



INFLUENCE OF HEPARINOID FROM PAEONIA LACTIFLORA ON HEMOSTATIC SYSTEM WITHIN CONDITIONS OF PRETHROMBOSIS

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The search and development of direct and rapid anticoagulants used *per os*, is an urgent problem in physiological and medical science. A number of plants contain heparin-like components with a positive effect on the hemostatic system, both within normal and in some pathological conditions of the body.

The aim of the work was to study the complex effect of fibrin, a heparin-like substance (heparinoid) from the roots of *Paeonia lactiflora*, on fibrinolytic, anticoagulant systems of the body and polymerization processes, when it is administered *per os* in animals within normal conditions and when modeling the state of prethrombosis.

Materials and methods. To carry out the research, the roots of *Paeonia lactiflora* growing in the Botanical Garden of Moscow State University, and laboratory animals – male Wistar rats – were used. To study the antithrombotic effects of the extract from roots containing heparinoid, the state of prethrombosis was modeled in rats. The determined parameters of hemostasis were: anticoagulant activity according to the tests of activated partial thromboplastin time and thrombin time, fibrinolytic activity according to the test of total fibrinolytic activity, fibrin polymerization according to the test of fibrindepolymerization activity of blood plasma.

Results. With repeated (every 24 hours within 3 days) oral administration of the extract containing heparinoid, in animals within normal conditions and with prethrombosis, the following anticoagulant effects were established in the blood: an increase in anticoagulant, fibrindepolymerization and fibrinolytic plasma activity. Possible mechanisms of the activating effect of heparinoid on fibrinolysis and anticoagulant properties of plasma due to the excretion of tissue plasminogen activator into the bloodstream from the endothelium, thrombin inhibition, and fibrin polymerization are described. Moreover, the anticoagulant effect of the use of the extract from the peony roots was equivalent to that of the reference drug of low molecular weight heparin from Celsus (USA). For the first time, it was revealed that when modeling experimental prethrombosis, the administration of heparinoid in rats at the dose of 37.5 IU/kg ME/кг body weight restored impaired hemostasis, which requires a further study.

Conclusion. The ability of heparinoid from peony roots to normalize the functional state of the anticoagulant system during the development of prethrombosis in animals has been established. The restriction of fibrin polymerization during oral administration of heparinoid from peony in animals by increasing the enzymatic fibrinolytic and fibrindepolymerization activity of blood plasma was revealed. In the future, heparinoid can be used as an antithrombotic agent.

Keywords: *Paeonia lactiflora*, peony extract, hemostatic system, prethrombosis, anticoagulant, heparinoid

List of abbreviations: CLA – conjugated linoleic acid; TFA – total fibrinolytic activity; GAGs – glycosaminoglycans; LMWH – low molecular weight heparin; PTT – partial thromboplastin time; FDPA – fibrindepolymerization activity; TT – thrombin time; TFA – total fibrinolytic activity, ACS – anticoagulative system; TTPA – tissue-type plasminogen activator

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ВЛИЯНИЕ ГЕПАРИНОИДА ИЗ ПИОНА (*PAEONIA LACTIFLORA*) НА СИСТЕМУ ГЕМОСТАЗА В УСЛОВИЯХ ПРЕДТРОМБОЗА

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Поиск и разработка антикоагулянтов прямого и быстрого действия, применяемых пероральным методом, является актуальной задачей физиологической и медицинской науки. Ряд растений содержит гепариноподобные компоненты с положительным влиянием на систему гемостаза как в норме, так и при некоторых патологических состояниях организма.

Цель исследования – изучение комплексного воздействия на фибринолитическую, антикоагулянтную системы организма и процессы полимеризации фибрина гепариноподобного вещества (гепариноида) из корней пиона молочноцветкового при его пероральном введении животным в норме и при моделировании состояния предтромбоза.

