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The Effect of Sulphated Cellulose on Haemostasis

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Abstract

Background: Experimental studies and analyses of new compounds with different mechanisms of action on systemic haemostasis are relevant for the identification and development of potential pharmacological preparations. Objective: The modified sulphated polysaccharides with anticoagulant and antithrombin activity were studied for haemostasis. Methods: Platelet-rich plasma was obtained by centrifugation at 200 g for 10 minutes. The remaining citrate blood was further centrifuged at 1500 g for 10 min to obtain platelet-poor plasma. The antithrombin activity of the compounds was evaluated in vitro by their effect on the recalcification time, thrombin and prothrombin time of rabbit and human blood plasma stabilized with a 3.8 % sodium citrate solution in the ratio 9:1. Results: The results showed that the anticoagulant activity of the studied sulphates increased with an increasing degree of sulphation. Sulphated polysaccharides showed strong anticoagulant activity *in vitro*. The experimental results showed a significant increase in the coagulation time of blood plasma in tests for prothrombin and thrombin time. Conclusion: These properties of these components are of particular interest, and further detailed studies of the physicochemical characteristics and mechanisms of action of these molecules should be performed, which will eventually allow them to be used as heparin-like drugs.

Keywords: modified sulphated polysaccharides, activated partial thromboplastin time (APTT), prothrombin time, anticoagulant, thrombin time, platelet aggregation, ADP.

1. Introduction

Currently, modified sulphated polysaccharides, which are a complicated group of macromolecules possessing a wide spectrum of biological characteristics, have attracted increased attention. Along with antiviral, antimicrobial, and anti-inflammatory properties, these molecules also have anticoagulant properties (Alekseenko et al., 2007; Chen et al., 2009; Hayashi et al., 2008). Cellulose is the most widely available compound, is physiologically related to polysaccharides in human and has a well-known structure, which has been extensively studied. The basic materials for the synthesis of cellulose are various structural modifications of cellulose: a cotton cellulose fibre with a degree of polymerization from 1000 and higher or other pulp forms obtained from cotton, wood, and other raw materials (Athukorala et al., 2008). To increase the anticoagulant activity, researchers have introduced strong electronegative groups into cellulose, such as sulphate, phosphate, carboxylic, and amine groups, indicating that the mechanism of action is associated with electrostatic complex formation between blood proteins and cellulose

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(Wang et al., 2005). Fan. L et al. also noted that with the anticoagulant activity increased with the introduction of many sulphate groups in the starch molecule (Fan et al., 2013).

Anticoagulants are used, inter alia, for the prevention and treatment of thrombus in humans (Geerts et al., 2008; Ageno et al., 2014). Anticoagulants with indirect and direct types of action are distinguished (Molteni et al., 2014). The mechanism of action of direct anticoagulants is associated either with the direct inhibition of the activity of thrombin or factor Xa or with the activation of the plasma inhibitor antithrombin (Vo et al., 2014).

The anticoagulant drugs that are currently being used do not satisfy the clinical requirements. Due to their indirect effects, indirect anticoagulants and heparin preparations are limited. One of the most common side effects of all modern anticoagulants is bleeding (Levi et al., 2012; Tosetto et al., 2013; Liotta et al., 2013). Along with preparations of heparin and anticoagulants with direct action, modern inhibitors of thrombin/factor Xa for oral and parenteral use are administered (Ganetsky et al., 2014; Limdi et al., 2013; Roskell et al., 2013; Scaglione, 2013). Therefore, the development of new anticoagulants based on compounds with different chemical structures and low haemorrhagic activity is needed.

Thus, the search for new chemical compounds for the development of drugs possessing anticoagulant activity is important. In vitro experiments can be used to determine the specific activity of the compounds, select an treatment, provide data for in vivo experiments, evaluate the pharmacodynamics, and determine the dosage of the anticoagulants needed in experimental animals.

Experimental research and analysis of new compounds with different mechanisms of anticoagulant action are needed for the identification and development of potential pharmacological preparations. Investigation of the bond structure and the activity of cellulose sulphate will allow the most active polysaccharides to be obtained.

The purpose of the study is to investigate the heparin-like activity of various chemically modified sulphated polysaccharides in systemic haemostasis.

