MOLECULAR MECHANISMS OF INHIBITION OF INTRAVASCULAR, AND ACTIVATION OF EXTRAVASCULAR THROMBUS FORMATION

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The hemostasis system is designed to maintain a balance between the processes of blood clotting, anticoagulation, and fibrinolysis, as well as to ensure constant effective blood circulation in the body and rapid cessation of bleeding in the event of their occurrence. The procoagulant potential of the hemostasis system is based on molecular mechanisms that lead to the formation of fibrin in the bloodstream, which is the framework of the thrombus, and to the aggregation of platelets — the basis of the thrombus body. The anticoagulant potential of blood plasma is provided by mechanisms aimed at inhibiting blood coagulation processes.

Thorough study of these mechanisms will open up numerous treatments for pathologies associated with both intravascular thrombosis and bleeding of various origins. The purpose of this review is to analyze ways to prevent intravascular thrombosis and stimulate extravascular thrombosis. The review describes and analyzes available data on thrombosis prevention, in particular, direct and indirect anticoagulants and antiplatelet, as well as methods of effective stimulation of thrombosis, which is necessary in case of vascular damage.

This analysis will determine the nodal points of the protein network of the hemostasis system, whose action by specific molecular effectors will control the process of thrombosis.

Key words: anticoagulants, antiplatelets, activator, blood clotting, thrombosis.

The hemostasis system is designed to maintain a balance between the processes of blood clotting, anticoagulation and fibrinolysis, to ensure constant efficient blood circulation in the body and rapid cessation of bleeding in the event of their occurrence. To do this, the enzyme-cell complex of the hemostasis system must at any time, depending on the needs of the body, initiate either coagulation, anticoagulant mechanisms, or fibrinolysis. The basis for providing procoagulant and anticoagulant potentials of the hemostasis system are molecular mechanisms, which have been the subject of research for many years. Such studies are not only fundamental but also important in practice, as they allow influencing the pro- and anticoagulant potential of the hemostasis system, which opens the possibility of treating a number of serious diseases associated with intravascular thrombosis and bleeding of various origins.

Intravascular thrombosis occurs due to pathological activation of the blood coagulation system and is manifested in the formation of fibrin deposits, fibrin clots, and thrombi, which completely or partially block the lumen of the vessel, impeding the blood supply to vital tissues and organs. Intravascular thrombosis is the cause of diseases such as myocardial infarction, pulmonary embolism, ischemic stroke, deep vein thrombosis, and often accompanies a number of cardiovascular, metabolic and cancer diseases, surgical, gynecological and obstetric diseases. It is one of the main causes of patient mortality. Timely proper antithrombotic and sometimes

fibrinolytic therapy is required to correct such complications.

The hemorrhages are no less dangerous for human life and health, if the extravascular thrombosis which is designed to stop the bleeding is not effective enough. Bleeding that cannot be stopped in time is the cause of death from various injuries, catastrophes, combat injuries, in surgery, obstetrics and in patients with hemophilia.

That is why this review is devoted to the analysis of available and promising ways to prevent intravascular thrombosis and stimulate extravascular thrombosis in case of vascular damage.

Intravascular pathological thrombosis and basic antithrombotic agents

Intravascular pathological thrombosis is one of the leading causes of death in the world. It can occur in cardiovascular diseases, surgery, injuries and burns, cancer and metabolic diseases, sepsis, immobilization, infectious and inflammatory processes, including COVID-19 [1], etc.

Intravascular thrombosis occurs due to the following pathogenic factors (Virchow's triad): 1) damage to endothelial cells with exposure to thrombogenic subendothelial structures and factors; 2) hyperactivation of the blood coagulation system, which leads to the appearance of active thrombin in the bloodstream, pathological activation of platelets, decreased fibrinolytic potential; 3) decrease in blood flow velocity [2].

Understanding the mechanisms associated with hyperactivation of the blood coagulation system and pathological platelet activation is the basis for the development and improvement of antithrombotic agents (Fig. 1) [3, 4]. Fig. 1 presents a generalized scheme of the hemostasis system, which combines the system of blood clotting, which leads to the formation of a three-dimensional network of fibrin, the framework of the thrombus; platelet link, the activation of which leads to the formation of platelet "body" of the thrombus; the protein C system, which regulates the activity of the blood coagulation system; the fibrinolysis system, which provides hydrolysis of polymeric fibrin.

The following symbols are used in the legend of Fig. 1: Fg — fibrinogen; Fn — fibrin; fXIIIa — activated factor XIII; Plat — platelets; Plat$_a$ — activated platelets; fIXa — activated factor IX; fXIa — activated factor XI; fXIIa — activated factor XII; KI — kallikrein; TF — tissue factor; fXa — activated factor X; fVIIa — activated factor VII; PCa —
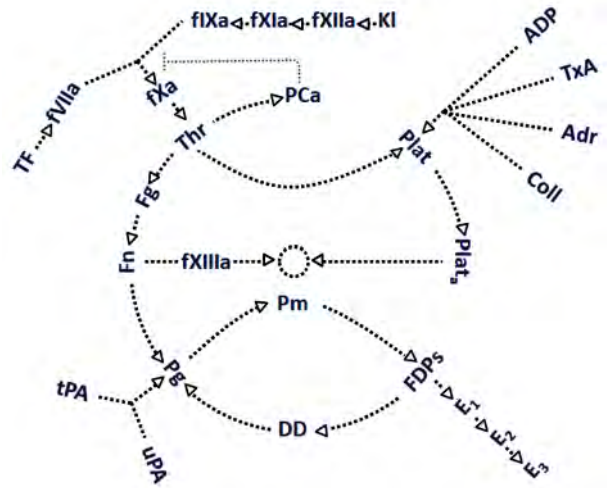

Fig. 1. **Generalized scheme of the hemostasis system, combining blood clotting, platelet, protein C and fibrinolysis systems**

activated protein C; Coll — collagen; Adr — adrenaline; TxA — thromboxane A; ADP — adenosine diphosphate; Pg — plasminogen; Pm — plasmin; tPA — tissue plasminogen activator; uPA — urokinase; FDPs — fibrin degradation products; DD — D-dimer; E_1 — high molecular weight E-fragment; E_2, E_3 — hydrolyzed E-fragment.

When the blood coagulation factor X is activated as a result of a cascade of successive enzymatic reactions through either the external or internal pathway, it then activates prothrombin to thrombin by limited proteolysis (Fig. 1) [5–8].