Материалы и методы. Корни пиона молочноцветкового, произрастающего в Ботаническом саду МГУ. Использовались лабораторные животные – крысы-самцы линии Wistar. Для изучения противотромботических эффектов экстракта из корней, содержащего гепариноид, моделировали у крыс состояние предтромбоза. Определяемыми параметрами гемостаза служили антикоагулянтная активность по тестам активированного частичного тромбопластинового времени и тромбинового времени, фибринолитическая активность по тесту суммарной фибринолитической активности, полимеризации фибрина по тесту фибриндеполимеризационной активности плазмы крови,

Результаты. При многократном (в течение 3-х сут. через каждые 24 ч.) пероральном введении животным в норме и при предтромбозе экстракта, содержащего гепариноид, в крови установлены противосвертывающие эффекты: повышение антикоагулянтной, фибриндеполимеризационной и фибринолитической активности плазмы. Описаны возможные механизмы активирующего действия гепариноида на фибринолиз и антикоагулянтные свойства плазмы вследствие экскреции в кровоток из эндотелия тканевого активатора плазминогена, ингибирования тромбина и полимеризации фибрина. При этом антикоагулянтный эффект от применения экстракта из корней пиона был равноценен таковому у препарата сравнения низкомолекулярного гепарина фирмы Celsus (США). Впервые выявлено, что при моделировании экспериментального предтромбоза введение крысам гепариноида в дозе 37,5 МЕ/кг массы тела восстанавливало нарушенную функцию гемостаза, что требует дальнейшего его изучения.

Заключение. Установлена способность гепариноида из корней пиона нормализовать функциональное состояние противосвертывающей системы при развитии предтромбоза у животных. Выявлено ограничение процесса полимеризации фибрина при пероральном введении животным гепариноида из пиона путем увеличения ферментативной фибринолитической и фибриндеполимеризационной активности плазмы крови. В перспективе гепариноид может применяться как антитромботический агент.

Ключевые слова: экстракт пиона, система гемостаза, предтромбоз, антикоагулянт, гепариноид

Список сокращений: АЧТВ – активированное частичное тромбопластиновое время; ГАГ – гликозаминогликаны; ГП – гепариноподобное вещество (гепариноид); МЕ – международная единица гепарина; НМГ – низкомолекулярный гепарин; ПСС – противосвертывающая система; СЛК – сулодексид; СФА – суммарная фибринолитическая активность; ТАП – тканевой активатор плазминогена; ТВ – тромбиновое время; ФДПА – фибриндеполимеризационная активность

INTRODUCTION

The search and development of direct and rapid anticoagulants used *per os*, is an urgent problem in physiological and medical science. Currently, new oral anticoagulants are widely used for the prevention of thrombosis and the intensive care without a laboratory control [1], which are inferior to low molecular weight heparins in terms of speed of action. Low molecular weight heparin (LMWH) preparations have universally recognized advantages, which include: 1) ease of administration – once a day; 2) no need for laboratory monitoring; 3) relative safety without adverse side effects in the form of bleeding [2]. Despite the creation of ever new drugs for anticoagulant therapy, LMWHs remain the preparations of choice for the

prevention and long-term treatment of thrombosis. Traditionally, LMWH is considered a variety of interchangeable drugs of the same type. However, their relative antithrombin activity varies depending on the average molecular weight. It is known that LMWH drugs are used only subcutaneously or intravenously [3]. Of all the clinic drugs containing LMWH, only Sulodexide can be administered *per os*. It is a highly purified mixture of glycosaminoglycans consisting of LMWH (80%) and dermatan sulfate (20%). The low molecular weight of both fractions of conjugated linoleic acid (CLA) provides a high absorption of the drug when taken *per os* [4]. The pharmacological properties of CLA are significantly different from other glycosaminoglycans (GAGs) and are characterized by a long half-life and

a limited effect on the parameters of the hemostatic system. Due to the presence of two fractions of GAG, CLA potentiates the activity of antithrombin III and cofactor heparin II simultaneously. When taken orally, CLA additionally detects a fibrinolytic activity due to the release of tissue plasminogen activator from vascular endothelium [5]. The antithrombotic and antithrombin activity of CLA is of significant pharmacological interest and makes it possible to use it for the prevention and treatment of diseases complicated by thrombosis [6, 7]. However, this drug is obtained from tissues of the animal origin, namely, from the mucous membrane of the pig's small intestine. At the same time, the search for drugs of a similar action of a plant nature is of an undoubted interest. Many plants serve a source of medicinal raw materials for the isolation of anticoagulants, fibrinolytics and thrombolytics [8–11].

