Experimental Thrombosis in Rabbit Marginal Ear Vein and Evaluation of the Thrombolytic Action of Longolytin

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A model of venous thrombosis on the rabbit marginal ear vein was developed. It is based on occlusion of a segment of the left or right ear vein followed by injection of thrombin into this segment. Thrombolytic activity of longolytin applied externally onto the thrombotic venous segment was evaluated. The drug 2-fold shortened the time of clot lysis and 4-4.5-fold increased the rate of thrombolysis. The effect of longolytin was potentiated by heparin. The rate of lysis was higher in large thrombi and in the left vein (effect of asymmetry).

Key Words: *thrombus; thrombolysis; longolytin; thrombolytic asymmetry*

Thrombosis is a dangerous pathological state initiating and/or accompanying severe human diseases: myocardial infarction, stroke, and atherosclerosis. Various aspects of thrombosis development are now discussed: clinical, physiological, biochemical, genetic, ecological, epidemiological, and psychosocial. However, the study of the very process of thrombus formation on the experimental *in vivo* and *in vitro* models remains most important, because it yields information on complex processes underlying thrombosis and thrombolysis and approaches at modification of these processes. In animal *in vivo* models, thrombi are usually induced in vessels, primarily in surface veins: in the femoral vein in dogs and rabbits and in the jugular vein in rats and mice. Our study was carried out on rabbit marginal ear vein, a large convex vessel passing along the ear edge. This vein can be easily observed via a thin layer of auricular cartilage and epithelium, which makes it accessible for manipulations either from outside or inside, for example, for induction of experimental thrombi and subsequent visual control of their lysis [8,9].

Our aim was to develop a method of clot formation in rabbit marginal ear vein and evaluation of the possibility of using the developed clot as the substrate for the thrombolytic action of longolytin applied externally alone or in combination with heparin.

MATERIALS AND METHODS

Experiments were carried out on rabbits (n=40) for 5 years. Each animal was used in the experiments no more than 3 times. The clots were formed on both ears. After complete formation of the thrombus, 0.1-3.0% thrombolytic drug longolytin (a mixture of proteases dissolved in glycerol) was applied to the skin above the clotted vein segment. Longolytin is extracted from the culture medium of saprophyte fungus *Arthrobotrys longa*. This drug can dissolve the thrombi *in vitro* and *in vivo* after intravenous injection [7] or external application onto the clotted segment of rat jugular vein [6]. In a special series of experiments, we examined the effi-

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cacy of longolytin in comparison to the control application of glycerol. In experimental series, longolytin was applied in combination with heparin (5-10 U/ml) to prevent rethrombosis and to promote lysis (heparin was used as the control). Thrombus formation was assessed by its area (mm^2), while thrombolysis was estimated by the time of clot lysis (min) and by the rate of lysis (the ratio of the difference in clot area over a certain period to this period, mm^2/min).

RESULTS

The thrombi were formed according to the routine method used on rats [9]. The rabbit ear was shaved along the marginal vein and its 5-7-mm-long medium segment with minimum number of collaterals was clamped at both ends. Thrombin (30-50 μl, ≅20 U/ml) was injected into this segment. After 1.0-1.5 h, the presence of the clot was confirmed using powerful electric lamp directing focused light onto the internal side of the ear. The clot looked like a dark strand filling the vein segment with clear boundaries. Its size was determined as the product of its length by width (mm^2). This value varied from 4 to 52 mm^2, and such a scatter resulted from individual peculiarities rather than from methodical errors. The distribution of thrombus area was as follows: 4-12 mm^2 (23%), 13-24 mm^2 (50%), and 25-52 mm^2 (27%).

The dynamics of longolytin-induced thrombolysis was assessed over 5-7 h (by the decrease of thrombus size). Thrombolysis developed persistently usually during experimental day 1, in some cases over 2-3 days (primarily in control groups). Thrombolysis was documented as recovery of blood flow and normalization of vein pattern, albeit in some cases the vessels remained damaged for 7-10 days.