Thrombin converts fibrinogen, which circulates freely in the blood, to fibrin, capable of spontaneous polymerization. Fibrin polymerizes with the formation of oligomers and polymers of different lengths, which then laterally associate, forming fibrils, branching and eventually forming a three-dimensional fibrin network, which serves as the framework of the thrombus [9].

The body of the thrombus, which fills the fibrin framework, is formed by aggregated platelets. Inactive non-nuclear round cells are activated by direct thrombin action and by the vascular endothelial damage. Active platelets change shape and aggregate due to multipotent binding to fibrinogen molecules and fibrin of the blood clot.

The main known antithrombotic agents are anticoagulants, which prevent the formation and polymerization of fibrin, and antiplatelet agents, which prevent excessive activation and aggregation of platelets.

Anticoagulants

Anticoagulants are substances that inhibit the rate of fibrin formation in the bloodstream, and therefore thrombosis.

According to the mechanism of action, there are direct- and indirect-acting anticoagulants.

Direct-acting anticoagulants immediately affect coagulation factors. According to the mechanism of action, they are divided into those that depend on or do not depend on antithrombin III.

Antithrombin-dependent anticoagulants are unfractionated high molecular weight heparin (UFH); low molecular weight fractionated heparins (LMWH), such as enoxaparin sodium, nadroparin calcium, bemiparin sodium, dalteparin sodium, tinzaparin sodium, etc., factor Xa inhibitors (fondaparinux sodium, hydraparinux sodium), and heparinoids.

Antithrombin-independent anticoagulants are preparations of antithrombin III and hirudin; preparations that directly inhibit thrombin, such as dabigatran etexilate, ximelagatran, melagatran, argatroban, bivalirudin and others; drugs that directly inhibit factor Xa (rivaroxaban, apixaban, edoxaban, betrixaban, otamixaban) and preparations that inhibit factors Va and VIIIa (human protein C, drotrecogin alpha) [10, 11].

Indirect anticoagulants include substances that inhibit the formation of coagulation factors, in particular prothrombin, in the liver and thus reduce the coagulation potential. They are also called vitamin K antagonists. These are coumarin derivatives (mono- and dicoumarins: warfarin, marcumar, syncumar, acenocoumarol, dicoumarin, tromexane) and indandione derivatives (phenindione, phenylin, dipaxin, omefin) [11].

Indirect anticoagulants

Indirect anticoagulants differ in that they do not immediately affect any of the links in the cascade of the blood coagulation system. They act only indirectly, inhibiting the synthesis, namely the carboxylation of vitamin K-dependent factors of the blood coagulation cascade, mainly prothrombin [12], the precursor of thrombin. The main preparation in this group is an affordable drug warfarin. Today, warfarin therapy is widely used in patients at risk of intravascular thrombosis.

Warfarin blocks the formation of vitamin-K-dependent coagulation factors in the liver, namely the factors II, VII, IX and X. The concentration of these components in the blood is reduced and the coagulation process is slowed down. Regular intake of warfarin lessens the risk of thrombosis, but does not eliminate the risk of bleeding, requires certain dietary restrictions and constant laboratory monitoring of the hemostasis system, MHO, to adjust the dosage, which is difficult to constantly perform in an outpatient setting [13, 14].

Direct coagulants
Heparins

The most common direct-acting anti-coagulants are high molecular weight unfractionated heparin (UFH) and low molecular weight fractionated heparins (LMWH) [11–15].

Unfractionated heparin is a sulfated glycosaminoglycan (mucopolysaccharide) of mixed polysaccharide nature. It consists of polymers derived from D-glycosamine and L-iduronic or D-glucuronic acid [16, 17], with a molecular weight of from 3,000 to 30,000 Da, in commercial medicinal heparin preparations mainly from 12,000 to 16,000 Da [18].

Heparin has a negative charge in the solution, which promotes its interaction with proteins involved in blood clotting. In combination with antithrombin III, UFH primarily inhibits activity of thrombin and factor Xa. Binding of antithrombin III to heparin leads to conformational rearrangements in the molecule, which provide greater affinity of antithrombin III to thrombin and other coagulation factors and, consequently, increase the inhibitory properties of antithrombin by 1000 to 5000 times [19].

The heparin molecule contains a large number of reducing agents, and therefore has the ability to bind to free plasma proteins and blood cells. Heparin is also moderately related to fibrinogen and prothrombin, which also contributes to the inhibition of plasma procoagulant activity. In addition, UFH acts on other factors of hemocoagulation, in particular on factors IX, XI, Va and XII. Thus, the action of heparin extends to virtually all parts of the blood clotting system [20, 21]. However, the main mechanism of action of UFH on the coagulation system is still the inhibition of coagulation factors Xa and thrombin.

Smaller heparin molecules are able to inhibit factor Xa activity well and do not potentiate the effect of antithrombin in inhibiting thrombin. Longer-chain heparins increase antithrombin activity, leading to thrombin inhibition. Heparins that activate antithrombin are a third of those that are part of "unfractionated" heparin [22].

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Heparin drugs injected into the blood remain active for 4-6 hours, during which time the substance is inactivated by the enzyme heparinase produced by the liver and kidneys. As heparinase activity of blood of patients is not defined, careful control of indicators of blood coagulation at the introduction of heparin is necessary for timely correction of a dose of drug [23]. Therefore, UFH is more convenient and safer to use in a hospital.

Low molecular weight heparins (LMWH) are a heterogeneous mixture of sulfated glycosaminoglycans with molecular weight of mostly 4,000 to 5,000 Da (ranging from 2,000 to 9,000 Da) [24]. LMWH are purified fragments of natural heparins that have anticoagulant activity. Unlike UFH, LMWH inhibit mainly the coagulation factor Xa, because LMWH bind only to antithrombin III (ATIII) in the complex [heparin + ATIII + Xa]. Inhibition of thrombin requires the formation of a triple complex involving at least 18 saccharide residues in the heparin molecule, which is possible at a molecular weight of at least 5400 Da [18, 25, 26].

LMWH have a number of advantages over UFH due to more favorable pharmacokinetics and fewer side effects. Their use can more accurately predict the dose-dependent anticoagulant effect, they have increased bioavailability when administered subcutaneously, longer half-life, low incidence of thrombocytopenia, so there is no need for regular monitoring of hemostasis and outpatient administration is possible. All LMWH have a similar mechanism of action, but different molecular weights cause different activity for factors Xa and thrombin, as well as different affinities for plasma proteins [18, 27, 28].