2. Material and methods

2.1. Experimental biological part

Plasma for research was used without platelets. Platelet-rich plasma was obtained by centrifugation at 200 g for 10 minutes. The remaining citrate blood was further centrifuged at 1500 g for 10 min to obtain platelet-poor plasma. The antithrombin activity of the compounds was evaluated in vitro by their effect on the recalcification time, thrombin and prothrombin time of rabbit and human blood plasma stabilized with a 3.8 % sodium citrate solution in the ratio 9:1 (Menshikov, 1987). To isolate platelets used 150 μM NaCl, 2,7 μM KCl, 0,37 μM NaH_2Po_4 ' $2H_2O$,1 μM $CaCl_2$, 5 μM glucose, 10 μM HEPES. In coagulation tests, sulfated compounds were used at a dose of 10-50 μg / ml. Thrombin (1 unit) was used as a control. Prothrombin activity was tested by the Quick method with thromboplastin (Quick). To determine the effect of compounds on thrombosis, it was evaluated by their effect on known haemostasis tests. Thrombin (1 unit) was also used as a control. For experiments using sulfate cellulose 1 mg/6 ml of H_2O. The effect of anticoagulants on the coagulation of human and rabbit plasma in vitro was evaluated using the following tests: activated partial thromboplastin time (APTT) (Scaglione, 2013), prothrombin time (PT) (Rosenberg, 1977) thrombin time (TT) (Teien, 1975), and the ReaClot – Heparin (NPO Rename Russia, Moscow) tests (Yin, 1973) for analysis of the effects on fibrinogen polymerization (for thrombin and buffer, Cypress Diagnostics, Belgium). All coagulation tests (with human plasma) were performed on a single channel coagulometer (CYANCoag, Belgium.CY003, SN:5400439). For evaluation of the anticoagulation potential of the compounds, the effective concentrations in the APTT, PT, TT, and ReaClot tests, which were found on the abscissa of the curves showing the dependence on the concentration of the anticoagulant, were graphically determined. We detected a 2-fold increase in plasma clotting time compared with that of the control, which had no anticoagulants.

2.2. Experimental chemical part

In this work, various modified sulphated polysaccharides (MSPs) with different molecular masses and linear compounds obtained via homo- and heterogeneous sulphation, were used.

The modified sulfated polysaccharides studied in this research was cellulose sulfates with different molecular parameters that synthesized, purified and characterized as described in

(Muhitdinov et al., 2017; Muhitdinov et al., 2019). The information regarding the cellulose sulfates is included in the revised manuscript.

The molecular structure of the cellulose sulfates are added into the manuscript. The molecular structure of the cellulose sulfates is as follow (Figure 1):

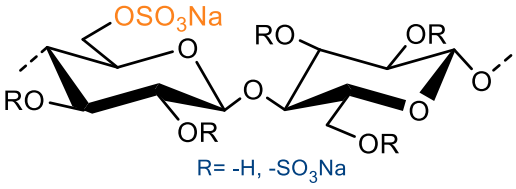

Fig. 1. Molecular structure of the cellulose sulfates

The polysaccharide sulfates used in this study were purified. Sulfate content of the samples were evaluated by elemental analysis and ^{13}C NMR spectroscopy methods. The sample BOS-122 is depolymerized, low molecular weight cellulose sulfate degree of polymerization (DP) and degree of substitution (DS) values of 59 and of 2.01, respectively. The sample GSC-14 is cellulose sulfate having DP and DS values of 724 and 1.98, respectively.

2.3. Statistical analysis

The measurements were made using a universal spectrometer (USB-2000). Statistical significance of differences between control and experimental values determined for a number of data using a paired t-test, where the control and the experimental values are taken together, and unpaired t-test, if they are taken separately. The value of $P < 0.05$ indicated a statistically significant differences. The results obtained are statistically processed to Origin 7,5 (Origin Lab Corporation, USA).

3. Results and discussion

In this study, the effect of various MSPs on blood coagulation in laboratory tests, as well as the comparison of the anticoagulant effect to that of heparin, was studied. The effects of the studied compounds on the plasma clotting time in the APTT, PT, TT, and ReaClot tests were determined.

