laser irradiation in the same doses was studied in samples which contained no PS. As can be seen from the data in Table 2, laser irradiation of PRP (especially at a dose of 25 J/cm^2) led to a significant increase in the intensity of platelet aggregation; however, the kinetics of the process did not change significantly. After irradiation of PRP previously incubated with radachlorine, a decrease in the intensity of aggregation and a slowdown in disaggregation were observed, especially at a dose of 25 J/cm^2. Thus, photoactivation of radachlorin enhanced its inhibitory effect on ADP-induced rat platelet aggregation, whereas no stimulating effect of irradiation on aggregation was observed.

Таблица 1
Влияние радахлорина на АДФ-индуцированную агрегацию тромбоцитов *in vitro*
Table 1
The effect of radachlorin on ADP-induced platelet aggregation *in vitro*

Группа Group	Число крыс (N) Number of rats (N)	Максимальная амплитуда агрегации (MA), % Maximal aggregation amplitude (MA), %	Скорость агрегации ($V_{агр}$), %×с$^{-1}$ Aggregation rate (V_{aggr}), % × s^{-1}	Скорость дезагрегации ($V_{дезагр}$), %×с$^{-1}$ Disaggregation rate ($V_{disaggr}$), % × s^{-1}
Контроль Control	10	55,5 (51–61,7)	0,42 (0,39–0,45)	0,31 (0,26–0,34)
Радахлорин, 10 мкг/мл Radachlorin, 10 μg/ml	5	46 (44–49)*	0,35 (0,29–0,37)*	0,15 (0,15–0,19)*
Радахлорин, 20 мкг/мл Radachlorin, 20 μg/ml	5	36 (30–41)*	0,23 (0,23–0,29)*	0,12 (0,11–0,12)*
Радахлорин, 40 мкг/мл Radachlorin, 40 μg/ml	5	21 (21–28)*	0,2 (0,17–0,22)*	0,08 (0,08–0,09)*

* – p<0,01 по сравнению с контролем (без радахлорина)
* – p<0.01 compared to control (without Radachlorin)

Таблица 2
Влияние фотоактивированного (12,5 и 25 Дж/см²) радахлорина (14 мкг/мл) на АДФ-индуцированную агрегацию тромбоцитов
Table 2
The effect of photoactivated (12.5 and 25 J/cm²) radachlorin (14 μg/ml) on ADP-induced platelet aggregation

Показатель Criterion	Контроль (n=10) Control (n=10)	Лазерное облучение, Дж/см² Laser irradiation, J/cm²		Радахлорин (n=5) Radachlorin (n=5)	Радахлорин + лазерное облучение, Дж/см² Radachlorin + laser irradiation, J/cm²	
		12,5 (n=5)	25 (n=5)		12,5 (n=5)	25 (n=5)
Максимальная амплитуда агрегации (МА), % Maximal aggregation amplitude (MA), %	55,5 (51–61,7)	76 (76–77)*	81 (81–82)*	37 (36–39)*	29 (26–30)*#	18 (8–18)*#
Скорость агрегации ($V_{агр}$), %×с$^{-1}$ Aggregation rate (V_{aggr}), % ×s^{-1}	0,42 (0,39–0,45)	0,59 (0,57–0,64)	0,8 (0,74–0,82)	0,28 (0,26–0,29)	0,09 (0,0,08–0,15)#	0,07 (0,02–0,09)#
Скорость дезагрегации ($V_{дезагр}$), %×с$^{-1}$ Disaggregation rate ($V_{disaggr}$), % ×s^{-1}	0,31 (0,26–0,34)	0,34 (0,32–034)	0,3 (0,29–0,33)	0,13 (0,13–0,14)*	Нет дезагрегации No disaggregation	

* – p<0,01 по сравнению с контролем; # – p<0,01 по сравнению с радахлорином без лазерного облучения.
* – p<0.01 compared to control; # – p<0.01 compared to Radachlorin without laser irradiation.

