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Original Article

Venom-induced consumption coagulopathy in rats following *Leiurus macroctenus* (*Scorpiones: Buthidae*) envenomation

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ABSTRACT

Leiurus macroctenus belongs to the genus of one of the most dangerous scorpions on the globe. Among the variety of symptoms caused by *Leiurus* scorpion stings, not much attention is paid to the hemostasis disturbances in patients, and nothing much is known about the coagulation disorders associated with *L. macroctenus* envenomation. This study is dedicated to the investigation of the alterations in hemostasis system in a *L. macroctenus* envenomation rat model. The findings of our work suggest about the thrombin time prolongation, decreased levels of circulating fibrinogen, reduced activities of antithrombin-III and α2-antiplasmin, as well as the increased levels of fibrinogen/fibrin degradation products, prothrombin abnormal forms, and elevated activities of heparin and plasmin, all of which indicate the development of venom-induced consumption coagulopathy and anticoagulative properties of the venom. The major changes occurred mainly within 24 hours of envenomation, indicating the peak of envenomation, following the return to the normal values in the next 48 hours. The pattern of venom toxins' enzymatic activities and the precise molecular mechanisms of their interaction with hemostasis components should also be studied in the future in order to facilitate the appropriate treatment development and to consider the venom as the potential source of bioactive compounds.

KEYWORDS: envenomation, scorpion, VICC, coagulopathy, *Leiurus*.

1. INTRODUCTION

The *Leiurus* genus is comprised of one of the most dangerous scorpion species, which mainly populate the Middle East and North Africa regions [1]. The venom of these creatures contains a mixture of ion channel blockers, enzymes, bioactive peptides and other compounds, that are responsible for the devastating effects in the victim's body [2]. The most common manifestations of the *Leiurus* scorpions' sting include local pain, oedema, hypertension, cardiac, renal and respiratory failure; the most severe symptoms are usually observed in children [3]. Although venom of many scorpions may cause hemorrhages and coagulation disturbances, which is a significant threat to the victim's health [4], not many studies had been dedicated to the hemostasis disorders during the *Leiurus* scorpions envenomation.

Leiurus macroctenus as many other *Leiurus* species has been misperceived as *Leiurus quinquestriatus*

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for a long time until Lowe *et al*. [5] recently described *Leiurus macroctenus* as a separate species. Therefore, very little is known about the biochemical effects of its venom on victim's body. In their recent studies Gunas *et al*. showed the significant effects of *L. macroctenus* venom on the proteolytic activity and levels of matrix metalloproteases [6], as well as effects on the protein and peptide profiles and middle-mass molecule levels in various organs of rats [7].

Taking into account the aforementioned, this study's purpose was to broaden the knowledge on the effects of *L. macroctenus* venom on biochemical levels by investigating the possible alterations of key parameters concerning the hemostasis system in response to *Leirus macroctenus* envenomation.

2. MATERIALS AND METHODS

2.1. Scorpion specimen collection and maintenance

Ten mature *Leiurus macroctenus* scorpions were used in this work. The scorpions were identified by Mark Stockmann and kept in Ibbenbüren private collection (Germany). Animals were kept separately in constant conditions (25 °C – 35 °C, 50 – 60% humidity, natural lighting conditions) in transparent plastic boxes (10x5x5 cm) layered with sand (Exo Terra «Desert Sand»). Water source as well as the aeration holes were provided. Once a week scorpions were fed with one *Shelfordella lateralis* cockroach, and each box was cleaned once a month.

2.2. Venom collection

The procedure for obtaining the venom, its storing and LD_{50} estimation comply with the protocols, described previously [6].

2.3. Envenomation modelling and plasma sample obtaining

All the procedures involving laboratory animals, including their keeping conditions, envenomation modelling and euthanasia were done in compliance with the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPSEA) and the study was approved by the Ethical Committee of Taras Shevchenko National University of Kyiv (protocol №2 approved 19.08.2021). Overall, 73 albino non-linear male rats (180 g ± 3 g), divided into control group (13 rats) and experimental group (60 rats), were used in the experiments. Experimental animals were injected intramuscularly with 0.5 ml venom solution (LD50), dissolved in the saline solution (0.9%), while control animals were injected with 0.5 ml saline solution. All rats in the experimental group were sub-grouped by 5 animals per cage and kept in constant vivarium conditions. The euthanasia of corresponding subgroups was performed in time frames of 1 hour, 3 hours, 24 hours and 72 hours after the venom injection in order to evaluate the dynamics of the assessed parameters during envenomation.