Table 1 shows the data on thrombolytic action of longolytin applied onto the skin over the thrombus every hour during experimental day 1 (usually 3-5 times per day). By all indices characterizing thrombolysis, the effect of longolytin was pronounced: it shortened the time of lysis by more than

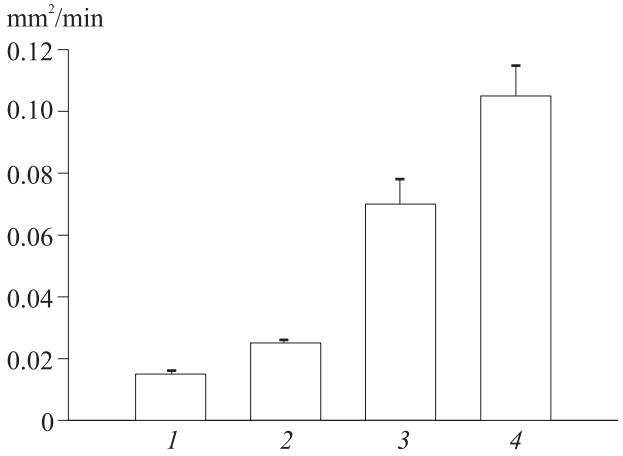

Fig. 1. Effect of external application of longolytin alone or in combination with heparin on lysis rate. *1*) control group (glycerol application); *2*) control group (heparin application); *3*) longolytin; *4*) longolytin+heparin.

2-fold, increased lysis rate by 4.5 times on day 1, and enhanced it during the entire observation period. As expected [3], heparin potentiated the thrombolytic activity of longolytin. It is noteworthy that heparin increased the rate of lysis (Fig. 1).

Therefore, the new method based on thrombus formation in rabbit marginal vein allows modeling of venous thrombosis, estimation of thrombus parameters using simple technical tools, and evaluation of the thrombolytic properties of the test drug (longolytin). This model confirmed the co-factor effect of heparin established in other experimental models [2]. Our original method is a comprehensive model of thrombosis allowing analysis of thrombolytic activity of various external drugs applied to the thrombotic vein.

When analyzing the dependence of thrombolysis on thrombus size, we obtained a result similar to that described on a thrombosis model in rat jugular vein: large thrombi were dissolved more rapidly than small ones [6]. Figure 2 shows the relation between the thrombus size and the rate of thrombolysis. This dependence was observed when longolytin was applied alone or in combination with heparin: the larger were the thrombi, the smaller was the lysis time. This was due to high affinity of

TABLE 1. Thrombolytic Effect of Longolytin Applied Externally over Thrombus in Rabbit Marginal Ear Vein

Group	Time of thrombus lysis on day 1, min	Rate of thrombus lysis on day 1, mm^2/min	Thrombolysis on day 1, %	Total lysis time, min	Total lysis rate, mm^2/min
Control (n=30)	No lysis	0.015±0.005	17.0±2.8	1382±121	0.014±0.004
Experiment (n=36)	255±31**	0.070±0.081*	72.0±8.5**	632±87*	0.060±0.007*

Note. *p<0.01, **p<0.001 compared to the control.