When the samples were supplemented with citrate human plasma in vitro, the time of appearance of a fibrin clot in the APTT, TT, PT and ReaClot-Heparin tests was increased; thus, the compounds are anticoagulants with direct action; BOS-122 SC and SC GSC-14 antithrombin activity reached 2.9 ± 1.1 and 7.6 ± 0.6 mcg/mL, respectively, and showed efficacy in the tests at concentrations not substantially different for those of unfractionated heparin (heparin) and were equal to 6.3 ± 0.1 and 7.6 ± 0.5 μg/ml, respectively (Figure 2 A, B, C).

In addition, it was noted that the anti-factor Xa activity among the most promising compounds was several times less than the antithrombin activity of these compounds and was similar to that of heparin (Linhardt et al., 2012).

We showed that the more complicated the coagulation test is, the more SC is required to achieve the same efficiency. Consequently, the effect of the SCs is not excluded at the stages preceding the coagulative transformation of fibrinogen, when the sulphated components are consumed. In contrast, their concentrations in different tests would be equal or at least comparable. This reduction is enhanced by the fact that, according to the electrocautery data, SCs extended the period before the formation of the fibrin clot.

To resolve this problem, we used a technique that allows us to isolate the coagulation of fibrinogenic coagulation from the overall cascade of coagulation reactions. This method is based on the fact that pulmonary donor plasma is released from fibrinogen by soft heat denaturation (56 °C, 3 minutes). If fibrinogen is added to such defibrinated plasma, followed by SC, and then mixed, SC can influence any plasma coagulation stage. If, however, in a defibrinated plasma, the activation of the coagulation cascade is first activated, and then, after forming thrombin, fibrinogen and SC are added, only the latter can influence the coagulation of fibrinogenic transformation.

