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Effect of the gasotransmitter nitric oxide on platelet aggregation: an *in vitro* light transmission study

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The influence of the gasotransmitter nitric oxide (NO) on the aggregation activity of platelets *in vitro* was analyzed using turbidimetric laser aggregometry, which records the light transmission signal of platelet-rich plasma. The aim of the study was to identify the dose-dependent and time-dependent effects of NO on the platelet aggregation process, as well as to establish the biochemical signaling cascades involved in these processes. Blood samples from 5 healthy donors were examined. It was shown that nitric oxide exerts a pronounced inhibitory effect on platelet aggregation, which is evident even at the minimum concentration 1 μM of the direct NO donor sodium nitroprusside. With an increase in concentration and incubation time, a statistically significant decrease in both the extent and the rate of platelet aggregation was observed. This indicates that the nitric oxide-mediated signaling pathway (NO/sGC/cGMP/PKG) exerts an inhibitory effect on platelet aggregation. The findings confirm the promise of using NO donors as agents for correcting platelet aggregation activity and demonstrate the high informativeness of laser aggregometry as a method for analyzing the microrheological properties of platelets.

Keywords: platelet aggregation, gasotransmitters, sodium nitroprusside, nitric oxide, light transmission, turbidimetry.

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1. Introduction

Platelets are involved in blood clotting and coagulation. They are critical elements of the hemostasis system that prevent blood loss when the vascular walls are damaged. After contact with a damaged vessel wall, platelets become active, attach to the vessel, and form an aggregate and a thrombus at the site of damage, thus, preventing the blood loss. Both conditions, bleeding and thrombosis, may result from impaired platelet aggregation [1]. Therefore, assessing platelet function is vital for diagnosing blood conditions and treating the abnormalities.

Nitric oxide (NO), in turn, serves as an essential physiological and regulatory biological mediator that modulates vascular wall tone and hemostatic-thrombotic balance. Impaired NO concentration in the human body leads to various cardiovascular diseases, including thrombosis, atherosclerosis, and others [2,3].

Platelet aggregation analysis is performed in the clinic to evaluate platelet function. The study allows to perform diagnostics of the bleeding risk, thrombophilia, evaluate the effectiveness of antiplatelet/anticoagulant therapy, and select the optimal dose of antiplatelet/anticoagulants, including during surgical interventions. In most cases, deviations from the normal platelet aggregation rate indicate a disorder of hemostasis and other functions in the human body. For example, today it is well-known that higher platelet aggregation rate is critical in the pathogenesis of both, the development and progression of cardiovascular diseases (ischemic heart disease, myocardial infarction, etc.), as well as in the occurrence of complications [4].

Recently, methods of instant diagnostics for studying the aggregation properties of red blood cells and platelets using optical techniques have been actively developed. The advantage of optical methods is their simplicity, low costs, and the possibility of using them directly in the clinic due to their small size and the need for small amounts of blood taken from the patient along with basic clinical tests. Moreover, optical methods allow us to obtain not only static characteristics of cell aggregation, but also to assess the process itself by recording the light scattering signal or the intensity of light passing through blood samples. Laser aggregometry and turbidimetry methods are highly accurate and repeatable [5,6].

The aim of this study is to investigate the process of platelet aggregation *in vitro* using laser aggregometry method. Studying the effect of NO gasotransmitter — sodium nitroprusside (SNP), which is a direct NO donor, on the cells aggregation is highlighted. The kinetics of platelet aggregation was registered at various concentrations of SNP and time of its incubation with platelet-rich plasma (PRP). The mechanism of NO gasotransmitter effect on platelet

