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Dose Dependency of Earthworm Powder on Antithrombotic and Fibrinolytic Effects

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The freeze-dried powder of *Lumbricus rubellus* earthworm was administered orally to rats and its fibrinolytic and antithrombotic effects were investigated. The fibrinolytic activity of plasma was determined by measuring the plasmin activity of the euglobulin fraction and was increased to two-folds of the control at a dose of 0.5 g/kg/day and five times with 1 g/kg/day after 4-day administration. The antithrombotic effect was studied in an arterio-venous shunt model of rats. The thrombus weight decreased significantly from 43.2 mg to 32.4 mg at a dose of 0.5 g/kg/day after 8-day treatment. The level of fibrinogen/fibrin degradation product (FDP) in serum was elevated in a dose-dependent manner during the treatment period. On the 8th day after administration, the FDP value was increased to 7.7 μg/ml compared with the control value of 3.3 μg/ml. These results support that earthworm powder is valuable for the prevention and/or treatment of thrombotic conditions.

Key words: *Lumbricus rubellus* earthworm powder, Antithrombotic activity, Fibrinolytic activity, Arterio-venous shunt model

INTRODUCTION

The dried earthworm has long been used in East Asia as a crude drug to treat hypertension and fever or to induce detoxification, sedation and bronchodilation (Kim and Xiao, 1995). One of other applications is the treatment of cerebral apoplexy related to cerebral thrombosis (Hong *et al.*, 1991). Recently, fibrinolytic enzymes were purified from *Lumbricus rubellus* (one of the species of earthworm) and their biochemical properties were characterized (Mihara *et al.*, 1983; Mihara *et al.*, 1991; Nakajima *et al.*, 1996). An intravenous injection of the purified enzyme was reported to show an antithrombotic effect on thrombin-induced thromboembolism in mice (Park *et al.*, 1991) and the strong evidences were presented that orally administered earthworm could exert thrombolytic effects *in vivo* (Mihara *et al.*, 1992). The anticoagulant and fibrinolytic potentials were also observed with the oral administration of earthworm powder in the previous reports (Chang *et al.*, 1995; Hahn *et al.*, 1997). The purpose of this paper is to evaluate the effects of earthworm powder on the experimentally induced thrombus formation *in vivo*. The weights of thrombus formed inside the arterio-venous shunt tube and the serum FDP level were measured.

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MATERIALS AND METHODS

Materials

The freeze-dried earthworm powder (EWP) was provided by Daedo Pharmaceutical Co. (Seoul, Korea). ADP and collagen were purchased from Chrono-Log (Haevertown, U.S.A). Thrombin, fibrinogen, urokinase and D-val-leu-lys-*p*-nitroanilide were products of Sigma (St Louis, U.S.A.). The reagents for the measurement of activated partial thromboplastin time were also from Sigma. Sprague-Dawley male rats weighing 200~220 g were bred at the Laboratory Animal Care Facilities of Natural Products Research Institute, Seoul National University.

Administration of EWP and blood collection

The suspension of EWP (0.1, 0.5 and 1 g/5 ml/kg) was administered to rats orally once every day for 4, 8 and 12 days. On the specified days, rats were anesthetized with ether and blood was drawn from heart using syringes containing 0.1 volume of 3.8% sodium citrate.

Preparation of euglobulin fraction

The euglobulin fraction was prepared by adding 4.5 ml of 3.8 mM acetic acid to 500 μl of citrated rat plasma. After standing for 1 hr at 4°C, the precipitate was collected by centrifugation. It was dissolved in 300 μl of 50 mM Tris-HCl (pH 7.5) and used for the determination of plasmin activity.

Determination of plasmin activity

The absorbance change was monitored at 405 nm using Jasco UV/VIS spectrophotometer (Model V550, Japan). In brief, each cuvette containing 500 μl of 5 mM D-Val-Leu-Lys-p-nitroanilide in 0.01 M Tris-HCl buffer (pH 7.4) and 50 μl of euglobulin fraction was incubated at 37°C and the absorbance was read at 10 sec intervals for 120 sec. The plasmin activity was defined as the initial velocity from the slope of 30 sec to 90 sec. One arbitrary unit was defined as the change of absorbance (0.01) in 1 min.

Thrombus formation in the arterio-venous shunt tube

One hour after the final administration of EWP, each rat was anesthetized with ketamine (250 mg/kg, *i.m.*) and an arterio-venous shunt tube was installed between an abdominal aorta and the renal vein. The method developed by Umetsu and Sanai (1978) was modified for the present study and the shunt tube was prepared from the polyethylene tubing of a scalp vein set. The tube (18 cm, containing 5 cm cotton thread) was filled with saline before installation. After the circulation of blood through the shunt tube for 20 min, both ends of the tubing were pinched, blood was collected from the heart for the determination of FDP level and the cotton thread was taken out from the shunt tube. The wet and the dried weights of thrombus formed on the cotton thread were determined by subtracting the wet and the dried weights of thread soaked with blood from the total wet and dried weights of the threads taken out from the shunt tube. Urokinase (500 U) was intravenously injected for the positive control.

