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Influences of earthworm extract G-90 on haematological and haemostatic parameters in Wistar rats

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Abstract. – *Objectives:* **A balance between fibrinolysis and coagulation is crucial for maintaining haemostasis. A haemostatic disorder leads to various physiological changes and causes different diseases. Glycolipoprotein mixture (G-90), prepared from the tissue homogenate of the earthworm Eisenia foetida, was examined in vivo, in an animal study (conducted on Wistar rats) in order to determine its fibrinolytic and anticoagulation activity.**

Materials and Methods: **The influence of G-90 treatment on haematological and homeostatic parameters was monitored as well.**

Results: **Statistical analysis has shown the most pronounced effect of G-90 to be exerted on bleeding and coagulation times; the effects in reference were proven to be statistically significant (p=0.03 and 0.005, respectively). A statistically significant effect of G-90 was also seen with thrombin time (p=0.02) and plasminogen level (p=0.004).**

Discussion and Conclusion: **The results have shown the influence of G-90 on blood coagulation to be very similar to that of heparin, a known anticoagulant. Thus G-90 could be considered as a new thrombolytic agent of use in veterinary and human medicine.**

Key Words:

Earthworm, *Eisenia foetida*, G-90, haemostasis.

Introduction

Fibrinolytic system is responsible for proteolytic degradation of fibrin, and therefore may play a role in haemostasis and the onset of thrombosis[1,2]. Intravascular thrombosis, a consequence of intra-arterial fibrin aggregation, is one of the main causes of cardiovascular disease. Fibrin is the primary protein component of blood clots, which are formed out of fibrinogen by virtue of thrombin mediation[3]. In biological systems, fibrin clot formation and fibrinolysis are normally well-balanced. However, if fibrin is not hydrolyzed due to some disorder, thrombosis may occur. The most common form of such thrombosis is myocardial infarction. Fibrinolytic enzymes are agents which dissolve a fibrin clot. Nowadays, fibrinolytic agents most widely in use are plasminogen activator (t-PA), urokinase, and streptokinase[4]. However, they exhibit low fibrin specificity, undesired side effects, and are relatively expensive. Therefore, the search for an alternative fibrinolytic agent to be obtained from various sources is still underway. The presence of fibrinolytic activity in earthworm ceolomic fluid or tissue homogenate was reported earlier[5-10]. In addition, earthworm tissue homogenate is an attractive source of various physiologically active compounds[11-17].

Heparin is a naturally- occurring anticoagulant produced by basophiles and mast cells[18], capable of preventing the formation of clots and their further haematogenic spread. Though heparin fails to break down the clots that have already been formed, (unlike tissue plasminogen activator) it allows the natural clot lytic mechanisms to work normally and break down the clots that have not yet been fully formed[19,20]. Heparin binds to the enzyme inhibitor antithrombin (AT), causing a conformational change what results in its activation through the increase in flexibility of the reactive site loop[21]. Activated AT then inactivates thrombin and other proteases involved in blood clotting, most notably the Xa factor. The rate of inhibition of these proteases, exhibited by AT, can increase by up to 1000-fold due to heparin binding. The effects of heparin can be measured in the laboratory using Activated Partial Thromboplastin Time (APTT)[22].

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This paper describes *in vivo* influence of glycolipoprotein mixture G-90 on haemostatic and haematological parameters, and compares it to that of heparin. Glycolipoprotein mixture G-90 was obtained from the tissue homogenate of the earthworm *Eisenia foetida*[23].

Materials and Methods

Animals

Adult Wistar rats (b.w. 200-300 g) were bread at the Institute for Medical Research and Occupational Health, Zagreb, Croatia. The procedure used in this study was in accordance with the provisions of the National Law on Care and Use of Laboratory Animals, and was approved by the Board of Ethics in charge of Animal Care & Use, operating under the wing of the Institute for Medical Research and Occupational Health, Zagreb, Croatia. The animals were kept in steady-state micro-environmental conditions (22 ± 1^{0}C, 50-70%-humidity), had received standard laboratory food and water *ad libitum*, and were exposed to alternating 12h-lasting light/darkness cycles. As known from the literature[24], there exist some indications of sex-linked determinants affecting the response to anticoagulants administered to rats; therefore, only male rats were used in this study. The animals were treated intraperitoneally (i.p.) during 7 days, as follows: Group 1, i.e. the control group (no treatment) (10 animals); Group 2 – treated with G-90 in the concentration of 10 ng/ml (6 animals), and Group 3 – (6 animals) treated with heparin (250 IU per 300-400 g of body weight)[25].