Таблица 3
Влияние фотоактивированного (5, 10, 20 Дж/см²) радахлорина (через 3 ч после внутривенного введения в дозе 5 мг/кг) на АДФ-индуцированную агрегацию тромбоцитов
Table 3
The effect of photoactivated (5, 10, 20 J/cm²) Radachlorin (3 hours after intravenous administration at a dose of 5 mg/kg) on ADP-induced platelet aggregation

Группа Group	Число крыс (N) Number of rats (N)	Максимальная амплитуда агрегации (МА), % Maximal aggregation amplitude (MA), %	Скорость агрегации ($V_{агр}$), %×с$^{-1}$ Aggregation rate (V_{aggr}), % × s^{-1}	Скорость дезагрегации ($V_{дезагр}$), %×с$^{-1}$ Disaggregation rate ($V_{disaggr}$), % × s^{-1}
Контроль Control	**10**	**55,5 (51–61,7)**	**0,42 (0,39–0,45)**	**0,31 (0,26–0,34)**
в/в-введение радахлорина за 3 ч до забора крови IV-administration of Radachlorin 3 hours before blood sampling	5	68 (67–74)*	0,44 (0,41–0,46)	0,22 (0,18–0,23)*
Облучение 5 Дж/см² Irradiation 5 J/cm²	5	65 (64–68)	0,41 (0,36–0,49)	0,21 (0,15–0,22)*
Облучение 10 Дж/см² Irradiation 10 J/cm²	5	56 (56–57)	0,45 (0,43–0,46)	0,12 (0,1–0,13)#
Облучение 20 Дж/см² Irradiation 20 J/cm²	5	36 (32–37)#	0,08 (0,06–0,18)#	Нет N/A

* – p<0,05 по сравнению с контролем; # – p<0,01 по сравнению с контролем.
* – p<0.05 compared to control; # – p<0.01 compared to control

In the third group of experiments, 3 hours after intravenous administration of radachlorin to rats, blood was collected, PRP was produced, and samples were subjected to laser irradiation in different doses, and platelet aggregation activity was determined.

Based on the blood volume in rats (63 ml/kg), the maximum plasma concentration of radachlorin immediately after its administration was 160 μg/ml. 3 hours after intravenous administration, the residual concentration of PS in rat PRP blood was (38.0±1.3) μg/ml, or 24% of the initial concentration, which is consistent with the published data (10–30%) [11–14]. In this case, platelet aggregation activity did not change, and the rate of disaggregation significantly decreased. *In vitro* experiments with comparable concentrations of radachlorin (20, 40 μg/ml) showed a significantly more pronounced effect ($p < 0.01$) (Table 3.).

As can be seen from the data in Table 3, after the irradiation of PRP at a dose of 5 J/cm^2, no changes in the kinetics of platelet aggregation activity were observed, and at a dose of 10 J/cm^2, the rate of disaggregation significantly slowed down, and after irradiation at a dose of 20 J/cm^2, the intensity and spead of platelet aggregation significantly decreased, while disaggregation was not observed.

The above studies showed that radachlorin (which has chlorin e_6 as its main active substance) has a direct effect on platelet aggregation in rat blood, the effect being more pronounced in *in vitro* than in *ex vivo* experiments.

In vitro, the addition of radachlorin dose-dependently reduced the intensity of ADP-induced aggregation, which is consistent with the literature [15], and also led to a slowdown in the rate of disaggregation. This effect has not been described before. According to J.Y. Park et al. [15], chlorin e_6 affects almost all processes that are activated after the interaction of ADP with purine receptors, i. e., it acts similar to P_2Y_{12}-receptor blockers. However, this mechanism is not the only one, since we previously described the inhibitory effect of radachlorin on collagen-induced platelet aggregation in rats, which is not associated with purine receptors activation [16].

Platelet disaggregation is currently believed to be an active process. The mechanisms initiating it constantly occur in platelets, preventing their activation and aggregation in the blood flow [17]. As can be seen from the data in Table 2, radachlorin dose-dependently inhibited the rate of both aggregation and, to an even greater extent, disaggregation. A model of ADP-induced reversible platelet aggregation in rats showed that the P_2Y_{12} receptor inhibitor (CS-747) decreased the intensity of aggregation and did not affect the rate of disaggregation [18]. This confirms our assumption that the point of application of radachlorin is not only purine receptors, but also other platelet receptors.