LMWH preparations differ in chemical structure, production methods, half-life, specificity of action and therefore they are not interchangeable. LMWH are obtained by depolymerization of heparin isolated from pig mucous membranes by various methods. As a result of the depolymerization process, the formed LMWH are mainly enriched with molecules with less than 18 monosaccharide subunits [29].

LMWH are widely used in the treatment of acute coronary syndrome, deep vein thrombosis of the lower extremities, pulmonary embolism, for the prevention of thromboembolic complications in persons at risk. In clinical practice, LMWH preparations such as enoxaparin and nadroparin are used as the first choice, followed by dalteparin, tinzaparin etc.

Enoxaparin is a LMWH with an average molecular weight of about 4500 Da. This is the first LMWH approved in the United States. It shows high anti-Xa activity and low antithrombin activity. It can ATIII-dependently inhibit factor VIIa, activate the inhibitor of tissue factor pathway (TFPI), reduce the release of von Willebrand factor (vWF) from the vascular endothelium into the circulatory tract [30].

Nadroparin is a LMWH containing glycosaminoglycans with an average molecular weight of 4300 Da. It exhibits a high level of binding to antithrombin III, which causes accelerated inhibition of factor Xa. Other mechanisms that provide the antithrombotic effect of nadroparin include activation of tissue factor pathway inhibitor (TFPI), activation of fibrinolysis by direct release of tissue plasminogen activator from endothelial cells and modification of rheological properties of blood (decrease in blood viscosity and increase in permeability of membranes of thrombocytes and granulocytes). Compared with UFH, nadroparin has less effect on platelet function and their ability to aggregate and less pronounced effect on primary hemostasis [31].

Deltaparin is a LMWH with an average molecular weight of 4000−6000 Da. It binds plasma antithrombin, thereby inhibiting some coagulation factors, primarily factor Xa, and slightly inhibits thrombin formation. It has virtually no effect on blood clotting time. It weakly affects platelet adhesion and may also act on the vascular wall and fibrinolysis system. Tinzaparin has a molecular weight of 4500−5500 Da, lower efficiency and limited indications [32, 33].

Heparins are also important regulators of the activity of protein C (PC), which cause the interaction between pC and its inhibitors, forming a complex with activated pC. The formation of such a complex occurs in three binding sites, also specific to factor Va [34].

The most common side effect of heparin therapy is bleeding, and the most serious is heparin-induced thrombocytopenia type II (HIT II), caused by the induction of antibodies to neoantigen complexes "heparin-platelet factor 4" (HPF4) which provokes serious bleeding [35]. That is why during UFH heparin therapy it is necessary to perform the platelet count at least every four days [36−38]. An important feature of LMWH is their low ability to sorb on the surface of vascular endothelium and blood cells and interact with proteins of the hemostasis system. Therefore, LMWH are much less

likely than UFH to lead to thrombocytopenia, and have no inhibitory effect on fVa [39]. The anticoagulant effect of UFH is removed if necessary by the introduction of protamine sulfate or protamine chloride. This is not always effective in the case of LMWH, but due to milder and predictable dose-dependent inhibitory effect on blood coagulation, LMWH are much less likely to cause massive bleeding and do not require frequent monitoring of the state of the blood coagulation system, so they can be safely used not only in the hospital but also in an outpatient setting [40].

It should be noted that heparin therapy may distort the results of coagulation tests. In this case, the greater the number of proteins of the blood coagulation system involved in the diagnostic test, the greater the error may be in the presence of heparin. For example, the minimum amount of unfractionated heparin introduced into the body (5000 units) leads to a decrease in prothrombin index by 30% [41].

Synthetic inhibitors of coagulation cascade factors

The listed shortcomings, high cross-selectivity of heparin, as well as the need for parenteral administration, led to the search for new effective means of reducing the procoagulant potential of blood plasma. To this end, compounds that selectively inhibit thrombin or activated coagulation factor Xa have been developed [42]. Direct inhibitors of these coagulation factors have been shown to be quite effective and are used increasingly in conjunction with indirect anticoagulants.

The success of coagulation inhibitors of this type is due to the fact that thrombin is key in the coagulation system due to its ability to catalyze the conversion of fibrinogen to fibrin and stimulate platelet activation and aggregation. Factor Xa combines the "external" and "internal" pathways of coagulation cascade, and converts prothrombin to the active enzyme thrombin [43].

Synthetic low molecular weight inhibitors of thrombin have a number of advantages over other anticoagulants currently used in practical medicine. These are the speed of action, high efficiency, predictable pharmacokinetics, the lack of need for continuous monitoring of hemostasis [44].

All inhibitors of factor Xa have the suffix "Xa-ban" [Ten-A-Ban] to indicate their mechanism of action.

Dabigatran is one of the best-known direct inhibitors of thrombin activity. Dabigatran inhibits free thrombin, fibrin-bound thrombin, and thrombin-initiated platelet aggregation. Dabigatran prolongs activated partial thromboplastin time (APTT), blood clotting time and thrombin time (TT). Today, several preparations have been developed on the basis of dabigatran, many of which are used clinically [45–46].

Indeed, inhibition of thrombin is one of the most obvious ways to prevent thrombosis, but there are a number of reasons why inhibition of factor Xa may be more effective. Activation of the blood coagulation system leads to the formation of a prothrombinase complex on cell membranes. Factor Xa, as part of this complex, is the only enzyme responsible for the continued formation of thrombin in the bloodstream. Unlike thrombin, which acts on various protein substrates, including fibrinogen and PAR receptors, factor Xa has one physiological substrate, namely prothrombin [47, 48].

Factor Xa is a key point of "enhancement" in the coagulation cascade: one factor Xa molecule generates activation of more than 1,000 thrombin molecules. Thus, the direct inhibition of factor Xa, as a method of indirect inhibition of thrombin formation, provides powerful control of fibrin formation (the basis of thrombus). Being part of the prothrombinase complex, factor Xa has a catalytic activity 10 times higher than the free enzyme. To achieve the anticoagulant effect, it is necessary to inhibit a much smaller amount of factor Xa than thrombin, due to the concentration of their zymogens in the blood (1.4 µM prothrombin against 150 nm factor X) [49, 50].