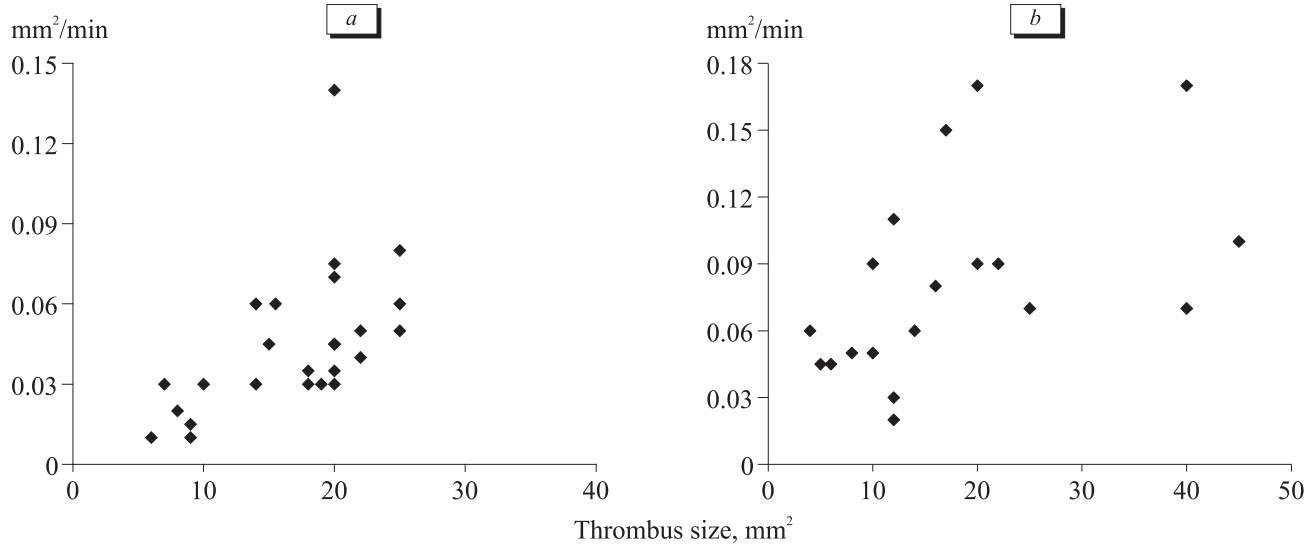

Fig. 2. Rate of thrombolysis as a function of thrombus size after external application of longolytin alone (*a*) or in combination with heparin (*b*).

TABLE 2. Effect of Drugs Applied Externally over Throumbus in Left and Right Rabbit Marginal Ear Veins on Rate of Thrombus Lysis

Drugs	Left vein	Right vein
Glycerol	0.013±0.001* (*n*=15)	0.015±0.001 (*n*=15)
Heparin	0.030±0.001* (*n*=40)	0.020±0.001 (*n*=39)
Longolytin	0.090±0.009* (*n*=18)	0.050±0.006 (*n*=16)
Longolytin+heparin	0.130±0.012** (*n*=47)	0.075±0.008 (*n*=44)

Note. *$p<0.01$, **$p<0.001$ compared to the right vein.

tissue plasminogen activator (largely determining longolytin activity) to fibrin (the main component of venous thrombus) [7]. As a result, the greater thrombus surface adsorbs larger amount of longolytin, thereby increasing the rate of thrombolysis. Of particular interest was asymmetry of the thrombolytic process in the right and left marginal veins: the lysis rate was higher in the left vein than in the right one. Indeed, among all modeled thrombi (left vein, *n*=120; right vein, *n*=114), lysis rate in the right and left veins was similar in 21 rabbits, was higher in the right vein in 28 animals, and was higher in the left vein in 68 cases.

Table 2 shows the rate of thrombolysis in the left and right marginal veins in all experimental situations. The data showed that in most cases, the lysis rate was higher in the left vein than in the right vein and in only one of two control groups (application of glycerol) no significant differences were observed, which confirms previous data [10] on inactivity of glycerol in processes of correction of biophysical damages in rabbit marginal ear veins. Hence, asymmetry is determined by vasoactive ingredients such as longolytin, heparin, or their mixture, but not inert supplement (*i.e.* asymmetry in our experiments was functional phenomenon and is a multilevel reaction of the endothelium in the right and left veins to active substances). The morphological and functional asymmetry between the microcirculatory beds in rabbit ears was reported previously [4,5].

Thus, our method enables modeling of clot formation in rabbit marginal ear vein with clearly visible shape and study of its lysis induced by chemical preparations (applied externally over the thrombotic area) that could be potential drugs to treat thrombophlebitis in humans. Moreover, the described method can be used for evaluation of thrombolytic process (synergism, antagonism, and inactivity of the preparations together with asymmetry of these peculiarities and individual sensitivity).

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