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Evaluation of the *in vivo* Antithrombotic, Anticoagulant and Fibrinolytic Activities of *Lumbricus rubellus* Earthworm Powder

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A saline suspension of *Lumbricus rubellus* earthworm powder (EWP) was administered to rats (1 g/kg/day) orally for 15 days to evaluate an oral effectiveness for thrombotic disorders. Blood was drawn at 2-day interval after the administration. Several parameters for antithrombotic, anticoagulant and fibrinolytic activities were measured, including platelet aggregation, clotting time, plasmin activity and the levels of FDP (fibrin/fibrinogen degradation products), D-dimer, and t-PA antigen. It did not affect platelet aggregation induced by ADP and collagen but anticoagulant activity (aPTT and TT) was gradually increased to two-folds for the first 5 days of administration and back to normal. Fibrinolytic activity of euglobulin fraction was highest on the 11th day after the administration. The level of FDP was elevated to be comparable to the positve control (5-10 μg/ml) after 9-day treatment. Oral administration of the EWP could also reduce the formation of venous thrombus induced with viper venom. Complete blood count (CBC) profiles were within normal ranges except for a slight increase in white blood cells after the oral administration for 15 days. These results suggested that the EWP may be valuable for the prevention and/or treatment of thrombotic diseases.

Key words : *Lumbricus rubellus* earthworm powder, Antiplatelet activity, Anticoagualant Activity, Fibrinolytic activity, Oral administration

INTRODUCTION

During the past decade, many significant developments in the therapeutics of thrombotic and vascular disorders have been introduced (Prous, 1995). Recent applications of such drugs are ticlopidine, low molecular weight heparin, and tissue-plasminogen activator (t-PA). Nevertheless, many new drugs are currently being tested in various clinical trials for higher specificity and fewer side effects. Hirudin, hirulog, GP IIb/IIIa targeting antibodies, ancrod, and tissue factor pathway inhibitor (TFPI) are some examples. Many cases were developed from natural products, especially animal crude drugs. The earthworm has been used in East Asia as a crude drug for hypertension, antipyretic, detoxification, sedation and bronchodilation (Kim and Xiao, 1995). One of the other applications is the treatment of cerebral apoplexy related to cerebral thrombosis (Hong *et al.*, 1990). Recently, fibrinolytic enzymes of earthworm were purified and characterized biochemically (Mihara *et al*, 1991, Mihara *et al.* 1992, Nakajima *et al.* 1996). Intraveneous injection of the purified enzyme showed an antithrombotic effect on thromboembolism in mice (Park *et al.*, 1991). As the application of the earthworm is mostly in an oral manner, its effectiveness is not clear *in vivo*. Strong evidence was shown that a portion of the orally administered earthworm could be absorbed from the intestine into blood (Mihara *et al.*, 1992). To determine whether the crude drug of earthworm used for thrombotic disease is effective, we administered a saline suspension of the powder to rats orally. In addition, acute and venous thrombosis model were induced and the effect of a crude drug was evaluated. The goal of this study is to investigate antiplatelet, anticoagulant and fibrinolytic activities of the earthwarm powder (EWP) *in vivo*.

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MATERIALS and METHODS

Materials

The freeze-dried EWP was provided by Daedo Pharmaceutical Co. (Seoul, Korea). ADP and collagen were purchased from Chronolog (Haevertown, U.S.A). Thrombin, fibrinogen and D-val-leu-lys-*p*-nitroaniline were products of Sigma (St Louis. U.S.A.). The

reagents for the measurement of activated partial thromboplastin time (aPTT), prothrombin time (PT) and thrombin time (TT) were also from Sigma. The assay kit of FDP was obtained from Murex (England). TintElize D-dimer kit was from Biopool (Sweden). ELISA kit for the measurement of t-PA antigen were products of Chromogenix (Sweden). The rats (Sprague-Dawley) and ICR mice were breeded at the Animal Station of Natural Products Research Institute, Seoul National University.

Sample preparation and blood collection

EWP dissolved in saline (1 g/5 ml/kg) was administered to rats orally every day for 15 days, unless otherwise specified. On days 3, 5, 7, 11, 13 and 15 blood was drawn using syringes containing 3.8% citrate buffer via heart puncture.

