



EXPERIMENTAL STUDY OF ANTI-THROMBOTIC ACTIVITY OF PENTOXIFYLLIN MICROPARTICLES: BASED ON POLY-DL-LACTIDE-CO-GLYCOLIDE IN COMPARISON WITH PENTOXIFYLLIN

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The aim of the work was a comparative experimental study of the effect of oral administration of Pentoxifylline microparticles based on PLGA, and “standard” Pentoxifylline, on the ADP-induced platelet aggregation process in rats.

Materials and methods. Pentoxifylline substance (100 mg/kg) was used as a reference drug, and PLGA-based Pentoxifylline microparticles with an average dynamic radius of 175.4 nm were used as the object in study. In the experiment, male Wistar rats ($m = 300\text{--}330$ g), the same age group (9 months) were used. They were divided into 3 groups, each of 6 animals. The antiplatelet activity was assessed by determining the degree and rate of platelet aggregation in 1, 3, 5, 8 and 24 hours after a single oral administration of the reference drug and the object under study. Adenosine diphosphate (ADP) at the concentration of $5\ \mu\text{M}$ was used as an aggregation inducer. The aggregation process was recorded using a two-channel laser platelet aggregation analyzer ALAT-2, wavelength of $0.785\ \mu\text{m}$. by determining the average conventional size of the aggregates.

Results. The experiment has proved the following: PLGA-based Pentoxifylline microparticles are more effective at reducing the possibility of platelets to aggregate within 24 hours of the investigation (more than 40%) conventional to the control group value. Besides, it should be noted that according to the effectiveness of the pharmacological action during AD-induced platelet aggregation, the microparticles are commensurate with the standard sample - Pentoxifylline. The action of the microparticle object under study lasts for 24 hours, while the effect of the reference drug is over after 3 hours and then the indicators of the reference group do not differ from those of the control one.

Conclusion. When administered per os, PLGA-based Pentoxifylline microparticles prolong the pharmacological effect significantly – up to 24 hours.

Keywords: Pentoxifylline, poly-DL-lactide-co-glycolide, Pentoxifylline microparticles, rheological properties of blood, antiplatelet agents

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ЭКСПЕРИМЕНТАЛЬНОЕ ИЗУЧЕНИЕ АНТИТРОМБОЦИТАРНОЙ АКТИВНОСТИ МИКРОЧАСТИЦ ПЕНТОКСИФИЛЛИНА НА ОСНОВЕ ПОЛИ-DL-ЛАКТИД-КО-ГЛИКОЛИДА В СРАВНЕНИИ С ПЕНТОКСИФИЛЛИНОМ

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Цель – сравнительное экспериментальное изучение влияния перорального введения микрочастиц пентоксифиллина на основе PLGA и «стандартного» пентоксифиллина, на процесс АДФ-индуцированной агрегации тромбоцитов крыс.

Материалы и методы. В качестве препарата сравнения использовалась субстанция пентоксифиллина (100 мг/кг), в роли исследуемого объекта – микрочастицы пентоксифиллина на основе PLGA (100 мг/кг) со средним динамическим радиусом 175,4 нм. В эксперименте использовались крысы-самцы линии Wistar ($m = 300\text{--}330$ г), одной возрастной группы (9 месяцев), разделенные на 3 группы по 6 животных. Антиагрегантную активность оценивали путем определения степени и скорости агрегации тромбоцитов через 1, 3, 5, 8 и 24 часа после перорального однократного введения препарата сравнения и исследуемого объекта. Аденозин дифосфат (АДФ) в концентрации 5 мкМ применяли в роли индуктора агрегации. Процесс агрегации регистрировали с применением системы двухканального лазерного анализатора агрегации тромбоцитов «АЛАТ – 2», длина волны 0.785 мкм, методом определения среднего относительного размера агрегатов.

Результаты. В ходе эксперимента было доказано следующее: микрочастицы пентоксифиллина на основе PLGA более эффективно уменьшают способность тромбоцитов к агрегации в течении 24 ч. исследования (больше, чем на 40%) относительно значений контрольной группы, кроме того следует отметить, что по эффективности фармакологического действия во время АДФ-индуцированной агрегации тромбоцитов микрочастицы соизмеримы со стандартным образцом – пентоксифиллином. Действие исследуемого объекта микрочастиц продолжается в течение 24 ч., в то время как действие препарата сравнения заканчивается через 3 часа и далее показатели группы сравнения не отличаются от показателей контроля.

