10.1053.paor.2001.0306 available online at http://www.idealibrary.com on IDEAL

ARTICLE

# Effect of Earthworm (G-90) Extract on Formation and Lysis of Clots Originated from Venous Blood of Dogs with Cardiopathies and with Malignant Tumors*

Maja POPOVIÆ,[1] Terezija Mihaela HRŽENJAK,[1] Tomislav BABIÆ,[2] Josip KOS,[2] Mira GRDIŠA[3]

[1]Department of Biology, Faculty of Veterinary Medicine; [2]Department of Surgery, Faculty of Veterinary Medicine; [3]Division of Molecular Medicine, Ruder Boškoviæ Institute, Zagreb, Croatia

**The stability of homeostasis is important to keep a balance between coagulation and fibrinolysis. A disorder of homeostasis leads to different physiological changes and causes different diseases such as cardiopathies and malignant tumors. Cardiopathies is characterized by a hypercoagulation. In the malignant tumors, besides the hypercoagulation due to plasminogen activators (PA) formed inside the tumor, a disorder of homeostasis leads also to acceleration of the fibrinolysis. The variety of internal and external factors in both cases determine the deviation of time for the clots formation, as well as the lyses of blood and fibrin clots. In this study the venous blood as well as the blood and the fibrin clots, derived from healthy dogs, the dogs with cardiopathies and with malignant tumors, were examined for the time of coagulation and fibrinolysis by adding different substances. In these experiments we used a glycolipoprotein extract from earthworm tissue homogenate (G-90) and the proteolytic enzymes P I and P II, isolated from G-90. The efficacy of the tested substances was comparable with the clinically administered anticoagulants. The most significant differences in clotting time among the three tested groups of dogs were obtained by application of the original G-90. The results suggest a possibility that G-90, along with the fibrinolytic enzymes and other biologically active factors, also contains a factor that decelerates the formation of clot in a specific medium, such as the blood from the dogs with malignant tumors.** (Pathology Oncology Research Vol 7, No 3, 197–202, 2001)

*Keywords:* homeostasis, cardiopathy, malignant tumors, earthworm extract (G-90)

## *Introduction*

Homeostasis, with the physiologically normal parameters, is dependent on interactions of the activators of coagulation and their inhibitors on the one side, and the fibrinolytic activators and their inhibitors on other side. In these interactions the increase of fibrin formation, formed by activated thrombin, is directly proportional to the increase in disintegration of the fibrin network as a result of the activation of plasmin. The initial moment of, either external or internal pathway of the coagulation mechanisms, is the release of tissue factor, named thromboplastin (TF). It activates the thrombocytes to excrete the integrin receptors (IIb/IIIa) for fibrinogen. The TF-thrombocyte-fibrinogen link, assisted by $Ca^{2+}$ ions, triggers a cascade pathway through the activation of factors $X \rightarrow Xa$ and thrombin, which is a precondition for starting of the coagulation. A Xa factor is also the site of TFPI (tissue factor pathway inhibitor) activity. The stability of homeostasis depends also on the antithrombin inhibitor. An increase of the activity of this mechanism causes a shift in the direction of hypercoagulation, what represents the beginning of trombotic state. The fibrin plaques are formed and they are bounded to the endothelium of blood vessels, the thrombocytes and a fibrinogen. These pathologic layers cause the myocardial and cerebral infarctions, the thrombosis of deep venous, etc. The malignant formations and their products, which stimulate a secretion of TF and the activation of intrinsic pathway, may also initiate the hypercoagulation. However, many malignant tumors synthesize and secrete the urokinase-type plasminogen activators (uPA), whose activity is opposite to

*Received:* Jan 29, 2001; *revised:* Jul 12, 2001; *accepted:* August 21, 2001

*Correspondence:* Dr. sc. Maja POPOVIÆ, Department of Biology, Faculty of Veterinary Medicine, Heinzelova 55, 10000 Zagreb, Croatia; Tel.: +385-1-239-0143, fax.: +385-1-244-1390; E-mail: mpopovic@vef.hr