Effects on platelets

Platelet rich plasma (PRP) was prepapred by centrifugation of the citrated blood at 200 g for 10 min and platelet poor plasma (PPP) were obtained from the residue by centrifugation at 900 g for 30 min. Platelet number was adjusted to 3×10^{11}/L by mixing PRP and PPP with the aid of platelet counter (PLT-1, Texas International Lab.). PRP (500 µl) was incubated at 37°C for 8 min and then 10 µl of 1 mM ADP or collagen (1 mg/ml) was added to induce platelet aggregation. The reduction in turbidity of PRP was observed and the degree of aggregation was monitored using a Chronolog aggregometer (Model 490-2D) interfaced with a personal computer.

Effects on collagen-induced intravascular platelet aggregation in rat

EWP (1 or 2 g/kg) was given orally to rats for two days. On 1 hr after the second EWP, rats were anesthetized with ketamine (250 mg/kg, i.m.). A canula was inserted into a carotid artery for blood collection before and 3 min after collagen injection (60 µg/kg, i.v.) (Arruzazabala, *et al.* 1993). Blood (900 µl) was collected with a syringe containing 100 µl of 2.2% sodium citrate (including 0.7 mg/ml indomethacin). Platelet of each blood sample was counted with the platelet counter mentioned above. Reduction in platelet counts was observed with the formation of platelet aggregates.

Effects on coagulation system and blood cells

Platelet poor plasma prepared above was used for the measurement of clotting time for aPTT, PT and TT. The clotting time tests were performed on a Beckton Dickenson BBL Fibrosystem (Cockeysville, USA) according to the specifications set by the manufacturer. Complete blood count (CBC) profiles were determined on a Coulter automatic blood cell counter (Model STKS)

Preparation of euglobulin fraction

Euglobulin fraction was prepared by adding 4.5 ml of acetic acid (3.8 mM) to 500 µl of rat plasma. The precipitate was left for 1 hr at 4°C and collected by centrifugation. It was dissolved in 300 µl of 50 mM Tris-HCl (pH 7.5).

Fibrin plate assay

The mixture of 10 ml of 0.7% (w/v) of bovine fibrinogen solution and 5 NIH units of human thrombin was poured to petri dishes and left for 30 min at room temperature. Plasma or euglobulin fractions were dropped onto the plate and incubated at 37°C overnight. Fibrinolytic activity was assessed by measuring the lysis zone (Astrup and Mullerrtz, 1952).

Determination of plasmin activity and t-PA antigen levels

Absorbance change was monitored at 405 nm using Jasco UV/VIS spectrophotometer (Model V550, Japan). In brief, the 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 fractions were incubated at 37°C and the absorbance was read over 10 sec intervals for 80 min. Initial velocity was calculated from the slope from 30 sec to 90 sec. The t-PA antigen levels in rat plasma were measured employing an ELISA kit with human t-PA as standard.

Determination of FDP levels

Thrombo-Wellcotest kit was used for FDP assay. The assay was perfomed according to manufacturer's procedure. Latex suspension was mixed with the serum diluted with saline in 1:5 and 1:20. The agglutinations were viewed and compared with the postive (5-10 µg of FDP/ml) or negative control (less than 2 µg of FDP/ml) serum.

Quantitative determination of D-dimer

Biopool TintElize kit was used for the determination of D-dimer. Plasma sample or standard containing D-dimer was added to a microtest well which was coated with a monoclonal antibody against D-dimer. After an incubation for 30 min on a microtest plate shaker at 600 rpm, HRP labelled Fab fragments of anti-D-dimer IgG were added. The wells were emptied and washed to remove unbound conjugate after which peroxidase substrate (*o*-phenylenediamine/H_2O_2) was added. The

yellow color developed was measured at 490 nm.

