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COAGULOPATHIES IN *NAJA NAJA KARACHIENSIS* (BLACK PAKISTAN COBRA) BITES AND ITS EFFECT ON COAGULATION TESTS UPON STORAGE OF PLATELET-POOR PLASMA

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Abstract: The aim of this study was to evaluate the effect of venom from *Naja naja karachiensis* on platelet-poor plasma, activated partial thromboplastin time (aPTT), prothrombin time (PT) / international normalized ratio (INR), thrombin time (TT) and to evaluate its effect on clotting time upon storage of plasma for a specific time period with possible mechanism responsible for that. Prolongation of PT / INR, aPTT and TT was observed when different concentrations of venom were introduced due to degeneration of fibrinogen. Preservation of plasma for three months further prolong clotting time for coagulation tests, however, difference of PT and TT results were not very prominent as compared to aPTT. Minute concentrations of cobra venom and short as well as long storage of platelet-poor plasma badly affects the INR ratio.

Keywords: *Naja naja karachiensis*, coagulation tests, coagulopathies, INR.

Snake bites cause high mortality rates all over the world. In Pakistan, 20,000 annual deaths are reported due to snake envomination (1). Snakes belonging to genus Naja are represented by two species in Pakistan: *Naja naja naja* (Indian cobra) and *Naja naja oxiana* (Brown cobra). *Naja naja karachiensis* (Black Pakistan cobra) is a subspecies of *Naja naja oxiana* and found widespread in southern Pakistan (2). Several enzymes present in cobra venom specifically act by degeneration of fibrinogen. The most important groups of these include metalloproteinases and serine proteinases. The metalloproteinases (α-fibrinogenase or β-fibrinogenase) act by physical cleaving of fibrinogen while serine proteinases may be thrombin like enzymes (firinogenolytic) or plasminogen activating that cleaves fibrin and fibrinogen (3).

Literature review revealed that a large number of snake venoms have been tested *in vitro* for their clotting effects on human plasma (4–6). However, the effects of venom from *Naja naja karachiensis* have not yet been investigated**.** Therefore, the current study was undertaken for the first time in order to test venom from *Naja naja karachiensis in vitro* on human plasma. The anticoagulant effects of this venom were explained scientifically from the outcomes of general purpose coagulation tests including prothrombin time (PT) / international normalized ratio (INR) activated partial thromboplastin time (aPTT) and thrombin clotting time (TT). As reported earlier, storage of plasma samples for a specific time period results in a significant prolongation of clotting time along with INR (7). To the authors' knowledge, this is the first *in vitro* study to evaluate the effect of various storage conditions on PT / INR, aPTT and TT measurements in samples collected from healthy volunteers with different concentrations of black Pakistan cobra venom.

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EXPERIMENTAL

Venom

Cobra snakes (*N. naja karachiensis*) were purchased from the Jogi Colony of Haram Gate (Baba-Shero Wala) Multan, Pakistan. Venom from *Naja naja karachiensis* was collected by protocol as reported earlier (2). The glands of the snake below its eyes were compressed in dark environment at room temperature (38°C) to collect venom, followed by lyophilization of venom. The lyophilized venom was then stored in an air-tight, sterilized, colored glass container at 4°C until further use. Before use, venom was reconstituted in phosphate buffer saline (PBS) and its concentration was used in terms of dry weight. All experimentation was carried in Dr. Tahir Razi Laboratory for Toxicology, Bahauddin Zakariya University, Multan, Pakistan.

Platelet poor plasma (PPP) preparation and its storage

PPP was prepared from the blood of five healthy human male donors (mean age 23 ± 0.8 years; mean weight 61 ± 0.3 kg), collected in a tube containing liquid K_3-EDTA and centrifuged at ambient temperature for 15 min at 200 × *g*. The obtained platelet rich plasma was subjected to centrifugation for 20 min at 2000 × *g* in order to obtain fresh PPP on which different coagulation tests were performed. A portion of fresh PPP was stored at –20°C for 3 months and again aPTT, PT and TT tests were performed on thawed samples immediate-

Table 1. Effect of cobra venom on aPTT, PT/INR and TT of the platelet poor plasma (PPP) from five healthy volunteers collected freshly, immediately thawed (0 h), after 24 h and after 48 h at room temperature.