At a high concentration of radachlorin, hardly any aggregation of rat platelets activated by ADP and collagen occurred. The aggregatogram in this case had the same pattern as in the case of Glanzmann's disease (genetic deficiency of GPIIb/IIIa receptors). Perhaps the main mechanism of the effect of radachlorin on platelet aggregation and disaggregation is associated with its effect on membrane receptors, including GPIIb/IIIa. The severity of the expression of GP receptors and the strength of their bounding with fibrinogen determine the intensity of aggregation and the possibility of disag-

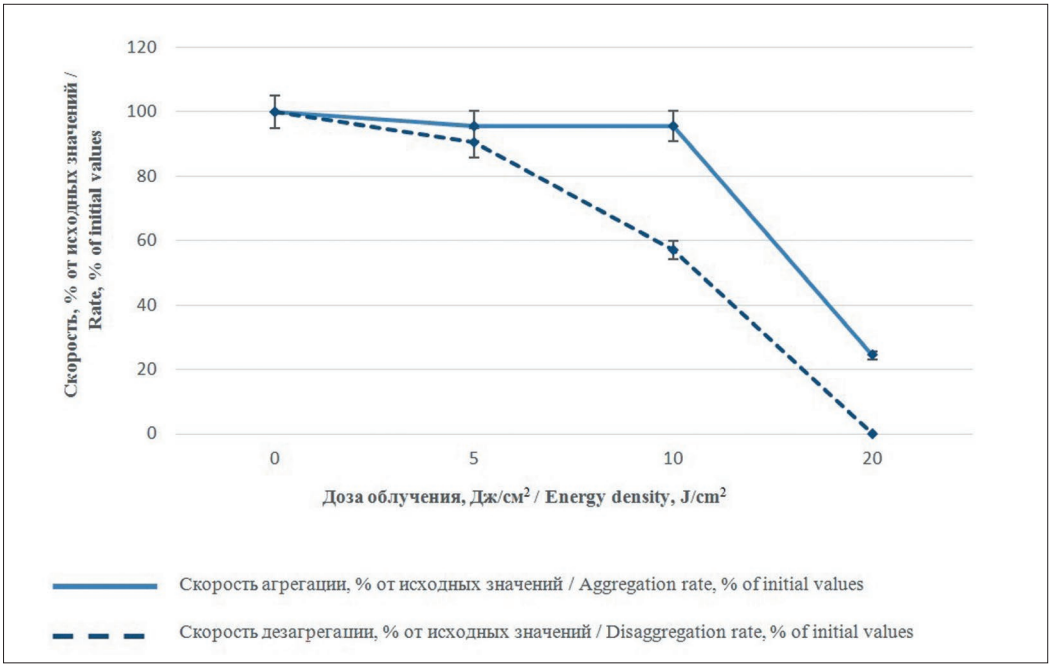

Рис. 1. Изменение скорости агрегации и дезагрегации тромбоцитов в зависимости от дозы облучения
Fig. 1. Change in the rate of aggregation and disaggregation of platelets depending on the dose of radiation

gregation. It is possible that with a high concentration of radachlorin, fibrinogen becomes irreversibly bound to GP receptors. One of the mechanisms of sharp inhibition of platelet aggregation *in vitro* at high concentrations of radachlorin, especially after light activation, may be the loss (shedding) of GP receptors. A similar effect on the platelet GP receptors is produced by a temperature increase [19].

Upon photoactivation of radachlorin, its effect on platelet aggregation and disaggregation was significantly stronger, with the process of disaggregation being more sensitive.

During PDT, the PS circulating in the blood penetrate into blood cells, including platelets; after irradiation, PS photoactivation occurs, probably resulting in photodynamic damage to platelets. It remains unclear to what extent this may affect the formation of blood clots and impaired microcirculation in the area of PDT. Very tentatively, the answer to this question can be obtained in *ex vivo* experiments by studying the aggregation activity of platelets after their prolonged contact with PS in the bloodstream.

According to our data, 3 hours after the intravenous administration of radachlorin, the rate of aggregation did not change, and the rate of disaggregation significantly decreased. Subsequent *in vitro* laser irradiation had an inhibitory effect on both processes, with a more significant impact on the disaggregation rate (Fig. 1). Based on these data, it can be assumed that irradiation of photosensitized platelets in the area of PDT can lead to a change in their functional activity, but the severity of these changes is unlikely to be significant for microcirculation disturbance. A decrease in disaggregation activity can be considered as a factor contributing to thrombosis during platelet activation by thrombogenic factors (ADP, thromboxane A2, etc.), which are released from damaged endothelial cells in the PDT zone.

Conclusion

In vitro, radachlorin dose-dependently inhibits ADP-induced rat platelet aggregation; after laser irradiation, this effect significantly increases. The effect of radachlorin on circulating platelets leads to a change in their functional state, which is manifested in a slowdown in disaggregation. After laser irradiation, the functional changes become more severe. Decreased platelet disaggregation activity may be significant in the mechanism of vascular thrombosis in the area of PDT exposure.

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