RESEARCH PAPER

MECHANISMS OF THE INFLUENCE OF MODIFIED SULFATED POLYSACCHARIDE K-F-γ ON THE HEMOSTASIS SYSTEM

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ABSTRACT

This article discusses the effect of modified sulphated polysaccharide MSP K-F-γ (K-F-γ) isolated from potato starch on the coagulation-platelet haemostasis system. It is shown that, in vitro, K-F-γ does not affect blood coagulation and platelet aggregation under normal conditions, but when activated by blood coagulation factors (in APTT, PTT, TV, LET) or when activated by platelet inducers (ADP or adrenaline), it exhibits varying degrees of anticoagulant and platelet aggregation inhibitory effects. Experiments conducted using fluorescence methods with Fura 2AM and chlorotetracycline (ChTC) probes have shown that the action of K-F-γ is associated with the manifestation of chelating ability in relation to intracellular and membrane-bound calcium ions. Analysis of the results obtained indicates that the action of K-F-γ in binding Ca^{2+} ions prevents its participation as a blood coagulation cofactor, inhibits the activation of the prothrombinase complex [Xa-Va-Ca^{2+}-phospholipid membrane] and suppresses platelet activation processes.

Keywords: modified sulphated polysaccharide, modulation of experimental thrombosis, thromboelastin, anticoagulant, platelet aggregation inhibitor.

How to cite this article: Musaeva MK, Ruzumova GK, Usmanov PB, Nasirov KE, Ikromova P, Erkinova NE, Yusupov AAA, Ortikov MMO, Akhmedov OR. Mechanisms of the Influence of Modified Sulfated Polysaccharide K-F-γ on the Hemostasis System. Int J Drug Deliv Technol. 2026;16(5): 1370-1378. DOI: 10.25258/ijddt.16.5.127

Source of support: Nil.

Conflict of interest: None

Introduction

Polysaccharides containing sulphate groups in their macromolecular chains are an important class of natural high-molecular compounds with a wide range of pharmacological properties. Due to the presence of negatively charged sulphate groups, such polysaccharides are capable of interacting complementarily with biomolecules in the body, including blood plasma proteins and cell receptor components, which determines their biological activity. Such interactions underlie a variety of physiological effects, including anti-inflammatory, immunotropic, antitumour and antiviral actions.

Sulfated polysaccharides are of great scientific and practical importance in the development of new anticoagulants. It is known that taking anticoagulant drugs based on sulfated polysaccharides can reduce the risk of acute vascular complications, including ischaemic disease [1–3,4].

The mechanism of action of sulphated polysaccharides differs from that of heparin. Modified sulphated polysaccharides (MSP). Modified sulphated polysaccharides directly inhibit thrombin without the involvement of plasma serine protease inhibitors, unlike heparin. MSP inhibit the intrinsic coagulation pathway at low doses and the extrinsic coagulation pathway at high doses. It has been established that MSP do not have an acute toxic effect, but have a broad therapeutic effect. It is likely that the anticoagulant activity of sulphated polysaccharides is due not only to the presence of sulphate groups and their distribution, but also to the structure of the carbohydrate chains of polysaccharides, the presence of other functional substituents, their position and distribution. [5]

Therefore, modified sulphated polysaccharides exhibit anticoagulant properties associated with the inhibition of fibrinogen coagulation and the amidolytic activity of thrombin and factor Xa by antithrombin. The specific anticoagulant activity of synthesised sulphated derivatives depends on the number of sulphate groups.[6,7] At the same time, some polysaccharides used to treat thrombosis can cause undesirable side effects by suppressing normal platelet function [8,9]. In this regard, it is important to evaluate the effect of sulphated polysaccharides not only on coagulation but also on platelet function. Therefore, the aim of this study was to investigate the effect of modified K-F-γ on coagulation and platelet haemostasis in vitro and in vivo.