Determination of fibrinogen/fibrin degradation product (FDP) level

Thrombo-Wellcotest kit (Murex Diagnostics, England) was used for FDP assay and the assay was performed semi-quantitatively. Each blood sample (2 ml) was mixed well with soybean trypsin inhibitor and Bothrops atrox venom in a sample collection tube and incubated at 37°C for 30 min. After centrifugation at 1200 g for 5 min twice, the supernatant serum was separated and stored in the freezer for at least 12 hours before FDP testing. The serum was diluted with glycine saline buffer (0.1 M glycine, 0.15 M NaCl, 0.1 % sodium azide, pH 8.2) with the up and down dilution method for the determination of FDP concentration semi-quantitatively. The diluted serum (50 μl) was mixed with a drop of latex suspension on a test slide and the slide was rocked gently to and fro for two min. The degree of macroscopic agglutination was judged using the results obtained from the positive and negative control sera and the FDP concentration was determined as the follows: with 1:0 dilution (-):0 μg/ml, (+):1 μg/ml, (++):2 μg/ml; with 1:1 dilution (+):3 μg/ml, (++):4 μg/ml; with 1:2 dilution (+):5 μg/ml, (++):6 μg/ml etc.

Statistical analysis

Data were summarized as mean±SD. For analysis of two groups the unpaired Student's t-test was performed. For analysis of three or more groups, one-way analysis of variance (ANOVA) was perfomed. Then, Newmann-Keul's multiple range test was used to determined which means were significantly different from the mean of the control. A value of $p<0.05$ was considered statistically different.

RESULTS

Effects on fibrinolytic activity

The results of plasmin activity of euglobulin prepared from the plasma are described in Fig. 1. The activities are represented as specific activities (U/mg protein). One unit is defined as the change (0.01) of ab-

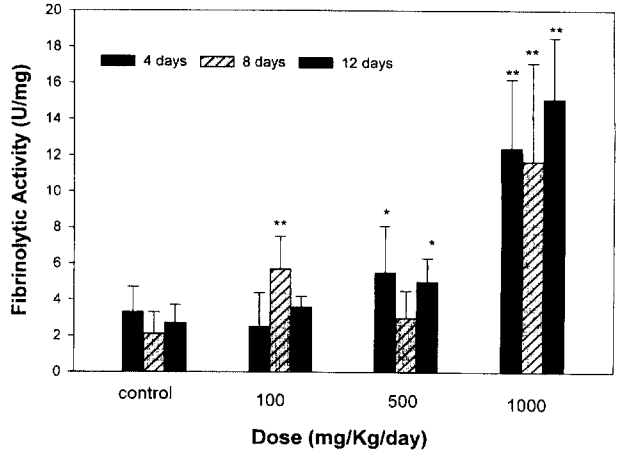

Fig. 1. Fibrinolytic activity of euglobulin fractions after the administration of earthworm powder. Activity was expressed as U/mg of protein. One unit was defined as the change (0.01) of absorbance at 405 nm. The values dependent on dose are significantly different at $p<0.001$ and the results according to treatment period are significantly different at $p<0.05$. -Each group consisted of 6 rats. -A significant difference between test and control group is indicated by *$p<0.05$ or **$p<0.01$.

sorbance at 405 nm. Total fibrinolytic activities of all the euglobulin fractions of the treated rats were increased compared to those of control. Fibrinolytic activities were increased to two-folds of the control at a dose of 0.5 g/kg and five times higher than the control value at a dose of 1 g/kg after 4-day administration. The specific activities were increased from 2.7± 1.0 U/mg of control value to 5.0±1.3 U/mg at the dose of 0.5 g/kg and to 15.1±3.4 U/mg at the dose of 1 g/kg after 12 day treatments. Statistical significances were found among the period of treatment at the 95% level and among the dose of treatment at 99.9% level.

Effects on the thrombus formation in the arterio-venous shunt model

The wet and dry weights of thrombus formed inside the shunt tube were measured (Table I). The weight of thrombus formed inside the A-V shunt tube was much influenced by the weather condition (atmospheric pressure, humidity etc.) of the day of experiment and the wet weights of the control groups varied from 38.4 ±2.6 mg to 44.8±3.5 mg. The oral administration of EWP caused a considerable reduction of thrombus in all the experiment groups. The thrombus weights were significantly decreased at both doses of 0.5 g/kg and 1 g/kg administration for 8 days. The wet weight of thrombus was decreased significantly from 43.2 mg to 32.4 mg at a dose of 0.5 g/kg after 8-day administration. The highest inhibition (31%) was observed when 1 g/kg of EWP was administered for 8 days and its wet weight was about 30 mg. The wet thrombus weight was significantly reduced to 28.3 mg with the positive control, i.e. the intravenous injection of urokinase (500 U), compared to that of control value of 44.8 mg. The dried weights behaved similarly.