Preparation of G-90

Glycolipoprotein mixture (G-90) was obtained from the tissue homogenate of *Eisenia foetida* according to the procedure described by Hrzenjak et al[23]. Water-soluble powder containing 40 µg of proteins/ mg powder was dissolved in a sterile saline (0.9 % NaCl) and diluted to the final concentration of 10 ng/mL.

Heparin injection, heparinum natricum, 1ml 5000 UI was from Belupo (Koprivnica, Croatia).

Animal Blood Sampling

The rats were deeply anaesthetised using the i.p. injection of Narcetan and Xylapan (Vetoquinol, Switzerland) (1 ml + 0.75 ml), 1.4 ml/kg body weight; upon that, whole blood samples were collected via heart puncture.

In order to complete haematological tests, 7.5% Preanalytical Solution vacutainers® (BD vacutainer® K3E, Plymouth, UK) were used. In order to determine haemostatic parameters, nine parts of freshly-drawn blood were mixed with one part of trisodium citrate, using BD vacutainer® (0.105 M •9NC Tubes 2.7 ml, Franklin Lakes, NY, USA).

Haematological Parameters

Haematological testing included the determination of white blood cell count (WBC), red blood cell count (RBC), haemoglobin concentration (Hb), haematocrit (HT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelet count (PLT), and mean thrombocyte volume (MPV). To the goal of obtaining these parameters, Serono Baker System 9120 Impendance Principle was put in use. Differential cell count was obtained using May Grünwald Giemsa staining.

Haemostatic Parameters

Blood samples were withdrawn into vacutainers containing citrate anticoagulant; plasma was obtained by centrifugation of the test tubes at 1000xg for 15 minutes. Coagulation assays like prothrombin time, activated partial thromboplastin time, thrombin time, plasminogen share, and platelet aggregation, were completed on a fully-automated system called the ACL-ELITE-PRO (Instrumentation Laboratory IL, Lexington, MA, USA):

1. Prothrombin time (PT): the reagent: PT-Fibrinogen HS PLUS, lyophilized rabbit brain thromboplastin, high sensitivity, used for the evaluation of extrinsic coagulation pathway (F II, V, VII, X) (ISI value 1.12). The results are expressed in seconds.
2. Activated partial thromboplastin time (APTT): the reagent: SynthASil (synthetic, high sensitivity), which was used for the evaluation of intrinsic coagulation pathway. The results are expressed in seconds.
3. Thrombin time (TT): the reagent: thrombin (lyophilized bovine thrombin, 15 UNIH/vial), used for the evaluation of common coagulation pathway and fibrinogen levels. The results are expressed in seconds.
4. Plasminogen (PLG): the reagent: Plasminogen, kit (Streptokinase reagent, Chromogenic

substrate). The results are expressed in percent shares (%) of activity.

5. Platelet aggregation (AGR): the reagent: 0.2 mM ADP (adenosine dyphosphate) in platelet function analyzer (Multiplate R, Dynabyte, Munich, Germany). The results are expressed in: U (area under curve), AU (RUO, aggregation), and AU/min. (RUO, the velocity of aggregation).

Bleeding Time (BT)

Bleeding time was measured according to the previously reported methods[26]. The rat's tail was kept in the warm water (40° C) for 1 minute and then dried up. A small cut was made in the middle of the tail. Bleeding time was defined as the period of time elapsing between the first drops of blood touched the filter paper until no further bleeding was observed. According to the literature, normal range to be observed with the bleeding time of Wistar rats is 60-110 seconds (mean ± SEM; 88 ± 4)[26].