* Supported by the Ministry of Science of Croatia

 1219-4956/01/030197+06 $ 12.00/0

the primarily induced hypercoagulation.[9,13] Tumor uPA, besides the lysis of the fibrin filaments and the pathological fibrin plaques, also lyses the proteins of extracellular matrix. Through the action of tumor uPA, insulinase and collagenase, the tumor cells could be included into circulation, and produce the metastases.[2,13] If the etiology of hypercoagulation is not caused by malignant tumors, fibrinolysis is insufficient. Clinical prevention of hypercoagulation, in these cases, is based on blocking of separate phases of the process itself. The prophylaxis can be therapy with heparin, hirudin or coumarin (warfarin), etc. In the therapy of acute pathological conditions fibrinolytics are used, which penetrate into the clots and lyse them. The enzymes, such as β streptokinase and urokinase are very often used for these purposes. After fibrinolysis, the therapy is continued with the anticoagulants.[1] However, the hypercoagulation in the patients with malignancies is accompanied by accelerated fibrinolysis, and the activity of inhibitors. The states of such disturbed stability have not been sufficiently explored in terms of cause and effect. Because of this, they are hardly accessible to prophylaxis and therapy.[3,10]

Concentrations and activities of the fibrinolytic enzymes can be monitored in the processes of coagulation and fibrinolysis. Their activity has been the subject of numerous studies. Our study describes the potential of a glycolipoprotein mixture (G-90) as a modulator in the processes of coagulation and fibrinolysis. It was obtained from the tissue homogenate of the earthworm Eisenia foetida. G-90 possesses numerous biological activities. It is neither mutagenic nor cancerogenic.[6] Recent studies showed that it is not toxic, not allergenic, and it has antibacterial activity. G-90 mixture contains the growth factors of the insulin superfamily, adhesins of immunoglobulin superfamily, and proteolytic enzymes of the trypsin family.[6,7,8,12] The latter is confirmed by the fact that the fibrinolytic activity of G-90 *in vitro* is specific, considering the site of the primary malignant process of the serum donor, whereas uPA detected in G-90 has no such specificity.[9]

We have evaluated the fibrinolytic activity of G-90 in relation to the β streptokinase activity, and its possible *in vitro* action on the permeability of erythrocyte membranes. We have also examined whether or not there is a difference in the action of G-90 on: the clots from whole venous blood and the fibrin clots from venous blood of clinically healthy dogs, dogs with malignant tumors and the dogs with cardiopathies.

### *Materials and Methods*

Glycolipoprotein mixture (G-90) was obtained from the tissue homogenate of the earthworm Eisenia foetida (Annelida, Oligochaeta, Lumbricidae) (Patent: "Glycolipoprotein mixture from tissue homogenates of the Lumbricidae family earthworm – Procedure for its obtaining and its application in medicine" (P920481A), Republic of Croatia, State Patent Office 1992).[6]

The proteolytic enzymes P I (32 kDa) and P II (23 kDa) were isolated from G-90.[8]

Venous blood was taken from dogs (n = 60): a) clinically healthy dogs (n = 20); b) dogs with malignant tumors (mammary carcinoma) (n = 20); c) dogs with cardiopathies (dilative cardiopathies) (n = 20). Both disorders were diagnosed at the Clinics for Internal Diseases of the Faculty of Veterinary Medicine, Zagreb, Croatia, using a clinical examination such as ECG, ultrasound and histological pathoanalysis. The dogs examined in our Clinic very often suffer from mammary carcinoma and dilative cardiopathies, and that was reason that we examined the blood samples from these groups (without distinguishing the type breed, sex and age of dogs). The blood samples were taken simultaneously with the blood for routine tests. All procedures were performed according to the European guidance on keeping and handling laboratory animals (86/609/EEC).[14]

Fibrin clots were obtained from whole venous blood of examined dogs. Before use in the tests, the fibrin clots were stored at –20°C.

Anticoagulative activity was checked by clotting test: to 1 ml of freshly withdrawn venous blood 10 μl of tested substances were added (G-90 or P I or P II or P I+ PII) in the concentrations from 1 pg/ml to 100 μg/ml, dissolved in physiological solution. A mixture P I + P II was prepared with equal amount of each component (1:1). The time of blood clotting was monitored at room temperature.

Fibrinolytic activity was checked by test of lysis of whole blood clots: a clot derived from 2 ml of venous blood was kept at room temperature for 15 minutes. After that, 1 ml of physiological solution with G-90 or P I or P II or P I+P II in the concentrations of 10 μg/ml or 10 ng/ml or 10 pg/ml, was added to the clots. To the control clot 1 ml of the physiological solution was added. Clot lysis time was monitored at room temperature.