Заключение. Микрочастицы пентоксифиллина на основе PLGA при пероральном введении существенным образом пролонгируют фармакологическое действие до 24 ч.

Ключевые слова: пентоксифиллин, поли-DL-лактид-ко-гликолид, микрочастицы пентоксифиллина, реологические свойства крови, антиагреганты

INTRODUCTION

In pathological conditions such as strokes and heart attacks, platelet thrombi play a triggering role. [1]. The task of pharmacy is the development and study of highly effective drugs that comprehensively affect vascular-platelet hemostasis [2–5]. In a number of products aimed at improving the rheological properties of blood, Pentoxifylline has been used most widely [6, 7]. In patients with complex cardiovascular pathology, Pentoxifylline has the most convincing basis for the correction of perfusion disorders [8, 9]. In cerebrovascular and peripheral vascular diseases of atherosclerotic genesis, Pentoxifylline is included in the treatment standards [10–13]. Its use is pathogenetically and clinically validated for the treatment of patients with systemic atherosclerosis [14–16].

Pentoxifylline is known to play the role of a weak P2Y-receptor antagonist, thus it competes with ADP for

the ability to bind to these receptors, resulting in a decrease in the proaggregant effect of ADP on the purine receptors and the assembly process of integral receptors. Pentoxifylline helps to reduce platelet aggregation and adhesion, and also has a vasodilating effect. It also has a weak cardiostimulant effect, caused by the process of blocking phosphodiesterase of type III in cardiomyocytes [1].

It should be also noted that Pentoxifylline reduces the process of fibrinogen synthesis, and reinforces the occurrence of tissue plasminogen activator (t-PA). This leads to the increased activity of the fibrinolytic system [1, 17–20].

The primary metabolism of Pentoxifylline occurs in the blood. Up to seven metabolites are formed during that process, two of them are characterized by a pronounced antiaggregant activity. The final metabolism of Pentoxifylline occurs in the liver [21].

Pentoxifylline is characterized by good tolerance, due to this it is possible to combine its use with many other drugs. Pentoxifylline preparations existing on the pharmaceutical market today, require a triple administration per day, which makes the treatment process rather compliant. In addition, if a patient does not take the drug in time, there is not only a decrease in the effectiveness of therapy, but also a risk of an impairment increase of hemorheological blood properties. In this regard, the creation of Pentoxifylline with prolonged properties is relevant and promising [3, 22, 23].

According to the literature data, the use of prolonged forms based on PLGA (Somatuline, Sandostatin Lar and others), allows increasing the bioavailability of the drug and its delivery to the target organ, maintaining a constant therapeutic concentration in the blood and reducing the frequency of administration. The advantages of PLGA should also include the fact that it has low toxicity, and when ingested, it is completely biodegradable [23]. Considering the above, Pyatigorsk Medical and Pharmaceutical institute outlines the research to create an innovative, prolonged dosage form of Pentoxifylline based on PLGA.

The aim of the work is to study the effect of oral administration of Pentoxifylline microparticles basing on poly-DL-lactide-co-glycolide, on ADP-induced aggregation process, in comparison with Pentoxifylline.

MATERIALS AND METHODS

Animals

The lab rats were obtained from the vivarium of Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. Keeping experimental animals complied with the current regulatory documentation, i.e., the “Sanitary rules for the design, equipment, and maintenance of experimental biological clinics (vivariums)”. The animals were kept on a standard diet that complies with current regulations. Feeding was carried out at a fixed time. For drinking, the laboratory animals were supplied with drinking bowls. The environmental factors (temperature, humidity, light intensity and air exchange rate, litter composition) met the requirements for keeping laboratory animals. The cages, drinking bowls, litter were changed at least once a week. [24]. The contents and all animal manipulations complied with the requirements of the European Convention for the Protection of Vertebrate Animals used for experiments and other scientific purposes (Strasbourg, 1986).