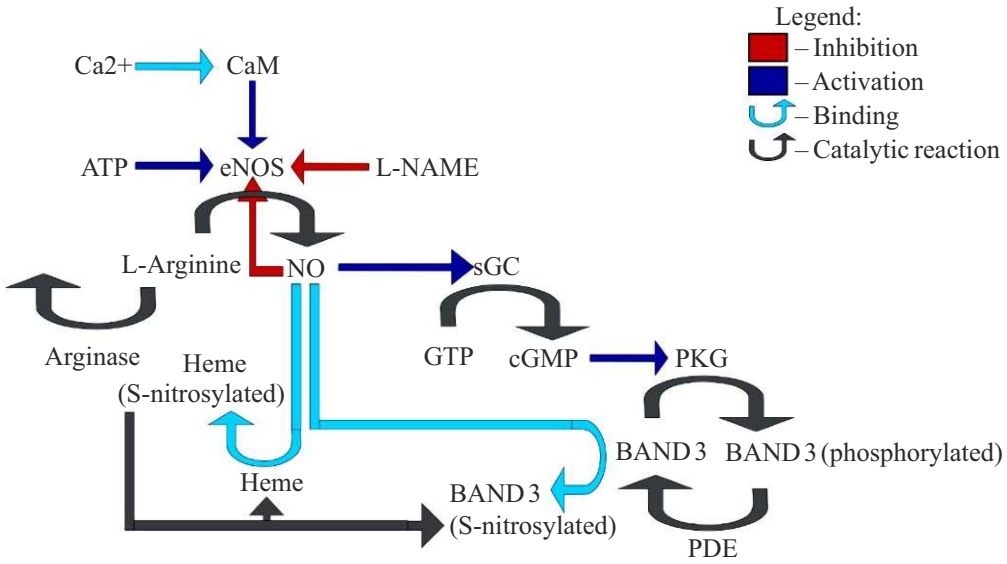

Figure 1. The main NO-mediated signaling pathway (sGC — soluble guanylate cyclase [s-GC], eNOS — endothelial nitric oxide synthase, CaM — calmodulin, ATP — adenosine triphosphate, GTP — guanosine triphosphate, cGMP — cyclic guanosine monophosphate [cGMP], PKG — protein kinase G [PKG], PDE — phosphodiesterase).

aggregation and the signaling pathway mediated by this molecule was analyzed.

2. Nitric oxide in blood circulation

Recently, a method of intercellular and intracellular communication using gas mediators, or gasotransmitters (GT), namely, signaling molecules, has been of particular interest among scientists. Currently, three molecules are the most well-studied: nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S) [7–9].

For a long time, the above-mentioned gas mediators were described as toxic gases that had a damaging effect on the human body. However, it has recently been found that these compounds are endogenously synthesized by the body cells and serve as signaling molecules that perform both autocrine and paracrine regulation in many tissues and organs of the human and animal bodies [10]. Studying the mechanism of GT action — is a pivotal issue in today's physiology.

The object of regulatory impact is the cellular structures of the blood circulatory system. The arterial pressure and tissue perfusion are known to be largely regulated by arterioles and arteries. Paracrine regulatory effects, including those involving GT, dilate small arteries and arterioles, thereby ensuring effective tissue perfusion [11]. NOs are known to stimulate formation of the cyclic nucleotides (cAMP/cGMP) and activate intracellular protein kinase (PKG/PKA) which causes vasodilatation (Fig. 1).

Gasotransmitters are involved in the regulation of blood circulation, including the vascular component, as well as the blood flowing through vessels, by controlling its fluidity and transport potential through the regulatory effect on the endothelial layer of cells that line the vessels, on the microrheology of blood cells, and especially red blood cells [12]. In the circulatory system, NO regulates blood pressure and vascular tone, increases the deformability of red blood cells, and inhibits the aggregation of red blood cells, platelets, and leukocytes [13]. Reduced NO bioavailability is considered one of the main factors in cardiovascular diseases. Exogenous NO donors and substances that stimulate intracellular NO synthase (L-arginine, L-citrulline, statins) are widely used in clinical practice and serve as the basis for the development of new generations of drugs based on NO metabolism [14].