After euthanasia, blood plasma was obtained using the standard method [8]. The blood samples were placed into plastic tubes containing 3.8% sodium citrate (9:1 blood-sodium citrate proportion). Afterwards, the samples were centrifuged at 900 g for 40 min, and plasma samples were aliquoted and stored at -20 °C.

2.4. Thrombin time determination

The changes in the thrombin time (TT) during the envenomation were determined using the Test Thrombin Reagent commercial kit (Siemens Healthineers, Germany). All procedures were performed strictly according to the manufacturer instructions.

2.5. Fibrinogen concentration measurement

The concentration of fibrinogen in the plasma samples were measured according to the method described elsewhere [9]. The mixture of plasma (0.2 ml), thrombin (2 NIH units, 0.1 ml), 0.1 M phosphate buffer (1.7 ml, pH = 7) and 0.04 M monoiodoacetic acid (0.1 ml) were prepared and incubated at +37 °C for 30 min. The formed fibrin clot was removed, washed several times with cooled 0.13 M NaCl, and dissolved in 5 ml of 1.5% acetic acid solution. The concentration of fibrinogen was measured spectrophotometrically, and calculated using the formula:

$$\text{Fibrinogen concentration, g/L} = [(E_{280}-E_{320})*255]/1.506,$$

where E_{280} and E_{320} are the extinction levels of the sample at wavelength of 280 nm and 320 nm, respectively; 255 is the conversion coefficient of the fibrinogen concentration in the reaction mixture to its actual concentration in plasma sample, and

1.506 is the coefficient representing the extinction of 1% fibrinogen acidic solution at the wavelength of 280 nm.

2.6. Fibrin/fibrinogen degradation products levels determination

The measurement of fibrin/fibrinogen degradation product (FDP) concentration was based on the ability of these molecules to prolong the polymerization time of monomer fibrin. The concentration of FDPs in plasma is directly proportional to the polymerization time, and can be calculated by using the calibration curve. The calibration curve was built using the plasma samples with known concentrations of FDPs and the monomer fibrin solution.

2.7. Assessment of plasmin, antithrombin-III and α2-antiplasmin activities

The relative activities of plasmin, antithrombin-III (AT-III) and α2-antiplasmin (α2-AP) were measured using the chromogenic assay kits (Diapharma, USA). All of the procedures were done following the manufacturer's instructions. For the plasmin activity determination, the plasma sample was diluted in 0.05 M Tris-HCl buffer (pH = 7.4), containing 0.13 M NaCl (henceforth: Tris-HCl buffer) in 1:49 plasma-buffer proportion, and mixed with the S_{2251} chromogenic substrate (final concentration 0.003 M). For the α2-AP activity determination, plasma samples were diluted in Tris-HCl buffer (1:3 plasma-buffer proportion), mixed with 40 μL of plasminogen, and S_{2251} chromogenic substrate (final concentration 0.003 M). For the AT-III activity determination plasma samples were diluted in Tris-HCl buffer (1:29 plasma-buffer proportion), and mixed with thrombin, heparin (final concentration 0.2 IU) and S_{2238} chromogenic substrate (final concentration 0.003 M).

The reaction mixtures' volumes were adjusted to 250 μL by Tris-HCl buffer, and were incubated at 37 °C for 30 min. After incubation, the activity of plasmin, AT-III and α2-AP in samples were measured using microplate spectrophotometer (BioTek Instruments, USA). The activity of plasmin in the sample is proportional (while the activities of AT-III and α2-AP are inversely proportional) to the amount of released para-nitroaniline from the chromogenic substrate molecule, which is detected at a wavelength of 405 nm. The relative activities of plasmin, AT-III and α2-AP in experimental groups were calculated by considering the activity in control group as 100%.