Materials and Methods. Synthesis of starch derivative (K-F-γ). (Figure 1.) 3 g of potato starch dried to constant weight was placed in a 500 L dark glass jar, then 150 mL of acetate buffer with a pH of 4.5 and 100 mL of 0.25 M NaIO4 solution were added. The periodate oxidation reaction of potato starch continued for 8 hours at t=20°C. The resulting modified starch was filtered on a Schott filter and washed with 500-700 ml of water with the addition of hydrochloric acid solution to pH 1.2-1.5, then 150-200 ml of acetone until a negative reaction to IO4- and IO3- ions (control by reaction with silver nitrate solution) and dried at t=50°C. The weight content of aldehyde groups in the modified starch is 21.6 wt.%. In the next stage, oxidised starch with an aldehyde group content of 21.6% by weight was placed in a flask and 100 ml of water was added. The resulting reaction mixture was heated to 70-80°C and stirred until a clear solution was formed. 1.4 g of sodium formamidine sulphate was added to the clear solution at a temperature of 70-80°C and held for 60 minutes. The solution was placed in semi-permeable membranes with a protein permeability limit of 3000 Da, dialyzed against distilled water for 48 hours and isolated by lyophilization. The resulting starch derivative is an odourless, white powder, highly soluble in water (>10 g per 100 g of water), with an average molecular weight of 12,000 Da, a ζ-potential of -28 mV and a pKa value of 3.8.

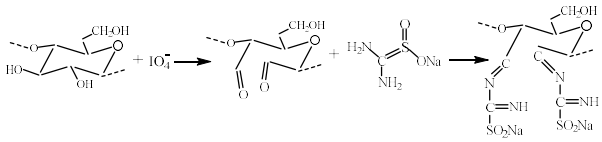


Figure 1. Synthesis pathway of a starch derivative containing anionic sulphate groups.

Platelet-rich plasma obtained from human donors was used to assess changes in platelet coagulation and aggregation activity. All coagulation tests were performed on a single-channel coagulometer (CYANCoag , Belgium.CY003, SN:5400439).

Platelet aggregation was recorded using the Born method [10] on an ALAT-2 Biola aggregometer (No. FSR2007/01301, Russia). A fluorescent method was used to measure the amount of intracellular Ca2+ in platelets. The measurement was performed using a USB-2000 spectrometer (USB2E7916.OceanOptics.USA.2010) [11].

Simulated experiments were conducted on white mice of both sexes weighing 20±2 g, in vivo. A suspension of thromboephrin in 0.9% sodium chloride solution (25 mg/kg) was used as a thromboforming agent, which was injected into the tail vein of the animal in a volume of 0.02 ml. K-F-γ at a dose of 25 mg/kg and 50 mg/kg was administered intraperitoneally 10 minutes before the administration of the thrombus-forming agent. The number of dead animals and macroscopic examination of the lungs of dead and surviving mice one day after administration of the thromboforming agent and the compounds under study were recorded as criteria for thrombus formation.

The following model experiments were conducted on white rats of both sexes weighing 180-200 g. Thromboplastin was administered intravenously at a dose of 10 mg/kg against a background of prior administration of K-F-γ into the abdominal cavity at a dose of 5 mg/kg 10 minutes before the administration of the thrombus-forming agent thromboplastin. Control rats were injected with a 0.9% NaCl solution into the same vein instead of the thromboplastin suspension. Ten and 30 minutes after the administration of thromboplastin, blood taken from the rat's gums was centrifuged on a coagulant (3.8% sodium citrate solution) 9:1 at 3000 rpm for 10 minutes (OPN-8 centrifuge (rotor RU180 L, 8000 rpm) 2 2007-2008. Russia). The resulting plasma was tested for activated partial thromboplastin time (APTT) and other coagulation tests.

Results. Studies have shown that K-F-γ SML, in activated partial thromboplastin time (APTT), prothrombin time (PT), prolonged clotting time to varying degrees, depending on the dose. These studies

yielded the following results: K-F-γ MSP led to a dose-dependent increase in prothrombin time and effective prolongation of APTT. It should be noted that the dose-dependent prolongation of prothrombin time and APTT occurred up to a certain dose (50 µg/ml). K-F-γ at concentrations of 5-50 µg/ml prolonged APTT from 32 to 500 seconds, and PT from 12 to 60 seconds, respectively. Further increases in dose did not lead to an increase in coagulation parameters. As can be seen, at these concentrations, the polysaccharide K-F-γ prolonged APTT much more than PT (**Figure 2. (A and B**)).