A lysis of fibrin clot was performed by euglobulinic test: 1 ml of tested substances (G-90 or P I or P II or P I+P II), in the concentrations of 10 pg/ml – 100 μg/ml, was added to the fibrin clot derived from 1 ml of plasma. A clot lysis time was monitored at room temperature and at 37°C.

The hemolytic activities of tested substances: 2 ml of freshly drawn venous blood were left to clot at room temperature. A 1 ml of each tested substances (G-90 or P I or P II or P I+P II), in the concentrations of 10 μg/ml or 10 ng/ml or 10 pg/ml, were added to the clots. Then the samples were centrifuged for 10 minutes at 1500 rpm. The clear supernatants were checked for the presence of hemoglobin by spectrophotometer at 546 nm. A negative control was provided by the physiological solution, whereas a positive control was provided by hemolyzed blood.

The evaluation of fibrinolytic activity of the testing substances (G-90 or P I or P II or P I+P II) in relation to β

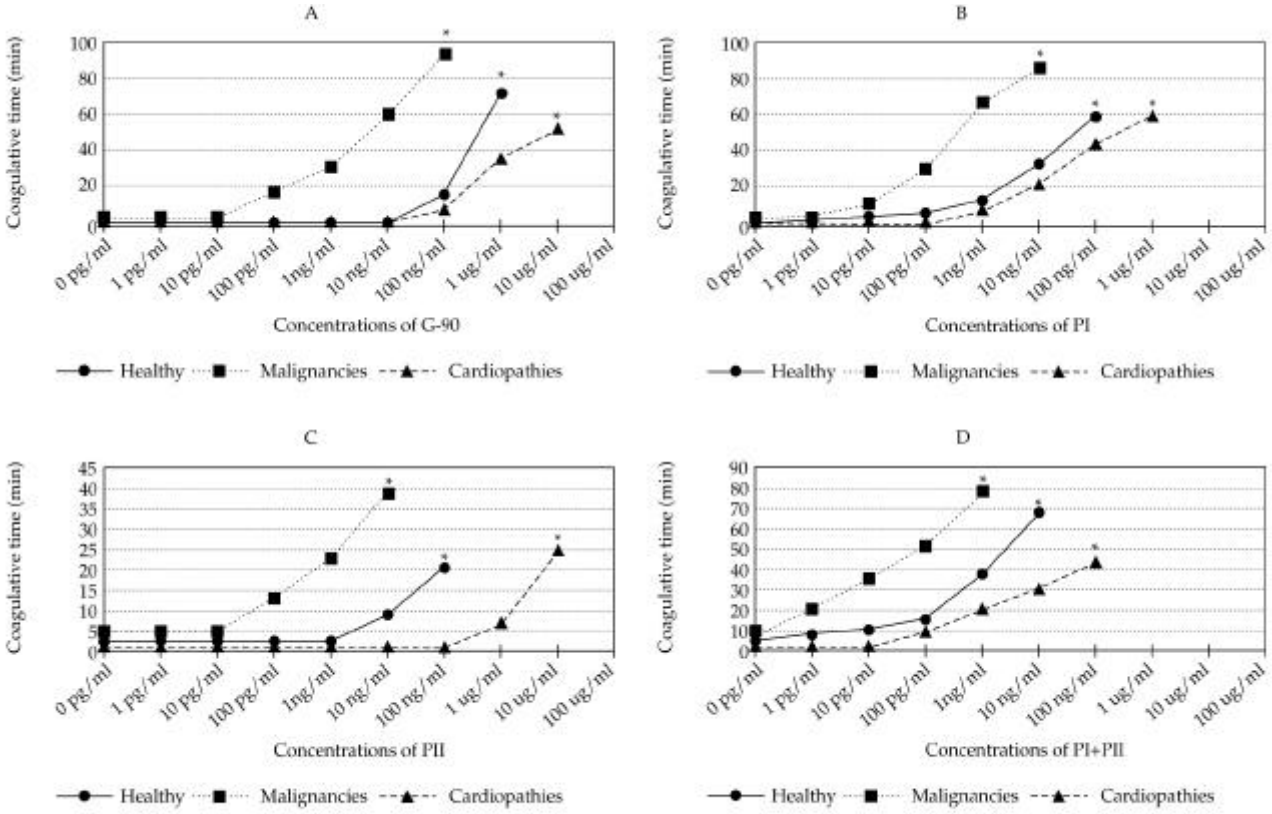


***Figure 1.*** *A time of coagulation (min) of the dog's venous blood in depending on the concentration of added substances.*
** – Stop of coagulation*

