The aim of the study was to evaluate the effect of L-arginine (L-Arg) on haemostasis in stasis model of venous thrombosis in renal hypertensive rats. The effect of the single dose (i.v. 300 mg/kg bolus+300 mg/kg/h) and of the 10-day application (p.o. 1 g/kg, once daily) of L-Arg was determined. L-Arg reduced the blood pressure both in the acute and long-term application. The single dose of L-Arg decreased the occurrence rate of the thrombus whereas long-term administration reduced significantly the thrombus weight. There were no differences in prothrombin time and activated partial thromboplastin time while the fibrinogen concentration decreased both in the acute and the long-term experiment. L-Arg shortened euglobulin clot lysis time and bleeding time in the long-term application. The chronic L-Arg treatment also inhibited significantly collagen-induced platelet aggregation. The overall haemostasis and coagulation potentials were inhibited and the fibrinolysis potential was higher in the group receiving this amino-acid. The results show that L-Arg, in a complex way, evokes the antithrombotic effect in the model of venous thrombosis in hypertensive rats.

**Key words:** L-arginine, haemostasis, venous thrombosis, rats

**INTRODUCTION**

The vascular endothelium produces a large number of biological substances, of which nitric oxide (NO) is the best known. NO originates in a complex reaction of L-Arg oxidation, in which also L-citrulline is produced (1-3).

Nitric oxide has been shown to be an extremely important signaling molecule in cardiovascular system (3,4). Although, in the last decade many attempts were done to prevent cardiovascular disease by changing life style and diet, sufficient therapies have not been found yet. Thus, the possibility to increase nitric oxide
availability and to enhance the enzymatic activity of NO synthase by the simultaneous treatment with L-Arg has been postulated. Both in vitro (5,6) and in vivo (7-9) studies have demonstrated that L-Arg as a precursor for nitric oxide can augment vascular dilation under certain conditions. According to these findings, it was shown that a long-term administration of L-Arg ameliorated peripheral (mainly muscle) insulin sensitivity through a normalization of NO/cGMP pathway in lean type 2 diabetic patients (10) and that an increased NO availability, after an acute infusion of L-Arg, significantly improved forearm oxidative glucose metabolism in subjects with microvascular angina (11).

Besides blood pressure, the majority of literature reports refer to the interaction of L-Arg with blood platelets (9,12-14). L-Arg via enhancement of nitric oxide synthesis in endothelium activates intraplatelet guanylate cyclase, thus increasing cGMP concentration, and prevents platelet adhesion and aggregation (15-17).

Besides that, up to now, only few reports have appeared concerning L-Arg and haemostasis. Haemostasis is a complex processes that involves: vascular wall, blood platelets, coagulation and fibrinolytic system interactions. Stief et al. have observed a prolongation of PT and APTT after L-Arg incubation with human plasma (18). It has been also shown that L-Arg inhibits PAI release from platelets and stimulates the activity of endothelium-derived t-PA (13).

Taking the above data into account as well as the fact that L-Arg is the only substrate for NO synthesis, it can be assumed that this amino acid may also modify coagulation and fibrinolysis in vivo. The present study is an attempt to find the potential antithrombotic action of L-Arg, including the effect of L-Arg on primary haemostasis, coagulation and fibrinolysis in the model of venous thrombosis in hypertensive rats.

MATERIAL AND METHODS

Animals and renovascular hypertension induction

Male Wistar rats (200g) were used in the experiments. Two kidney, one clip model of hypertension was induced by partial, standardized clipping of the left renal artery under pentobarbital anesthesia (45 mg/kg, i.p.) (19). After 6 weeks all the animals developed hypertension which was confirmed by the blood pressure measurement using "tail cuff" method (20). The animals were housed in a room with a 12h light/dark cycle, in group cages as appropriate, were given tap water and fed a standard rat chow. Twenty four hours before the induction of venous thrombosis, the rats were deprived of food but had free access to water. Only animals with blood pressure higher than 140 mm Hg were included in the experiments. Procedures involving the animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and Guidelines for the Use of Animals in Biomedical Research (Thromb Haemost 1987; 58: 1078-84).
**Drugs and reagents**

L-arginine (L-arginine Hydrochloride, Sigma Chemical Co., USA), pentobarbital (Vetbutal, Biovet, Poland), buffer Tris [Tris(hydroxymethyl)-aminomethane hydrochloride (Sigma Chemical Co., USA) and Tris(hydroxymethyl)-aminomethane (Merck, Germany)], collagen (Chronolog, USA), trisodium citrate (Polish Chemical Reagents), calcium chloride (CaCl2) (Polish Reagents Chemical) were used in the experiments.