Effect on venous thrombosis model

The animal model of venous thrombosis was designed by a modification of the previous reports (Fareed *et al.* 1985; Hladovec, 1984). Rats were divided into four groups (eight rats per each group). EWP and saline were administered to rats for 3 days. Heparin was administered into the femoral vein intravenously 5 min before the injection of 0.01 U of Russel's viper venom (RVV), a thrombogenic agent, for the first positve control. Urokinase was injected 10 min after venom injection for the second positive control. Then, ketamine was administered intramuscularly. After 30 min they were operated under ether anesthesia and the abdominal cavity was opened. One minute later the inferior *vena cava* was isolated and a tight ligature was applied with a cotton thread below the left renal vein. The abdominal cavity was closed provisionally, reopened 10 min later and the *vena cava* was ligated 2 cm below the first ligature. After ligating the remaining 2-3 bigger branches leading to the vascular segment, the latter was removed, washed in a saline and left for 24 hrs at 4°C. The dissected thrombi were weighed.

Effects on acute thrombosis induced by a combination of collagen and epinephrine in mice

EWP (1 g/kg or 2 g/kg) or aspirin (50 mg/kg) as positive control was administered to mice orally for two days. On the second day the mixture of collagen (700-750 μg/kg) and epinephrine (70-75 μg/kg) was injected to the tail vein of mice to induce acute thrombosis 1 hr after the administration of samples. Then, each mouse was carefully watched for 15 min to determine whether the mouse was paralyzed, dead, or recovered due to thrombotic challenge (Diminno *et al.*, 1983).

Statistical analysis

Data were summarized as mean±SD. To analyze the data statistically, we performed a one-way analysis of variance (ANOVA) for repeated measurements of the same variable. We then used Duncan's multiple range t test to determine which means were significantly different from the mean of the control. We considered difference significant at $p<0.05$.

RESULTS

Effects on platelets

The oral administration of EWP gave essentially no effects on the platelet counts in whole blood or PRP. The platelet aggregabilities of PRP in response to ADP or collagen were also not affected regardless of days of administration (Fig. 1). Moreover, no significant reduction in intravascular platelet aggregation induced by collagen was observed in EWP treated groups of rats in comparison with the control rats (Table I).

Effects on blood coagulation

When suspension of EWP (1 g/kg) was administered

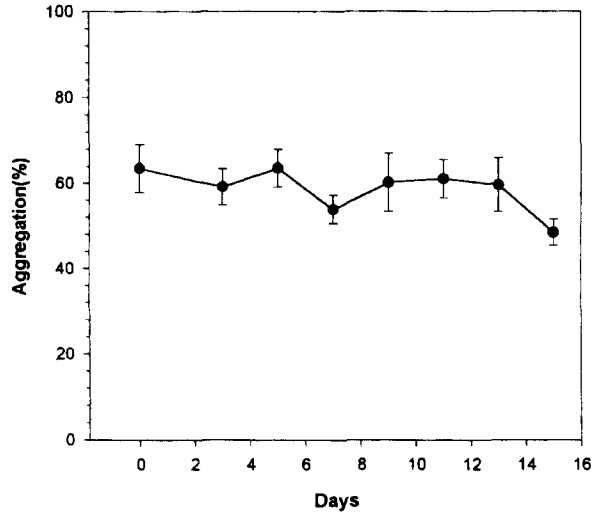

Fig. 1. Platelet aggregability of PRP in response to ADP after oral administration of EWP. EWP dissolved in saline (1 g/5ml/kg) was administered to rats orally every day for 15 days. Bood was drawn and platelet rich plasma (PRP) was prepapred as described in Materials and Methods. PRP was incubated at 37°C for 8 min and then 10 μl of 1 mM ADP was added to induce platelet aggregation. The reduction in turbidity of PRP was observed and the degree of aggregation was monitored.

