Biological Activity and Pharmacological Properties of Anticoagulant Complex (Hirudin, Plasma Kallikrein Inhibitor, Prostaglandin) from *Hirudo Medicinalis*

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Antiprocoagulant complex isolated from lyophilized medicinal leeches exerted pronounced antithrombotic, thrombolytic, and hypotensive effects in experimental animals after intravenous injection and showed antithrombotic activity after oral administration in combination with hirudone, the source of hyaluronidase and inhibitors of digestive proteolytic enzymes. The antiprocoagulant complex can be used as a specific medicinal preparation.

Key Words: *medicinal leech; hirudin; kallikrein inhibitor; prostacyclin; thrombosis*

A long history of hirudotherapy testifies to the efficiency of this method in the prevention and treatment of various diseases [2].

Leech salivary gland secret contains unique bioactive components produced by this species only [2]: hirudin, a highly specific thrombin inhibitor [8]; destabilase [4] selectively hydrolyzing the isopeptide bonds in stabilized fibrin; plasma kallikrein inhibitor [5]; bdellins and eglins, the inhibitors of plasmin, trypsin, chymotrypsin, and neutral proteases of human granulocytes; hyaluronidase [2], prostaglandins, similar to prostacyclin by their physiological activity [1], and others. However, both physicians and patients feel uncomfortable using medicinal leeches, which necessitates the development of medicinal preparations on the basis of bioactive compounds produced by medicinal leeches. The first generation preparations such as hirudon and piyavit (gelatin capsules for oral administration), hirudo ointment and gel, as well as various cosmetic creams contain a complete collection of bioactive compounds produced by medicinal leeches, which determines their complex effect on the organism. These preparations are used for prevention and treatment of thrombosis, atherosclerosis, hypertension, inflammatory processes, cardiovascular and dermatological diseases, *etc.* [2].

At the same time, some pathologies associated with disorders in the blood coagulation system as well as prevention of disseminated intravascular coagulation and postoperative clotting require specific anticoagulant treatment, which does not affect the fibrinolysis system, complement, and the extrinsic pathway of blood coagulation. In this connection we attempted to develop a preparation with selective anticoagulant activity.

MATERIALS AND METHODS

Medicinal leeches from BIOKON (Russia — Ukraine) and Rosfarmatsiya (Russia) plants were used.

Antiprocoagulant complex (APC) was isolated from hirudon water solution as described elsewhere [11]. Protein concentration was measured by Lowry's method [7]. Hirudin activity was measured by fibrinogen clotting time in the presence fo thrombin using "Test-Thrombin" kits (Behringwerke). Kalllikrein inhibitor activity was measured by plasma recalcification time: 2-fold prolongation corresponded to 1 unit. The content of hirudinal prostaglandins was determined by measuring the concentration of 6-keto-prostaglandin $F_{1\alpha}$ using a "6-Keto-Prostaglandin $F_{1\alpha}$ (^{125}I) Assay

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TABLE 1. Comparative Characteristic of Preparations ($M \pm m$)

Parameter	Hirudon	APC
Antithrombin activity, unit/mg	20.0±05	80±2.5
Plasma recalcification time, unit/mg	60±15	540±24
Isopeptidase activity, mm^2/mg	25±5	0
Amidolytic activity, mol/mg/sec	$(1.0\pm0.2)\times10^{-3}$	$(0.2\pm0.1)\times10^{-3}$
Inhibition of: trypsin, unit/mg	6.5±1.0	—
chymotrypsin, unit/mg	1.8±0.5	0
kallikrein, unit/mg	5.0±1.5	42.0±6.5
Content of 6-keto-prostaglandin $F_{1\alpha}$, ng/ml	500±100	3670±280
Activity of trigliceride lipase, nmol/mg/h	7.7±0.3	0
Cholesterol esterase activity, nmol/mg/h	2.9±0.2	0

System" (Amersham). Destabilase activity was determined using plates of stabilized fibrin [4] and spectrophotometrically at 405 nm using L-γ-Glu-pNA chromogenic substrate [11]. The inhibition of trypsin and chemotrypsin activity was measured using S-2302 and Succinyl-L-α-Phe-pNA (Kabi-Diagnostica) chromogenic substrates. Electrophoresis was performed in a Laemmli's system using 12.5 and 16% PAAG with 2M urea, mercaptoethanol, and SDS.

Antithrombotic and trhombolytic effects were evaluated on the model of venous thrombosis [13]. Thrombosis was stimulated with glass-activated human serum followed by stasis of the jugular vein. Thrombosis was assessed visually and by the weight of formed thrombus.

Blood pressure was measured in the caudal vein of spontaneously hypertensive rats (SHR strain).

RESULTS

APC was isolated from hirudon (Biokon), lyophilized powder of medicinal leeches. After pH correction, and heating at 60°C for 25 min, the initial extract from hirudon water solution was filtered through a Sephadex-75 gel to obtain a fraction enriched with plasma kallikrein inhibitor and prostaglandins. This fraction (APC) contains 60 times more hirudin, 9 times more plasma kallikrein and 5.7 times more prostaglandins than the initial hirudon solution (Table 1). Only trace amounts of destabilase and no other bioactive compounds produced by medicinal leeches are present in APC.

Since APC bioactive components inhibit different stages of the hemostatic process (prostaglandins inhibit platelet-vascular stage, kallikrein inhibitor — plasma stage of the intrinsic pathway, hirudin — late plasma stage of coagulation) it was reasonable to assume that intravenous APC can prevent clot formation inder conditions of prethrombosis.