streptokinase activity: the clots were obtained from 2 ml of fresh venous blood. β streptokinase (Hoechst, Austria AG, Vienna) with 150,000 IE/ml was used to prepare a standard curve. Each point was made in duplicate, and the lysis time of the clots from healthy dogs was determined. To assess the number of EI of tested substances, the clots from healthy dogs and the dogs with malignancies and cardiopathies, were lysed by adding of G-90 or P I or P II or P I + P II in the concentrations of 10 μg/ml or 10 ng/ml or 10 pg/ml. Their lysis times were compared with the lysis time of β strptokinase in the standard curve, which served for determination of the number of EI of tested substances.

The results are presented as a mean values ± SD, using Mann-Whitney test and t-test (software ANOVA).

### *Results*

The anticoagulative activities of the tested substances, examined on blood samples from the dogs with malignant tumors, cardiopathies and healthy dogs are shown in *Figures 1a-d*. The results represent the mean values from the 20 examined blood samples in each group. Clotting time was expressed in min. A physiological clotting time for the blood from the healthy dogs was 3 min, and this was used as a control value. The blood from the dogs with cardiopathies showed an accelerated clotting (1.5 min), whereas a blood taken from the dogs with malignant tumors showed a slower clotting time (6 min). Addition of the examined substances (G-90, P I, P II, P I + P II) disrupted this ratio depending on their concentrations, and delayed clotting time in all examined groups.

To prevent coagulation of the samples from healthy dogs, the concentrations of P I and P II as well as their combination (P I + P II) was 1 μg/ml (*Figures 1b-d*), but for G-90 it was 10x more (10 μg/ml) (*Figure 1a*). The blood samples from the dogs with cardiopathies were more sensitive in the presence of combination P I + P II. At a concentration of 1 μg/ml there was no coagulation (*Figure 1d*). P I alone prevented coagulation at concentration of 10 μg/ml (*Figure 1b*), but P II and G-90 exerted the same effect at 10 x higher concentration (1 μg/ml) (*Figures 1a,c*). In the blood from dogs with malignant tumors P I (10 ng/ml) decelerated clotting to 87 min (*Figure 1b*) and G-90 (10 ng/ml) to 60 min (*Figure 1a*). At a higher concentration (100 ng/ml), P I did not exerted any anticoagulative activity (*Figure 1b*), but G-90 delayed a clotting time to 95 min (*Figure 1a*). P II was less effective than P I (*Figure 1c*). But, the combination of P I and P II was more effective than each enzyme alone (*Figure 1d*). The mixture of P I and P II at a concentration of 1 ng/ml, delayed a clotting time of blood from dogs with malignant tumors up to 78