Coagulation Time (CT)

In order to determine blood clotting time, rat blood samples were taken in BD vacutainers®: 1 ml of whole blood only; b) 1 ml of whole blood mixed with G-90 in the concentration of 10 ng/ml: and c) 1 ml of whole blood mixed with 30 UI/ml of heparin. The interval between blood sampling and clot formation was considered to be the coagulation time[27]. According to the Pass et al[28], normal range pertaining to the rat whole blood clotting time is 85-140 seconds, while Manzano et al[26] suggest that time-frame to be between 113 and136 seconds (mean ± SEM; 125 ± 3.86).

Statistical Analysis

Statistical significance of the differences observed between the experimental and the control groups were assessed by analysis of variance (Kruskal-Wallis, ANOVA). *P*-values of < 0.05 were considered statistically significant. The significance of difference among the groups was estimated using Mann-Whitney test.

Results

Blood test results, including those pertaining to haemostatic (bleeding time, clotting time, prothrombin time, activated partial thromboplastin time, thrombin time, plasminogen level, and platelet aggregation) and haematological parameters, are summarized in Tables I and II, respectively, together with the pertaining statistical data (median value and the significance of inter- and intra-group differences .

Bleeding and Coagulation Time

In G-90 treated rats, the bleeding time was 2.5 folds longer in comparison to that observed in the control and heparin-treated rats (Table I). A non-parametric Kruskal-Wallis ANOVA failed to reveal statistically significant differences in bleeding time duration, but Mann-Whitney test applicable to independent samples revealed the differences between the control group and G-90 treated group to be statistically significant ($p<0.05$) (Figure 1A).

Respective of the coagulation time, a non-parametric Kruskal-Wallis ANOVA revealed statistically significant ($p<0.05$) differences across the groups. The differences between the groups were also analyzed by Mann-Whitney test applicable to independent samples. The control group was shown to significantly differ from G-90- and heparin-treated groups. In addition, G-90 and heparin were demonstrated to have a similar effect (Figure 1B). The comparison of median values pertaining to blood coagulation in G-90 and heparin-treated rats, revealed the G-90 arm to have a 32%-prolonged blood coagulation time, while that of heparin-treated arm was found to be prolonged by 42 %.

On the other hand, if the blood was taken in vacutainer® filled with G-90, the coagulation time significantly ($p<0.05$) differed across the experimental groups (Figure 1C). Coagulation time obtained in the control rats was 28%-longer, while that in the heparin arm was prolonged by 15 %. It seems that heparin and G-90 tend to exhibit some sort of synergy. However, in oppose to that, under the same conditions, blood coagulation time in G-90 treated rats was shortened by 10%.

Haematological Parameters

Treatment with G-90 and heparin was not shown to have any remarkable influence on haematological parameters estimated within the framework of this study. Only a few parameters were affected (Table I). Platelet count (PLT) differed among the examined groups, that difference being proven to be statistically significant by virtue of Kruskal-Wallis test ($p<0.05$). How-

Table I. Haematological parameters determined in the blood samples withdrawn from Wistar rats treated as follows: 1- control group (no treatment), 2 – G-90 treated group, and 3 – Heparin-treated group (see details in the Methods section).

Tests	Kruska-Wallis test $p < 0.05$	No of rats	Control group – 1		G-90 group – 2		Mann Whitney 1-2 $p \leq 0.05$	Heparin group – 3		Mann Whitney 1-3 $p \leq 0.05$	Mann Whitne 2-3 $p \leq 0.05$
			Median	(Min-max)	Median	(Min-max)		Median	(Min-max)		
WBC × 10^9 L	0.086	6	4.2	(0.3-5.8)	2.94	(2.1-5.1)	–	2.05	(1.2-3.1)	–	–
RBC × 10^{12} L	0.805	6	5.9	(4.2-6.2)	5.87	(5.54-6.98)	–	6.2	(5.17-6.87)	–	–
HBG g/L	0.467	6	133.5	(85-145)	137	(129-156)	–	138	(128-161)	–	–
HTC L/L	0.610	6	0.34	(0.24-0.38)	0.34	(0.33-0.43)	–	0.36	(0.3-0.4)	–	–
MCV fL	0.493	6	58.6	(55.4-62.3)	59	(57.5-61.4)	–	58.15	(57.2-59.2)	–	–
MCH fL	0.021*	6	22.3	(20.3-23.5)	23.05	(22.3-23.3)	0.230	25.5	(22.7-28)	0.016*	0.037
MCHC g/L	0.021*	6	384	(350-403)	392	(364-407)	0.810	415	(395-474)	0.020*	0.016
RDW %	0.804	6	13.7	(11.9-14.19)	13.6	(13.1-14.5)	–	13.5	(13.2-14.3)	–	–
PLT × 10^9 L	0.003*	6	603	(275-644)	745.5	(651-793)	0.004*	766.5	(679-811)	0.004*	0.423
MPV fL	0.094	6	6.5	(5.3-7.1)	6.5	(6.2-6.8)	–	6.9	(6.5-7.3)	–	–