3. Materials and methods

Venous blood for the experiments was taken from healthy donors in the fasted state who had not taken any medication for at least 2 weeks before donating blood. All volunteers were informed about the purpose of the study and gave informed consent in accordance with the World Medical Association Declaration of Helsinki. The study was approved by the Ethics Committee of the Medical Research and Educational Center at Lomonosov Moscow State University. Sodium citrate 3.8% was used as an anticoagulant, which has minimal effect on platelet aggregation. To prepare the platelet-rich plasma (PRP), whole blood was centrifuged at 200 g in „Eppendorf" tubes for 7 min. 800 μL supernatant (top layer) was collected from each tube. The PRP prepared for the study was poured into cuvets and placed in a thermostat at a temperature of 37° to heat it to physiological PRP values for 3 minutes. The viscosity of the prepared blood samples was measured using a rotational viscometer RM100 CP1000 (Lamy Rheology Instruments, France)

purchased as a part of Lomonosov Moscow State University development program. All the prepared samples met the viscosity requirements, as specified in the measurement method. The platelet suspension was mixed and allowed to aggregate using a rotating magnetic rod, which was washed in distilled water and placed in the upper part of the cuvette using a magnet. After that the cuvette was transferred to the measurement cell. Further, prior to the start of measurements they added sodium nitroprusside (SNP) which is a direct NO donor (i.e. it is a chemical substance which generates NO by mechanisms which do not depend on NO-synthase), in volume of 15 μL to reach the next final concentrations 1, 10, 25 50 and 100 μM. The required concentration was achieved by adding distilled water to the SNP solution in the appropriate proportions. Incubation with SNP was hold for 0, 5, 15, 25 and 40 min at 37 °C.

In 10 s after the start of measurements 15 μL of ADP (adenosine diphosphate) which is the platelet aggregation inductor was added to the measuring cuvette in the concentration of 5 μM. During the measurement process, an aggregate diagram was recorded, which is a dependence of the OTP cuvette light transmission signal on time. The higher the light transmission signal, the lower the optical density of the medium, which depends on the characteristic sizes of the scatterers — platelet aggregates. Additionally, an aggregatogram may be used to determine the rate of aggregates formation during their formation process, as this affects the increase in the light transmission signal registered in the experiment. Based on the obtained dependence, the degree of aggregation (maximum light transmission signal during a 5 min aggregation process) and the aggregation rate (the maximum slope of the light transmission curve for 5 min) were calculated (Fig. 2).

The experiments were conducted using ALAT-2 laser platelet aggregation analyzer „Biola“, where a turbidimetric method is used to assess platelet aggregation parameters (Fig. 3). The change in the optical properties of the studied suspension during platelet aggregation is caused by a decrease in the total scattering surface of the cells and an increase in the scattering cross-section of characteristic particles as a result of the formation of platelet aggregates [15]. When analyzing aggregateograms, the degree of aggregation (getting maximum aggregation) and the rate of aggregation (change in plasma optical density in the first minute or the curve slope during rapid aggregation stage) are evaluated [15]. A semiconductor laser with a power of about 1–50 mW and a wavelength of 785 nm, which lies in the optical transparency window of platelets, was used as a light source. Thus, heat effect on PRP may be neglected.

During the experiments, curves (aggregatograms) were obtained that show the intensity of forward-scattered light (transmission) versus time (Fig. 2). 0 % of aggregation correspond to the initial state — the first 10 s after the start of measurement before ADP is added — i.e. the state of the platelet-rich plasma, and 100 % correspond to the platelet-poor plasma (PPP). At the same time, with an