Indirect evidence for the hypothesis of the predominance of factor Xa as a therapeutic target over thrombin can also be found in clinical trials for the prevention of deep vein thrombosis. Fondaparinux (ATIII-dependent factor Xa inhibitor) has been shown to be superior to LMWH in anticoagulant activity [51]. The antithrombotic activity of fondaparinux is the result of selective inhibition of factor Xa mediated by antithrombin III. By selectively binding to ATIII, fondaparinux potentiates (approximately 300-fold) the initial neutralization of factor Xa by ATIII. Neutralization of factor Xa interrupts the coagulation chain and inhibits both thrombin formation and thrombus formation. Fondaparinux is not inactivated by thrombin and does not affect platelets.

Today, preparations based on factor Xa inhibitors are widely used to prevent intravascular thrombosis in most cardio-vascular and other pathologies. These drugs reduce the risk of blood clots, but in some

cases can also cause bleeding. Clinical studies suggest that they are comparable to warfarin in terms of efficacy, however, unlike indirect anticoagulants, direct anticoagulants have an antidote that makes them safer [52, 53].

The first oral factor Xa inhibitor, rivaroxaban, was approved in the United States in 2011 and is currently one of the most studied and widely used oral anticoagulants [54]. Rivaroxaban is a highly selective direct factor Xa inhibitor, which has a fairly high bioavailability when taken orally and a rapid onset of action. The preparation is used primarily for the prevention of venous thromboembolism (VTE) after elective surgery on the knee or hip joint, therapy and secondary prevention of VTE, prevention of ischemic stroke and thromboembolism in persons diagnosed with valvular fibrilla-tion. In Europe, rivaroxaban is also used for the prevention of atherothrombotic episodes after acute coronary syndrome in patients with elevated cardiac biomarkers. Rivaroxaban is relatively easy to use compared to LMWH and fondaparinux, which require subcutaneous administration, or vitamin K antagonists (VKA), which require regular monitoring of the international normalized ratio, though dose adjustment is required in people with renal impairment [55]. Factor Xa inhibitors apixaban [56] and edoxaban [57, 58] have been approved and are still under investigation.

Apixaban inhibits free and thrombus-associated factor Xa and inhibits prothrom-binase activity. Apixaban does not directly affect platelet aggregation, however it indirectly inhibits thrombin-induced platelet aggregation. By inhibiting factor Xa, apixaban prevents thrombin formation and thrombus formation. Preclinical studies of apixaban in animals have shown the effectiveness of antithrombotic action of the drug for the prevention of arterial and venous thrombosis in doses that do not disrupt hemostasis.

Edoxaban, a direct inhibitor of factor Xa, is the last of the oral anticoagulants that are not vitamin K antagonists (NOACs). Its use is now widespread in modern clinical practice, indicated for thromboprophylaxis in patients with non-valvular atrial fibrillation (NVAF) and for the treatment and prevention of venous thromboembolism (VTE).

There is ample data in the literature on the development of low molecular weight inhibitors of thrombin and factor Xa. The studies include those of molecular modeling, rational design and synthesis of a new series of carboxylate compounds of bisphenylamidine, which are inhibitors of factor Xa. Approaches to the effective search for new inhibitors using *de novo* software solutions and data on the X-ray crystal structure of factor Xa have been studied [59].

There are also attempts to create direct inhibitors of coagulation factors located in the coagulation cascade before prothrombinase. In particular, the main component of the tenase complex, factor Va, can be inhibited by a recombinant analogue of protein C — drotecogin [60]. Such preparation will have not only anticoagulant action but also anti-inflammatory properties. It is already offered as part of complex therapy for the treatment of sepsis [61, 62].

Anticoagulant action that targets fibrinogen

Decades ago, attempts were made to directly reduce the procoagulant potential of blood plasma by defibrination. For this purpose, it was proposed to use ancrod, an enzyme from the venom of *Calloselasma rhodostoma* [63, 64]. Created on the basis of this enzyme, the drug "Arvin" has been widely used in clinical trials [65]. As a thrombin-like enzyme, ancrod caused desA-fibrin to appear in the bloodstream, which was not stabilized by factor XIIIa and therefore had to be rapidly and efficiently removed from the bloodstream due to the fibrinolysis system [66]. However, the deliberate generation of polymeric fibrin in the bloodstream, even if unstabilized, is associated with many risks because of the possibility of its further polymerization in the presence of thrombin in the bloodstream. That is, the use of Arvin to prevent thrombosis primarily threatens thrombosis. In addition, the amount of fibrinogen in the bloodstream is so high that it is probably impossible to reduce it so much that it cannot be converted under action of thrombin to fibrin in an amount sufficient for thrombosis. In addition, defibrinogenizing the blood plasma completely is extremely risky due to the inability to stop bleeding if it occurs.

Special mention should be made of enzymes specific primarily for fibrinogen, which circulates in the bloodstream. Limited proteolysis of fibrinogen by such enzymes reduces the ability of fibrinogen to convert to fibrin and polymerize, while not leading to the removal of fibrinogen from the bloodstream, which allows the partially hydrolyzed molecule to perform other physiological functions [67, 68]. The idea of using fibrinogenases as a

means of reducing procoagulant potential has not yet received widespread support [69].

Today, many studies are concerned with creating antithrombotic drugs that would directly target the final stage of thrombosis by directly inhibiting the polymerization of monomers and oligomers of fibrin [70, 71]. In particular, it is proposed to use certain low molecular weight compounds that block fibrin polymerization centers, silver nanoparticles [72] and peptide inhibitors that completely or partially mimic fibrin polymerization centers, in particular GPRP conjugated with albumin [73], etc. [74]. These include calix[4]arenes, which have low molecular weight and are potentially non-immunogenic compounds of non-protein nature. Calixarenes are promising antithrombotic agents because they inhibit fibrin polymerization centers and thus inhibit the formation of polymeric fibrin network, which is the framework of thrombus [75]. The study of the action of calix[4]arene C-145 *in vivo* with intravenous administration showed a significant antipolymerizing effect [76].

Antiplatelet agents as inhibitors of platelet aggregation

Platelet activation required for their aggregation is a complex process regulated by changes in metabolic and biochemical mechanisms, change in the shape of platelets, activation of platelet surface receptors, and change in the orientation of membrane phospholipids [77].

Changes in the orientation of phospholipids near the plasma membrane create the possibility of association of coagulation factors on the activated surface with the formation of a catalytic prothrombinase complex. This leads to increased thrombin secretion and strengthening of the thrombus with transverse fibrin insertion [78].