Table I. Effects of EWP on intravascular platelet aggregation

Treatment	No of animals	Platelets count		B/A (%) (mean±SD)
		before (A) (mean±SD)	after (B) (mean±SD)	
Control	4	563.7±102.9	480.0±93.6	(85.3± 6.0)
EP (1 g/kg)	4	678.3± 64.4	604.0± 93.6	(88.8± 8.7)**
EP (2 g/kg)	4	728.8± 92.6	651.0±54.6	(89.7± 3.9)**

*Intravascular platelet aggregation was caused by collagen (60 μg/kg, *i.v.*) and platelets were counted from blood samples collected before and 3 min after the injection of collagen

**Values are not significantly different at $p<0.05$

orally for 15 days, the clotting times of aPTT and TT were increased from 39 to 59 sec and 44 to 65 sec, respectively, on the 5th days of administration (Fig. 2). Afterwards clotting times of both cases gradually decreased to normal. In contrast, the oral administration of EWP gave no influence on PT at all.

Effects on fibrinolysis

Euglobulin was prepared from rat plasma by acid treatment. The result of plasmin activity and fibrin lysis zone of euglobulin prepared from the plasma collected each day are described in Table II. Total fibrinolytic activities of all euglobulin fractions were increased compared to control. The highest fibrinolytic activity was shown on 13-day treatment. The specific activity of plasmin in the euglobulin was within the range of 0.23 to 0.55 U/mg, while the control value was 0.17 U/mg (Table II). The initial velocity of each fraction was also higher than that of control value (data not shown). The agglutinations of FDP were clearly observed in the sera collected on 11 and 13-day treatment (Table III). Especially the serum from 5 animals on 11-day treatment showed an agglutination of which the level was between 2 and 10 μg/ml. Weak agglutinations were also seen in the sera collected at 9 and 13 days after an oral administration. In contrast, D-dimer values were not increased according to the days (Fig. 3).

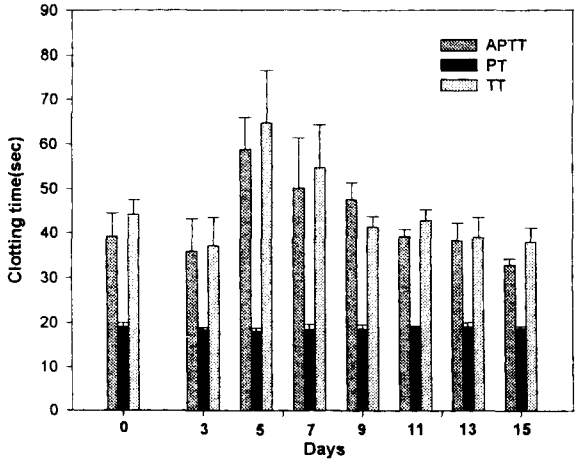

Fig. 2. Effects on blood coagulation parameters after the oral administration of EWP to rats. After the oral administration of EWP to rats (1 g/kg) blood was drawn and coagulation tests (aPTT, PT and TT) were perfomed according to manufacturer's procedures. Results were espressed as mean±SD (n=5).

Table II. Lysis area of fibrin plate and plasmin activity of euglobulin fraction

Days	Area (mm^2)	Specific Activity (U/mg)
Control (0)	0	0.19
3	7	0.52
5	6	0.23
7	8	0.55
9	7	0.55
11	28	0.29
13	30	0.31
15	10	0.42

Effects on blood cells

The white blood cell count was slightly increased in all rats after the administration of EWP at a dose of 1 g/kg. The other cellular parameters such as MCV and RBC and the other respective hemogram indices did not significantly change as shown in Fig. 4.

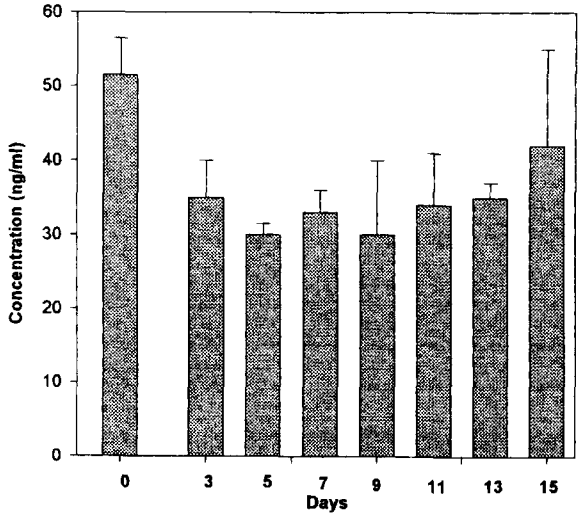

Fig. 3. Plasma levels of D-dimer after the adminstration of EWP. After the oral administration to rats, blood was drawn and the level of D-dimer in plasma was measured as described in Materials and Methods. Results were expressed as mean±SD (n=5).