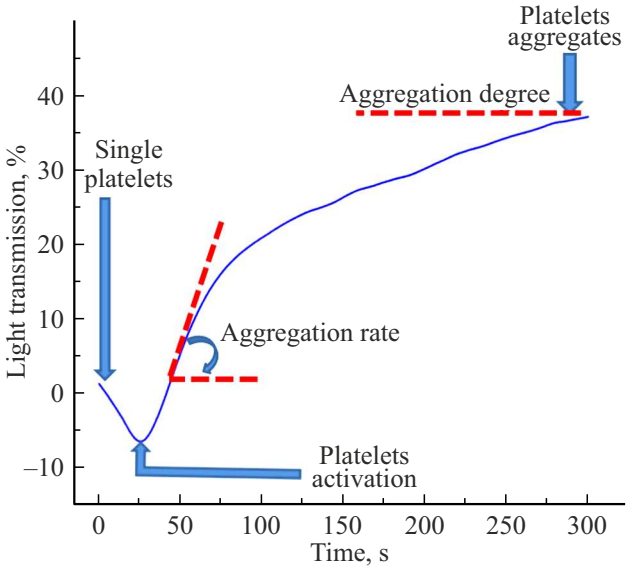

Figure 2. Example of an aggregogram obtained using the turbidimetry method when ADP is added to the platelet-rich plasma.

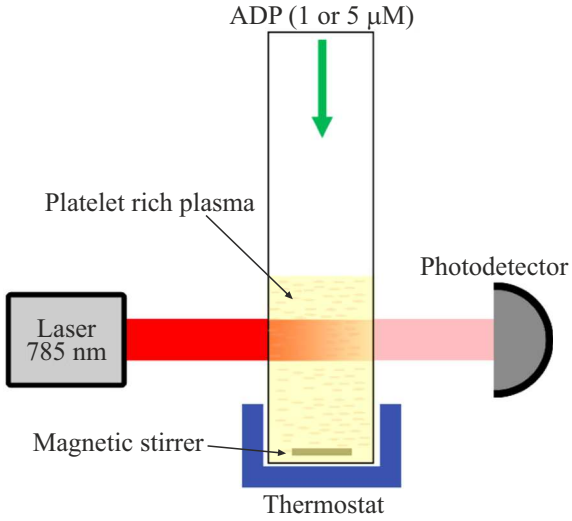

Figure 3. Diagram of the platelet laser aggregometry method.

accuracy sufficient for practice, it can be assumed that the optical properties of PPP and distilled water are equivalent (on ALAT-2 device calibration 100 % is made using distilled water). A decrease in light transmission by 10 s is associated with platelet activation, during which they change their disc shape into a spherical one, forming pseudopodia. As a result, the light transmission becomes less than it was initially (the graph clearly shows a decrease in light transmission). It should also be noted that aggregation is already in progress when ADP is added. This is followed by active platelet aggregation (corresponding to the steepest part of the graph), after which the aggregation gradually reaches saturation.