Study design

Pentoxifylline substance (100 mg / kg, “TCI”, USA, Lot. BRDTB-FM, P 2050) was used as a reference drug, and PLGA-based Pentoxifylline microparticles with an average dynamic radius of 175.4 nm were used as the object under study. They were obtained on the base of Pyatigorsk Medical and Pharmaceutical Institute – branch Volgograd State Medical University. An optimal technol-

ogy has been developed for obtaining a prolonged dosage form of Pentoxifylline based on PLGA, namely the ratio of Pentoxifylline and poly-DL-lactide-co-glycolide (50:50), mol.wt 40,000-75.000 (Sigma) – 1: 3. Accurate portions of the polymer and Pentoxifylline substance are dissolved in 2 ml of solvent (chloroform), then the finished composition is added dropwise to the aqueous solution of polyvinyl alcohol at the concentration of 0.3%. The process takes place with continuous operation of the homogenizer at 20,000 rpm for 15 minutes. The finished solution is centrifuged at 6000 rpm for 40 minutes, then the supernatant is decanted and returned for the subsequent analysis.

The recovered sediment of microparticles is washed with purified water. After that it is centrifuged again (4 times). The finished microparticles are transferred to a 25 ml flask, and brought to the mark with purified water. This dosage form is used for pharmacological studies [22, 23].

During the experiment, healthy adult male Wistar rats ($m = 300\text{--}330$ g) of the same age group (9 months), which had been quarantined for 14 days, were used.

With the help of the method of random sampling three groups of 6 animals were created:

- Group 1 – the animals which received a 0.9% sodium chloride solution *per os* in an equivalent volume (control group);
- Group 2 – the animals, which were given a single dose of Pentoxifylline at the dose of 100 mg/kg *per os* (experimental group);
- Group 3 – the animals, which were given a single dose of a prolonged form of Pentoxifylline at the dose of 100 mg/kg *per os* (experimental group).

The objects of the study were administrated at the fixed time of the day (8-00 - 8-30). Considering the fact that Pentoxifylline is widely used in clinical practice *per os* [25], this route of administration was used in the further study. For that, a suspension was prepared in a 0.9% solution of sodium chloride, which was then administered to the animals using a special probe in a volume of 10 ml/kg. The effect of the objects of the study on platelet aggregation was studied at the dose of 100 mg/kg (in terms of Pentoxifylline).

Based on the scientific data on effective therapeutic doses of Pentoxifylline, as well as taking into account the coefficient of conversion of the dose from human to rat, this dose was determined by calculation. [26, 27].

Blood sampling from the animals was carried out on an empty stomach in the morning. To prevent the blood clotting process, a 3.8% solution of sodium citrate was added at the ratio of 1:9. Silicone dishes were used to exclude a contact platelet activity. The induced platelet aggregation was investigated immediately after taking blood for analysis.

Platelet rich plasma (PRP) was obtained and platelet counts were calculated using the standard method [28, 26]. With the help of the centrifuging method (a PC-6

centrifuge was used in the experiment) at 400 g and 1800 g, respectively, PRP was obtained from the blood samples taken for the analysis.

In the Goryaev chamber platelet counts in PRP were performed with the use of the microscopic method with phase contrast. Normally, in the blood of a rat, the number of platelets varies widely - from 430,000 to 1 million in 1 mm^3 - after the analysis of the number of platelets in PRP. To analyze the platelet count of PRP, standardization of the platelet count was carried out, for which the PRP was diluted with the necessary number of PRP to 400 ± 30 thousand platelets in 1 mm^3 in the sample.

Defined indicators

The antiplatelet activity of the prolonged form of Pentoxifylline was evaluated by the degree of platelet aggregation. The indices were recorded after 1, 3, 5, 8 and 24 hours after a single administration of Pentoxifylline microparticles based on poly-DL-lactide-co-glycolide. ADF ADP (NPO "RENAM", Russia) acted as an inducer of aggregation with a total concentration of $5 \mu\text{M}$ [26].

By laser aggregometry, platelet aggregates and a detection and determination of their sizes were held. An assessment of the degree of dispersion of the light beam and fluctuations in the analysis of the optical density were carried out taking into account the light transmittance curve and the size of the aggregates.

This method allows to investigate the platelet ag-

gregation process, size and shape of aggregates. When adding an inductor, the degree of aggregation has a maximum value of the average size of the units [29, 30]. According to the obtained aggregatogram the extent of platelet aggregation was determined.

The conditions in the study of platelets on the aggregometer were close to physiological, namely: a constant mixing speed was maintained, simulating blood circulation, the experiment was conducted at the temperature of $+37^\circ\text{C}$.

Statistical processing

the data obtained were processed by the application package STATISTICA 6.0 (StatSoft, Inc., USA, for the Windows operating system) and Microsoft Excel 2010. The mean value and its standard error ($M \pm m$) was determined. The normal distribution was evaluated by the Shapiro-Wilk criterion. In the normal distribution of the data, the Student's t-test for multiple comparisons was used to compare means. The differences were considered significant at $p < 0.05$. Student's t-parameter was used for normal data distribution of [28].