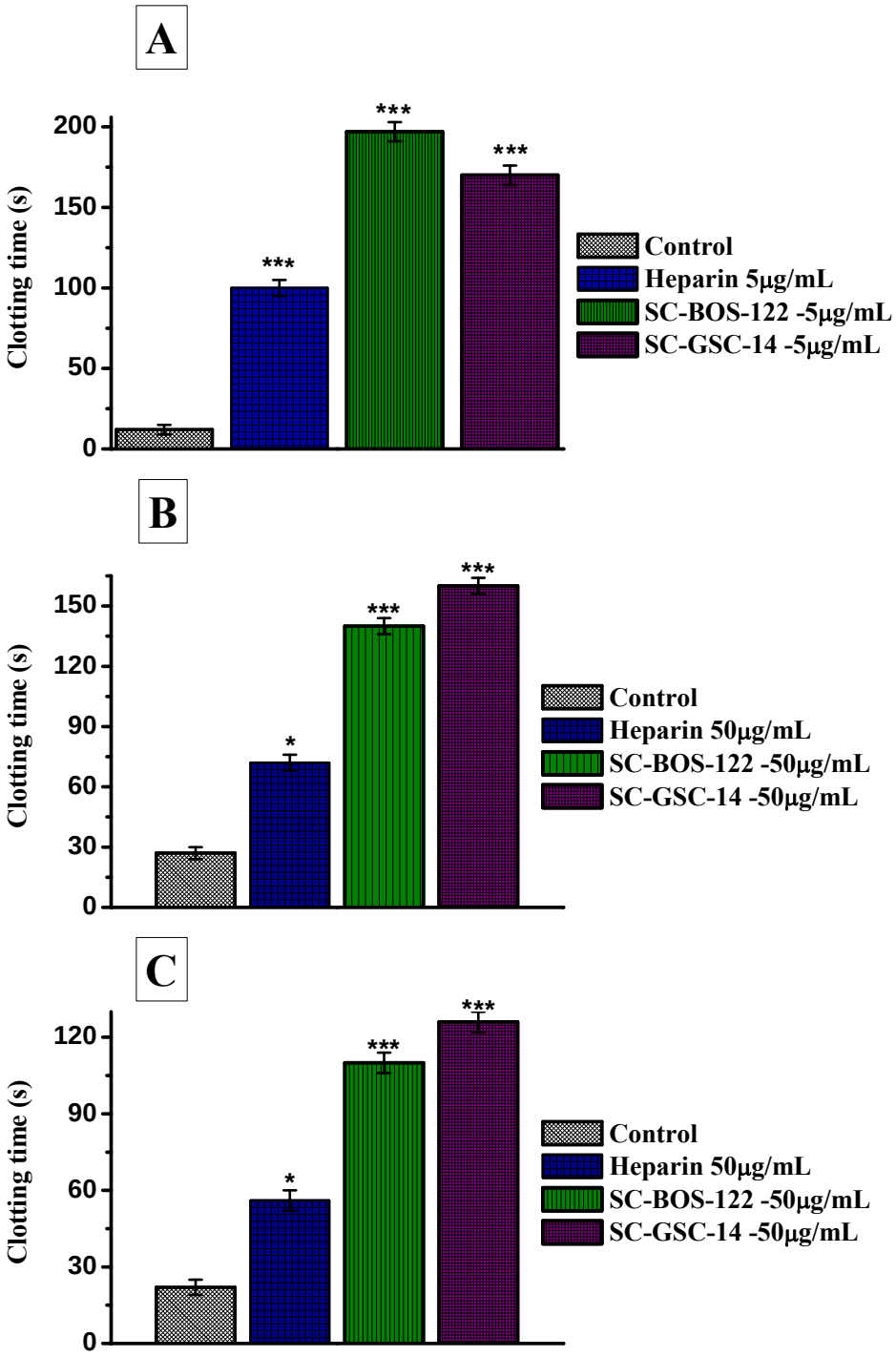

Fig. 2. Anticoagulant activity of SC BOS-122 and SC GSC-14 in (A) APTT, (B) TT, and (C) PT versus heparin *- P < 0.05; **- P < 0.01; ***- P < 0.001. (n=6)

The results of this experiment showed that the inhibitory efficiency in such cases as those described above was almost equal to that when using both BOS-122 and GSC-14. Therefore, the initial assumption about SCs was confirmed at the level of coagulation of fibrinogen transformation, and the differences in the effects of SCs on coagulation were observed at the stage of fibrinogen transformation.

To confirm this conclusion, we determined the effectiveness of inhibition of the interaction between thrombin and the fibrinogen of the SCs and their additive effects compared to the expected effect. The expected (theoretical) effect was significantly lower (on average by 70 %),

indicating the synergistic effect of the SC. These data also indicate that the mechanism of the studied SCs on the coagulative transformation of fibrinogen is different: in the case of an identical mechanism, we would observe additive effects based on the deficiency of the SC in the system or antagonism due to the supersaturation of the SCs.

More information about the differences in the mechanism of action of the SCs was obtained by observing the coagulation of fibrinogen transformation with a nephelometer with an automatic recording of the stages of this process.

SC BOS-122, compared with the control, on average always delayed the formation of oligomers by 9.7 % and increased the time required for the formation of a fibrin clot by 81 %. Since the aggregation of protofibrils is almost instantaneous, SC BOS-122 mainly inhibits the formation of relatively mature oligomers. In contrast, GSC-14 SC predominantly blocked monomer and oligomer assembly; no critical increase in the aggregates was observed with a spectrometer. However, the formation of a fibrin clot increased by an average of 70 % compared to that of the control (Figure 3).

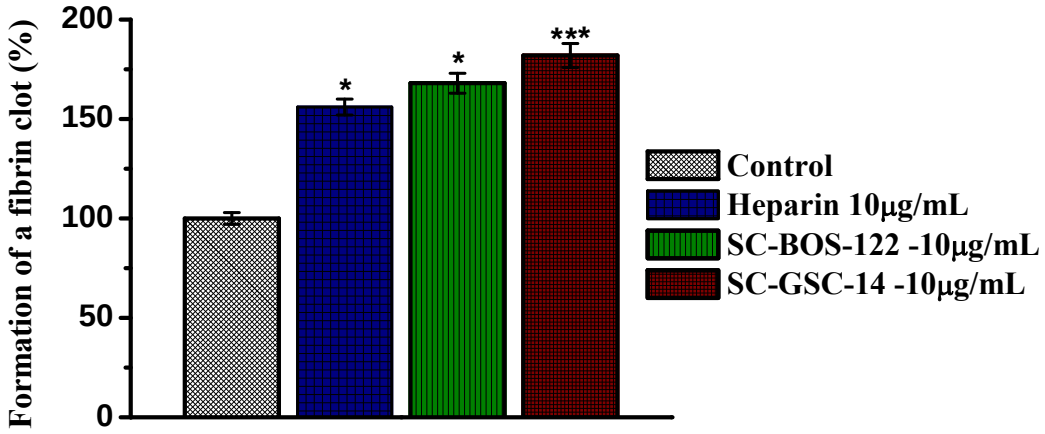

Fig. 3. The effect of SC BOS-122 SC and SC GSC-14 on the formation of fibrin clots in human blood. *- P < 0.05; **- P < 0.01; ***- P < 0.001. (n=6)