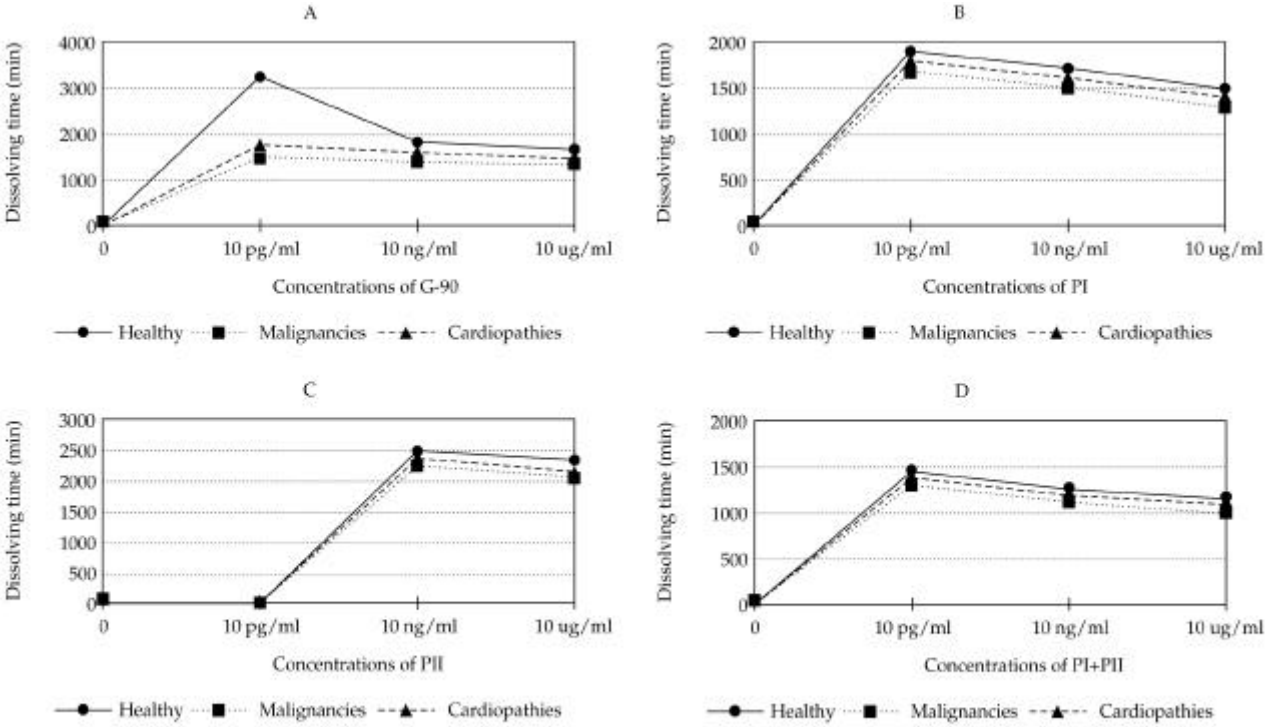


***Figure 2.*** *A dissolving time (in min) of the clots from the dog's venous blood in depending on the concentration of added substances.*

minutes. On the other hand, the combination of P I + P II at concentration of 10 ng/ml prevented clotting of blood from dogs with malignant tumors. Although, when P I and P II were added separately, their effects were weaker on the same sample (*Fig 1b,c*). For prevention of the coagulation in the same extent we needed 10x more (100 ng/ml) of P I or P II (*Figures 1b,c*). The effect of G-90 was even weaker than that of P I or P II, and was added at a concentration of 1 μg/ml (100x more than P I + P II) (*Figure 1a*).

To compare the significance of differences between the clotting times among the substances applied and blood samples, statistical analyses were performed. The above results show that all examined substances influence the time of clotting, depending on the source of blood sample and the concentration of substance. In comparison with clotting time of blood samples from healthy dogs, all substances delayed a coagulation of blood samples from the dogs with malignant tumors, and shortened it from the dogs with cardiopathies (*Figure 1a-d*).

Dissolving blood clots: The fibrinolytic activities of G-90, P I, P II and P I + P II were examined by measuring a dissolving time of blood clots. The blood clots of all examined blood samples of dogs (20 in each groups) were treated with of G-90 or P I or P II or P I + P II, at concentrations of 10 μg/ml, 10 ng/ml and 10 pg/ml. The efficacy of applied substances was expressed in the min. The dissolving of clots was accelerated proportionally to the concentration of tested substances (*Figure 2a-d*). At a concentration of 10 pg/ml, P II did not show any effect (*Figure 2c*), but the other substances examined dissolved the clots (*Figure 2a,b,d*). The best effect of all substances was achieved at a concentration of 10 μg/ml. The blood clots from the dogs with malignant tumors were more sensitive then the other clots. The efficacy of substances was as follows: (P I + P II) > P I > G-90 > P II (*Figure 2a-d*). At concentration of 10 pg/ml, P II did not show any fibrinolytic activity (*Fig 2 C*). In combination with P I, the fibrinolytic activity was even higher than that with G-90 or P I alone (*Figure 2a,b,d*). The control clots (with adding of physiological solution) were not dissolved during the experiments.

The lysis of fibrin clot (euglobulinic test)**:** The results of euglobulinic test were shown in *Figure 3*. The activity of examined substances was determined as a time for lysis of the fibrin clots. The results show that all tested substances, except P II are still active in the concentration of 1 pg/ml. At concentrations higher than 10 ng/ml, P II suppressed the reaction, which was reflected as the prolongation of a time required for lyses, above the physiological lysing time. If the tests were carried at 37°C, the activities of all substances increased for 12%. The fibrin clots lysis also depended on the origin of blood samples. The clot from the dogs with malignant tumors lysed faster (220 min) than the clots from healthy dogs (248 min) and dogs with cardiopathies (279 min).