RESULTS AND DISCUSSION

These amplitudes of ADP-induced platelet aggregation in a standardized plasma in the control group of animals after 1 h amounted to 41.8 ± 4.8 conventional units (Table 1).

Table 1 – The effect of Pentoxifylline microparticles based on poly-DL-lactide-co-glycolide and a standard sample of Pentoxifylline when administered per os at the dose of 100 mg/kg per ADP-induced platelet aggregation process

Group of animals, conventional units	Observation time				
	1 h	3 h	5 h	8 h	24 h
Control	41.8 ± 4.8	47.0 ± 5.1	39.4 ± 2.9	37.8 ± 2.3	42.7 ± 4.8
Pentoxifylline	$23.9 \pm 1.9^*$ x=57.2%	$23.8 \pm 1.9^*$ x=50.6%	40.8 ± 6.8 x=103.6%	42.5 ± 2.8 x=112.4%	40.4 ± 3.9 x=95.7%
Microparticles of Pentoxifylline on the basis of PLGA	$24.2 \pm 1.8^*$ x=57.9%	$27.0 \pm 2.2^*$ x=57.4%	$27.9 \pm 2.3^{\#}$ x=70.8%	$27.2 \pm 3.2^{\#}$ x=72%	$27.2 \pm 1.7^{\#}$ x=63.7%

Note:

* – statistically significant (t - Student's criterion) relative to the control group;

– statistically significant (t - Student's criterion) relative to the Pentoxifylline group

After a single intragastric administration of Pentoxifylline (at the dose of 100 mg/kg), the degree of ADP-induced platelet aggregation in a standardized plasma after 1 h of administration was 23.9 ± 1.9 conventional units (Table 1), i.e. 42.8% lower than in the control group rats. A similar effect on the platelet aggregation activity after 1 h after the introduction of the observation was also established with the introduction of PLGA-based Pentoxifylline microparticles: the studied parameter was 24.2 ± 1.8 conventional units (Table 1) 42.1% lower than in the experimental group. The high efficacy of Pentoxifylline, as well as microparticles of Pentoxifylline on the basis of PLGA as an antiplatelet agent for ADP-induced

platelet aggregation was also recorded 3 hours after the administration (Table 1).

It should be noted that the antiplatelet effect in both experimental groups in the considered time interval was comparable. Thus, in the group with Pentoxifylline, the indicator under study was 23.8 ± 1.9 conventional units (Table 1), and in the group of Pentoxifylline microparticles on the basis of PLGA it was 27.0 ± 2.2 conventional units, while in the control 47.0 ± 5.1 it was conventional units (Table 1). Thus, the reduction in platelet aggregation was 49.4% for the "standard" Pentoxifylline and 42.6% for the PLX-based Pentoxifylline microparticles.

In the study of other time periods (5 h, 8 h and 24 h),

the process of platelet aggregation established significant differences in the effects on the object under study from the comparator drug.

Thus, in the group of the animals which received Pentoxifylline *per os* at the dose of 100 mg/kg, there were no statistically significant differences from the animals of the control group (Table 1). At the same time, in the animals which received oral PLGA-based microparticles *per os* in a similar dose, significant differences were recorded during the analyzed observation period. So, after 5 hours, the degree of platelet aggregation was 27.9 ± 2.3 conventional units, after 8 h – 27.2 ± 3.2 conventional units, and after 24 h – 27.2 ± 1.7 conventional units (Tab. 1). In the control, the studied indicator was respectively 39.4 ± 2.9 conventional units, 37.8 ± 2.9 conventional units, and 42.7 ± 4.8 conventional units (Table. 1).

Thus, the prolonged form of Pentoxifylline on the

basis of PLGA in the dose of 100 mg/kg taken intragastrically once, unlike the standard Pentoxifylline in a similar dose, has a pronounced antiaggregant activity not only for 1 hour and 3 hours of the experiment, but for 5 hours, 8 hours and 24 hours of the observation, respectively, inhibiting the process of platelet aggregation by 29.2%, 28.04% and 36.3%.

Analyzing the influence of the objects of study on the rate of platelet aggregation, it was established that Pentoxifylline microparticles on the basis of PLGA, unlike Pentoxifylline, significantly inhibit this process during the entire observation period. It was experimentally shown that in the 1st and 3rd hours of the observation, the studied parameter in both experimental groups was statistically significantly lower than in the animals without pharmacological correction (Fig. 1).