At these concentrations, K-F-γ had practically no effect on thrombin time (TT). It should be noted that the resulting thrombus was weak and loose.

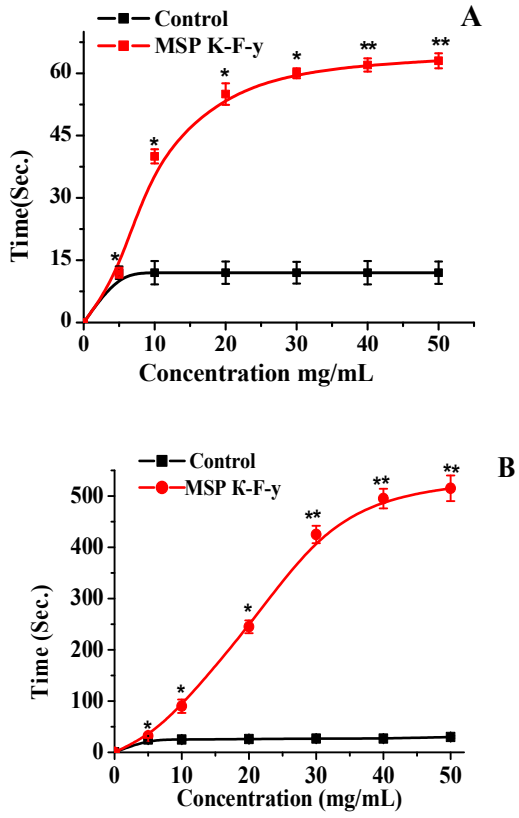


Figure 2 (A). PTT tests at concentrations of -50 mg/mL . **(B)**. The effect of K-F-γ on APTT *- p<0.05. **- p<0.01 (n=6).

The following experiment investigated the effect of polysaccharide K-F-y on the lebetox test (LTT) and echitox test (EChT).

The LET based on the venom of the Central Asian viper Vipere lebetina is used to determine the activation of factor X in the presence of Ca^{2+} and factor V and diagnoses disorders in the haemostasis system, starting with factors X and V, II, I, XIII. When exposed to K-F-γ , the cause of the prolongation of both APTT and PTT is the inhibition of one of the factors II, V, VII, X, XIII + Ca^{2+}. Since the cause of PTT prolongation when exposed to anticoagulants may be the deactivation of factors II, V, VII, X. Factor X can be activated by factor IXa, requiring Ca2+, phospholipid and factor VIIIa, or factor VIIa, requiring Ca^{2+} and tissue factor.

It is known that some snake venoms contain factor X activators and prothrombin activators, which are serine proteases and can cause blood clotting disorders [8,19,32,33].

EChT based on Echis multisquamatus venom is used to determine the activation of the prothrombin complex and factors II, I, XIII, regardless of the presence of Ca^{2+} ions.

In this table 1. In different tests, the causes of blood clotting disorders depending on factor inactivation.

This table 1 shows the causes of blood coagulation disorders depending on the inactivation of factors.

APTT	PT	The lebetax test	The echitax is test	Cause of violations Factor disorders
elongation	norm	norm	norm	XII, XI, IX, VIII
norm	elongation	norm	norm	VII
elongation	elongation	elongation	norm	X, V, XIII + Ca^{2+}
elongation	elongation	elongation	elongation	X, V, II, I, XIII + Ca^{2+}

Table 2. Effect of polysaccharide on coagulation tests. The table shows that K-F-γ is more effective in prolonging clotting time in the APTT test than LET, PTT, and EChT.

APTT	PT	The lebetax test	Echitax test	Cause of violations Factor disorders
Extends significantly Up to 350-500 seconds	Lengthens Weakly 55-60 seconds	Lengthens dramatically to 300-325 seconds.	lengthens weakly 35-40 seconds	

elongation	elongation	elongation	elongation	X, V, II, I, XIII + Ca^{2+}
norm	elongation	norm	norm	VII
elongation	elongation	elongation	norm	X, V+ Ca^{2+}
elongation	elongation	elongation	elongation	II, I, XIII+ Ca^{2+}

As is known, APTT allows one to assess the overall integrity of coagulation factors, identify deficiencies or disorders in the functions of individual coagulation factors and contact factors of the internal pathway (VIII, IX, X, XI, XII, and XIII), and determine the degree of deficiency or disorder in the functions of individual coagulation factors and contact factors of the external pathway.