It was previously established that a number of plants contain the components that are an integral part of heparin and other GAGs [12, 13] with a positive effect on the hemostatic system both within normal and in some pathological conditions of the body [14]. An anticoagulant agent of the heparin nature with an inhibitory activity against factor Xa and thrombin was obtained from birch bark [15]. A heparin-like substance was found in the roots of herbaceous peonies. It exerts anticoagulant effects *in vitro* and when administered in animals intravenously [16].

The aim of the work was to establish a comprehensive effect on the fibrinolytic and anticoagulant systems of the body, as well as on the polymerization of fibrin of a heparin-like substance from the roots of *Paeonia lactiflora* administered in animals *per os* both within normal conditions and when modeling the state of prethrombosis.

MATERIALS AND METHODS

Animals

The experiments were performed on 66 laboratory animals – white male Wistar rats weighing 190–210 g. The animals were obtained from the nursery of the Stolbovaya station in the Moscow region. Before and during the experiments, the rats were on a normal laboratory diet and kept under standard conditions in the vivarium of the Faculty of Biology of Moscow State University with a free access to water and food and observing a 12-hour light regime of the day. All the animal experiments were carried out in accordance with the generally accepted ethical principles and standards recommended by the European Convention for the Protection of Vertebrate Animals (Strasbourg 06/15/2006), the Basel and Helsinki Declaration on the Humane Treatment of Animals.

Raw Materials

Paeonia lactiflora roots were obtained from environmentally friendly plants growing in the Botanical Gar-

den of Moscow State University. (Moscow, Russia). This peony species was determined by the employees of the Botanical Garden of Moscow State University chaired by M.S. Uspenskaya. The raw materials were harvested in autumn (from late August to mid-October 2016) and stored at the temperature of + 3...5°C. For the experiments, the primary 5% extract was prepared from dry, clean roots, which were ground in a porcelain mortar to a powder state. The presence of a heparin-like substance was determined in the extract [16] by photoelectrocolorimetric method using Azur A (a dye for heparin) and protamine sulfate (a heparin inhibitor). The heparin-like substance in the amount of 0.5 mg, dissolved in 0.5 ml of physiological solution of sodium chloride, contained 37.5 IU of heparin.

Study Design

The heparin-like substance (heparinoid) in the indicated dose (0.5 mg) and volume (0.5 ml) was administered orally in experimental rats weighing 200 g every day.

The first series of experiments

The studies were conducted on healthy rats kept under standard vivarium conditions. The rats were divided into three groups: the 1st group received heparinoid *per os* once, the 2nd group received heparinoid for 3 days every 24 hours and the 3rd (control) one – a 0.85% sodium chloride solution.

The second series of experiments

The study was carried out under experimental prethrombosis. The state of prethrombosis in rats was modeled by creating depression of the anticoagulative system function by administering a 2.5% solution of chlorpromazine (0.06 ml/200 g of body weight) to turn off the autonomic nervous system, followed (after 30–40 minutes) by intravenous injection in rats of a 1% solution of coagulant – tissue (brain) thromboplastin in the volume of 0.6 ml/200 g of body weight.

Rats with experimental prethrombosis were divided into 3 groups: the 1st (experimental) group preliminarily received -heparinoid three times in the indicated dose and volume; the 2nd one (control) was given a 0.85% sodium chloride solution – at the same time and in the same volume; the 3rd (comparison) group received commercial low molecular weight heparin (LMWH) from Celsus (release date 2016 with antifactorial Xa activity) three-times —on the same time and in the same volume (37.5 IU of heparin per 200 g of a rat body weight). At the same time, in this series of experiments, 2 groups of healthy rats were used for comparison, one of which did not receive any drugs (Norm group), and the second one was treated with heparinoid only three times.