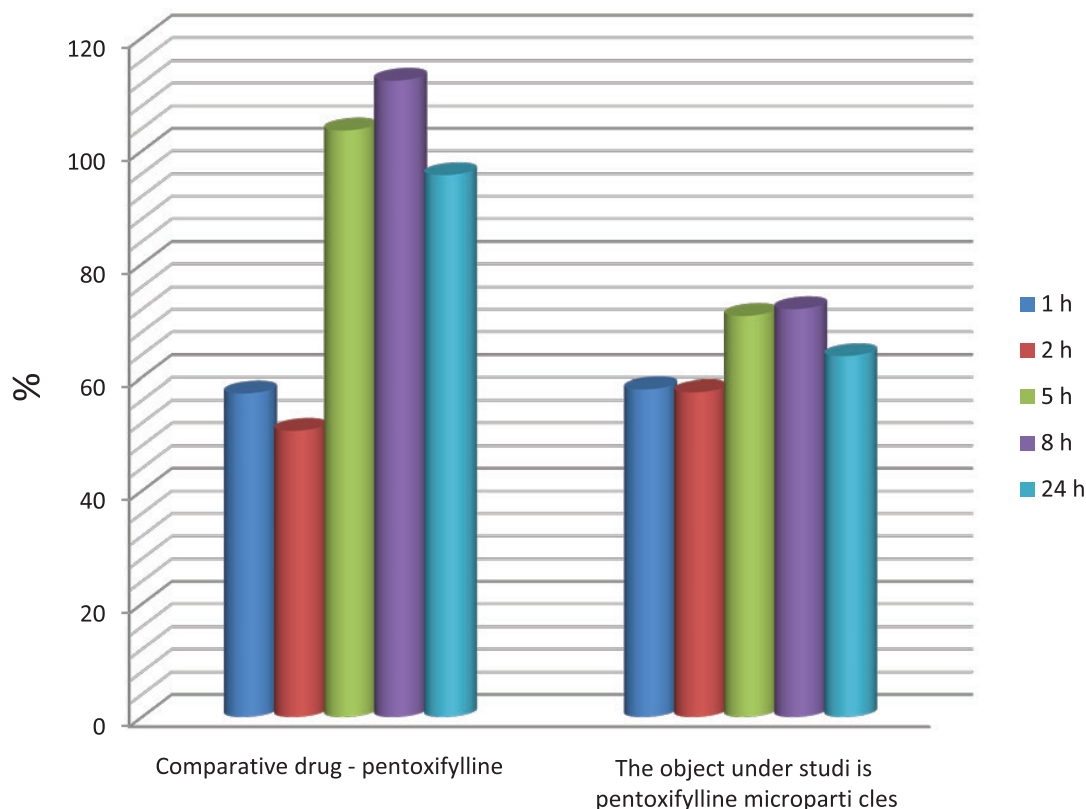


Figure 1 – The effect of Pentoxifylline and Pentoxifylline microparticles on the basis of PLGA on the rate of ADP-induced platelet aggregation

Thus, in the control, the rate of platelet aggregation after 1 hour and 3 hours was 52.71 ± 2.12 conventional units and 57.48 ± 1.44 conventional units, respectively. In the group of the animals that received microparticles of Pentoxifylline on the basis of PLGA it was 30.90 ± 1.37 conventional units (after 1 h) and 29.02 ± 1.63 conventional units (after 3 hours), while in the group of the animals receiving the “standard” preparation, the analyzed indicator was 32.20 ± 0.82 conventional units and $33, 62 \pm 1.36$ conventional units, respectively, after 1 h and 3 h of observation.

Further study of the rate of platelet aggregation indicates a significant difference in the action of Pentoxifylline microparticles based on PLGA and the reference drug, on the dynamics of the process under consideration. In the group of the animals to which the “standard” Pentoxifylline was administered, no statistically significant differences from the control animals on the effects on the platelet aggregation rate at 5, 8 and 24 hours of the experiment were revealed, i.e. the drug’s effect was over (Figure 1). At the same time, the use of the innovative

form of Pentoxifylline significantly limits the process of platelet aggregation during the entire observation period (Fig. 1). In addition, it should be noted that the severity of this process in this group of the animals throughout the experiment was comparable.

