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INTERFERENCE OF *Bothrops jararacussu* AND *Bothrops cotiara* VENOMS UPON BLOOD COAGULATION PATHWAYS

Interferência dos venenos de Bothrops jararacussu *e* Bothrops cotiara *sobre as vias da coagulação sanguínea*

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Abstract

Hemostasis includes mechanisms for the hemodynamic balance and integrity of the cardiovascular system. Overflow of blood provoke as an answer the activation of coagulation cascade which leads to a clot of fibrin. Snake venoms affect different interaction processes within the haemostatic system. Coagulation time (CT) were determined by the addition of venom samples to human plasma at 37OC. Minimum coagulant dose (MCD) was measured by the incubation of human plasma to venom sample in decreasing concentrations in two-fold serial dilutions (from 50 µg/ml). For thrombin (TT) and prothrombin time (PT) 100 µl of citrated plasma were incubated at 37° C for 60 sec followed by the addition of 200 µl specific reagent (bovine thrombin and thromboplastin, respectively). *B. jararacussu* venom presented TC of 29.4 sec and DMC of 250 mg/l. For *B. cotiara* venom TC was 30.2 sec and DMC 125 mg/l. Results suggested that both snakes venoms acted upon the common pathway.

Keywords: Blood coagulation. Bothropic venom. Thrombin. Prothrombin.

Resumo

Hemostasia inclui mecanismos de balanço hemodinâmico e integridade do sistema vascular. O vazamento de sangue provoca como resposta a ativação da cascata da coagulação que leva à formação de uma malha de fibrina. Os venenos de serpentes afetam diferentes processos de interação no sistema

hemostático. O tempo de coagulação (CT) foi determinado pela adição de amostras do veneno ao plasma humano a 37 ºC. A dose mínima coagulante (MCD) foi medida pela incubação do plasma humano a amostras dos venenos em concentrações decrescentes em diluições seriadas (a partir de 50 μg/ml). Para o tempo de trombina (TT) e de protrombina (PT) 100 μl de plasma citratado foi incubado a 37 ºC por 60 seg, seguida da adição de reagente específico (trombina bovina e tromboplastina, respectivamente). O veneno de B. jararacussu *apresentou CT de 29,4 s e MCD de 250 mg/l. Para o veneno de* B. cotiara*, TC foi de 30,2 s e DMC de 125 mg/l. Ambos venenos agiram sobre a via comum da cascata da coagulação.*

***Palavras-chave**: Coagulação sanguínea. Veneno botrópico. Trombina. Protrombina.*

INTRODUCTION

Hemostasis includes mechanisms for the hemodynamic balance and integrity of the cardiovascular system. Overflow of blood provoke as a final answer platelet aggregation and simultaneously the activation of coagulation pathways. The classic coagulation cascade can proceed through the intrinsic pathway, initiated by surface activation due to exposure to subendothelial components of the vessel walls. Another more critical route of initiation is through the extrinsic pathway, triggered by a lipoprotein exposed from damaged tissue, called tissue factor. Both of these pathways result in the activation of factor X to Xa, and the subsequent steps which leads to the formation of the fibrin clot from the activation of fibrinogen by thrombin (1). The main action of thrombin is to catalyse the proteolysis of fibrinogen, a soluble plasma protein, to form fibrin monomers that are still soluble. Fibrin monomers then polymerise to form a gel of fibrin polymers that traps blood cells. Thrombin also activates coagulation factor V, factor VIII and factor XIII (2). After clot formation, once bleeding has been stopped, the continuity of the endothelium is restored and the fibrinolytic system is then activated and gradually dissolves the clot.

Snake venoms are complex mixtures of a large variety of proteins and peptides affecting the hemostatic system. Those components can present different action and can be classified as procoagulant, anticoagulant, fibrinolytic, vessel wall interactive, platelet inducers or inhibitor, and plasma protein activators (3). Although many snake venoms contain a number of hemostatically active components, no single venom contain all categories. *Bothrops* species are outstanding for the high clotting activity of their venoms, due to the presence of thrombin-like enzymes which transform fibrinogen into fibrin. Most of these enzymes differ from thrombin itself, because they can liberate only fibrinopeptide A from the fibrinogen molecule and do not activate coagulation factor XIII, resulting in the formation of a type of fibrin which is instable and can be easily destroyed by fibrinolytic system (4, 5). The investigation of the effects of different snake venoms on blood clotting is important in order to understand the mechanism of action of venom components aiming their use in therapeutics or laboratory assays of blood clotting.

The objective of the present investigation was to identify the *in vitro* interference of *B. cotiara* and *B. jararacussu* venom on plasma coagulation pathways. Eventually, the isolation of active compounds from these venoms will permit the study of their mechanism of action on blood coagulation itself and therapeutic applications.