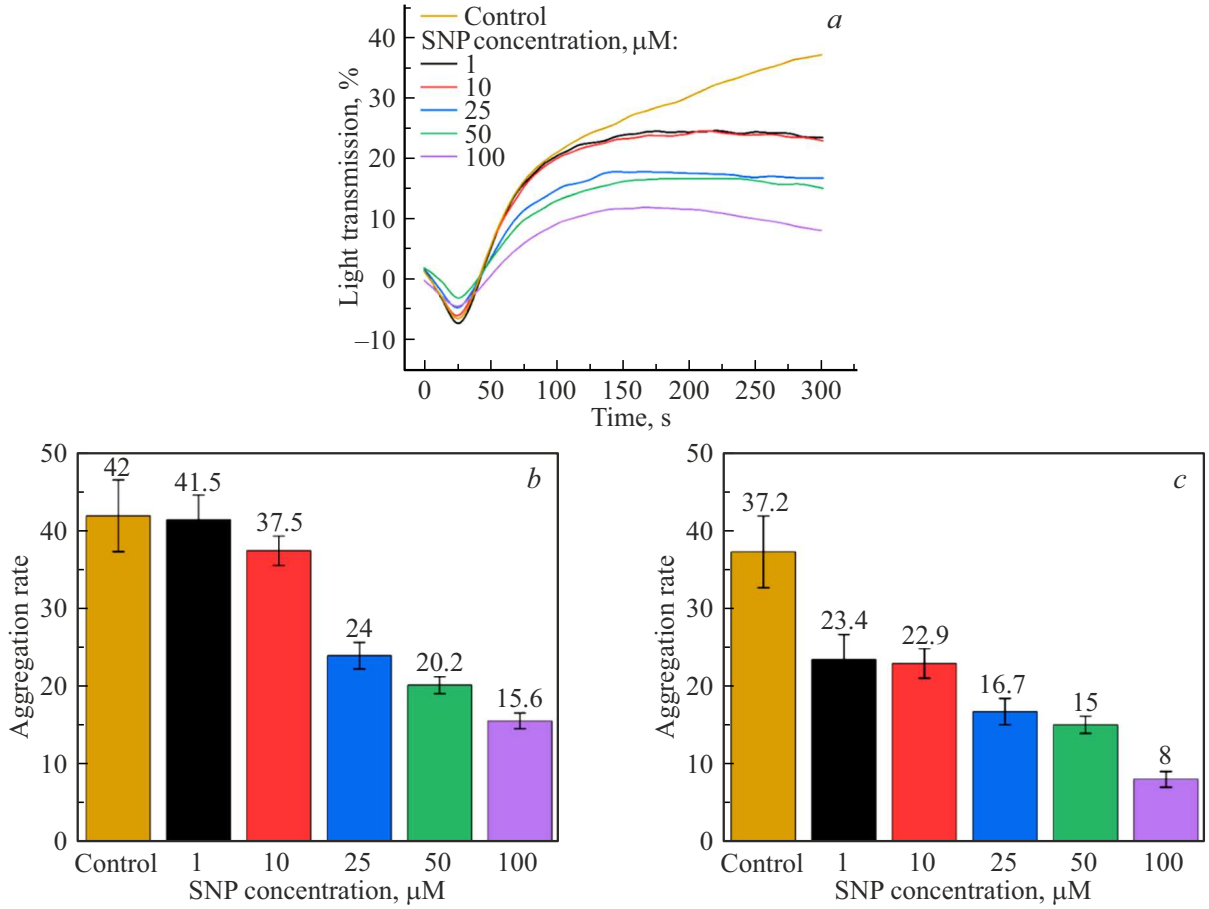

Figure 4. Light transmission kinetics (a), rate (b) and degree (c) of platelet aggregation under the action of NO at different concentrations of SNP.

4. Results and discussion

The degree and rates of platelets aggregations versus SNP concentration were measured within $1-100\,\mu$M. The results obtained for PRP incubated with SNP, as well as for plasma with platelets without SNP, are illustrated in Fig. 4. The graphs in Fig. 4, *a* show smoothed curves of the light transmission versus time, averaged over 5 measurements, and the absolute differences in measurements for each SNP concentration. The statistical significance of the differences in the obtained results was evaluated using the two-sided Mann-Whitney test.

The findings evidence that SNP — as a donor of NO — has great effect on the parameters of platelets aggregation *in vitro*. It is worth noting that even at the lowest concentration of $1\,\mu$M used in the experiment, NO has an inhibitory effect on platelet aggregation. The aggregation degree for a sample incubated with SNP of $1\,\mu$M goes down by $(37-7)\,\%$, however, the *aggregation rate* doesn't change within the measurement accuracy (Fig. 4, *b, c*). At higher concentrations of SNP, there is even more pronounced inhibition of platelet aggregation, and the rate of platelet aggregation also declines (Fig. 4, *b, c*). With SNP of $100\,\mu$M, the degree of aggregation decreases by $(70 \pm 9)\,\%$, and the rate of aggregation goes down by $(27 \pm 5)\,\%$ relative to values without SNP. A statistically significant decrease in platelet aggregation rate is observed with SNP concentration higher than $25\,\mu$M ($p < 0.05$) (Fig. 4, *c*).