2.8. Heparin activity determination

To measure the activity of heparin, we used the commercial chromogenic assay kit (RENAU, Ukraine). All of the procedures were done strictly according to the manufacturer's instruction, and all reagents used were included in the kit. The plasma samples were diluted by the running buffer in 1:4 proportion, mixed with AT-III solution, factor Xa solution and incubated for 5 min at 37 °C. After that, the chromogenic substrate was added and the mixture was incubated again for 5 min at 37 °C. The reaction was stopped by adding 50-% acetic acid to the mixture. The activity of heparin was measured using microplate spectrophotometer (BioTek Instruments, USA), and it was inversely proportional to the amount of para-nitroaniline released from the chromogenic substrate molecule, which is detected at a wavelength of 405 nm. The relative activities of heparin in experimental groups were calculated by considering the activity in control group as 100%.

2.9. Evaluation of the levels of prothrombin abnormal forms

To measure the relative levels of prothrombin abnormal forms (PAFs), we used a method based on the measurement of the difference between ecamulin time (ET) and prothrombin time (PT). ET is proportional to the levels of normal and functionally inactive prothrombin, or PAFs, while the PT represents the levels of normal thrombin. Therefore, the difference between ET and PT is proportional to the PAFs levels in plasma samples. The relative levels of PAFs in experimental groups were calculated by considering the activity in control group as 100%.

2.10. Statistical analysis and data interpretation

Data was analyzed *via* the OriginPro v9.5 software, and expressed as mean ± standard error of mean (M±m), n = 5. The significance of the differences between the control and experimental groups was determined by two-sample independent t-test using the OpenEpi software. The presented results were considered statistically significant when $p < 0.05$.

3. RESULTS

As the screening test of the hemostasis disturbances after scorpion envenomation we used the TT determination method. According to the results, presence of the venom has been constantly increasing the TT since the very beginning of the envenomation (Fig. 1). The peak of TT elevation (32 ± 0.2 s) happens in the first 24 hours of envenomation, followed by the decrease in the next 48 hours.

In order to study hemostasis disruption more closely, we determined the levels of fibrinogen and fibrin/fibrin degradation products (FDPs), and observed a gradual decrease in the concentration of fibrinogen after venom injection, reaching only 65% of control levels in the 24th hour of envenomation (Fig. 2A), while the concentration of FDPs in the same period amounted to 199% of the control values (Fig. 2B), which is an obvious correlation between these parameters. We also observed the subsequent rapid decrease in the concentration of FDPs, while the concentration of fibrinogen remained low even 72 hours after venom injection.

Next, we measured the changes in the activities of plasmin, α2-AP as well as the AT-III and heparin, all of which are related to the anticoagulation processes in the hemostasis system. As it can be seen in Fig. 3A, the elevation of relative plasmin activity symmetrically reflects the reduction in the activity of α2-AP. In the period of 24 hours after venom injection, the activity of plasmin is increased by 34%, compared to the control values, while activity of the α2-AP is reduced by 31% of the control values. It also can be seen that in the next 48 hours the activities of plasmin/α2-AP tend to decrease and increase respectively, indicating the steady return to the control values. Quite similar results are shown in Fig. 3B, which is notable, considering the property of heparin to potentiate the AT-III activity. The proportional decrease in the AT-III activity and increase in the heparin activity in the first 24 hours of envenomation can be traced. After this period, the relative activity of

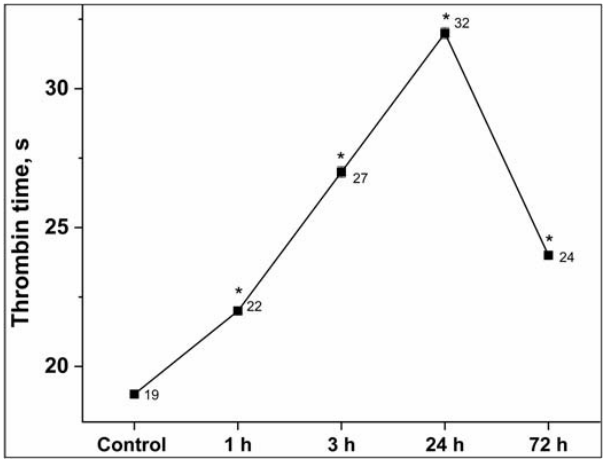

Fig. 1. Thrombin time changes in the course of *L. macroctenus* envenomation. Data presented as mean ± SEM, n = 5; *p < 0.05.