The generation of TXA_2 by platelets and a decrease in the level of cAMP is associated with the fact that ADP is a weak agonist. The recorded effect of PLGA-based Pentoxifylline microparticles on cell aggregability must be associated with the changes in platelet membrane properties.

On the platelet membrane, ADP binds to 3 purinoreceptors (P2Y₁₂, P2X₁ and P2Y₁). The ionotropic receptor, P2X₁, is responsible for the entry of exogenous Ca^{2+} and Na^+ into the cell; the remaining two P2Y receptors are associated with G-proteins, which carry a stimulation

signal inside the cell. In order to develop a complete aggregation when exposed to ADP platelets, a compound of this agonist with both P2Y receptors is required [1]. The fact that under the influence of ADP there is a clear aggregation of platelets, as well as due to the effect of Pentoxifylline, its significant suppression occurs, which proves a significant role of purinergic receptors in the implementation of the pharmacological response to this drug once again.

The comparability of the antiplatelet action of the “standard” Pentoxifylline and its innovative form based on PLGA, which were identified at 1 and 3 hours after oral administration of the objects of study, indicate the preservation of biophase during the implementation of the antiplatelet effect of Pentoxifylline microparticles (Fig. 2).

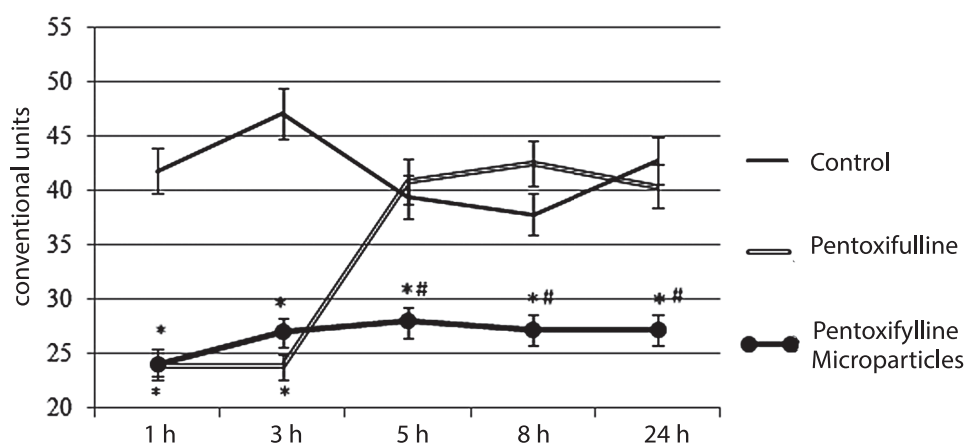


Figure 2 – The effect of Pentoxifylline and Pentoxifylline microparticles on the basis of PLGA on the process of ADP-induced platelet aggregation

Note:

* – marked statistically significant ($p < 0.05$) shifts of parameters compared with the control;

– marked statistically significant ($p < 0.05$) parameter shifts compared to Pentoxifylline.

At the same time, the duration of the pharmacological response (24 hours), which was observed in the study of Pentoxifylline microparticles based on PLGA and under the conditions of ADP induction of platelet aggregation, makes it possible to suggest that pharmacodynamic changes are due to the pharmacokinetics of the object of study. The results of the study indicate that PLGA-based Pentoxifylline microparticles effectively reduce (by more than 40%) platelet aggregation in the first 3 hours of the experiment, while the effectiveness of the pharmacological action of ADP induced platelet aggregation is comparable to “standard” Pentoxifylline (Fig. 2).

According to the data obtained during the experiment, Pentoxifylline microparticles based on PLGA (unlike Pentoxifylline) significantly inhibit ADP-induced platelet aggregation within 24 hours.

CONCLUSION

The use of PLGA-based Pentoxifylline microparti-

cles significantly contributes to prolongation of the action of Pentoxifylline as an antiaggregatory agent for 24 hours.

The results of the experiments showed that PLGA-based Pentoxifylline microparticles are more effective at reducing the ability of platelets to aggregate in the first 3 hours of the study (more than 40%). Besides, it should be noted that the effectiveness of pharmacological action during ADP-induced microparticle platelet aggregation commensurate with the standard sample. According to the data obtained during the experiment, Pentoxifylline microparticles based on poly-DL-lactide-co-glycolide (unlike Pentoxifylline) significantly inhibit ADP-induced platelet aggregation within 24 hours.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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