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COAGULATION STUDIES OF BUNGARUS CAERULEUS VENOM *IN-VIVO*

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ABSTRACT

Bungarus caeruleus is a venomous snake that is responsible for most of the snakebites in India. In the present study, we report the coagulative study of *B. caeruleus* venom. The purpose of this study was to characterize the coagulation in crude krait venom. The results of this study indicate that hydrolysis of human fibrinogen, hydrolysis of casein and hydrolysis of gelatin. *B. caeruleus* venom proteases hydrolyse Aα and Bβ subunits of human fibrinogen. *B. caeruleus* venom upon re-calcification of citrated human plasma induces clot formation and reduces the clotting time. *B. caeruleus* venom also reduces the prothrombin time. These data indicates the involvement of *B. caeruleus* venom in inducing clot formation by activating Factors X and / or subsequent clotting factors in the down stream of coagulation pathway.

Keywords: Bungarus caeruleus, coagulation, fibrinogen, thrombin, prothrombin and re-calcification.

1. INTRODUCTION

The incidence of snakebite is high in India. About 15,000 patients are reported to be dying every year from venomous snakebites in India. Apart from mortality is the morbidity due to various complications [1]. Approximately 15% of 3000 species of snakes found worldwide are considered to be poisonous to human. There are about 216 species of snakes in India of

which 52 species are reported to be poisonous [2].The other common poisonous snakes, Naja naja and Daboia russelii are distributed all over India including Southern region of Western Ghats. However, none of these snakebites have been reported to cause local tissue damage due to myonecrosis leading to gangrene at lethal or sub lethal doses. Though myotoxicity is observed with D. russelii and other viperine snakes, the severity is less when compared to B.caeruleus. The agronomic conditions of Southern region of Western Ghats in the state of Karnataka, Tamilnadu and Kerala are suitable plantation crops such as coffee, cardamom, rubber and tea. They primarily feed on frogs, lizards and small rodents [3]. The four major venomous snakes chiefly responsible for the injuries and fatalities in India are krait (B.caeruleus), cobra (Naja naja), saw-scaled or carpet viper (Echis carinatus) and Russell's viper (Daboia russelli russelli) [4,5].

Various toxins and hydrolytic enzymes have been characterized from many snake venoms. The systemic toxins that have been characterized from snake venoms are neurotoxins (pre/post), cardiotoxins, myotoxins, cytotoxins, hemotoxins, toxins interfering in blood coagulation pathways (pro/anti coagulants) and toxins inducing hemorrhage in vital organs [6,7,8]. Hydrolytic enzymes commonly detected in all snake venoms are proteases, PLA2, hyaluronidases, 5'-nucleotidases, diesterases and acetylcholine esterases [9]. Among these enzymes protease and PLA2 enzymes are known to induce diversified pathological symptoms and are responsible for local tissue damage [10]. The anticoagulant effects of venoms have been mainly attributed to proteases and PLA2s [11,12]. 5'-nucleotidase an ubiquitous enzyme of snake venoms has been shown to act as a co-factor of hemorrhagic toxins and also known to affect hemostasis by modulating platelet functions [13,14,15].

Snake venom induced mortality and morbidity is a major health hazard worldwide, most prevalant in rural regions. Snake venom are Highly specialized saliva, Immobilize, kill and digest the prey, Enzymatic and non enzymatic toxins and Affects various organs including Haemostatic system. "Big Four" venomous snakes of India-*Naja naja, Daboia russelli, Echis carinatus* and *Bungarus caeruleus. Bungarus caeruleus* (Common Indian Krait) is the most deadliest of the "Big Four". Mainly consists of neurotoxic peptides. The haemostatic interference of *B. caeruleus* is least studied. Hence in this study the crude venom of *B. caeruleus* was studied for the interference in blood coagulation cascade.

2. MATERIALS AND METHODS

2.1. Materials

Fat free casein, gelatin, bovine serum albumin (BSA), *Escherichia coli*, W (ATCC 9637) strains, *p*-tosyl-L-arginine methyl ester (TAME), human fibrinogen, hyaluronic acid, and bis-*p*-nitrophenyl phosphate (bis-pNPP) phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma Chemical Company, St. Louis, MO, USA. ^{14}C-oleic acid and Ultima Gold Scintillation cocktail was obtained from Packard Bioscience, USA. Adenosine 5`-monophosphate (5`AMP) was purchased from SRL Chemical Company, Bangalore, India. Molecular weight markers were from Genei Private Limited, Bangalore, India. Lactate dehydrogenase, creatine kinase and prothrombin kits were purchased from Auto Span Diagnostic Center, Bangalore, India. Male Swiss Wistar albino mice weighing 20-25 g were obtained from Central Animal House facility at Department of Studies in Zoology, University of Mysore, Mysore, India. The animal care and handling were conducted in compliance with the national regulations for animal research. The animal experiments were carried out after reviewing the protocols by the animal ethical committee of the University of Mysore, Mysore. Fresh human blood samples were collected from healthy volunteers from the Department of Studies in Biochemistry, University of Mysore, India. All other reagents and chemicals used were of analytical grade. All the solvents were redistilled before use.