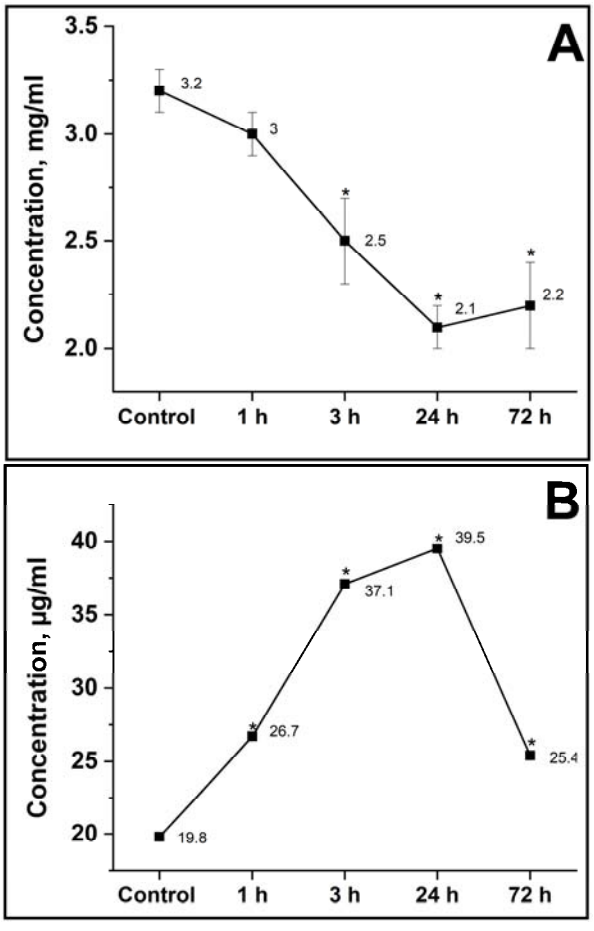

Fig. 2. Changes in concentrations of fibrinogen (A) and fibrinogen degradation products (B) in the plasma of rats during *L. macroctenus* envenomation. Data presented as mean ± SEM, n = 5; *p < 0.05.