Hemolytic activity: To examine if G-90 or P I or P II or P I + P II lysed the erythrocytes, a hemolytic test was perform (results not shown). The activity was expressed as a percentage (%) of released hemoglobin from the erythrocytes.

All tested substances in concentrations from 10 pg/ml to 10 ng/ml did not lyse the erythrocytes in all examined groups. At higher concentration (10 μg/ml) only P I exerted some hemolytic activity (40-50 %) in all type of blood samples.

Evaluation of tested substances as the fibrinolytics in relation to the β streptokinase activity: Fibrinolytic activities of G-90, P I and P II were tested on the clots of venous blood from 10 healthy dogs, in comparison with the activity of β streptokinase. The time required for complete lytic effect of β streptokinase, with a certain number of IE was compared with the time required for the same effect by the tested substances. The values, expressed in IE, were determined from the calibration curve obtained with β streptokinase. A β streptokinase, with 147.2 IE/ml, completely lysed a clot for 5 hours and 22 min. A P I (10 μg/ml) lysed a clot from the same source for 22 hour, which would correspond to 7.0 IE/ml. G-90 (10 ng/ml) needed 27 hours for the same effect, which would be about 3.5 IE/ml. P II lysed a clot for 45 hours, a value did not fit a basic curve of β streptokinase.

### *Discussion*

This paper describes the investigation of possible clinical use of the substances prepared from the earthworm's tissue (Eisenia foetida). In these studies we examined a G-90 and the proteolytic enzymes P I and P II, isolated from G-90.[7] P I and P II possess a strong fibrinolytic and anticoagulative activities.[7] A G-90 is neither mutagenic nor cancerogenic. Recently, it was shown that G-90 is not allergic, nor toxic, and possesses antibacterial activity and helps in wound healing (own results, not published).

To assess the fibrinolytic activity of tested substances, their activities were compared with the activity of β streptokinase. The results pointed out that malignant tumors and cardiopathies are rather common antagonists of the homeostatic stability either to the side of fibrinolysis (in malignant tumors), or hypercoagulability (in cardiopathies). The value of negative controls, obtained in applied fibrinolysis and the coagulation tests, were dependent on the origin of blood (healthy dogs or dogs with cardiopathies / malignant tumors). The clots from blood of the dogs with malignant tumors dissolved faster, due to tumor plasminogen activator already present in the clot. The slowest rate of dissolving had the clots of blood from healthy dogs with the most stable homeostasis. The euglobulinic tests showed that in dissolving the fibrin clots of the blood from dogs with cardiopathies were very resistant. The pathology of cardiopathy is associated with increasied coagulation factors, that could cause the resistance of a clot on dissolving, seen in our experiments. By application of the test substances we