Platelets are activated by collagen and the first portions of thrombin, which is formed at the site of damage to the vessel wall. The products of these reactions activate protein kinase C, as well as increase the concentration of calcium in the cytosol of platelets. In addition to thrombin, platelet activation is caused by such soluble agents as platelet activating factor (PAF) and ADP, which are released from the damaged cell, as well as catecholamines, serotonin and others. All of these agents have specific receptors on the platelet plasma membrane. The result is a series of successive reactions [79]:

1. The shape of platelets changes, they form long pseudopodia.

2. On the surface of the platelet membrane, a combined GPIIbIIIa receptor is formed from GPIIb and GPIIIa receptors, to which fibrinogen and other adhesive proteins bind, causing platelets to adhere together [80].

3. Arachidonic acid is released from membrane phospholipids. It oxidizes to form a number of derivatives, including prostaglandin PGH2, which is a platelet activating cofactor, and thromboxane A2, which is also able to activate platelets [81].

4. ADP is secreted, which has the ability to activate platelets and attract more of them to thrombus formation.

5. The membrane surfaces of platelets are reorganized, exposing phospholipids, which are necessary for the further formation of coagulation enzyme-cofactor complexes. Secretion of platelet factor V from -granules of platelets provides a key component for the formation of one of the enzyme-cofactor complexes. As a result, an additional amount of thrombin is formed, which leads to the activation of fibrinogen and the formation of fibrin threads that radially depart from the platelet aggregate and contribute to the formation of platelet thrombus, which closes the vessel [82].

6. Inside platelets, the mechanism of platelet actomyosin contraction is activated. The platelet clot compresses, providing a more effective attachment to the site of vascular damage.

Each of these stages can potentially be targeted by antiplatelet agents.

Platelet aggregation inhibitors are divided into groups depending on the targets of inhibition.

P2Y12 receptor antagonists are known to be activated by ADP [83]. Such preparations include ticagrelor, clopidogrel and prasugrel. All of these drugs are widely used in treatment of diseases associated with the risk of intravascular coagulation, including the myocardial infarction [84].

Nevertheless, the most common inhibitor of platelet activation is aspirin, a cyclooxygenase inhibitor [85]. This is due to the high efficiency, low cost and comprehensive information on possible side effects of aspirin [86]. However, some patients have innate resistance to aspirin, which is very difficult to predict [87, 88], so the search for and development of new inhibitors of platelet aggregation continues.

There are also phosphodiesterase inhibitors that inhibit the hydrolysis of cAMP and cGMP. Anti-ischemic potential was shown by drugs of this group, namely cilostazol [89] and dipyridamole [90].

Another large group of inhibitors of platelet aggregation are GPIIbIIIa receptor antagonists. An intracellular signal is required, which is induced by an external signal (ADP, collagen, etc.) and realized through G-proteins, to build and activate the glycoprotein complex of the fibrinogen receptor GPIIbIIIa on the platelet surface [91, 92]. There is an allosteric equilibrium between the affine and non-affine forms of GPIIbIIIa [93, 94]. The affinity form is able to bind fibrinogen and support aggregation. At this stage, the binding of fibrinogen to GPIIbIIIa can be reversed [95]. Interaction of fibrinogen with GPIIbIIIa causes exposure of ligand-induced binding sites that are involved in platelet aggregation — antibodies to these sites inhibit platelet aggregation [96]. Multipoint binding of fibrinogen and GPIIbIIIa leads to irreversible intermolecular interactions [97], as well as to clustering of receptors on the platelet surface and triggering a signaling that enhances platelet activation. The final stage of the interactions of fibrinogen and GPIIbIIIa is the retraction (sealing) of the fibrin-platelet thrombus [98].

The main class of GPIIbIIIa receptor antagonists are RGD-containing protein preparations [99]. However, it should be noted that they cannot inhibit the interaction of platelet receptors and fibrinogen, which is realized most effectively at the C-terminal dodecapeptide γ-chain of the fibrinogen molecule. Development of RGD-containing drugs continues [100]. This is a topical issue because disintegrins are potential antiproliferative agents, preventing

the adhesion of cancer cells [101]. Some of them have been successfully tested in models of carcinogenesis in laboratory animals [102].

The drugs based on antibodies to GPIIbIIIa receptors are promising for the inhibition of platelet aggregation. In particular, abciximab is used in coronary heart disease [103]. The synthetic heterocyclic compound tirofiban was an active inhibitor of platelet aggregation [104].

Thus, it is possible to identify the main targets for the inhibition of intravascular thrombosis: factors of the blood coagulation cascade, primarily factor Xa and thrombin, as well as platelets. Thrombin or factor Xa inhibitors have been shown to be more effective in preventing thrombosis than platelet aggregation inhibitors, but the use and research of platelet aggregation inhibitors is ongoing. Specific inhibition of a single cascade or mechanism, as well as several mechanisms simultaneously, is possible. The effect on platelets does not lead to disruption of the protein part of the blood coagulation system, but reduces the overall procoagulant potential of the blood.

Fig. 2 presents a generalized scheme of the hemostasis system with the indicated nodal points, which are targeted by the action of the most common and promising anticoagulant agents: 1 — indirect anticoagulants; 2 — fibrinogenases; 3 — fibrin polymerization inhibitors; 4 — activated protein C; 5 — direct thrombin inhibitors; 6 — direct inhibitors of factor Xa; 7 — activators of fibrinolysis; 8 — inhibitors of platelet activation and aggregation; 9 — heparins.

The following symbols are used in Fig. 2: Fg — fibrinogen; Fn — fibrin; fXIIIa — activated factor XIII; Plat — platelets; $Plat_a$ — activated platelets; fIXa — activated factor IX; fXIa — activated factor XI; fXIIa — activated factor XII; KI — kallikrein; TF — tissue factor; fXa — activated factor X; fVIIa — activated factor VII; PCa — activated protein C; Coll — collagen; Adr — adrenaline; TxA — thromboxane A; ADP — adenosine diphosphate; Pg — plasminogen; Pm — plasmin; tPA — tissue plasminogen activator; uPA — urokinase; FDPs — fibrin degradation products; DD — D-dimer; E_1 — high molecular weight E-fragment; E_2, E_3 — hydrolyzed E-fragment.