Recently, new hypotheses about the stimulating or dual functions of PKG in platelets have appeared in the literature. A group of Chinese scientists raised the issue of cGMP/PKG functions and their role in the platelet activation [16,17]. This work shows that the NO/s-GC/cGMP/PKG signaling pathway is one of the key pathways in inhibiting platelet activity, which completely contradicts the theory of its activating effect on platelet aggregation.

We also studied the effect of NO on platelet aggregation depending on the incubation time of SNP with PRP at different concentrations in the range of $0-40$ min. The results obtained from the aggregatograms are presented in Fig. 5, 6.

Based on the results obtained, it can be seen that at a concentration of SNP in $1\,\mu$M, platelet aggregation is practically independent of the incubation time (Fig. 5, *a*). Thus, it can be assumed that at this concentration, most of

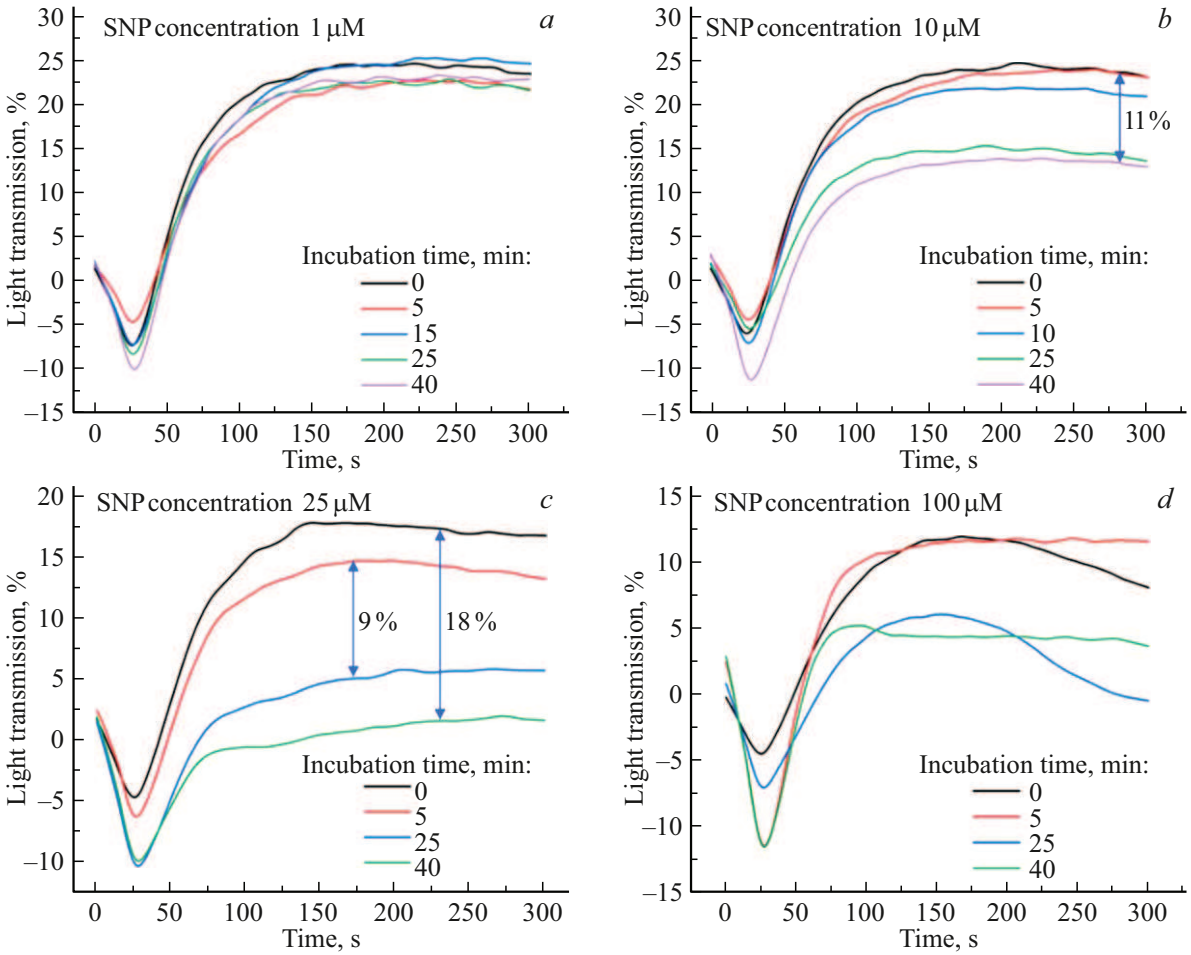

Figure 5. Light transmission kinetics during platelets aggregation under the action of NO at various periods of SNP incubation with PRP and SNP concentrations 1 μM (*a*), 10 μM (*b*), 25 μM (*c*), 100 μM (*d*).

the NO that affects platelet aggregation is released in less than 10 s (the time of ADP addition).

However, at a concentration of SNP 10 μM, there is a significant effect of NO on platelet aggregation depending on the incubation time, and the resulting aggregograms can be divided into two groups based on the incubation time (0–10 min and 25–40 min) where the difference in the platelets aggregation makes (54 ± 7) % (Fig. 5, *b*).

At SNP concentrations of 25 and 100 μM, there is an even more pronounced effect of incubation time on platelet aggregation, and the aggregograms may also be divided into two groups, but when SNP 25 μM, the interval between the groups is greater than at concentrations of 100 μM (Fig. 5, *c, d*). The graphs show a trend of increasing inhibitory effect on platelet