To study the effect of SC BOS-122 on the anticoagulant activity of rabbit plasma, we intravenously injected the test compound at various doses into the marginal ear vein. After the plasma was collected at different time intervals after SC administration, the clotting time was determined in the APTT/ReaClot-Heparin tests, and the plasma Xa was determined compared with that with heparin.

To evaluate the effectiveness of the anticoagulants in practice, clinics have used the rate of double the plasma clotting time or human blood counts in some coagulant tests compared with the readings before drug administration (Ng et al., 2003). *In vitro*, we showed that the effective concentrations of the studied compounds (APTT, PT, TT, ReaClot) decreased with an increase in the degree of sulphation; similar findings for sulphates of polysaccharides from various types of vegetation have been found in multiple works (Almedia-Lima et al., 2010; Nasirov et al., 2020; Cipriani et al., 2009; Nadjimova et al., 2020; Khoshimov et al., 2015; Luo et al., 2013; Maas et al., 2012; Silva et al., 2012).

For monitoring of heparin therapy, blood clotting time, plasma clotting time in the APTT test, activated blood clotting time, plasma antithrombin and aXa activities in the plasma are determined. APTT is the most widely used test for determining the degree of anticoagulation after administration of heparin at therapeutic doses (Kleinjan et al., 2013; Marlar et al., 2013).

In the 1970s, the anticoagulant effect of the APTT range (1.5–2.5 times that of the control) was established to reduce the risk of recurrent thrombosis in patients When the concentration of heparin in plasma was 0.3 aXa U/ml, the average time in the APTT test was 48 to 108 seconds, depending on the laboratory test method. Therapeutic levels of heparin (0.3 - 0.7 aXa U/ml) in modern tests of APTT demonstrate ratios of 1.6-2.7 and 3.7-6.2 times (Baglin et al., 2006).

In this work, a definite increase in plasma clotting in the APTT and ReaClot-Heparin tests were performed after an increase in the dose of SC (3, 5 and 7 mg/kg) or heparin (0.75 and 1 μg/kg) after intravenous administration. The time of action (for APTT and ReaClot-Heparin) after the introduction of heparin at doses of 0.75 and 1 mg/kg reached 72 minutes and 113 minutes, respectively. The greater prolongation of the anticoagulant effect of SC BOS-122 compared with that of heparin was associated with higher doses. To achieve the same effect at the time of plasma coagulation in the APTT test (15 minutes after the injection), SC BOS-122 required a 7 times greater concentration than heparin.

The maximum plasma aXa activity was noted in the 5th minute after the administration of SC BOS-122 and heparin into the blood, which coincides with the literature on the intravenous administration of the direct anticoagulant heparin (James, Coller, 2012). The complete disappearance of the aXa activity in the rabbit plasma was observed with the introduction of only heparin at doses of 0.75 and 1 μg/kg after 170 and 110 minutes, which is consistent with the data on the plasma coagulation time in the APTT and ReaClot-Heparin tests.

We observed a naturally greater antithrombin activity in the rabbit plasma compared with the aXa activity after the introduction of SC BOS-122 at different doses. This finding is expected since the specific activity of SC BOS-122 is greater than the aXa activity.

To neutralize the anticoagulant effect of heparin, in clinical practice, researchers administer protamine sulphate (Suryanarayan, Schulman, 2014). Consistent administration of sulphate protamine for sulphate cellulose or unfractionated heparin (in the same doses) caused a reduction in the clotting time of plasma in the APTT/ReaClot Heparin-aXa tests in rabbits and reduced plasma activity. Therefore, 15 minutes after the SC and protamine sulphate (PS) were injected, the plasma coagulation time in the APTT test depending on the dose was 2.3 - 4.4 times shorter than that with the administration of only SC; for heparin and SP, this difference was 15 and 6 times for 15 minutes, depending on the dose. An analysis of the plasma 15 minutes after the SC and SP were injected with the ReaClot-Heparin test showed that the coagulation time was 1.5-2.0 times lower, depending on the dose; for heparin and PS, this difference was 15 minutes, 1.9 and 3.4 times, depending on the dose. The effect of PS on heparin has long been known (Montalescot, 1990).