As is well known, APTT allows one to assess the overall preservation of coagulation factor functions, identify deficiencies or disorders in the functions of individual coagulation factors and contact factors of the internal pathway (VIII, IX, XI, XII, prekallikrein, high-molecular-weight kininogens) and the common pathway (including factors II, V, X and I). This test determines the time required for clot formation after the addition of a mixture of kaolin, kefalin and $CaCl_2$. [12,30
].

A study of the effect of K-F-γ on haemostasis in experimental rats showed that two subcutaneous injections led to a significant reduction in APTT and recalcification time compared with intact animals.

The data obtained indicate that K-F-γ effectively acts on the blood coagulation system, primarily by inhibiting Ca^{2+}-activated factors such as the prothrombin complex (Xa + Va complex, which ensures the conversion of prothrombin to thrombin II → IIa) and the final stage of fibrin clot formation under the action of fibrin-stabilising factor XIII (which, with the participation of Ca^{2+} ions, changes from an inactive form to an active form, XIIIa) [13,14].

As is known, when coagulation factors are activated, a disruption in the platelet haemostasis system plays a special role. In this regard, the effect of K-F-γ on platelet functional activity was investigated. In these experiments, the effect of K-F-γ on spontaneous and ADP, adrenaline and collagen-induced platelet aggregation was evaluated [15,16,29,31].

These studies showed that K-F-γ at a dose of 50 μg/ml does not affect spontaneous platelet aggregation, but causes a significant inhibitory effect when induced with ADP and adrenaline. At the same time, the pronounced inhibitory effect of K-F-γ was manifested when platelets were induced with ADP. K-F-γ significantly altered the dynamics of the first phase and almost completely inhibited the second phase (90%). K-F-γ had practically no effect on collagen-induced platelet aggregation **(Figure 3. (A and B))**

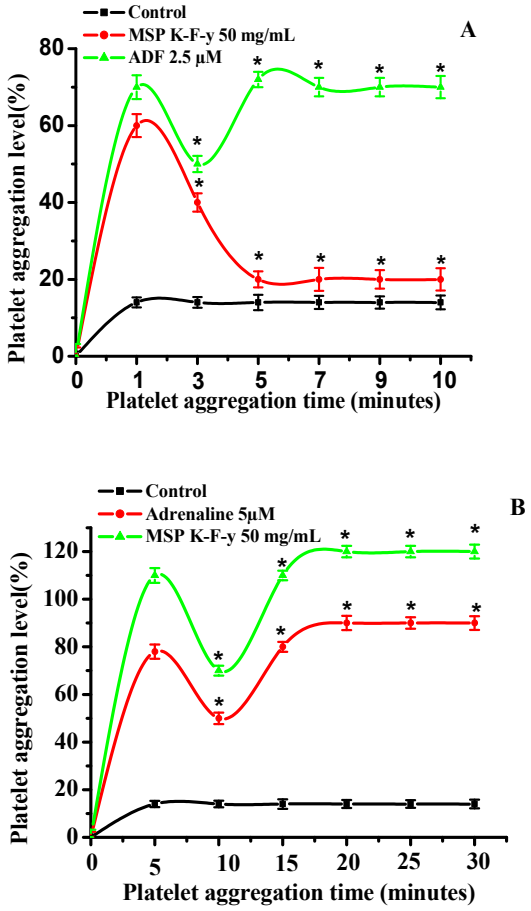


Figure 3. Effect of K-F-γ (at a concentration of 50 mg/ml) on ADP **(A)** and adrenaline **(B)** induced platelet aggregation. *- $p<0.05$. (n=6)

As is known, the action of ADP is mediated through binding to the P2Y12 receptor. When platelets interact with ADP, their shape changes, the GPIIb–IIIa complex (a receptor for fibrinogen) is exposed on the membrane, and primary calcium-dependent aggregation occurs. Secondary aggregation is mediated by intracellular signal transmission via G proteins with an increase in intracellular Ca^{2+} concentration [17,18].