Table II. Haemostatic parameters determined in the blood samples withdrawn from Wistar rats treated as follows: 1- control group (no treatment), 2 – G-90 treated group, and 3 – Heparin-treated group (see details in the Methods section).

Tests	Kruska-Wallis test $p < 0.05$	No of rats	Control group – 1		No of rats	G-90 group – 2		Mann Whitney test 1-3 group	No of rats	Heparin group – 3		Mann Whitney test 1-3 group	Mann Whitne test 2-3 group
			Median	(Min-max)		Median	(Min-max)			Median	(Min-max)		
BT (s)	0.055*	10	101.5	(86-109)	6	245	(75-339)	0.03*	6	103.5	(63-260)	0.59	0.09
CT (s)	0.005*	9	96	(25-105)	6	122	(102-143)	0.01*	6	138	(61-152)	0.01*	0.18
CT- G90 (s)	0.038*	4	141	(118-162)	6	112	(94-118)	0.01*	6	154	(60-183)	0.52	0.06
PT-s	0.147	10	23.3	(20.6-23.9)	6	23.2	(20.9-27)	–	6	22.2	(20.7-22.8)	–	–
APTT-s	0.148	10	15.5	(12.2-16.7)	4	13	(11.4-15.1)	–	6	15.2	(12.6-17.3)	–	–
TT-s	0.017*	10	43.5	(36.5-50.3)	6	37.3	(28.5-41.1)	0.02*	6	36.5	(31.0-40.7)	0.02*	0.82
PLG %	0.003*	10	3.9	(3.6-4.5)	4	3.1	(2.9-3.5)	0.004*	6	4.1	(4.0-4.4)	0.08	0.01*
AGRT-ADP-U	0.277	9	41.0	(25-48)	6	34	(9-45)	–	6	35.0	(9.0-39)	–	–
AGR-ADP-RUO	0.360	9	64.9	(39.4-77.3)	6	49.3	(13.9-70.1)	–	6	55.8	(15 -66)	–	–
AGR-RUO-VEL	0.882	9	13.9	(7.4-18.4)	6	13	(6.3-17.4)	–	6	13.3	(2.8-17.1)	–	–
Eugl fibrinolizamin	0.138	4	101	(101-101)	6	101	(101-102)	–	6	101	(60-101)	–	–