aggregation with increasing incubation time of SNP with PRP at concentrations of SNP greater than 10 μM. It can be assumed that at low concentrations of SNP, the formation of NO molecules that have an inhibitory effect on platelet aggregation occurs within the first seconds of the experiment, which is why the effect related to the incubation time in this period is not observed.

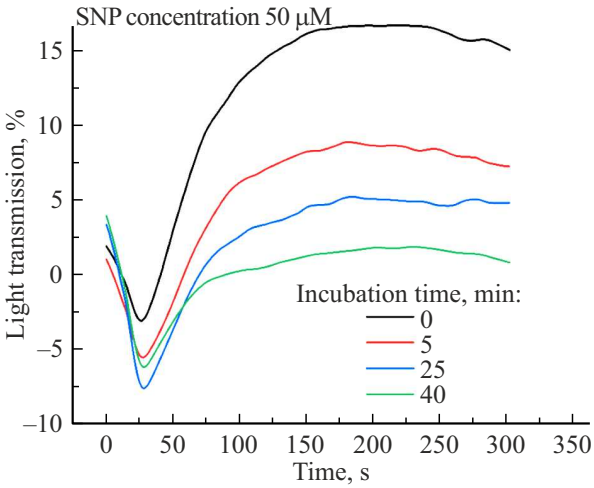

Figure 6. Light transmission kinetics during platelet aggregation under NO action at SNP of 50 μM and different incubation times of SNP with PRP

It can be seen that at a concentration of 50 μM, each aggregatogram is formed along its own individual trajectory

(Fig. 6) without forming two groups in terms of incubation time. Therefore, it can be suggested that the incubation time significantly affects the amount of nitric oxide produced, which affects platelet aggregation.

5. Conclusions

In this study, the turbidimetric method used demonstrated high sensitivity to changes in the platelet aggregation parameters when exposed to NO gasotransmitter — statistically significant differences in the kinetics and parameters of platelet aggregation were found when incubated with SNP. The analysis of NO effect on the aggregation properties of platelets showed an inhibitory effect of this effect on the NO-mediated signaling pathway. This effect doesn't depend on the incubation time and SNP concentration. Based on the analysis of the micro-rheological responses of platelets to NO gasotransmitter donor, it can be concluded that nitric oxide effectively inhibits platelet aggregation *in vitro* through the NO/s-GC/cGMP/PKG signaling pathway and is a promising compound that can be included in important therapeutic recommendations for a number of diseases.

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Conflict of interest

The authors declare that they have no conflict of interest.

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