**L-arginine administration**

In the acute experiment, rats received a single dose of L-Arg by intravenous infusion (300 mg/kg bolus + 300 mg/kg/h for 2h). Control rats were given physiological saline in the same way and in the same volume. In the chronic experiment, L-Arg, dissolved in distilled water was administered by intragastric probe always at the same time for 10 days (1g/kg, once daily). Control group received distilled water also per os, at the same time and in the same volume as the L-Arg group.

**Venous thrombosis**

The animals were anaesthetized with pentobarbital (45mg/kg, i.p.). Venous thrombosis was performed as previously described by Reyers et al. (21). Briefly, the abdomen was opened, the vena cava was carefully separated from surrounding tissues and then ligated tightly with a cotton thread just below the left renal vein. Subsequently, the abdomen was closed with a double layer of sutures (peritoneum with muscles and the skin separately). After two hours the animals were re-anaesthetized and the abdomen was then reopened, the vena cava was carefully dissected and inspected for the presence of thrombus. The thrombus was kept at 37°C and after 24 hours its dry weight was measured.

**Direct blood pressure measurement**

Mean blood pressure in rats receiving L-Arg infusion was measured directly through a cannula filled with heparin solution, placed in the right common carotid artery and connected to a pressure transducer (PD-23 Gould) and monitor Trendoscope 8031, Unitra Biazza S&W Medico Teknik, A/S Denmark).

**Bleeding time (BT)**

"Template" bleeding time was measured just before venous thrombosis induction by longitudinal incision of a rat tail according to Dejana et al. (22).

**Haemostatic parameters (PT, APTT, ECLT and fibrinogen concentration)**

Blood samples were taken from the heart two hours after venous thrombosis induction. All samples were mixed with 3.13% trisodium citrate in a volume ratio 10:1. Prothrombin time (PT), activated partial thromboplastin time (APTT), euglobulin clot lysis time (ECLT) and fibrinogen concentration were determined by routine laboratory assays.

**Whole blood platelet aggregation**

The blood was collected and added to 0.9% NaCl (1:1), poured to cuvettes (1 ml in each) and incubated for 15 minutes at 37°C. Then, it was mixed for 1 minute and collagen (5µg/ml) was
added. Platelet aggregation was measured for 6 minutes according to the method of Cardinal (23) using a Lumi-aggregometer (Chrono-log, USA). A change in blood resistance was expressed in ohms.

**Haemostatic potentials (HP)**

The haemostatic potential was assessed according to the method of He S et al. (24,25). From pentobarbital-anaesthetized (45 mg/kg) control and drug-receiving rats blood was collected, mixed with trisodium citrate (10:1) and centrifuged at 2000 x g for 20 minutes at 4°C. In the plasma so prepared, the haemostatic potentials (HP) were determined or the 0.5 ml plasma samples were frozen at -70°C and defrosted just before HP assay.

a) **Assessment of the overall coagulation potential (OCP)**

120 µl of plasma was poured to the first rows of microplatelets. 100 µl of 66 mM buffer Tris, pH 7.4, as well as thrombin, CaCl, and NaCl (final concentrations: 0.005 IU/ml, 17 mM and 65 mM, respectively) were added to each sample. Referential samples (blank) contained only 120µl of plasma and 100 µl of buffer Tris. The mixtures, 4 minutes after the beginning of buffer addition, were placed in a microplatelet reader (Dynex Technologies Revelation II, USA) and fibrinous thrombus growth was measured every minute for 15 minutes at 405 nm wavelength. The OCP was evaluated as sum of the increment in optical density of the tested sample compared to the referential sample (blank).

b) **Assessment of the overall haemostatic potential (OHP)**

The OHP was determined at the same time and in the identical way as the OCP, using the same platelet (further rows), but to each plasma sample, buffer Tris was added with composition as for the OCP and additionally containing t-PA at the final concentration of 1500 ng/ml.

c) **Assessment of the overall fibrinolytic potential (OFP)**

The OFP was expressed in percentages and showed the difference between the OCP and OHP, which was demonstrated by the difference between OCP and OHP calculated using the formula:

\[
\text{OFP} = \frac{\text{OCP} - \text{OHP}}{\text{OCP}} \times 100\%.
\]

**Statistical analysis**

Multiple group comparisons were performed by one-way analysis of variance (ANOVA), and when significant intergroup differences occurred, were assessed by a Student-Neuman-Keuls test. Incidence of venous thrombosis was evaluated by Fisher's exact test. Student's unpaired t-test was also used to determine the significance between means for two groups. A value of p<0.05 was considered statistically significant.