Table III. Serum levels of FDP after the administration of EWP to rats

Rats\Days	0	3	5	7	9	11	13	15
1	---	---	---	---	---	++	+++	---
2	---	---	---	---	++	++++	++	---
3	---	---	---	---	---	++	+	---
4	---	---	---	---	---	+	---	---
5	---	---	---	---	+	+	---	---

Postive control (μg/ml): ++++ (8-10), +++ (6-8), ++ (4-6), + (2-4) Negative control (μg/ml): --- (0-2)

Effect on venous thrombosis models

The effect of earthworm powder on the inhibitory effect of thrombus formation of vein was tested. Heparin and urokinase were administered at the same time. As shown in Table IV, the groups taking EWP, heparin and urokinase demonstrated a decrease of thrombus weight compared to the control group. (from 0.0121 g to 0.0102, 0.0080, and 0.0064 g). Although the earthworm powder was administered orally, thrombus weight was decreased to 16%. But the statistical analysis using ANOVA indicated it was not significant at the level of $p<0.05$.

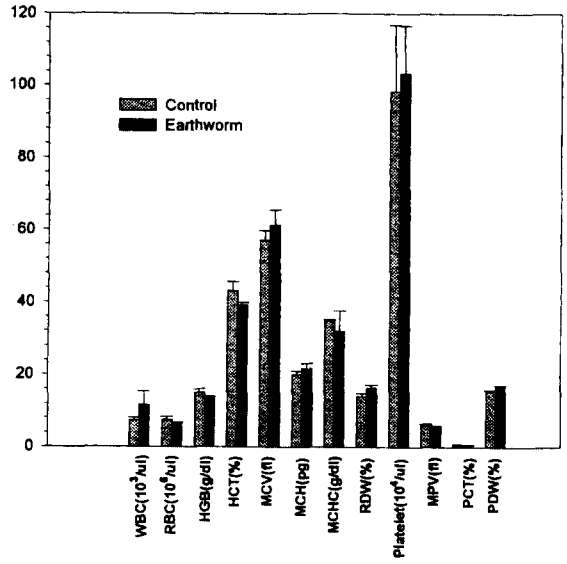

Fig. 4. Effects of EWP and saline solution on the CBC profile of blood samples obtained from rats administered 1 g/kg EWP. Results were expressed as mean±SD (n=3). WBC=white blood cells, RBC=red blood cells, HGB=hemoglobin, HCT=hematocrit, MCV=mean cell volume, MCH=mean cell hemoglobin, MCHC=mean cell hemoglobin concentration, RDW=red cell distribution width, MPV=mean platelet volume, Platelet=number of platelet , PDW=platelet distribution width, PCT=ratio of platelet volume to total volume of blood.

Table IV. Effect of earthworm powder on venous thrombosis

Treatment	Dose	No of animals	Thrombus weight
Saline	2 ml/kg (*p.o.*)	8	0.0121±0.007
Earthworm powder	200 mg/kg (*p.o.*)	8	0.0102±0.004*
Heparin	1 mg/kg (*i.v.*)	8	0.0080±0.001**
Urokinase	2000 U/kg (*i.v.*)	8	0.0064±0.007**

*Not statistically significant ($p<0.05$)
**Statistically significant ($p<0.05$)

Effects on acute thrombotic challenge induced by collagen and epinephrine in mice

An injection of the mixture of collagen and epinephrine in mice induces massive pulmonary thrombosis causing acute paralysis leading to a sudden death. The mortalities in EWP (1 g or 2 g/kg) treated groups were slightly lower than those in the control group, but the effects were far less than aspirin (50 mg/kg) treatment (Table V). And the survival rates of EWP group were as low as the control group of mice.