Adrenaline, interacting with α2-adrenoreceptors, causes inhibition of adenylate cyclase. It is possible that the mechanism underlying the effect of adrenaline and the development of the first wave of aggregation does not depend on the formation of thromboxane A2, the release reaction or the synthesis of platelet aggregation factor, but is associated with the ability of adrenaline to directly alter the permeability of the cell membrane to calcium ions. The second wave of

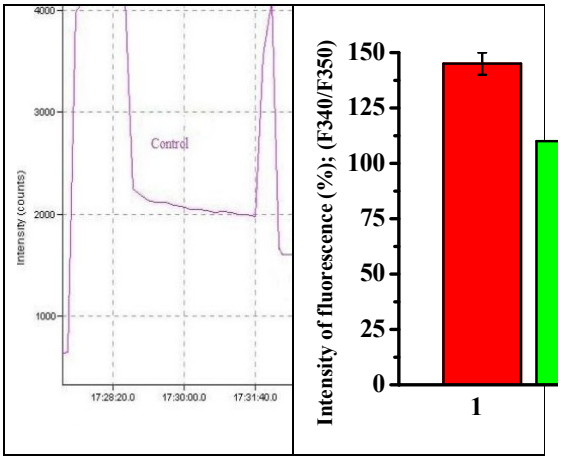
aggregation occurs as a result of the release reaction and production of thromboxane A2. Adrenaline reflects the TXA2-dependent pathway of platelet activation.

Based on the results obtained, it can be assumed that K-F-γ as an inhibitor causes inhibition of calcium-dependent processes, activation of platelet aggregation factor and secretion, and release of coagulation factors. It is known from the literature that Ca^{2+} ions are a very important intracellular regulator of platelet function . Extracellular Ca^{2+} ions are very important for platelet aggregation, since aggregation and the release of platelet granules are largely dependent on the level of this cation. About a quarter of all calcium is bound to platelet membrane structures, but most of it is located in dense granules and the tubular system [20,21,22].

Some platelet aggregation inducers, such as platelet-activating factor, thrombin, and others, cause Ca^{2+} to be released from plasma into platelets through receptor-controlled channels capable of passing divalent cations. To verify whether the antiaggregatory action of K-F-γ is related to the effect on calcium-dependent receptor-controlled channels, its action on the intracellular calcium level in platelets was investigated using the fluorescent probe fura 2A. In these excretions, ADP in platelet-enriched plasma increased fluorescence in accordance with intracellular Ca^{2+} concentration, whereas K-F-γ , on the contrary, decreased fluorescence. K-F-γ also dose-dependently reduced the ADP-induced increase in intracellular calcium levels in the cytoplasm.

These results show that K-F-γ can act on both extracellular and intracellular calcium-dependent processes. **(Figure 4. (A and B))**

A



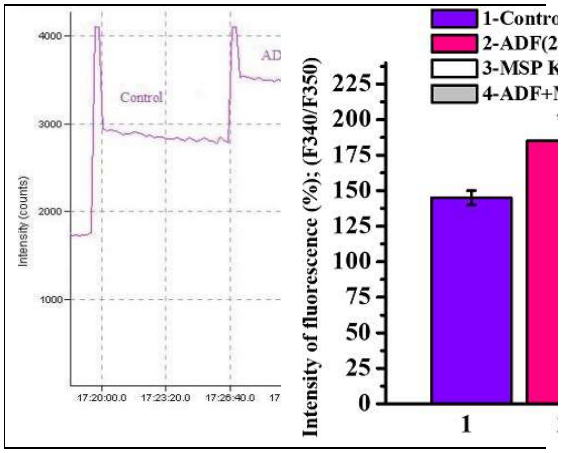


Figure 4. Effect of ADP and K-F-γ on fluorescence from fura 2AM.