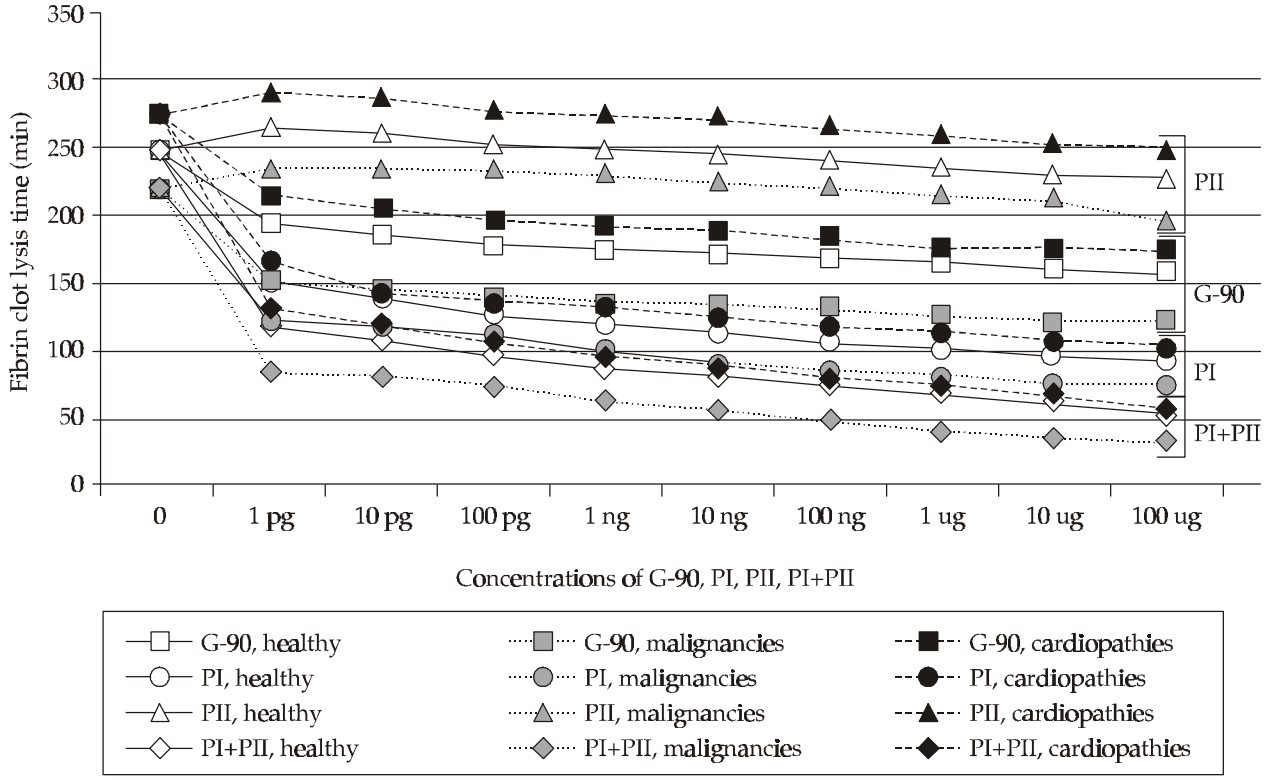


*Figure 3. The effects of G-90, PI, PII and the combination of P I + PII on the lyses of fibrin clots from plasma of clinically healthy dogs (20), the dogs with malignant tumors (20) and the dogs with cardiopathies (20) in depending on the concentrations of examined substances.*

have attempted to find out whether these substances could be applied *in vivo* in the processes of fibrinolysis and coagulation. Also we compared the doses and effects for the *in vitro* and *in vivo* tests. The test of anticoagulative activity *in vitro* showed that in the cases of mammary carcinoma, small doses were sufficient to ensure the effect (10 ng/ml of G-90 or 100 pg/ml of P I or 10 pg/ml of P I + P II).

The results obtained with testing of clots lysis show that P II contributed to the activity of P I, whereas its individual action was even weaker than G-90. The euglobulinic test provided interesting information about P II. In a concentration below 10 ng/ml it suppressed the reaction and prolonged the lysis time above the physiological values. From the above results it is evident that theoretically for anticoagulative treatment of dog of 20 kg (2 l of blood) a single 20 mg dose of G-90 would be required (1 mg/kg of body weight) or a corresponding lower dose by application of P I (2 ng; 0.1 mg/kg of body weight)) or P I + P II (0.2 ng; 0.01 mg/kg of body weight). This „theoretical" assumption corresponds to the statements in the literature.[4,5,11] Patients for cardiovascular bypass are usually treat with heparin (3 mg/kg), prior to intervention. To achieve the normal (physiological) values of all parameters, the treatment is usually continue with a dose of 1 mg/kg of heparin.[11] Other groups experimentally induced a hemorrhage in pigs with a recombinant hirudin (rH) in a dose of 0.3–0.5 mg/kg/hr, over three hours. The animals were pre-treated with the infusion of aspirin (20 mg/kg).[4] Also the fibrinolytic enzymes, isolated from earthworm, were tested in the dogs (oral administration 40 or 80 mg/kg) along with the treatment with aspirin (8 mg/kg). A significant increase in the activities of plasmin and an inhibition of the thrombocyte aggregation were observed. The effect was not directly dependent on the amount of dose administered.[5] The similar observation was made in our study. The results point to specific differences in clotting time among the three test groups rather than dependence on a concentration. All substances accelerated the coagulation of blood samples from the dogs with cardiopathies and decelerate the coagulation of the samples from the dogs with carcinoma, in comparison with healthy dogs. The differences between the concentration of particular substances were in concordance with the purity. A G-90 is a mixture, which contains P I and P II, and for some action we needed a higher concentration of G-90 than P I. The pattern of activity observed with G-90 suggests that G-90, besides the proteolytic enzymes, also contains some other factors responsible for its anticoagulative activity. A similar effect was seen by measuring the fibrinolytic activities. Because, P I and P II are the parts of G-90 mixture, the effect of P I was stronger than the effect of G-90 (at 10 ng/ml of substance). On the other hand, at a concentration of 100 ng/ml G-90 influenced the processes of fibrinolysis and anticoagulation, but P I did not. It should be a consequence of a high concentration of active substance for optimal reaction, which inhibits a process of fibrinolysis (feed back reaction). It is obvious that P II contributed to the actions of P I as the fibirnolytic and anticoagulative agents.