2.2. Source of venom

Bungarus caeruleus venom (500mg) was purchased from Irula Snake Catchers co-operative society. According to the need venom was weighed and diluted in 10mM Tris HCl pH 7.4. Further different concentrations of diluted venom was used for further experiments.

2.3. Human fibrinogenolytic activity

Fibrinogenolytic activity was assayed according to the method of Ouyang and Teng (1976) [16]. Human fibrinogen (50 µg) was incubated with purified protein in 10 mM Tris-HCl buffer pH 7.4 for different time intervals at 37 ^{o}C. The reaction was arrested by the addition of 20 µl denaturing buffer containing 1 M urea, 4% SDS and 4% β-mercaptoethanol and then subjected to 10% SDS-PAGE. Fibrinogen hydrolysis pattern was analyzed after staining with Coomassie Brilliant Blue R-250. For inhibition studies, purified protein was pre-incubated with or without specific protease inhibitors for 20 min followed by the addition of 50 µg fibrinogen. Further, the assay was carried out as described above.

2.4. Thrombin-like activity

Thrombin-like activity was determined according to the method of Denson (1971) [17]. The assay volume of 0.3 ml human fibrinogen (0.5 %) was treated with purified protein (2–20 µg) and the mixture was agitated gently against a light source to record the formation of the visible clot in seconds at room temperature. For control experiments, fibrinogen was incubated with the thrombin.

2.5. Re-calcification time

Re-calcification time was determined according to the procedure as described by Condrea *et al*., (1983) [18]. Fresh human blood sample was mixed with 0.11 M tri-sodium citrate in the ratio of nine parts to one. The mixture was centrifuged for 15 min at 500 *g*. The supernatant was used as platelet poor plasma (PPP) which was pre warmed to 37 ^{o}C before use. Different concentration of *B. caeruleus* venom in 10 mM Tris-HCl buffer pH 7.4 were separately added to 0.3 ml of PPP and incubated for one min. The clot formation was initiated by adding 30 µl of 0.25 M $CaCl_2$ and the time taken for visible clot to appear from the time of addition of $CaCl_2$ was recorded in sec. For control experiments Tris-HCl buffer alone was added instead of the venom sample.

2.6. Activated partial thromboplastin time (APTT)

Fresh citrated human plasma (0.1 ml) and various concentrations of purified protein were pre-incubated for 1 min by Chopin, N., et al., 2006 [19]. For APTT, 100 µl reagent (LIQUICELIN-E Phospholipids preparation derived from Rabbit brain with ellagic acid), which was activated for 3 min at 37 ^{o}C was added.

2.7. Prothrombin time

Prothrombin time was determined according to the method of Quick (1972) [20]. Fresh citrated human plasma (0.1 ml) was incubated separately with various concentrations of *B. caeruleus* venom. The plasma clotting time was recorded in sec after the addition of an optimal amount (0.2 ml) of brain thromboplastin.

2.8. Thromboelastography

Blood sample (typically 0.36 ml) is placed into a cuvette (cup) which is rotated gently through 4º 45´ (cycle time 6/min) to imitate sluggish venous flow and activate coagulation by Dirkmann D (2008) [21].

2.9. Statistics

Where appropriate, the results were expressed as mean±SD. Statistical differences were evaluated by Duncan's multiple range tests at $p<0.01$ using SPSS 11.0 statistical software.