Methods of stimulating extravascular thrombosis

The need to initiate thrombosis arises in the case of violation of the integrity of the walls of blood vessels, internal or external bleeding, which threaten to disrupt the blood
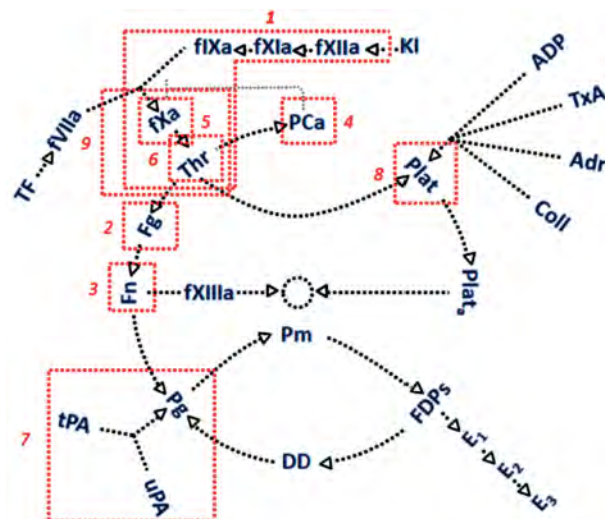

***Fig. 2.* The generalized scheme of the hemostasis system with the indicated nodal points targeted by the most common and promising anticoagulant agents**

supply to tissues and organs and should be stopped as soon as possible. Biotechnological challenges of creating opportunities to initiate thrombosis are an important issue of disaster medicine and surgery.

There are three basic ways to solve the problem of local bleeding: the introduction of exogenous activated blood clotting factors, and the use of non-specific or highly selective activators of blood clotting cascade factors.

The first method is the base of transfusion, the only effective antidote against bleeding caused by drug overdose or congenital pathologies of blood clotting [105, 106]. The patient can be transfused with blood, platelet mass, blood plasma, but the most effective is the use of prothrombin concentrate, which contains vitamin K-dependent coagulation factors, salted from human blood plasma [107]. Recombinant or purified plasma coagulation factors are also used [108].

Non-specific and highly selective agents that activate coagulation will be considered separately.

1. Nonspecific activators of the coagulation cascade

Under conditions of violation of the integrity of the vascular wall or in the case of pathological activation of blood clotting, the initiation of thrombosis occurs by physiological mechanisms of the external and internal pathways of the coagulation cascade [109]. Accordingly, it is possible to activate these mechanisms using non-specific physiological activators: thromboplastin for the external blood clotting pathway, and substances that have negatively charged negative surfaces for the internal pathway.

Thromboplastin is a preparation derived from tissues of various origins (most often the brain), which has procoagulant activity and is able in a matter of seconds to cause clotting of donor blood plasma. The composition of thromboplastin includes myelin membranes, individual membranes and even cell organelles. Coagulation activity of thromboplastin is determined by tissue factor [110].

Tissue factor is a membrane glycoprotein with a molecular mass of 45 kDa, which is found in the membranes of endothelial and smooth muscle cells, as well as in the membranes of monocytes and macrophages, and always functions exclusively in complex with the phospholipid matrix. The tissue factor molecule has three domains, the hydrophilic E- and C-domains and the hydrophobic domain represented in the membrane phase [111].

The process of damage to the outer cell membrane is associated with loss of the normal asymmetric distribution of lipids between its outer and inner surfaces. At the same time, tissue factor appears on the surface of the damaged cell, which, with the participation of Ca^{2+} ions, immediately forms a complex with factor VII, activating it. Bound to the lipid membrane, this complex effectively converts factors IX and X. In addition, the ability of tissue factor to form regulatory complexes is important, including the triple complex with VIIa and antithrombin III, as well as the complex with tissue factor inhibitor TFPI and factors Xa and VIIa [112].

Meanwhile, it is difficult to imagine the use of tissue factor of natural origin as a procoagulant agent. Recombinant tissue factor can be obtained, but as a transmembrane protein, it will require a lipid matrix.

Therefore, tissue factor is not considered as a basis for the creation of drugs with procoagulant action. But substances and biomaterials that can trigger the contact activation system are quite common in biomedicine.

A striking example of such preparations are kaolin-containing substances [113]. In particular, the most common APT test in clinical practice is performed using a reagent containing kaolin and ellagic acid [114]. Activation of the kallikrein-kinin system leads to the generation of tenase and prothrombinase complex and ultimately to the appearance of thrombin [115]. Moderate activation of this mechanism occurs to some extent in contact of blood with any negatively charged surface [116].

Hence, the action of thromboplastin or kaolin does not involve direct activation of prothrombin, instead, thrombin formation is carried out indirectly through a number of factors of the coagulation system, which in turn are activated in the presence of nonspecific activators (factors IX, VII, X, etc.). In view of this, the activity of these factors and their content in the studied plasma may affect the efficiency of thrombosis [117].

2. Activators of factor Xa

Describing specific activators, it is possible to imagine a hypothetical enzyme activator for each of the factors of the blood clotting cascade. However, selective activation of certain factors in the initial stages of the coagulation cascade will be less effective compared to the strong the avalanche-like action of kaolin or thromboplastin. In fact, activators of enzymes of the final links of the coagulation cascade,

namely the prothrombin and factor X, can be quite active procoagulants.

Activators of coagulation factor X have been found in the venoms of snakes of the families Viperidae and Crotalidae, and in the venom of some members of Elapidae. These include both serine proteinases and metalloproteinases [118]. The most well-known activator of factor X is RVV-X from the venom of Russell's viper *Daboia russelli*. RVV-X has found application in clinical laboratory diagnosis [119]. This metalloproteinase consists of a heavy chain that forms a catalytic domain and two light chains that are homologous to C-lectins [120].

Factor X activator has been described in particular in the venoms of desert cobra *Walterinnesia aegyptia* and Lebetine viper *Vipera lebetina* [121–123]. All of them act directly on factor X directly, regardless of the presence of phospholipids, which distinguishes the mechanism of their action from the mechanism of action of tenase. However, they have never been proposed for use as coagulation inducers.

3. Prothrombin activators

Activation of prothrombin seems to be the most promising way to stimulate thrombosis, as it leads to the generation of intravascular thrombin, which in turn not only converts soluble fibrinogen into insoluble fibrin, but also causes its covalent stabilization. However, there are ways to stop local bleeding by using directly exogenous thrombin, which is designed to immediately start the process of blood clotting, converting fibrinogen to fibrin with its subsequent polymerization and formation of a three-dimensional thrombus framework [124]. However, exogenous thrombin may be inhibited by endogenous anticoagulant proteins, including antithrombin, and lose its activity. In addition, exogenous thrombin can be applied to the wound surface only in limited quantities, which will not be sufficient to effectively stop the bleeding.