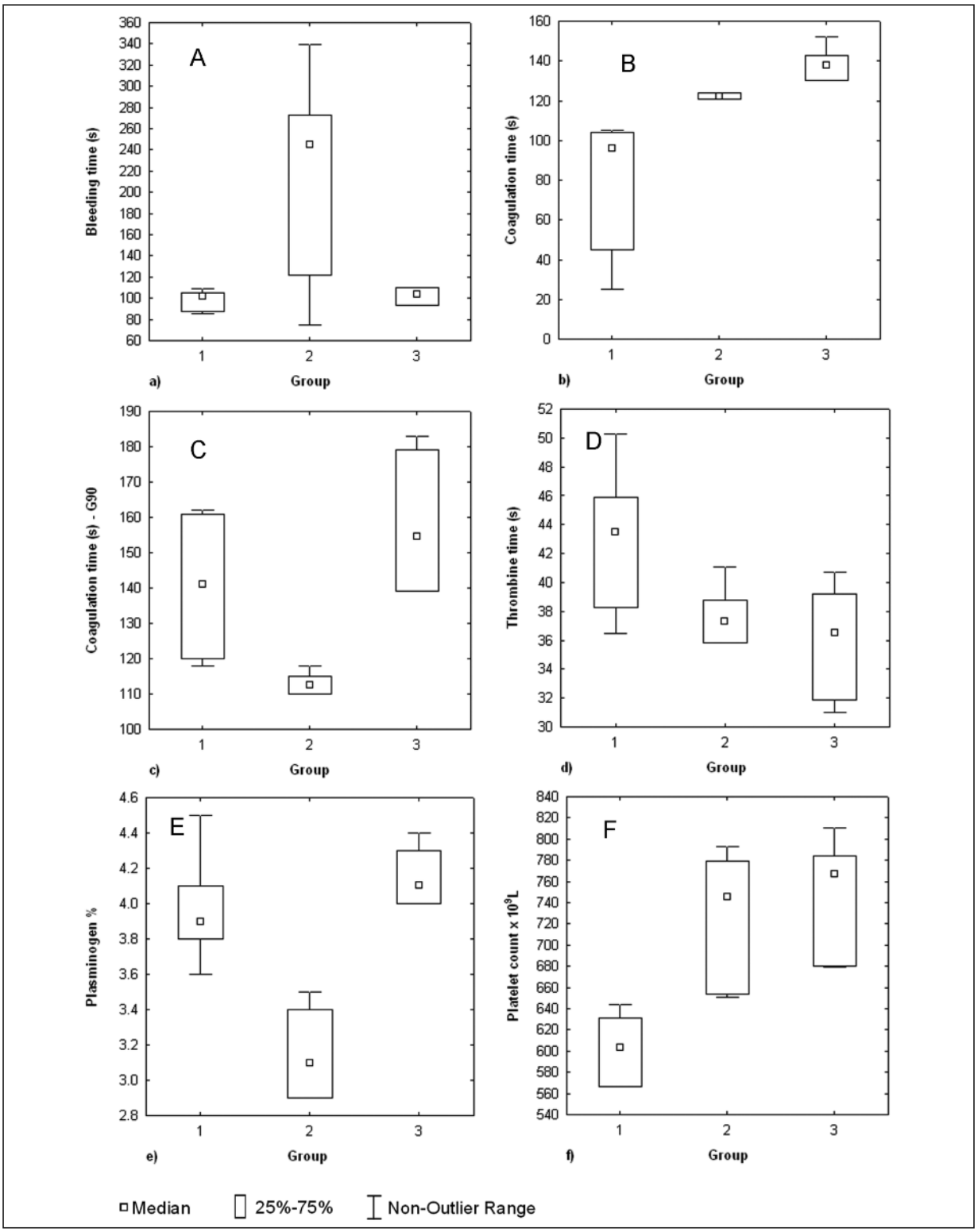

Figure 1. Influence of G-90 on haemostatic parameters in Wistar rats treated during 7 days with 1 mL (i.p.) of: group 1 – without treatment; group 2 – with G-90 in concentration of 10 ng/mL and group 3 – with heparin 250 IU/200-300 g/day. The results were statistically analyzed using Kruskal-Wallis ANOVA method and are displayed as median, minimum and maximum values (*$p < 0.05$). ***A,*** Bleeding time. ***B,*** Coagulation time: The blood samples were collected in empty vacutainers®. ***C,*** Coagulation time: The blood samples were collected in vacutainers® with 1 mL of G-90 (10 ng/mL). ***D,*** Thrombin time. ***E,*** Plasminogen value. ***F,*** Platelets count.

ever, Mann-Whitney test failed to show any remarkable difference between G-90 and heparin arms ($p>0.05$). Thus, we may conclude that G-90 has an effect similar to that of heparin. MCH and MCHC values obtained in heparin vs G-90 arm were statistically significantly different. Nevertheless, the same does not apply for the differences in these parameters obtained between the control and G-90 group.

Haemostatic Parameters

The influence of G-90 treatment on haemostatic parameters is shown in Table II. G-90 and heparin treatment influenced thrombin time (TT). Kruskal-Wallis ANOVA test showed significant intra-group differences in thrombin time ($p<0.05$) (Figure 1 D) in both treated groups, but no significant inter-group differences at all. This result points towards the similarity in G-90 and heparin effects. On the other hand, plasminogen value (PLG %) (Figure 1 E) statistically significantly differed across the groups ($p<0.005$), but with no remarkable difference between the control and heparin-treated animals. In G-90 treated rats, the level of plasminogen decreased, possibly due to its engagement in fibrinolytic process. However, when it comes to prothrombin time (PT), activated partial thromboplastin time (APTT), and platelet aggregation (Figure 1 F), no significant differences among the groups have been detected.