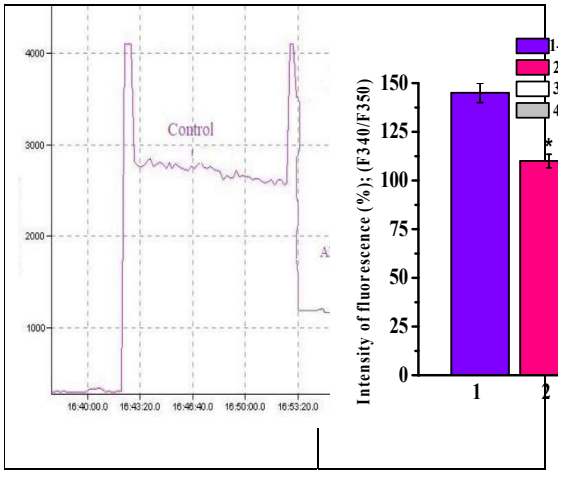
A. Dose dependent effect of K-F-γ on fluorescence from fura 2AM.

1.Control. 2.K-F-γ (10 mg/ml); 3.K-F-γ MSP (25 mg/ml); 4.K-F-γ (50 mg/ml); *- $p<0.05$(n=6)

B. Effect of K-F-γ MSP on ADP induced fluorescence from fura 2AM

1.Control. 2.ADP 2(μM); 3.K-F-γ (50 mg/ml); 4.ADP + K-F-γ (50 mg/ml) *- $p<0.05$. **- $p<0.01$ (n=6).

Experiments were performed in two steps in both the presence and absence of physiological Ca^{2+} concentrations in platelet-rich plasma. The chelating ability of K-F-γ MSPs towards calcium ions was investigated using fluorescent ChTC probes in the presence and absence of EGTA. Fluorescence with ChTC in the background of EGTA was significantly lower than its absence in fluorescence with ChTC relative to the control**.(Figure (5. A and B))**. Without calcium medium on EGTA background, ADP and K-F-γ MSP had practical no effect on fluorescence, respectively on mebran-bound Ca^{2+}.But in the absence of EGTA, ADP and K-F-γ alone and in combination caused an increase



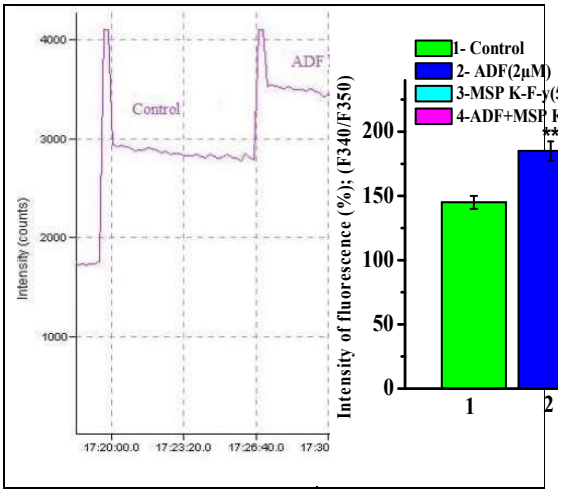


Figure 5. A and B. Effect of ADP and K-F-γ on Fluorescence with ChTC.
A. In the presence of EGTA
1.Control. 2.ADP; 3.K-F-γ; 4.ADP + K-F-γ ;
B. In the absence of EGTA.
1.Control. 2. ADP; 3. K-F-γ; 4.ADP + K-F -γ *- $p<0.05$. **- $p<0.01$ (n=6)

From the results obtained, it can be assumed that the action of K-F-γ is associated with the manifestation of chelating ability towards calcium ions, and the formation of a complex with membrane-bound calcium. Increased amounts of extracellular membrane-bound Ca^{2+}, under the action of K-F-γ inhibit aggregation and release reaction.

In vivo studies investigated the effect of K-F-γ on pulmonary thrombosis in model experiments on rats.Thrombosis was induced by administering thromboplastin in a 0.9% sodium chloride solution (25 mg/kg) into the tail vein of mice in a volume of 0.02 ml. [23-25,28-30]. As can be seen from the data presented in Table 3, after the administration of the thromboforming agent, 3 out of 4 mice died in the group of animals, which amounted to 75% mortality. When thromboplastin was administered against the background of K-F-γ at a dose of 25 mg/kg, 1 mouse out of 4, or 25%, died. When thromboplastin was administered intravenously against the background of K-F-γ at a dose of 50 mg/kg, there were no animal deaths (Table 1).