All these findings could be associated with the specificity of G-90, and its fibrinolytic activity, considering the site of the primary malignant tumor. A significant difference of G-90 on the clotting time in cardiopathies and malignant tumors could be also used as a additional differential test, showing whether these diseases may cause disturbed homeostatic stability.

## *References*


1. *Brommer EJP, Emeis JJ, Verheijen JH, et al:* Progress in Clinical Fibrinolysis. In: 7 Recent Advances in Blood Coagulation. (Eds. Poller L and Ludlam CA), Churchill Livingstone, 1997, pp 161-182.
2. *Gandolfo GM, Conti L, Vercillo M:* Fibrinolysis Components as Prognostic Markers in Breast Cancer and Colorectal Carcinoma. Anticancer Res 16:2155-2160, 1992.
3. *Glassman A:* Hemostatic abnormalities associated with cancer and its therapy. Ann Clini Labo Sci 27:391-395, 1997.
4. *Dickneite G, Nicolay U, Friesen HJ, et al:* Development of an anti-bleeding agent for recombinant hirudin induced skin bleeding in the pig. Thrombo Haemost 80:192-198, 1998.
5. *Du Z, Li X, Qian J, et al:* Effect of earthworm fibrinolytic enzyme given orally on the activity of plasmin and on platelet aggregation in beagles dogs. Acta Acade Medi Shang 25:229-231, 1998.
6. *Hrženjak T, Hrženjak M, Kašuba V, et al:* A new source of biologically active compounds-earthworm tissue (Eisenia foetida, Lumbricus rubelus). Comp Biochem Physiol 102A:441-447, 1992.
7. *Hrženjak M, Kobrehel Ð, Levanat S:* Mitogenicity of the earthworm's (Eisenia foetida) insulin-like proteins. Comp Biochem Physiol 104B:723-729,1993.
8. *Hrženjak T, Popoviæ M, Božiæ T, et al:* Fibrinolytic and anticoagulative activities from the earthworm Eisenia foetida. Comp Biochem Physiol 119B:825-832, 1998.
9. *Hrženjak T, Popoviæ M, Tiška-Rudman LJ:* Fibrinolytic activity of earthworms extract (G-90) on lysis of fibrin clots originated from the venous blood of patients with malignant tumors. Pat Oncol Res 3:206-211, 1998.
10. *Oberhoff C, Szymeczek J, Hoffmann O, et al:* Adjuvant antiestrogen treatment with tamoxifen in postmenopausal women with breast cancer: A longitudinal study of blood coagulation and fibrinolysis. Breast Canc Res Treat 50:73-81, 1998.
11. *Okita Y, Takamoto S, Ando M, et al:* Coagulation and fibrinolysis system in aortic surgery under deep hypothermic ciculatory arrest with aprotinin. The importance of adequate heparinization. Circulation 96:376-381, 1997.
12. *Popoviæ M, Grdiša M, Vukoviæ S, et al:* Adhesins of immunoglobulin-like superfamily from earthworm Eisenia foetida. Gen Pharmacol 30: 795-800, 1997.
13. *Sagripanti A, Capri A, Zacharski LR*: The Pathophysiology of the haemostatic system in cancer patients: Insights gained from studies using plasmatic markers of haemostatic system activation. In: Cancer and Blood Coagulation: basic and clinical aspect. (Eds. Sagripanti A, Carpi A and Zacharski LR), Ets Editrice, 1990, pp 69-92.
14. *Van Zutphen LFM, Baumans V, Beynen AC:* Principles of Laboratory animal science. (Eds. Van Zutphen LFM, Baumans V and Beynen A C), Elsevier Science Publishers, 1993.