With the introduction of heparin and the subsequent neutralization of its effect in the 15th minute after administration, we noted an 8-fold decrease in antithrombin activity. The subsequent maintenance of protamine sulphate led to a decrease in the plasma activity after the administration of BOS-122 SC by an average of 2 times compared to the results without treatment.

The introduction of an antidote after the administration of SC BOS-122 or heparin led to a decrease in the anticoagulation activity in the rabbit plasma. Therefore, in the 6th minute of aXa, plasma activity with the introduction of SC BOS-122 and SP at doses of 3, 5, and 7 mg/kg on average decreased almost 3 times compared with the introduction of SC BOS-122 only, with the disappearance of plasma aXa activity after 160 minutes. With the introduction of PS for heparin aXa, plasma activity decreased 13 times.

In vitro experiments showed that for neutralization of the anticoagulant effect of SC BOS-122, depending on the concentration, the addition of protamine sulphate at a ratio of 1 to 10 (by weight) to the anticoagulant may be needed. In our animal experiments, we showed that for neutralization of the anticoagulant activity of BOS-122 SC, it may be sufficient to use protamine sulphate and the anticoagulant at identical doses.

We conducted studies on the influence of SC BOS-122 and SC GSC-14 (at equal concentrations of 10–100 μM) on the aggregation function of thrombocytes. ADPs (2.5 and 10 μg/ml) and adrenaline (Tonogen Solution) were used as inducers, since the results of the aggregation intensity values, caused by the above inductors, are the most satisfactory, and the extent of the aggregation, caused by the exposure, is also determined by the application of adrenaline (Avenarius, Deinhardt, 1980).

Under these conditions, both studied celluloses inhibited the aggregation of thrombocytes, but their mechanism of action, as well as their effect on the coagulation of fibrinogen, was significantly different.

Thus, when using ADP with a concentration of 2.5 μg/ml SC BOS-122 and SC GSC-14 at 10-100 μM, the first wave of aggregation, which is characterized by the formation of a clot under the influence of an inductor, was not changed, but the same symptoms were observed. The maximum magnitudes of the second wave of aggregation for SC BOS-122 and SC GSC-14 were

similar and were lower than the control value by 31 % and 38 %, respectively. SC BOS-122 and SC GSC-14 shortened the time necessary to reach the maximum value of the second wave but showed a difference in intensity of 62.5 % and 37.5 %, respectively. In addition, SC BOS-122 activated disaggregation, and SC GSC-14 did not affect this process (Figure 4A).