Discussion

It is widely known that snake venom is the most abundant source of fibrinolytic enzymes[29,30]. In recent years, however, fibrinolytic enzymes have been discovered in a variety of organisms[31-33]. Fibrinolytic enzymes are agents that dissolve a fibrin clot. In biological systems, clot formation and fibrinolysis are normally well-balanced. Nowadays, fibrinolytic agents are in a widespread use[4], but with a number of limitations. Therefore, it seems as a very attractive idea to find alternative fibrinolytic agents originating from various sources. For that reason, the potentials of macromolecular mixture (G-90) prepared from the tissue homogenate of the earthworm *Eisenia foetida* was evaluated for its fibrinolytic and anticoagulation activity *in vivo*. The activity of molecules derived from G-90 was compared to that of heparin as a known anticoagulant. According to the literature, rat blood coagulation time is very short (125±3.86 sec)[26]. However, the addition of G-90 (10 ng/mL) prolonged that time to a marked extent. A similar effect has already been seen with a crude extract of earthworms *Lumbricus rubellus*[9,10,34]. It was shown that mixing the blood with that extract prolonged the clotting time; the effect that was attributed to the activity of fibrinolytic enzymes isolated from *Lumbricus rubellus*.

To examine G-90 as a potential fibrinolytic and anticoagulation agent *in vivo*, rats were treated for 7 days (i.p. injection) with G-90 and heparin. Statistical analysis pointed out that the administration of G-90 remarkably (by 2.5 folds) prolonged the bleeding time. In addition, coagulation time observed in the control and the treated group was statistically significantly different, with a similar effect observed after the administration of G-90 and that of heparin. It seems that G-90 also increased the effect of heparin. Therefore, one could speculate that these two agents might have exhibited a synergistic effect. But, the addition of G-90 to the blood of G-90 treated rats resulted in the decrease of median coagulation time. That might be a consequence of the presence of some component (inhibitor) in G-90 preparation that might either stimulate coagulation or inhibit fibrinolytic enzymes' activity. Earlier results have shown that G-90 exerts strong fibrinolytic and anticoagulation activity *in vitro*. G-90 was capable of lysing fibrin clots both in human and dog blood, and of preventing coagulation[12,17,35].

The results of haemostatic tests (BT, CT, PT, APTT, TT, PLT, AGR, and euglobulin fibrinoliysis) (Table I) have revealed different effects. The majority of the determined parameters were on the control group level. A remarkable effect was seen on bleeding time (BT), which was 2.5 times longer than in the control and heparin arms. Such a result pointed out that G-90 exhibits a hypo-coagulation effect, and could be useful in maintaining primary haemostasis. Because the function of thrombocytes (Figure 1 F; Table 1) (in terms of their number and aggregation) was not affected by G-90 treatment, we could speculate that G-90 influences the blood vessel wall, the effect also important for the process of primary haemostasis[26]. In the rats treated with heparin, the expected effect (prolonged TT and APTT) was not achieved[26], possibly due to less absorption or degradation of heparin after the i.p. injection. Some discrepancies were also noticed with

plasminogen levels (Figure 1 E, Table II). High plasminogen levels may indicate an increased activity of fibrinolytic system, which can induce an accelerated fibrinolysis. However, G-90 treatment resulted in a low plasminogen level, which might suggest its possible usefulness in the fibrinolytic process[36].

Haemostatic activity of G-90 was confirmed in an *in vivo* system. This macromolecular mixture obtained from the tissue homogenate of the earthworm *Eisenia foetida* exhibits a remarkable degree of fibrinolytic and anticoagulation activity. The most pronounced G-90 effect was observed with bleeding and coagulation times. Mann-Whitney test has shown that the effect exhibited by G-90 was very similar to that of heparin. Therefore, G-90 could represent a new source of fibrinolytic and anticoagulation enzymes suitable for future application in human and veterinary medicine.

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