**RESULTS**

The mean blood pressure of anaesthetized rats was 158 ± 13.12 mmHg. Infusion of physiological saline for 300 seconds had no effect on this parameter (**Fig. 1A**). L-Arg caused a sudden pressure drop between 40 and 150 second, with the maximum effect at 60 second (drop by 49 mm Hg). At 210 second the mean pressure returned to the initial level. The pressure reduction between 40 and 150 second was statistically significant (p<0.05). Further infusion (for two hours) of physiological saline or L-Arg did not change the blood pressure (data not shown). In the animals
receiving L-Arg per os, the systolic pressure decreased to 135.8 ± 3.29 mm Hg, in comparison to control group being 162 ± 12.74 mmHg (p<0.001) (Fig.1B).

Two hours after experimental induction of venous thrombosis, thrombus was found in all control and L-Arg chronically treated animals, whereas only 75% of rats receiving L-Arg intravenously were occluded (p<0.01). The mean dry weight of thrombus was slightly reduced in animals given L-Arg, but the difference was statistically insignificant. In chronic experiment, thrombus was found in all control rats, but the mean dry mass of venous thrombus in L-Arg treated group was markedly reduced from 4.98 ± 1.56 mg to 1.56 ± 0.73 mg (p<0.001) (Fig.2).

Fig. 1. Graph represents (A) the mean blood pressure in rats acutely treated with L-arg (300 mg/kg bolus and 300 mg/kg/h i.v. for 2 hours, n=8) in comparison to the control (statistical significance between 40 and 150 second: p<0.05 vs control) and columns (B) represent the systolic blood pressure in rats chronically treated with L-arg (1g/kg p.o., once daily, for 10 days, n=10) in comparison to control (**p<0.001 vs control). Data are expressed as means ±SD.
The PT and APTT were not changed by L-Arg administration both in acute and chronic experiments (Table I). Intravenous administration of L-Arg caused a decrease in fibrinogen concentration from 266.6 ± 36.3 mg/dl (control group) to

![Figure 2: Thrombus weight in rats acutely treated with L-Arg (300 mg/kg bolus and 300 mg/kg/h i.v. for 2 hours, n=8) in comparison to control and chronically treated with L-Arg (1g/kg p.o. for 10 days, n=10) in comparison to control [***p<0.001 vs control]. Data are expressed as means ±SD.](image1)

![Figure 3: Bleeding time in rats chronically treated with L-Arg (1g/kg p.o. for 10 days, n=10) in comparison to control [***p<0.001 vs control]. Data are expressed as means ±SD.](image2)
Table I. Activated partial thromboplastin time (APTT), prothrombin time (PT), fibrinogen concentration and euglobulin clot lysis time (ECLT) in rats treated with L-arginine in acute (300mg/kg bolus and 300mg/kg/h i.v. for 2 hours, n=8) or chronic (1g/kg p.o., for 10 days, n=10) manner in comparison to control. Data are expressed as means±SD. *p<0.05, ***p<0.001 vs control

<table>
<thead>
<tr>
<th></th>
<th>PT(s)</th>
<th>APTT(s)</th>
<th>Fibrinogen concentration (mg/dl)</th>
<th>ECLT (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL ACUTE</td>
<td>34.17±2.76</td>
<td>18.51±0.46</td>
<td>266.6±36.3</td>
<td>319.75±52.22</td>
</tr>
<tr>
<td>i.v.</td>
<td>34.59±1.37</td>
<td>18.17±0.1</td>
<td>227.5±23.5*</td>
<td>297.5±35.35</td>
</tr>
<tr>
<td>L-ARG ACUTE</td>
<td>34.87±2.43</td>
<td>18.82±1.17</td>
<td>454.2±114.2</td>
<td>393.0±128.41</td>
</tr>
<tr>
<td>p.o.</td>
<td>27.07±3.53</td>
<td>18.43±0.3</td>
<td>267.0±27.4***</td>
<td>287.0±29.0*</td>
</tr>
<tr>
<td>L-ARG CHRONIC</td>
<td>25.87±2.43</td>
<td>18.82±1.17</td>
<td>454.2±114.2</td>
<td>393.0±128.41</td>
</tr>
<tr>
<td>p.o.</td>
<td>27.07±3.53</td>
<td>18.43±0.3</td>
<td>267.0±27.4***</td>
<td>287.0±29.0*</td>
</tr>
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</table>

227.5 ± 23.5 mg/dl (p<0.05). Oral administration of the drug led to a statistically significant decrease in this parameter from 454.2 ± 114.2 mg/dl (control rats) to 267.0 ± 27.4 mg/dl (p<0.001). The ECLT after intravenous and oral L-Arg administration was shortened, although the difference was statistically significant only in chronic experiment (p<0.05). In chronic experiment the BT was 102.3 ±

![Platelet Aggregation](image_url)

**Fig. 4.** Columns represent the collagen-induced platelet aggregation in rats chronically treated with L-arg (1g/kg p.o. for 10 days, n=10) in comparison to control (***p<0.001 vs control). Data are expressed as means ±SD.
5.8 sec in control animals and it was shortened to 85.6 ± 10.6 sec. in the L-Arg treated group (p<0.001) (Fig.3).