Endogenous prothrombin activators are devoid of these disadvantages and can be used to stop bleeding locally. Promising agents that can directly activate prothrombin are snake venom enzymes [125].

Among the enzymes of snake venoms that can activate prothrombin, there are factor X-like and ecarin-like proteins. Factor X-like enzymes activate prothrombin via prethrombin stage 2, cleaving the Arg274-Thr275 bond in the molecule to form fragment 1,2. Such enzymes are obtained from the poisons of Australian elapids. Ecarin-like enzymes cleave the Arg320-Ile321 bond in the prothrombin molecule to form mesothrombin, which is converted to normal α-thrombin via the mesothrombin stage [126].

The last group of prothrombin activators includes, in particular, ecarin, derived from the venom of *Echis carinatus* of the family Viperidae. This metalloproteinase is widely used in clinical practice and is a commercially viable drug, not least due to the possibility of obtaining a highly purified fraction of the enzyme, which allows for its detailed biochemical and biophysical characteristics [125, 127, 128]. An analogue of ecarin is ecamulin which is an enzyme isolated from the venom of a viper from Central Asia, *E. multisquamatis*, a species close to *Echis carinatus* [129, 130].

Non-physiological prothrombin activators activate all forms of prothrombin, even those that could not be activated by the physiological activation pathway. Ecamulin and ekarin both perform such activation through formation of an intermediate product, mesothrombin, with splitting of the Arg320-Ile321 communication without release of peptide. Mesothrombin, in turn, is autocatalytically converted to mesothrombin 1 (by cleavage of the Arg 156-Ser157 bond), and then to α-thrombin [131].

Although ecamulin and ekarin have similar functions and are synthesized by related species of snakes, there are significant differences in their structure. Thus, ekarin is a single-chain glycoprotein with a molecular mass of 63 kDa, 17% of the mass of which is a carbohydrate component. In contrast, ecamulin gives two bands with molecular masses of 67 kDa and 27 kDa in SDS electrophoresis. The highest coagulation activity is seen in fractions containing both of these components in equal proportions. The 27 kDa component contains two chains (13 kDa, 14 kDa). Separate chains of ecamulin do not have their own coagulation ability. S2 and S3 fractions are distinguished by the number of peaks that characterize ecamulin in ion exchange chromatography. One *g* of whole poison contains about 3 mg of S2-form and 10 mg of S3-form. The coagulation activity of S3 form is twice as high as that of S2. The isoelectric point of ecamulin is 4.3–4.5 [132].

Ecamulin, the activator of prothrombin released from the poison of *E. multisquamatis*, is used to determine the content of prothrombin and detect its functionally inactive forms. This test, called "ecamulin time", is based on the ability of ecamulin to activate both prothrombin and its functionally inactive forms — decarboxylated forms of

prothrombin, formed in the absence of vitamin K, and prethrombin. The latter appears in the bloodstream under the action of thrombin and is one of the markers of intravascular coagulation [133].

4. Thrombin-like enzymes

Another alternative use of exogenous thrombin to induce extravascular bleeding is the use of thrombin-like enzymes. Their main source is also snake venom [134].

Among the thrombin-like enzymes of snake venom, proteins such as ancrod (thrombin-like enzyme from the venom of *Colloselasma rhodostoma*) and batroxobin (*Bothrops moojeni*), have been relatively widely used in clinical practice for defibrination and laboratory diagnosis [135]. These enzymes belong to the class of serine proteases. Acting by a similar mechanism, they, unlike thrombin, cleave only one of the fibrinopeptides [136]. In addition, thrombin-like enzymes, unlike thrombin, do not have the ability to activate coagulation factor XIII, resulting in the formation of lacking covalent binds, and therefore unstable, fibrin clot of low strength.

In recent years, hemocoagulase, a reptilase analog, has been proposed to stop local bleeding [137−139]. The efficacy of such a hemostatic agent and its ability to initiate the formation of desA-fibrin was noted.

When comparing the prospects for the use of prothrombin activators and thrombin-like enzymes, the former should be preferred because they: a) lead to the activation of endogenous prothrombin with unlimited thrombin production potential; b) generating thrombin, trigger platelet activation; c) lead to the formation of a covalently stabilized clot.

5. Transglutaminase

The final stage of thrombus formation is covalent stabilization of fibrin by factor XIIIa. It is a physiological transglutaminase, activated by thrombin. In humans, nine types of transglutaminases have been described, which play an important role in maintaining homeostasis and are important in the development of certain pathological processes. Transglutaminases are enzymes that catalyze the formation of covalent isopeptide bonds between glutamyl and lysine protein residues [140].

The idea of increasing the efficiency of extravascular thrombosis through the use of transglutaminases is to stabilize endogenous fibrin polymers and bind them with adjacent tissues.
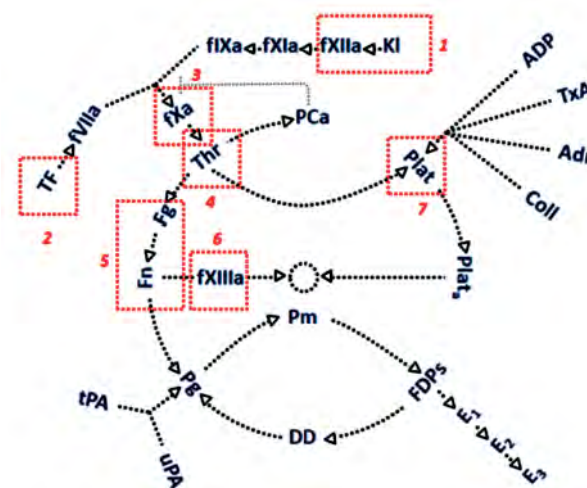

Fig. 3. **The generalized scheme of the hemostasis system with the indicated nodal points targeted by the action of the most common and promising procoagulant agents**

It is proposed to enhance thrombosis using factor XIIIa of the blood coagulation system [141] or its analogues with transglutaminase activity [142, 143]. However, although such enzymes act in the final stage of blood clotting, initiating covalent stabilization of polymeric fibrin, they are not able to trigger the conversion of fibrinogen to fibrin with its subsequent polymerization and formation of a three-dimensional thrombus framework. Thus, these enzymes can not serve as a basis for effective hemostatics.