DISCUSSION

In spite of several reports on the application of *Lumbricus rubellus* earthworm powder to thrombotic disorders, there have been few scientific approaches to figure out the mechanism as well as to characterize the active components. Recently several fibrinolytic enzymes have been isolated and characterized. Since the EWP is usually administerd in an oral route, it should be clarified whether enzymes are absorbed into the intestinal tract to diplay fibrinolytic activities

According to a recent report it contained two kinds of substances inhibitory of platelet aggregation induced by collagen and ADP (Mihara *et al.*, 1992). Our studies indicate the earthworm did not affect platelet aggregation in responce to ADP or collagen *in vitro* and *in vivo*. Instead it may contain anticoagulant substances acting on the intrinsic pathway, because it does not affect prothrombin time. It is not clear why the clotting time after 5 days of administraion dropped gradually.

The most remarkable thing of the earthworm is to show the fibirinolytic activity after oral administration. Earlier *in vitro* experiments could show that EWP strongly increased the fibrinolytic activity (Mihara *et al.*, 1991). *In vivo* there was a increased lysis time when the fibrin plate method was applied (Mihara *et al.*, 1992). The data in Table 2 strongly suggest EWP can enhance the fibrinolytic activity in the blood. We can speculate the release of t-PA on EWP or the ab-

Table V. Effect of the earthworm powder on acute thrombosis induced by collagen and epinephrine

Agent	Dose	Number of mice	Death[a] No (%)	Paralysis[b] No (%)	Survival[c] No (%)
Control		27	20 (74.1)	3 (11.1)	4 (14.8)
Aspirin	50 mg	28	15 (53.6)	3 (10.7)	10 (35.8)
Earthworm	1 g	30	20 (66.7)	6 (20.0)	4 (13.3)
Earthworm	2 g	31	21 (67.7)	4 (12.9)	6 (19.4)

*A mixture of collagen (700 μg/kg) and epinephrine (70 μg/kg) was injected.
[a]dead within 15 min, [b]paralysis persisted more than 15 min, [c]recovered from paralysis within 15 min

sorption of fibrinolytic enzymes of the earthworm into the blood and inhibit PAI-I (plasminogen activator inhibitor) activity. We have measured t-PA antigen level in the blood, but could not find the increase of t-PA. According to two human studies euglobulin fibrinolytic activity and t-PA antigen level showed a positive relationship after the administration of EWP (Hong *et al.* 1990, Mihara *et al.* 1991). The failure of our result may be due to the use of human antibody of t-PA in the kit. Another explanation for this fibirinolytic effect of EWP may be a direct action on fibrinogen or fibrin in plosma. There has been no evidence that EWP administered orally can pass the cell membrane without any modification of the molecule. In the present work, the agglutination of FDP was positive in the sera of treated groups but D-dimer values in plasma were not increased. It appears that the increase of FDP in the serum may have been caused by lysis by plasminogen activator and consumption of thrombi in the vascular system. The insensitivity for the detection of D-dimer may be due to the cross-reactivity of antibody depending sources or the pathological conditions may not be enough for yielding D-dimer (Sato *et al.*, 1995).

The effects of EWP were investigated in two animal models of thrombosis. In the rat model of venous thrombosis, viper venom induced the formation of thrombotic mass presumably by activating blood coagulation systems and the size of the thrombus formed in EWP-treated group of rats were smaller than those of control rats. However, EWP showed only very mild antagonistic effects against acute thrombotic challenge in mice which mostly involves platelet aggregation. The above results suggest that EWP (oral administration) has anticoagulant and fibrinolytic potentials. It is likely that the action of the EWP is mainly due to the mixture of proteases inside.

The absorption of proteins (fibrinolytic enzyme *in vitro*) administered orally remains a controversial question due to difficulty in accepting that molecules with high molecular weight and charge density can pass the gastric and intestinal mucosa. There are some similar reports to enhance fibrinolytic activity in the oral administration of urokinase or nattokinase (Sasaki *et al*, 1985; Sumi *et al*, 1990). It should be considered in balancing the level of t-PA and PAI-I. Otherwise there may be any low molecular weight substance to induce a fibrinolytic activity in blood. It should be studied in detail in the near future.

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