Collagen-induced platelets aggregation expressed as impedance measured between electrodes embedded in a whole blood sample was 6.72 ± 0.93 Ω in control group (Fig.4). In the L-Arg chronic treated group (p.o.) this value was significantly reduced to 4.56 ± 0.93 Ω (p<0.001).

The overall coagulation potential was presented as a sum of optical density increments in a 15 min period at 405 nm wavelength in relation to blind test, being respectively: 1040.1 (control) and 1006.8 (L-Arg chronic treated group). The overall haemostatic potential was analogically calculated and was 223.6 in control group and 143.8 in L-Arg group. The overall fibrinolytic potential in
control group was 78.3% and in L-Arg group - 85.5% of optical density (Fig.5). This change was statistically significant (p<0.05).

DISCUSSION

The aim of the present study was to evaluate the effect of L-Arg on the experimental venous thrombosis in rats. The study employed a simple and easy to perform model of venous thrombosis according to Reyers et al. (21). It is not only simple but also sensitive to study the action of haemostasis-affecting agents (19,26,27). The process of thrombus formation in this model is largely NO and PGI\textsubscript{2} dependent (19,26,27). In our study L-Arg administered per os for 10 days considerably suppressed the formation of venous thrombus in rats. Such characteristic changes were not observed when a single intravenous dose of L-Arg was used. The assessment of L-Arg involvement in haemostasis should take into consideration its effect on blood vessels, platelets, coagulation and fibrinolytic systems. Since L-Arg was used in doses possessing hypotensive effect, one might consider that its antithrombotic activity is a result of the reduction in blood pressure.

Indeed, our findings indicate that L-Arg administered i.v. in a single dose as well as chronically p.o. reduced arterial blood pressure. During intravenous infusion the pressure returned to the initial level already at 210 second and remained unchanged during two hours of observation. Thus, at the time of venous thrombosis induction (after two hours of L-Arg infusion) we did not observe any differences in BP between the control animals and the rats treated intravenously with L-Arg. Therefore, the contribution of a hypotensive effect in the antithrombotic effect of the tested amino acid should be excluded. This is in accordance with the findings of previous experiments where no correlation between hypotensive action of some drugs and antithrombotic effect was found (26,27).

Apart from vessels, blood platelets play a very important role in primary haemostasis. The literature provides well documented and defined antiaggregatory effect of L-Arg not only in whole blood but also in platelet-rich plasma. The aggregation has been induced by ADP (7,9,12,13,18), collagen (28) or thrombin (29). Our findings confirm that L-Arg suppresses platelet aggregation in whole blood. Other authors also demonstrated that this effect is associated with enhanced NO synthesis (15-17,30,31).

In the regulation of primary haemostasis, the interaction of blood vessel with platelets plays an essential role and its \textit{in vivo} evaluation involves bleeding time measurement. We found a significant shortening and not prolongation as expected of the bleeding time after 10-day L-Arg administration. A similar tendency was observed by Gruszecki et al. (32) during administration of propranolol. This may be associated with a considerable reduction in arterial blood pressure caused by
administration of drugs, being the result of a direct relaxatory effect on vascular smooth muscle cells.

Secondary haemostasis is another extremely important factor determining thrombus formation in vessels. Blood clotting can be inhibited by e.g. attenuation of plasma prothrombin activation system. Only few reports have appeared on the effect of L-Arg on the plasma clotting component. Stief et al. (18) exposed human plasma to the action of various concentrations of L-Arg in vitro, showing that the increasing amounts of this amino acid proportionally extend the prothrombin and activated partial thromboplastin time. Therefore, the influence of L-Arg on certain factors of the clotting system was examined. No significant differences were observed in the PT and APTT after i.v. and p.o. administration. However, fibrinogen concentration, especially in chronic administration of L-Arg was decreased, which was also confirmed by other authors (33).

The effect of L-Arg on the fibrinolytic system cannot be omitted here. We found that ECLT was considerably shortened, but only after 10-day L-Arg administration. We also noted the increased overall fibrinolytic potential. These results are in accordance with the data obtained by Gryglewski et al. (13) and Dambisya et al. (34).

Concluding, L-Arg administered chronically exerts an antithrombotic effect, which is associated with the suppression of platelet aggregation and increased fibrinolytic activity. This action seems to be indirect and occur via NO synthesis. Currently, L-Arg is used in the treatment of liver failure, where due to stimulation of the urea cycle it removes toxic ammonia from the organism. This amino acid is also used to prevent vascular disease proceeding particularly with endothelium impairment (10,11,35). The results of our study may contribute to the extension of L-Arg indication also to antithrombotic therapy or to the prophylaxis of thromboembolic disorders accompanying many systemic disorders in humans.

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