The antithrombotic effect of APC was studied in outbred albino rats using venous thrombosis model [13]. Experimental rats (n=10) received intravenous injection of APC (3.5 mg/kg, water solution), the control animals received the same volume of 0.85% NaCl (water solution). Clotting was stimulated in occluded jugular vein 12, 14, 36, and 48 h postinjection. APC containing inhibitors of blood clotting produced a powerful anticoagulant effect (Table 2), which determines its pharmacological specificity.

However, in our opinion a perfect anticoagulant should preserve its activity after oral administration. Since APC contains only anticoagulant compounds, it will degrade in the gastrointestinal tract. Hirudon preserving its activity in all administration routes [8], was

TABLE 2. Effect of APC and its Combination with Hirudon on Clotting ($M \pm m$)

Time from administration to the start of coagulation, h	Intravenous injection		Oral administration	
	APC	0.85% NaCl	APC+hirudon	0.85% NaCl+hirudon
12	0	90±5	10±10	95±5
24	10±5	95±5	30±10	90±10
36	20±5	90±10	45±5	90±5
48	35±5	90±5	65±5	90±5

TABLE 3. Thrombolytic Effect of Intravenous APC (*M*±*m*)

Time after administration, h	Thrombolysis			
	0.85% NaCl		APC	
	macroscopic examination, %	weight, mg	macroscopic examination, %	weight, mg
24	5	1.9±0.2	10	1.8±0.1
36	15	1.5±0.3	35	1.1±0.2
48	20	1.3±0.3	50	0.8±0.2

used as a source of proteolytic enzyme inhibitors. In the next experimental series, APC (3.5 mg/kg) was given perorally in combination with hirudon in a dose of 10 mg/kg. This dose has no antithrombotic effect, but protects APC components from proteolysis in the gastrointestinal tract and contains sufficient amount of hyaluronidase for supporting APC passage across intercellular junctions.

The experimental rats (n=9) perorally received APC water solution with hirudon suspension. The control rats (n=9) received an equal volume of hirudon suspension in 0.85% NaCl water solution. Clotting was stimulated by intravenous injection of glass-activated human serum 12, 24, 36, and 48 h after treatment. Peroral APC in combination with hirudon (the source of hyaluronidase and proteolytic enzyme inhibitors) prevented clotting (Table 2), but was 50% less efficient than intravenous APC due to partial splitting by digestive enzymes.

Thus, ACC can be considered as a selective anticoagulant efficient after both intravenous and oral administration in combination with proteolytic enzyme inhibitors (hirudon).

Since APC is proposed as a medicinal preparation, it was necessary to describe its properties determined by its other components — prostaglandins, hirudin, and kallikrein inhibitor. Since physiological activities of prostanoids produced by medicinal leeches are similar to those of prostacyclin, is was of interest to study the thrombolytic effect of APC. Prostacyclin interacts with vascular receptors and stimulates the release of tissue plasminogen activator (t-PA) [9] which activates the fibrinolytic and thrombolytic systems.

Taking into account that intravenous administration of leech prostaglandins elevates the blood content of t-PA [3], it could be expected that APC will increase fibrinolytic activity. The experimental rats (n=30) were intravenously injected with APC (water solution, 5.5 ml/kg) 18 h after the stasis of the jugular vein. The control rats (n=30) received the same volume of 0.85% NaCl. Thrombolysis intensity was evaluated 24, 36, and 48 h postinjection. Intravenous APC stimulated partial lysis of clots: 48 h postinjection the level of thrombosis in the experimental group was 50% of that in the control (Table 3).

Destabilase fibrinolytic activity does not contribute to the mechanisms of APC-stimulated fibrinolysis, since its insignificant content in APC caused no significant changes in amidolytic activity (assessed using a L-γ-Glu-pNA chromogenic substrate) of the blood plasma both in the control and experimental rats.

The polyfunctionality of the stable prostacyclin analog synthesized by medicinal leeches determine diverse physiological effects of APC. Prostacyclin is known to lower vascular tone [6] and leech prostaglandins exhibit the same activity [2].

In the next experimental series we studied the hypotensive effect of intravenous administration of APC to SHR.

The experimental rats (n=8) were intravenously injected with APC (water solution, 4.3 mg/kg). The control group (n=7) received the same volume of 0.85% NaCl. Blood pressure in the caudal vein was recorded 6,12, 24, and 36 h postinjection. Single intravenous injection of APC significantly lowered blood pressure (Table 4) and this effect persisted for 36 h.

Thus, APC possesses a pronounced antithrombotic activity due to the presence of hirudin, plasma kallikrein inhibitor, and prostacyclin analog. This activity is preserved after intravenous administration of APC alone and after its oral administration in combination with hirudon. In addition, APC exerts thrombolytic and hypotensive effects which are largely determined

TABLE 4. Blood Pressure in Rats after Single Intravenous Injection of APC (mm Hg, *M*±*m*)

Groups	Time after administration, h			
	6	12	24	36
Experimental	130±10	135±15	135±10	145±15
Control	165±15	170±15	160±10	165±10

by the presence of a stable prostacyclin analog produced by medicinal leeches. This effect should not be considered as a negative side effect, since increased vascular tone, high blood pressure, and local vasospasms contribute to the pathogenesis of prethrombosis condition.

Currently, APC is being prepared for clinical tests as a selective anticoagulant drug.

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