Research practice

The blood was taken from the jugular vein (*vena jugularis*) 20 hours after the last injection of drugs using 3.8% sodium citrate as a preservative in the ratio of 9:1. Then it was centrifuged at 3000g for 10–12 minutes to obtain platelet-poor blood plasma.

To characterize the parameters of the fibrinolytic anticoagulative blood unit, two types of fibrin plates were prepared: 1) unstabilized by factor XIIIa, characterized by the presence of hydrogen bonds in the soluble fibrin polymer; 2) stabilized by factor XIIIa, having strong covalent bonds in an insoluble fibrin polymer. The following biochemical parameters of hemostasis were determined in blood plasma: on the unstabilized fibrin plates there was a total fibrinolytic activity, including the activity of heparin complexes with blood plasma components and plasmin activity, as well as fibrindepolymerization activity, reflecting the processes of fibrin depolymerization; on the standard stabilized fibrin plates there was the activity of a tissue-type plasminogen activator and plasmin enzyme. The anticoagulant activity of plasma was determined by the partial thromboplastin time (PTT) test, which characterizes the internal mechanism of blood coagulation, and thrombin time (TT), which reflects the general path of blood coagulation. In addition, fibrinogen concentration was measured [17].

Statistical processing of results

All data were statistically processed using the non-

parametric Wilcoxon test and Student t-test (Statistica 7.0 software package).

RESULTS AND DISCUSSION

In the first series of experiments with a single oral administration of heparinoid after 20 hours, a significant increase by 28% in the anticoagulant activity (according to the PTT test) was found statically reliable; an increase by 37% in the total fibrinolytic activity (TFA) on unstabilized fibrin due to the total activity of heparin complexes and plasmin activity was established. As evidenced by an increase in the fibrindepolymerization (FDP) activity by 56% compared with the control, the fibrin polymerization process was significantly reduced. An additional confirmation of the inhibition of the fibrin polymerization process was a decrease in the concentration of fibrinogen by 33% compared with the control.

20 hours after a triple oral administration of heparinoid, an even sharper change in the studied parameters towards hypocoagulation was observed. Thus, anticoagulant activity increased by 50% (according to the PTT test) and by 23% (according to the TT test) compared to the control, and exceeded the same indices in the acute experiment by 22–13%, respectively. At the same time, TFA increased by 52%, and, according to the FDA test, the fibrin depolymerization increased by 49% compared with the control (Table 1).

Table 1 – Anticoagulant, fibrinolytic, fibrindepolymerization activities; fibrinogen concentration in 20 hours after once and triple oral administration of heparinoid from *Paeonia Lactiflora* roots in healthy animals (M ± m)

Blood values	Administered <i>per os</i>			
	Heparinoid, administered once (Group 1)	Heparinoid, administered 3 times (Group 2)	0.85% sodium chloride solution, administered once Control 1	0.85% sodium chloride solution, administered 3 times Control 2
Anticoagulant activity				
– (%) by PTT test	39.7 ± 0.4** (128%)	46.6 ± 2.7** (150%)	30.6 ± 1.7 (100%)	31.0 ± 0.7 (100%)
– by TT test	29.0 ± 0.6 110%	34.5 ± 1.4** (123%)	28.0 ± 1.1 (100%)	28.0 ± 1.1 (100%)
TFA, mm ² (%)	55.3 ± 1.3** (137%)	61.6 ± 1.1** (152%)	40.5 ± 1.0 (100%)	40.3 ± 1.5 (100%)
FDPA, mm ² (%)	36.0 ± 1.1** (156%)	38.9 ± 1.3** (149%)	23.0 ± 0.9 (100%)	26.0 ± 0.9 (100%)
TTPA, mm ² (%)	40.1 ± 1.3** (413%)	42.3 ± 1.3** (436%)	9.5 ± 0.8 (100%)	9.7 ± 1.0 (100%)
Plasmin activity, mm ² (%)	20.2 ± 1.5** (230%)	24.2 ± 1.1** (275%)	9.0 ± 0.6 (100%)	8.8 ± 0.8 (100%)
Fibrinogen concentration, mg (%)	187.0 ± 13.2* (67%)	210.0 ± 9.8 (70%)	305.0 ± 10.0 (100%)	308.0 ± 11.3 (100%)