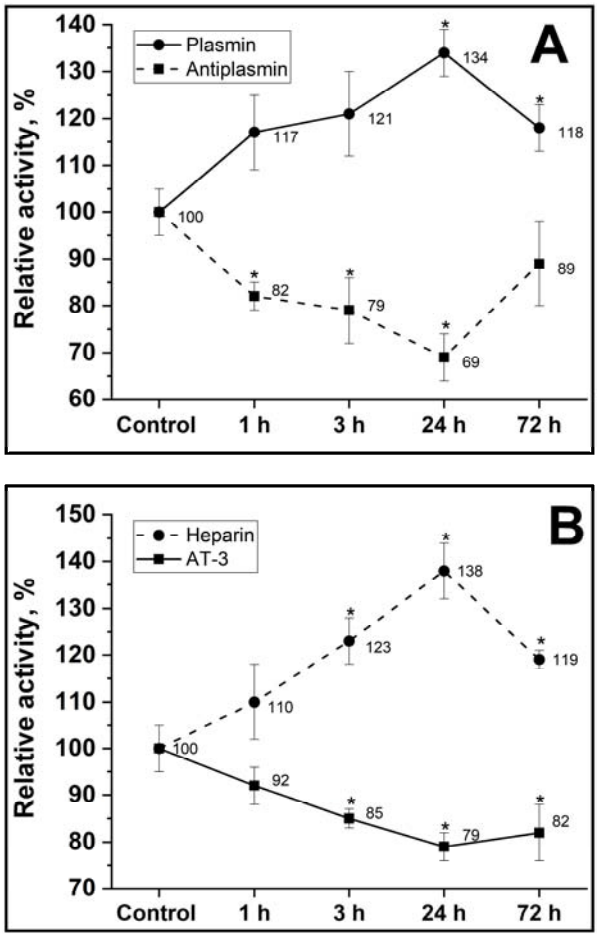

Fig. 3. Dynamics of relative activities (in % of control values) of plasmin, α2-antiplasmin (A); antithrombin-III and heparin (B) in plasma of rats, envenomated by *L. macroctenus*. Data presented as mean ± SEM, n = 5; *p < 0.05.

heparin tends to decrease, while the relative activity of AT-III did not show any statistically significant changes.

Expanding the relevance of the obtained result, we evaluated the relative levels of PAFs as one of the indicative parameters of the coagulation cascade impairments. Thus, we revealed that the formation of PAFs is inherent to the first 24 hours of *L. macroctenus* envenomation (Fig. 4). The maximal levels of this parameter in 24 hours are about 23% higher compared to the control group, yet after this period the presence of PAFs in the blood start to decrease, indicating the recovery process in the organism.

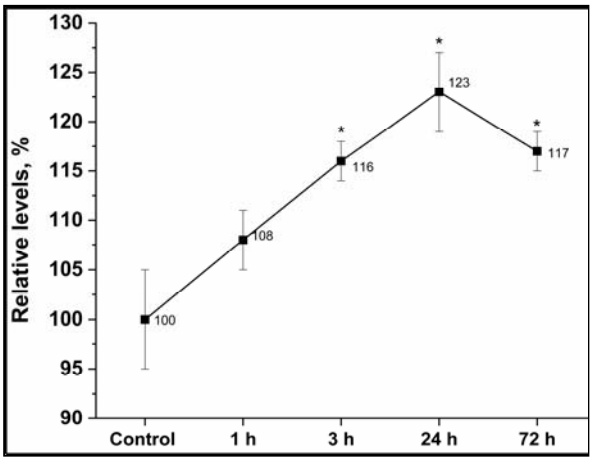

Fig. 4. Relative levels (in % of control values) of prothrombin abnormal forms in plasma of rats during *L. macroctenus* envenomation. Data presented as mean ± SEM, n = 5; *p < 0.05.

4. DISCUSSION

Coagulation system disturbances is one of many clinical manifestations of the scorpion sting [10]. Nevertheless, not many attention is being paid to the hemostasis system assessment during hospitalization after scorpion sting. For example, according to Al Abri *et al*., bedside clotting tests are done only in 11.5% of stung patients and coagulation tests are done in 5.5% of patients in one of the Oman hospitals [11]. In our study we have tried to shed light on the coagulation disturbances after being stung by *L. macroctenus*, which, to date, haven't been studied at all.

According to the results of this study, *L. macroctenus* venom causes a significant anticoagulation effect in rats which is common for *L. quinquestriatus*, the most studied member of the *Leiurus* genus [12, 13], as well as many other scorpion species [14].

TT is one of several parameters that reflect the coagulation system integrity and is indicative in the cases of hemostasis disturbances. In our experimental conditions, peak rates of TT were almost 1.6 times higher than in the control group, which is an average among studied scorpion venoms [14]. Taking into account that hemostasis system involves an extremely complicated biochemical cascades, venom can potentially affect a broad spectrum of hemostasis system components. Thus, we also examined the plasma concentration of