MATERIAL AND METHODS

Material

Bothrops jararacussu and *B. cotiara* dried venom were kindly provided by Instituto Butantan (São Paulo, Brazil) and stored at - 20 ºC until use. Briefly before coagulation assays venoms were reconstituted in saline solution (NaCl 0,9%). TT, PT and APTT reagents kits were from Dade Behring Inc. (Newark, USA). The coagulant tests were carried out at 37 ºC using human citrated plasma (0,38% sodium citrate solution) obtained from periferic blood from 20 volunteers.

Experimental procedures

Coagulation time (CT) were determined by the addition of 50 ml of venom sample (50 mg/

ml) or PBS to 100 ml of human plasma at 37 ºC. Minimum coagulant dose (MCD) was measured by the incubation of 100 µl of human plasma to 50 µl of venom sample in decreasing concentrations in two-fold serial dilutions (from 50 mg/ml), as described by EVANS e KINI (1997). For thrombin (TT) and prothrombin time (PT) 100 ml of citrated plasma were incubated at 37 ºC for 60 sec followed by the addition of 200 ml specific reagent (bovine thrombin and thromboplastin, respectively). On adding the reagent timer coagulometer was started to determine the coagulation time. In order to investigate venoms interference on coagulation tests, venom solution (50 mg/ml) was previously incubated with reagents before addition to test tubes.

Statistical analysis

Means and standard deviation of all data were obtained and compared by ANOVA followed by Tukey test, with significance probability levels of $p < 0.05$, using Statistica 5.1.1 program.

RESULTS AND DISCUSSION

Many snakes venom have long been known to affect blood coagulation. In the last few decades, a considerable amount of work on the mechanism of action of snake venoms in promoting or inhibiting blood coagulation process has been carried out (5, 6). In South America, venomous snakes comprise four distinct genera: *Bothrops, Crotalus, Lachesis* and *Micrurus*; among those only *Micrurus* produce venom which does not affect blood coagulation.

The effect of venom samples on coagulation of human plasma was analysed and our results confirm the procoagulation action of *Bothrops* venoms. Table 1 shows that at the concentration of 50 mg/ml both venoms induced coagulation in aprox. 30 sec. On the other hand serial dilution of venom samples in saline resulted in 125 mg/L for *B.cotiara* venom as the minimum coagulant dose (MCD) what points out *B. cotiara* venom twice more active than *B. jararacussu* venom. These results contrast from what has already been published in previous papers. Species from Costa Rica, *Bothrops atrox*, and *B.schlegellii*, exhibited MCD-P (minimum coagulant dose on plasma) of 1.4 and 13.2, mg/L, respectively, whereas *B. nasutus* showed no more than trace activity on plasma (7). Analysis of Brazilian species revealed MCD-P from 19.3 to 90.6 mg/L, specifically of 84.5 for *B. cotiara* and 42.8 mg/L for *B. jararacussu* (8). Such divergence may be explained by the the fact that the composition and the mechanism of blood coagulation activation by venoms varies among species. Theakston and Reid (1983) tested the activity of 45 Viperine venoms from diferent genera and showed that only 19 of them showed, at least, trace coagulant action on plasma. Even within the same species, variation can accur depending on the age of the snake (5, 9), season (10) and geographic distribution (11).

The last stage of the cascade is measured by thrombin time assay which consists in the conversion of fibrinogen to fibrin by the action of thrombin and the spontaneous polymerisation of the fibrin to form a soft clot. In the present work, thrombin time was significantly reduced by *B. cotiara* venom (12.47 sec) when compared to control (20.66 sec), whereas the venom of *B. jararacussu* promoted a minor decrease (18.16 sec), statistically similar to control. On the other hand, on prothrombin time investigation (PT) showed an slight but significant reduction on coagulation time promoved by *B. jararacussu* venom, whereas *B. cotiara* venom increased time of clot formation in aprox. 20%, compared to control (18.12 sec). This method indicates the integrity of the extrinsic pathway of the coagulation cascade, in which both thromboplastin (factor III) and calcium are added to plasma independently activating prothrombin into thrombin.

At the present time more than 100 different snake venoms are known to affect the hemostatic system by a variety of mechanisms (12). Venoms that promote clotting can act by activating fibrinogen (Factor I), prothrombin (Factor II) (13), Factors X, V, IX and factor VIII (12, 14). Our results imply that the venoms of *B. cotiara* and *B. jararacussu* promote coagulation by thrombin-like enzymes and prothrombin activators, respectively, but further investigation is required to a complete understanding of their action.

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