Note: statistical indicators are calculated relative to the corresponding control samples taken as 100%. *p < 0.05; **p < 0.01

As Table 1 shows, the most significant changes after the exposure to heparinoid compared with the control, were observed in enzymatic plasma fibrinolysis of the rats. So, 20 hours after the oral administration of heparinoid once or three times, the activity of plasmin significantly increased by 130–175%, respectively. But especially significant differences from the control under these conditions were found in the activity of TTPA, which exceeded the control level with single and triple use of heparinoid by 313 and 336%, respectively.

The second series of experiments involved the creation of a model of rats with the state of prethrombosis

against the background of depression of the ACS function. In pre-thrombosis (Control group), a sharp inhibition was compared with the Norm anticoagulant plasma activity group (by the PTT test) by 21.4%, TFA decrease – by 65%, FDPA – by 155%, TTP activity – by 115%, the plasmin level – by 69%. At the same time, in prethrombosis, the fibrinogen concentration was increased by 30% compared with the Norm group. In the experimental group of rats, in which prethrombosis was simulated 20 hours after the last triple use of heparinoid, 30 minutes after the administration of tissue thromboplastin in rats (Table 2), a protective antithrombotic effect was established.

Table 2 – Anticoagulant, fibrinolytic, fibrindepolymerization activities; fibrinogen concentration 30 minutes after modeling prethrombosis in rats against the background of triple oral administration of heparinoid from *Paeonia Lactiflora* (M ± m)

Blood values	Heparinoid, administrated 3 (Group 1 – before prethrombosis)	30 minutes after modeling experimental prethrombosis against the background triple administration of:			Healthy rats (Norm group)
		heparinoid – Experiment	0.85%-sodium chloride – Control	LMWH (Celsus) – reference drug	
Anticoagulant activity – (%) by PTT test	39.7 ± 0.4** (163%)	30.0 ± 1.7 (123%)	24.3 ± 2.2 (100%)	28.9 ± 0.7 (122%)	31.0 ± 0.7 (121.4%)
TFA, mm ² (%)	55.3 ± 1.3** (229%)	41.5 ± 1.0** (173%)	23.9 ± 2.1 (100%)	30.0 ± 1.1* (125%)	40.3 ± 1.5 (169%)
FDPA, mm ² (%)	36.0 ± 1.1** (400%)	26.0 ± 0.9** (279%)	9.0 ± 1.5 (100%)	–	23.0 ± 0.9 (255%)
TTPA, mm ² (%)	40.1 ± 1.3** (817%)	8.3 ± 0.8** (184%)	4.5 ± 0.1 (100%)	–	9.7 ± 1.0 (215%)
Plasmin activity, mm ² (%)	20.2 ± 1.5** (348%)	9.0 ± 0.6** (161%)	5.5 ± 0.2 (100%)	–	8.8 ± 0.8 (160%)
Fibrinogen concentration, mg (%)	187.0 ± 13.2** (45%)	326.0 ± 12.0** (78%)	415.0 ± 15.3 (100%)	400.0 ± 12.0 (96%)	308.0 ± 11.3 (70%)

Note: statistical indicators are calculated relative to the corresponding control samples taken as 100%. * $p < 0.05$; ** $p < 0.01$