Plasma sample	Venom (μg/mL)	aPTT (s)	PT (s)	TT (s)	INR
Fresh PPP	0/control*	30	12	17	1.0
	125	85	26	19	3.2
	500	Fails to clot**	41	Fails to clot***	6.3
	1000	Fails to clot**	100	Fails to clot***	24
	1500	Fails to clot**	140	Fails to clot***	39.8
	2000	Fails to clot**	180	Fails to clot***	58
Immediately thawed PPP (0 h)	0	35	13	20	1.1
	125	133	30	23	3.95
	500	Fails to clot**	47	Fails to clot***	7.75
	1000	Fails to clot**	113	Fails to clot***	28.89
	1500	Fails to clot**	163	Fails to clot***	50.06
	2000	Fails to clot**	209	Fails to clot***	72.68
After 24 h	0	36	15	22	1.4
	125	179	29	23	3.76
	500	Fails to clot**	49	Fails to clot***	8.25
	1000	Fails to clot**	110	Fails to clot***	27.75
	1500	Fails to clot**	174	Fails to clot***	55.21
	2000	Fails to clot**	214	Fails to clot***	75.31
After 48 h	0	40	18	25	1.84
	125	233	37	26	5.41
	500	Fails to clot**	53	Fails to clot***	9.28
	1000	Fails to clot**	117	Fails to clot***	30.44
	1500	Fails to clot**	185	Fails to clot***	60.53
	2000	Fails to clot**	231	Fails to clot***	84.46

* Indicates standard values for the respective coagulation tests established in our laboratory. ** Indicates plasma fails to clot within 36 s as normal range for aPTT is 25–36 s. *** Indicates plasma fails to clot within 25 s as normal range for TT is 15–25 s.

ly (0 h), after 24 h and after 48 h of storage at room temperature (8).

Anticoagulant activity of cobra venom on fresh platelet poor plasma

PPP (100 µL) was added to an equal amount of venom (125–2000 µg/mL) followed by addition of 100 µL of $CaCl_2$ (0.025 M). Clotting time was recorded at 37°C. Increased fold in clotting time was calculated and saline was used as a control (9).

Determination of activated partial thromboplastin time

Plasma (100 µL) was incubated with 100 µL of aPTT reagent (Hemostat aptt-EL, Germany) at 37°C for 3 min and then 100 µL $CaCl_2$ (0.025 M) was added followed by recording of clotting time**.** A 100 µL of venom (125–2000 µg/mL) was added to determine its effects in both fresh and thawed samples of PPP for aPTT (10).

Determination of prothrombin time

Plasma (100 µL) was incubated with 200 µL PT reagent (PlasmascannR, Germany) for 1 min at 37°C. A 100 µL of venom (125–2000 µg/mL) was added to determine its effects on PT (10). INR ratio was calculated by formula $INR = R^{ISI}$ where ISI for PT reagent was 1.5.

Determination of thrombin time

A 100 µL of calcium-thrombin (Hemoclot tt, Hyphen BioMed, Germany) incubated at 37°C was mixed with 100 µL of pre-incubated plasma and clotting time was recorded. A 100 µL of venom (125–2000 µg/mL) was added to determine its effects on PPP for TT (10).

Statistical analysis

Analysis of variance (ANOVA) was used to determine the differences between the calculated parameters using SPSS, version 12.0. The level of significance was set at 0.05.

RESULTS AND DISCUSSION

Clotting time for PPP significantly ($p < 0.05$) increased with the increase in concentration of venom in a dose dependant manner. Increase folds in clotting time for PPP are shown in Figure 1. Storage of plasma for a period of three months prolonged the clotting time while venom further accelerated the process. Overall effect of venom concentration as well as storage of plasma on coagulation tests has been summarized in Table 1. The outcome from coagulation tests showed that venom from *Naja naja karachiensis* significantly ($p < 0.05$) prolongs aPTT, PT and TT, which helped to understand why victims suffer from homeostasis in envomination. TT prolongs when there is a deficiency of blood plasma protein fibrinogen (factor I). In this work, the increase in TT clearly indicated that deficiency of fibrinogen occurs after addition of venom. It shows that this venom possesses some enzymes which degenerate fibrinogen or it possesses thrombin-like activity, which decreases fibrinogen concentration from 100 mg/dL leading to homeostasis