Fig. 3 presents a generalized scheme of the hemostasis system with the indicated nodal points, which are targeted by the action of the most common and promising procoagulant agents: kaolin (*1*); tissue factor (*2*); factor X activators (*3*); prothrombin activators (*4*); thrombin-like enzymes (*5*); transglutaminase (*6*); platelet activators (*7*).

The following symbols are used in Fig. 3: Fg — fibrinogen; Fn — fibrin; fXIIIa — activated factor XIII; Plat — platelets; $Plat_a$ — activated platelets; fIXa — activated factor IX; fXIa — activated factor XI; fXIIa — activated factor XII; KI — kallikrein; TF — tissue factor; fXa — activated factor X; fVIIa — activated factor VII; PCa — activated protein C; Coll — collagen; Adr — adrenaline; TxA — thromboxane A; ADP — adenosine diphosphate; Pg — plasminogen; Pm — plasmin; tPA — tissue plasminogen activator; uPA — urokinase; FDPs — fibrin degradation products; DD — D-dimer; E_1 — high molecular weight E-fragment; E_2, E_3 — hydrolyzed E-fragments.

Conclusions

Physiological (extravascular) thrombosis occurs in violation of the integrity of the vascular wall to prevent blood loss and is evidence of normal functioning of the hemostasis system. The pathological thrombosis (intravascular) is the result of imbalance in the hemostasis system. Both of these processes are based on the same molecular mechanisms: enzymatic coagulation cascade, platelet cell signaling, fibrinolytic and anticoagulant units. According to the basic concept, the researcher can, as needed, stimulate or inhibit the process of thrombosis, by acting on a certain part of the system.

The most effective way to inhibit intravascular thrombosis is to affect various parts of the coagulation cascade, primarily thrombin and factor Xa. The ways to affect platelet aggregation are less effective, but currently widely used. To inhibit the last link in coagulation (fibrin polymerization and the formation of a fibrin network, which is the framework of the thrombus), it is proposed to use peptide inhibitors that mimic the amino acid sequence of fibrin polymerization sites, or low molecular weight calixarenes that can block such sites.

The most effective way to stimulate extravascular thrombosis was at the stage of prothrombin activation. The use of enzymatic coagulation activators to modify biomaterials will make it possible to create effective means to prevent acute vascular and capillary bleeding.

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МОЛЕКУЛЯРНІ МЕХАНІЗМИ ІНГІБУВАННЯ ВНУТРІШНЬОСУДИННОГО І СТИМУЛЯЦІЇ ЕКСТРАСУДИННОГО ТРОМБОУТВОРЕННЯ

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Система гемостазу покликана підтримувати баланс між процесами зсідання крові, антикоагуляції, а також фібринолізу, забезпечувати постійний ефективний кровообіг в організмі та швидке припинення кровотеч у разі їх виникнення. В основі забезпечення прокоагулянтного потенціалу системи гемостазу лежать молекулярні механізми, що ведуть до утворення фібрину в кров'яному руслі, який є каркасом тромбу, та до агрегації тромбоцитів — основи тіла тромбу. Антикоагулянтний потенціал плазми крові забезпечується механізмами, спрямованими на інгібування процесів коагуляції крові. Ґрунтовне вивчення та розуміння цих механізмів дозволить відкрити численні засоби лікування патологічних станів, пов'язаних як із внутрішньосудинним тромбоутворенням, так і з кровотечами різного ґенезу.

Метою огляду є аналіз способів запобігання внутрішньосудинному тромбоутворенню та стимулювання екстрасудинного тромбоутворення. В огляді описано та проаналізовано доступні та перспективні засоби запобігання тромбоутворення, зокрема, прямі й непрямі антикоагулянти та антиагреганти, а також способи ефективного стимулювання тромбоутворення, що необхідно у разі пошкодження судин. Результатом такого аналізу є визначення вузлових точок протеїнової мережі системи гемостазу, дія на які специфічними молекулярними ефекторами дозволить керувати процесом тромбоутворення.

Ключові слова: антикоагулянти, антиагреганти, активатор, зсідання крові, тромбоутворення.

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Agrobacterium rhizogenes — ОПОСЕРЕДКОВАНА ТРАНСФОРМАЦІЯ ЯК СПОСІБ СТИМУЛЮВАННЯ СИНТЕЗУ АНТИОКСИДАНТНИХ СПОЛУК У *Artemisia absinthium* L.

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Рослини *Artemisia absinthium* L. відомі як продуценти речовин з антиоксидантними властивостями. Зокрема, в них виявлено поліфеноли та флавоноїди. Активізувати синтез цих сполук можна шляхом генетичної трансформації навіть без перенесення специфічних генів, які беруть участь у біосинтезі. Так, «бородаті» корені, одержані після *Agrobacterium rhizogenes*-опосередкованої трансформації, можуть бути продуцентами комплексу цінних метаболітів.

Метою роботи було одержати «бородаті» корені *A. absinthium* як продуценти поліфенольних сполук.

Методи. «Бородаті» корені одержували шляхом культивування листків з суспензією *A. rhizogenes* з вектором *pCB124*. Наявність перенесених генів підтверджували методом ПЛР. Для визначення вмісту флавоноїдів та поліфенолів використовували реакції з $AlCl_3$ та реактивом Фоліна-Чокальте. Антиоксидантну активність оцінювали за здатністю екстрактів відновлювати DPPH радикал.

Результати. ПЛР аналіз виявив наявність бактеріальних *rol* генів та відсутність генів плазміди pCB124. Лінії коренів відрізнялися між собою за швидкістю росту. «Бородаті» корені характеризувалися більшим вмістом поліфенолів, зокрема, флавоноїдів (до 4.784±0.10 мг/г ВМ) та вищим рівнем антиоксидантної активності (EC_{50} = 3.657 мг) у порівнянні з контролем (3.861±0,13 мг/г СМ та EC_{50} = 6.716 мг відповідно).

Висновки. Трансформацію *A. absinthium* із застосуванням *A. rhizogenes* може бути використано для одержання ліній з підвищеним вмістом поліфенольних сполук та більшою антиоксидантною активністю.

Ключові слова: Artemisia absinthium L., *Agrobacterium rhizogenes*-опосередкована трансформація, «бородаті» корені, флавоноїди, поліфенольні сполуки, антиоксидантна активність.