Table I. Effects of the oral administration of earthworm powder on the thrombus formation inside the arterio-venous shunt tube

	Dose (kg/day)	Days	Number of animals	Thrombus weight (mg) wet	dry
Control		4	7	38.44±2.64	10.49±0.47
		8	8	43.18±5.67	11.81±1.42
		12	7	40.55±3.33	11.34±0.86
EWP	0.5 g	4	7	32.00±3.97**	9.04±1.01**
		8	7	32.40±5.16**	9.37±1.44**
		12	5	35.96±4.40*	10.77±1.36
EWP	1 g	4	6	32.59±6.49**	9.55±1.60**
		8	8	29.77±3.76**	8.87±1.12**
		12	7	36.98±3.45*	10.72±0.96
Control	saline, *i.v.*		6	44.83±3.47	12.11±0.53
Urokinase	500 U, *i.v.*		4	28.27±2.69**	8.55±0.90**

A significant difference between test and control group is indicated by *p<0.05 or **p<0.01.

Table II. Effects of the oral administration of earthworm powder on the serum FDP level in the arterio-venous shunt model

	Dose (kg/day)	Days	Number of animals	FDP (μg/ml)
Control		4	6	3.33±1.03
		8	5	3.40±1.95
		12	6	3.33±2.42
EWP	0.5 g	4	7	4.57±2.23*
		8	7	5.57±4.54
		12	7	5.71±2.43*
EWP	1 g	4	5	6.00±3.16*
		8	7	7.71±1.38**
		12	7	6.29±3.73*
Control	saline, i.v		6	3.33±1.96
Urokinase	500 U, i.v		4	10.55±5.65*

A significant difference between test and control group is indicated by *p<0.05 or **p<0.01.

FDP values in the serum of treated rats were increased in a dose-dependent manner e.g. with the 8-day treatments, the value was increased from 3.3 μg/ml of control to 5.6 μg/ml with 0.5 mg/kg and 7.7 μg/ml with 1 g/kg (Table II). However, the values did not show significant differences with the different periods (4, 8 or 12 days) of treatment. The FDP level of the control rats were consistantly 3.3~3.4 μg/ml, although the weights of the thrombus formed varied much with the weather conditions. The statistical significances were found between the control groups and the group given at the dose of 1 g/kg.

DISCUSSION

The EWP is composed of many components including proteases with fibrinolytic activity *in vitro*. However, it has not been fully demonstrated that *in vitro* fibrinolytic activity of some proteases is represented *in vivo*. The previous observations presented some antithrombotic potentials of EWP (Chang *et al.*, 1995; Hahn *et al.*, 1997).

With varying the dosages and the periods of the oral administration, in the present study, the effects of EWP on the plasma fibrinolytic activity and on the thrombus formation and on the serum FDP level in the experimental thrombosis model were investigated. The fibrinolytic activity was increased while the thrombus weight formed in rat thrombus model was decreased with enhancing the dose of earthworm powder. Such a finding was consistent with the serum FDP level in the A-V shunt model.

The fibrinolytic system primarily consists of the precursor enzyme plasminogen, which circulates in the plasma as glu-plasminogen (Becker, 1991). The critical conversion of plasminogen to plasmin is medi-

ated by two plasminogen activators (tPA) and urokinase. The natural inhibitors of fibrinolysis block either plasmin or the two activators of plasminogen. Plasmin is rapidly inactivated by interaction via its lysine site with its inhibitor, α_2-antiplasmin. Plasminogen activator inhibitors (PAI) inhibit the plasminogen activators. Of the inhibitors, PAI-1 in plasma is known to be the main inhibitor of t-PA and urokinase (Vassali *et al.* 1991). Its expression and production are under complex control and the plasma level of PAI-1 has much relationship with thrombosis formation (Sawdey and Loskutoff, 1991). Considering these facts, it can be postulated that some of the components of earthworm, e.g. proteases, may act on fibrin, and/or modulate the levels of α_2-antiplasmin or PAI-1. The absorption of proteins (e.g. fibrinolytic enzymes) administered orally remains a controversial question because of the difficulty in accepting the fact that molecules with high molecular weight and charge density can, in intact state, pass the gastric and intestinal mucosa. There are some similar reports showing an enhancement of fibrinolytic activity after the oral administration of urokinase or nattokinase (Sumi *et al.*, 1980; Sumi *et al.*, 1990). However, the possibilities cannot be ruled out for small molecular components of the enzymes to enter the blood stream and act as inducers of plasminogen activator bound to endothelium. Such kinds of work need further investigations in the near future.

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