fibrinogen as one of the key components of coagulation cascade and concentration of FDPs as an indicator of active fibrinogen/fibrin degradation. The results provide evidence of the active consumption of fibrinogen and formation of significant amounts of FDPs. This pattern of coagulopathy is described as venom-induced consumption coagulopathy (VICC), and is usually inherent to snake envenomations [15]. In these cases, the consumption of fibrinogen can be caused either by the direct action of venom thrombin-like proteases, resulting in the formation of non-cross linked FDPs, or *via* the direct/ indirect formation of active thrombin. In the second scenario, activated thrombin cleaves the fibrinogen, forming the fibrin clot, which in turn can be degraded by plasmin-like venom proteases, or activated plasmin, resulting in formation of cross linked forms of FDPs such as D-dimer [16]. The results of VICC are obvious: consumption of coagulation factors, and as a result increased risk of bleeding with low circulating fibrinogen levels [17]. In addition to that, anticoagulative effect is aggravated by the presence of FDPs in high concentrations, which are able to inhibit coagulation by several mechanisms [18]. By conducting a set of additional experiments, we concluded that the fibrinogen consumption during the envenomation can be related to the increased activity of thrombin, since the activity of AT-III was decreased, that, in turn, can be the result of direct or indirect interaction with venom components. In this situation, the observed elevated activity of heparin could be the organism's compensatory response to the low AT-III activity. The involvement of thrombin in the fibrinogenolysis during the envenomation can also be proven by the elevated levels of PAFs, which are mostly generated during rapid and large-scale prothrombin cleavage. The activation of thrombin in high amounts with parallel decrease in AT-III activity quite logically leads to fibrinogen degradation, depleting its plasma pool. As mentioned before, the fibrinogenolysis during envenomation can be also related to the activity of thrombin-like venom proteases, or other proteases, affecting the coagulation cascade. In our experiments we measured the total concentration of total FDPs (including cross-linked and non-cross linked products); therefore, fibrinogen degradation in this case can be related to venom's thrombin-like activity. These findings support our insights about the VICC development, since the changes in the assessed parameters comply with the main laboratory findings of VICC patients [19]. Moreover, the pattern of *L. macroctenus* venom effects are highly similar to those observed in dogs with VICC during the *Bitis arietans*, *Naja annulifera* [20], and *Vipera palestinae* snake envenomations [21]. We may also assume that *L. macroctenus* envenomation can cause negative effect on kidneys, since the VICC in envenomated patients is strongly related to the development of kidney pathologies, including acute kidney injury (AKI) [22-24]. Taking into account that AKI and many other kidney pathologies are quite common in scorpion envenomations [25], it is possible that hemostasis disturbances following *L. macroctenus* envenomation may facilitate the renal impairments as one of the negative effects of the venom.

The measurement of activities of plasmin and α2-AP gave us an opportunity to assume that the hypercoagulation isn't the only effect of the *L. macroctenus* venom. According to the results, the envenomation provides a significant level of fibrinolysis by activated plasmin, since the activity of α2-AP was considerably low and the concentration of circulating FDPs were unprecedentedly high. Such effect could be the organism's response to the aforementioned coagulation processes or/and the direct effect of the venom components on the α2-AP, lowered levels of which made such activity of plasmin possible. We can't exclude the potential presence of enzymes with plasmin-like activity in the venom itself, yet the corresponding investigations should be made. Therefore, hypercoagulation and consumption of plasma fibrinogen is potentially the primary effect of envenomation, yet the activation of fibrinolysis by plasmin doesn't allow the progression of thrombosis or such effects, and hence the main effect of envenomation is simply the consumption of coagulation components and most likely – bleeding and blood coagulation disturbances for uncertain period in future. These findings allow us to state that *Leiurus macroctenus* venom possess anticoagulative properties in general, without excluding the presence of procoagulant properties of certain venom components, which also corelates