3. RESULTS AND DISCUSSION

The venom of highly evolved snakes is a well-balanced mixture of systemic toxins and hydrolytic enzymes, which helps in immobilizing the prey and simultaneously to initiate digestion [22]. Figure 1 showed the hydrolysis of human fibrinogen by *B. caeruleus* proteases. Hydrolysis of casein by venom proteases is very common and hence casein is used as a general substrate to measure protease activity. However hydrolysis of gelatin and fibrinogen by proteases is not common and only proteases that induce hemorrhage, myotoxicity and affect blood coagulation process hydrolyses gelatin and fibrinogen. *N. naja* and *D. russelii* venom shown to hydrolyses only casein and fibrinogen but not gelatin even at very high protein concentrations [23]. Gelatin zymogram of *B. caeruleus* venom exhibits three distinct translucent bands indicating the presence of multiple forms of gelatinases. Out of the three distinct bands the middle one is predominant. This type of proteases is known to induce local tissue damages like hemorrhage and tissue necrosis [23]. There is a distinct difference in the activity of gelatinase, and hemorrhagic activities in *B. caeruleus* venom when compared to the more lethal *N. naja* and *D. russelii* venoms. Among the three venom samples only *B. caeruleus* exhibited MHD at 5 µg concentrations. It has been reported that *D. russelii* at the same concentration failed to induce hemorrhage but at 50 µg concentration very faint hemorrhagic spot was observed. *N. naja* failed to show any hemorrhagic activity even at 50 µg of protein [23]. These studies strongly suggest that the hemorrhagic activity is probably due to the observed gelatinase activity.

The hydrolysis of human fibrinogen suggests that venom proteases affect blood coagulation cascade. Majority of the venom proteases affecting coagulation hydrolyse fibrinogen and induce/prolong clot formation [24,25,26,27]. Several venom proteases with fibrinogenolytic activity have been reported and they preferentially hydrolyse Aα and Bβ subunits of fibrinogen [28]. Similarly, *B. caeruleus* venom proteases hydrolyse Aα and Bβ subunits of human fibrinogen (Figure 1). It has been reported that *D. russelii* and *N. naja* venoms proteases hydrolyses only Aα subunits at 10 µg protein concentration. *D. russelii* and *N. naja* venoms proteases failed to hydrolyze Bβ and γ subunits of fibrinogen even at 50 µg [23].

Normal blood coagulation cascade is a tightly regulated pathway operating by combined action of both extrinsic and intrinsic pathways. These pathways converge at factor X activation and further become a common cascade in generating fibrin clot. Figure 2 shows the increasing activity of thrombin in *B. caeruleus.* The involvement of extrinsic pathway is elucidated by prothrombin time and intrinsic pathway by re-calcification time [29]. Venom proteases that affect coagulation process may act in either of these pathways or both [28,30]. In order to know the involvement of these venom proteases in the blood coagulation cascade, the coagulation assay was performed by determining re-calcification and prothrombin time. *B. caeruleus* venom upon re-calcification of citrated human plasma induces clot formation and increases the clotting time is shown in figure 3. Figure 4 shows the increasing activity of activated partial thromboplastin time in *B. caeruleus. B. caeruleus* venom also increases the prothrombin time is shown in figure 5. These data indicates the involvement of *B. caeruleus* venom in inducing clot formation by activating Factors X and / or subsequent clotting factors in the down stream of coagulation pathway. Since *B. caeruleus* venom affected both re-calcification and prothrombin time, the activation of other factors simultaneously in the extrinsic and intrinsic pathways cannot be ruled out at this point. *D. russelii* and *N. naja* venoms also known to hydrolyse fibrinogen but affect coagulation cascade differently. *D. russelii* venom advances both the re-calcification and prothrombin time and induce procoagulant activity. Whereas *N. naja* venom prolonged both re-calcification and prothrombin time exhibited strong anticoagulant activity and [23].

Proteases which hydrolyses wide range of substrates such as gelatin, fibrinogen are involved in the induction of various pharmacological activities. Metalloproteases which are capable of hydrolyzing gelatin, which is a denatured product of extracellular matrix protein; collagen [31,32,24] is known to be responsible the observed strong hemorrhagic activity. The type of proteases involved in hemorrhagic activity was studied by neutralizing it using specific proteases inhibitors. The hemorrhagic activity was neutralized completely in the presence of all the three metalloprotease inhibitors. The serine protease inhibitor failed to neutralize hemorrhage clearly demonstrate that the observed hemorrhagic activity is mainly due to metalloproteases. This data correlates with the observation that, proteases which act on gelatin usually belongs to zinc containing matrix metalloproteinases [33,34,35,36]. Even though several toxins responsible for hemorrhagic activity have been isolated from N. naja and D. russelii venom their contribution to the whole venom toxicity is negligible.

Figure 6 shows the normal level of thromboelastogram of *B. caeruleus*. The pathology of hemorrhagins involves mainly the degradation of proteins in the extracellular matrix, connective tissue surrounding blood vessels and capillaries leading to tissue necrosis [37,38,39].