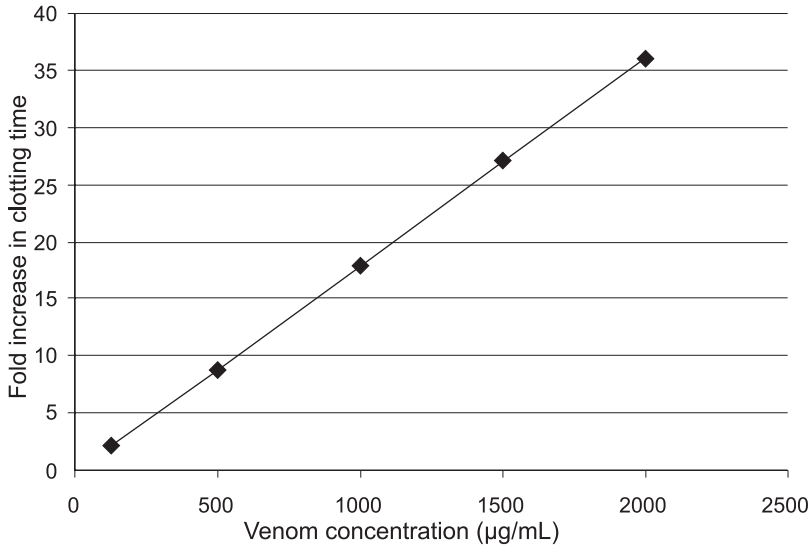

Figure 1. Folds increase in clotting time for platelet poor plasma (PPP) with different concentrations of venom from *Naja naja karachiensis*

as demonstrated previously for other venoms (11, 12). PT / INR and aPTT are used as screening tests and are surrogate markers of coagulation factor levels. The normal PT / INR was taken as presumptive evidence of normal factors II, V, VII and X, however, prolonged PT/INR due to cobra venom showed an evidence of deficiency of one or more coagulation factors. PT value of 12 s was equivalent to an INR of 01 while normal aPTT was 30 s. One stage clotting assays (PT and aPTT), and chromogenic and immunometric techniques are used to determine the deficiency of various coagulation factors. However, aPTT is widely used for measurement of coagulation factors VIII, IX and XI because the variable(s) alerting the aPTT of a test sample (relative to normal reference plasma) is the level of that coagulation factor (13). Thus, increased aPTT by venom in the current study is an evidence of deficiency of factors VIII, IX and XI. PLA_2 enzymes, which lead to turn down clotting factors in snake envomination, act on their specific target proteins (glycoproteins) present on cell surface by protein-protein interactions. PLA_2 binds to the γ-carboxyglutamic (Gla) domain of coagulation factors IX and X and thus impede their binding to phospholipid surfaces. Isothermal titration calorimetry technique has been widely employed to identify such target proteins (14).

Storage of blood samples for INR determination is not generally recommended because it affects coagulation tests unfavorably (8, 15). TT increased from 17 s to 20 s upon storage for 3 months when plasma was thawed immediately. Further delay in melting of congealed plasma for 24 and 48 h led to more prolongation of TT to 22 and 25 s, respectively. Significant ($p < 0.05$) increase in TT was recorded when various concentrations of venom were introduced. aPTT became more prolonged than PT/INR upon storage for 3 months under the same conditions as stated for TT. INR for freshly collected plasma was 01 which increased to 1.1, 1.4 and 1.84, respectively, when thawed immediately (0 h), after 24 h and after 48 h of storage. However, difference among PT results is not very clear compared to aPTT. *Naja naja karachiensis* venom caused further increase of clotting time.

CONCLUSIONS

From these studies it can be concluded that in all coagulation tests the addition of venom increased the clotting time in a dose dependent manner and also long time batching and freezing of plasma at $-20^{o}C$ prolongs the results for aPTT more drastically as compared to PT / INR and TT. Proteins' digesting enzymes or thrombin like activity of venom may be one of the causes for prolongation of general purpose coagulation tests.

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