So, in experimental rats (Experience), the anticoagulant activity, according to the PTT test, increased by 23% compared with the control, and approached the same indicator in healthy rats (Norm group). Moreover, TFA increased by 73% compared with the Control and also almost corresponded to the normal level (Norm group). According to the FDPA test, fibrin depolymerization increased by 179% under the influence of heparinoid, while in the Norm group this value exceeded the control level by 155%. In the Experience, TTPA increased by 84% compared with the Control but, however, did not reach (by 31%) the level noted in the Norm group. Plasmin activity in the Norm and Experience groups exceeded the control level by 60–61%. The fibrinogen concentration after the exposure to heparinoid corresponded to the normal values (Table 2). In the reference group (commercial LMWH was administrated), an increase in anticoagulant and the total fibrinolytic activity was found to be 22 and 25%, respectively.

Analyzing the results obtained, it should be noted that the extract from the roots of *Paeonia lactiflora*, like SLK [5], has not only an anticoagulant effect, like heparin [18] and the reference drug (Table 2), but also a wide

range of fibrinolytic effects when administered into the body *per os*. It enhances the activity of tissue plasminogen activator in the bloodstream more than 4 times, both with single and triple uses.

It indicates its high affinity for endothelial cells and indicates its ability to interact with endothelial receptors, expressing plasminogen activators into the bloodstream from the vessel wall, as was shown in other works, under the action of other plant anticoagulants [19]. It was also revealed that this process is associated with the increased plasmin activity under the influence of heparinoid. We are the first to have established the fact that plant-based heparinoid, when administered *per os*, interferes with the polymerization of fibrin, as a result of which its FDPA in blood plasma increases by more than 50–60%. Earlier [20], it was reported that thrombin interacts with fibrinogen under the influence of plant inhibitors, which was observed in our studies.

It is noteworthy that a protective antithrombotic effect, which was found when modeling the state of prethrombosis in animals, was revealed in heparinoid. The heparinoid studied by us when taken *per os*, like the SLK administered *per os* [6], has both antithrombin and an-

tithrombotic activity. Heparinoid, like SLK, is of pharmacological interest in terms of its use for the prevention of diseases complicated by thrombosis.

CONCLUSION

Based on the study of the effects of the plant heparinoid from *Paeonia lactiflora*, its anticoagulant effect is revealed even with a single administration *per os*. It is enhanced when administered every 24 hours at the daily dose of 37.5 IU (by heparin content) per 200 g of rat body weight for 3 days. Its protective and corrective role in restoring the impaired function of the anticoagulant system during the development of the state of prethrombosis in animals has been established. It was found out that heparinoid, when administered *per os*, reduces the plasma concentration of fibrinogen, limits the process of fibrin polymerization, increases the enzymatic plasma fibrinolytic activity by increasing the level of plasmin and the activity of tissue plasminogen activator, and enhances the total fibrinolytic activity, as well

as the fibrin-depolymerization activity caused by the complex of heparin with proteins, peptides and blood amino acids in the blood. Plant heparinoid has a functional similarity with low molecular weight heparin by the mechanism of an antithrombotic action. Moreover, the anticoagulant effect caused by the use of the extract from the roots of *Paeonia lactiflora*, was not inferior to that of the drug for comparison of low molecular weight, heparin from Celsus (USA). The studied preparation from the roots of *Paeonia lactiflora* is a complex agent with anticoagulant, fibrinolytic, fibrindepolymerization properties that protects the body from the development of thrombotic conditions. It can ensure the lysis of fresh fibrin clots that have just appeared in the bloodstream. It can do that both due to its direct effect on polymerizable fibrin and through an indirect effect on plasma hemostasis. All of these effects of plant heparinoid prove the effectiveness and prospects of studying this agent in order to prevent and treat thromboembolic diseases and thrombotic complications.

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AUTHORS' CONTRIBUTION

All the authors equally participated in physiological experiments and in determining the parameters of hemostasis. Lyapina M. G has made up the experimental strategy, Uspenskaya M. S. was responsible for the determination of the type of peony, harvesting and storage of raw materials; Maistrenko E. S. was responsible for the preparation of extract from peony, statistical processing of the results; Kalugina M. D. – for the literature search.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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