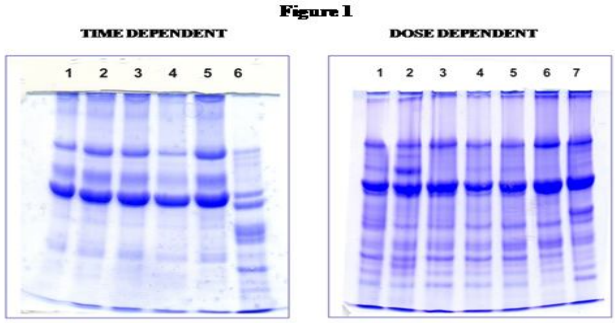

Figure 1 shows the human fibrogenolytic activity of *B. caeruleus* both dose dependent and time dependent.

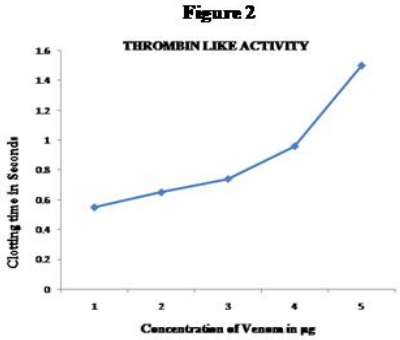

Figure 2 shows the increasing activity of thrombin time in *B. caeruleus.* Values are mean ± S.D. of three independent experiments.

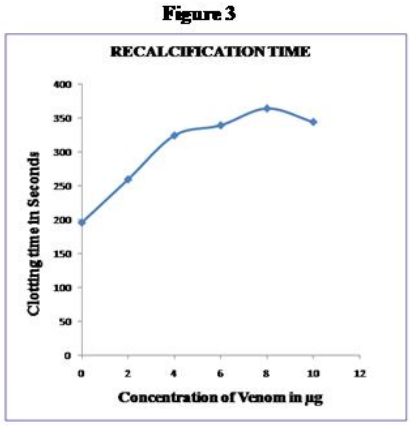

Figure 3 shows the Re-calcification time of *B. caeruleus:* *B. caeruleus* venom was pre-incubated with 0.3 ml of platelet poor plasma (PPP) at 37 ^{o}C for 1 min. 30 µl $CaCl_2$ (0.25 M) was added to the pre-incubated mixture to observe the clot formation. Plasma devoid of venom served as control.

Figure 4

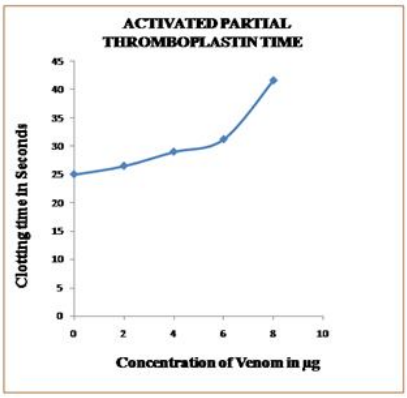

Figure 4 shows the increasing activity of activated partial thromboplastin time in *B. caeruleus.* Values are mean ± S.D. of three independent experiments.

Figure 5

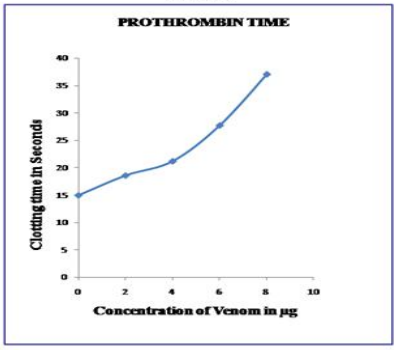

Figure 5 shows the Prothrombin time of *B. caeruleus: B. caeruleus* venom was pre incubated with 0.1 ml of human plasma and clotting time was recorded in sec after the addition of 0.2 ml of brain thromboplastin. Plasma devoid of venom served as control. Values are mean ± S.D. of three independent experiments.

Figure 6

THROMBOELASTOGRAPH

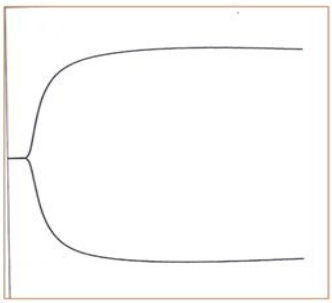

Figure 6 shows the normal level of thromboelastogram of *B. caeruleus.* Values are mean ± S.D. of three independent experiments.

DISCLOSURE OF INTEREST: The authors declare that they have no conflicts of interest concerning this article.

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