Table 3. Dose –dependent effect of K-F-γ on pulmonary thrombosis :

	Dose	Group	Burnt out	The deceased
Control NaCl solution	0,9%	4	4	0
Pulmonary thrombosis Thromboplastin	10 mg/kg	4	1	3
Pulmonary thrombosis against a background of K-F-γ	25 mg/kg	4	1	3
Pulmonary thrombosis against a background of K-F-γ	50 mg/kg	4	4	4

The death of animals was recorded after 15 minutes, as well as at other times during the first day of observation.
Thus, it was shown that intraperitoneal administration of K-F-γ increases the survival rate of animals with exogenous thrombophlebitis by limiting destructive changes in internal organs.

The following model experiments were performed on white rats of both sexes weighing 180-200 g. Thromboplastin was administered intravenously at a dose of 10 mg/kg against the background of prior administration of K-F-γ at a dose of 50 mg/kg 10 minutes before the administration of the thrombus-forming agent thromboplastin. Thirty minutes after the administration of thromboplastin, blood taken from the gums of the rats was examined in an activated partial thromboplastin time (APTT) test. In this experiment, it was found that 30 minutes after the administration of thromboplastin to experimental rats against the background of prior administration of isotonic sodium solution, APTT decreased sharply by 40% compared to the control group.

In this experiment, it was found that after the administration of thromboplastin against the background of prior administration of K-F-γ , there was a 3.5-fold increase in APTT at a dose of 50 mg/ml. Studies have shown that when polysaccharides are added to the plasma under study, factors VIII, IX, XI, and XII are completely inactivated. This indicates that the anticoagulant effect of K-F-γ is achieved through the blockade of factors in the intrinsic pathway of haemostasis. It is known that these sulphated polysaccharides affect factor Xa more than factor IIa (thrombin), which leads to a more pronounced suppression of thrombin formation.

The following conclusions can be drawn from the results obtained:
1.Findings from coagulation tests indicate that the anticoagulant effect of K-F-γ is associated with the inhibition of calcium-activated blood coagulation factors.
2.K-F-γ effectively inhibits the activation of coagulation factors and contact factors of the intrinsic (VIII, IX, XI, XII, prekallikrein, high molecular

weight kininogens) and common pathways (including factors II, V, X and I);

3.K-F-γ does not affect the venom of Echis multisquamatus, whose action is associated with the activation of the prothrombin complex and factors II, I, XIII, regardless of the presence of Ca^{2+} ions;

4.K-F-γ does not cause spontaneous platelet aggregation, but effectively inhibits ADP and adrenaline-induced platelet aggregation. As is known, these inducers increase intracellular Ca^{2+} concentrations during platelet activation;

5. Using fluorescence, it was established that K-F-γ can act on both extracellular and intracellular calcium-dependent processes.

6.A model experiment of pulmonary thrombosis conducted on rats established that, against the background of preliminary administration of K-F-γ at a dose of 50 mg/ml, APTT increases 3.5 times compared to its absence. K-F-γ acts by blocking factors of the internal haemostasis pathway.

7.The results obtained generally indicate that the action of K-F-γ is associated with the manifestation of chelating ability in relation to extracellular Ca^{2+} ions and the formation of a complex with membrane-bound and intracellular calcium, causing suppression of platelet function and inhibition of calcium-dependent blood coagulation factors.

Conclusion It is known that these sulfated polysaccharides affect factor Xa more strongly than thrombin, leading to a more pronounced inhibition of thrombin formation. Inactivation of one factor Xa molecule can prevent the synthesis of approximately 50 factor IIa molecules. Polysaccharides have a stronger effect on the release of plasminogen activator inhibitor, which enhances the complex antithrombotic effect.

The results obtained indicate that K-F-γ SMAs, which have an anticoagulant effect, can affect Ca^{2+}-dependent factors by inhibiting one of factors II, V, VII, or X. This is because the cause of increased PT under the influence of anticoagulants may be a deficiency of factors II, V, VII, or X. Thus, the compound K-F-γ can be classified as a direct-acting anticoagulant, as it prolongs APTT and PT at normal TT. These results indicate that K-F-γ , being a direct anticoagulant, limits the degree of plasma coagulation activation in thrombosis and accelerates the normalisation of haemostasis parameters. Their antithrombotic effect is of interest in terms of their use as an anticoagulant in medicine.

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