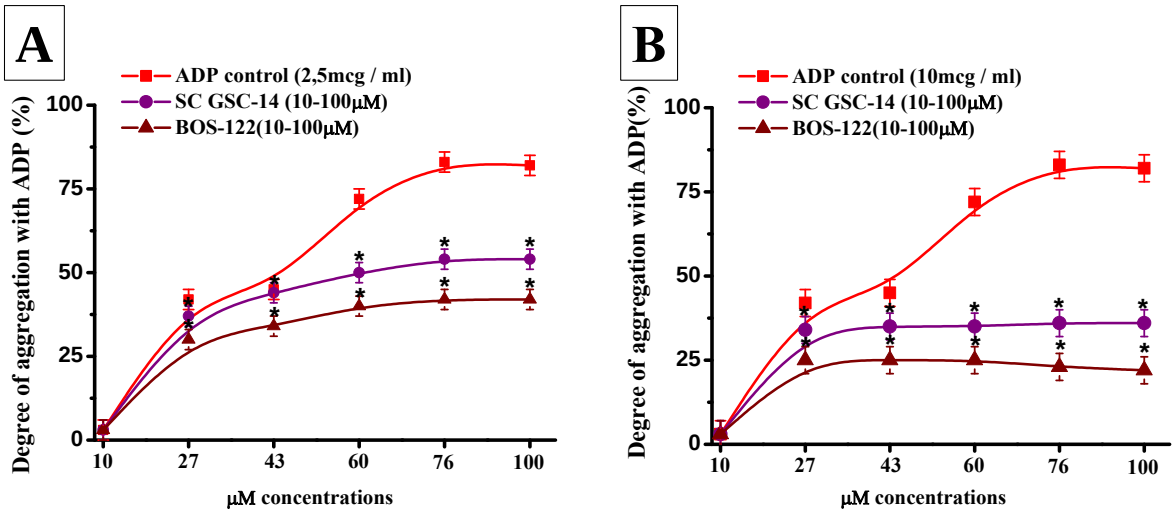

Fig. 4. The effect of sulphated cellulose on platelet aggregation with ADP 2.5 μg/ml (A) and 10 μg/ml (B). *- P < 0.05; **- P < 0.01; ***- P < 0.001. (n=6)

Significant differences in the mechanism of antiaggregant action were observed with an increase in the concentration of the inducer to 10 μg/ml. In this case, control of the first wave of aggregation was not observed. This finding was not observed in the presence of SC GSC-14, but the first wave of aggregation appeared in the presence of SC BOS-122.

The maximum values of the second wave of aggregation for SC BOS-122 and SC GSC-14 were lower than the control value by 44% and 32%, respectively. SC BOS-122 did not significantly change the time of occurrence of the maximum value in the second wave, and SC GSC-14 increased it by an average of 13 % (Figure 4B).

When used as an adrenaline inducer, SC GSC-14 smoothed the first aggregation wave, reduced the maximum value by a second, and lengthened its maximum time by 12.7 %. In contrast, SC BOS-122, without changing the maximum value of the first wave of aggregation, doubled the time required for its achievement, reduced the maximum value of the second by 50.4 % and lengthened the time of its maximum by 50.6 %.

When deciphering the mechanism of restriction of blood plasma clotting activity, we first used an objective method (determination of plasma coagulation with a CYANCOag coagulometer) and found that the SC had pronounced anticoagulant activity. In this case, pulverized donor plasma was used as a substrate, and BOS-122 SC and GSC-14 as anticoagulants were combined and added. In this case, some differences in the influence of carriers on blood plasma recalcification were revealed, which are especially reflected in the change in time before the start of coagulation, as well as in the rate of retraction and fibrinolysis.

Differences in the effect of the inhibitors on coagulation of fibrinogen transformation were found by another objective method: a USB-2000 spectrometer. SC GSC-14 had little effect on the early stages of the formation of protofibrils, and it predominantly slowed the autopolymerization of relatively mature oligomers. SC BOS-122, in contrast, had a pronounced inhibitory effect on the early stages of polymerization. With their joint influence on the coagulation of fibrinogen, a synergistic effect was observed, which confirmed the aforementioned differences in the mechanisms of action.

Thus, the current findings on the anticoagulant activity of these molecules in the final phase of coagulation showed the predominant influence of SC BOS-122 and SC GSC-14 on self-polymerizing fibrin.

We identified only those indicators that integrally reflect the total coagulation activity of the blood plasma (recalcification time), the speed of the interaction between thrombin and fibrinogen (thrombin time) and autopolymerization of the subject.

The properties of these components are of particular interest, and further detailed studies of the physicochemical characteristics and mechanisms of action of these molecules are needed, which will eventually allow them to be used as a heparin-like drug.

4. Conclusion

Some differences in the effects of SC BOS-122 and SC GSC-14 on the recalcification of blood plasma were revealed, especially the change in time before the start of coagulation, as well as in the rate of retraction and fibrinolysis.

The anticoagulant activity of directly acting cellulose sulphates, with a molecular mass of 21,500 kDa (sulphation degree of 2.01), is mediated through an interaction with antithrombin. The anticoagulant activity of the sulphate molecules studied increased with an increasing degree of sulphation. The maximum antithrombin activities of SC BOS-122 and SC GSC-14 reached 2.9 ± 1.1 and 7.6 ± 0.6 μg/ml, respectively.

Certain indicators generally reflect the total coagulation activity of blood plasma (recalcification time), the rate of interaction between thrombin and fibrinogen (thrombin time) and the autopolymerization of monomeric fibrin (time of self-scavenging of fibrin in plasma).

5. Acknowledgements

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