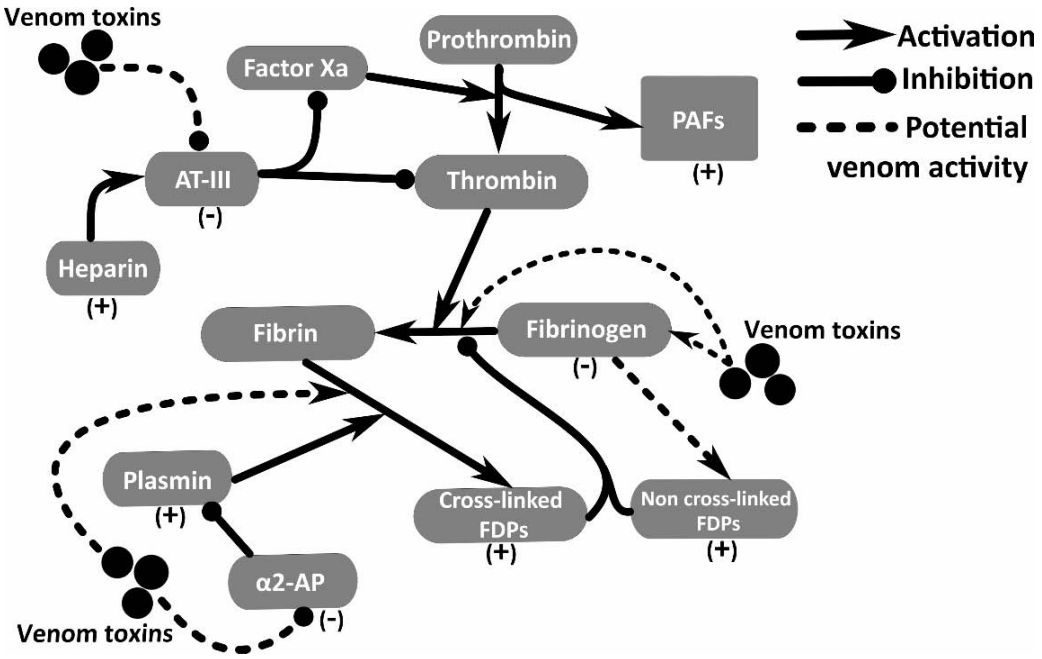

Fig. 5. An approximate scheme of the coagulation cascade, its main components, and potential effects of *Leiurus macroctenus* venom components. AT-III – antithrombin-III; PAFs – prothrombin abnormal forms; α2-AP – α2-antiplasmin; FDPs – fibrinogen/fibrin degradation products. '+' and '-' signs in brackets correspond to the increase and decrease in levels/activities of given parameters according to the findings of this study.

with the findings regarding *Leiurus* quinquestriatus envenomation [12, 13]. The simplified scheme of the coagulation cascade with implementation of present findings is provided in Fig. 5.

It is also notable that the peak values of the assessed parameters in this study occurred in the period of 24 hours after the venom injection, which correspond with previous findings regarding *L. macroctenus* envenomation provided by Gunas *et al.* [6, 7], strongly supporting the theory that this period of envenomation is challenging for the victim's homeostasis. After this period, most of the parameters changed towards the control values, which is most likely the result of neutralizing/excretion of venom toxins and post-envenomation recovery.

The findings of this study provide important information, exploring the insights of *L. macroctenus* envenomation course. However, the precise mechanisms of the venom-induced hemostasis disturbances still remain unclear. Therefore, considering the observations of this study, it is important to investigate the enzymatic activities of the venom components (plasmin-like, thrombin-like, etc.) not only to develop a strategy for *L. macroctenus* envenomation treatment, but also to obtain potential bioactive molecules from its venom, that affects hemostasis.

5. CONCLUSIONS

In his study we investigated the alterations in the rats' hemostasis system following *L. macroctenus* scorpion envenomation. As it turned out, the envenomation leads to the prolongation of thrombin time, increment of FDPs and PAFs levels, and declined levels of circulating fibrinogen, which is linked to the reduction of AT-III and α2-AP activities, as well as to the increment of plasmin activity. The pattern of these changes suggests about VICC development in the envenomated rats, and the anticoagulative effect of the venom in general. Moreover, the dynamics of envenomation shows major changes occurring mainly in the period of 24 hours after the venom injection, after which the levels of most of the assessed parameters were found to steadily return to the control values, indicating the post-envenomation

recovery period. The precise molecular mechanisms of venom action on the aforementioned parameters, as well as its enzymatic properties yet need to be investigated, which can underlie the basis of future studies. The exploration of *L. macroctenus* venom components acting on hemostasis would improve the strategies of scorpionism treatment, and also would be interesting from the biotechnological point of view.

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CONFLICT OF INTEREST STATEMENT

None to declare.

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