Summary and Conclusions

There are about 600 species of snakes are venomous among the 3000 known species. The venomous snakes are classified into five families, Viperidae, Crotalidae, Colubridae, Elapidae and Hydrophidae. Snakebite is the predominant cause for unaccounted mortality and morbidity among the rural populations of developing and under developed countries. The lethal effects of envenomation include flaccid paralysis, systemic myolysis, coagulopathy, hemorrhage, renal damage and failure, cardiotoxicity, and local tissue injury at the bite site [White, 2004]. There are several components that have been so far isolated from snake venoms, among them, proteases, phospholipases A₂, L-amino acid oxidases, 5’-nucleotidases and many more enzymes, while, disintegrins, c-type lectin proteins and three-finger toxins are the non enzymatic agents.

Hemostasis is the body’s own defense response to vascular injury to stop bleeding by forming the clot, and dissolution of clot during wound repair mechanism. Vasoconstriction is the immediate response following vascular injury to slow down the blood flow and to prevent blood loss. Initially platelets adhere to the exposed collagen through the collagen receptors and aggregate to form loose platelet plug. Platelet activation and aggregation is elicited by several ligands, including vWF, collagen, ADP, α-thrombin, epinephrine and arachidonic acid through several distinct platelet receptors such as GPIb, integrin α2β1 (GPIa/IIa), GPVI, and protease-activated receptors (PAR1 and PAR2). Receptor-mediated platelet activation finally results in aggregation of platelets due to cross linking of platelets. This cross linking is due to the binding of fibrinogen to its specific platelet surface receptor GPIIb/IIIa (integrin αIIbβ3). Simultaneously blood coagulation take place due to tissue factor exposure and exposure of coagulation factors to abnormal surface (collagen) to form the cross linked fibrin and stabilizes the initially formed loose platelet plug to stop the bleeding. The clot dissolution takes place by the activation of plasminogen to plasmin by a tissue plasminogen activator (t-PA) to restore the normalcy.

Snake venom proteases, both serine and metalloproteinases interfere strongly in hemostasis. They may be procoagulants, anticoagulants, inactivators of serine proteinase inhibitors (serpins), platelet modulating agents (activating/inhibiting), fibrinolytic activators and hemorrhagins. Serine proteases showing only fibrinogenolytic (thrombin-like enzymes) activity forms abnormal fibrin clots. Some
of them have kininogenase (kallikrein-like) activity releasing hypotensive bradykinin. A few venom serine proteases specifically activate coagulation factor V, protein C, plasminogen or platelets. The metalloproteinases, belonging to the metzincin family, generally show fibrin(ogen)olytic and extracellular matrix-degrading (hemorrhagic) activities. A number of metalloproteinases have chimeric structures composed of several domains such as proteinase, disintegrin, disintegrin-like, Cys-rich and c-type lectin-like domains. A few venom metalloproteinases show unique substrate specificity toward coagulation factor X, platelet membrane receptors or von Willebrand factor.

In the current investigation, the large molecular mass P-III class SVMP, the NN-PF3 from the Naja naja (Indian cobra) venom is extensively characterized for its effect on hemostasis, including the mechanism of anticoagulant [Kumar et al, 2010] and platelet aggregation inhibition [Kumar et al, 2011] properties and compared with the newly purified low molecular mass basic metalloprotease (NN-BMP).

NN-PF3 was purified according to the established protocol described by Jagadeesha et al. [2002]. The molecular mass of NN-PF3 was found to be 67.81 kDa by MALDI-TOF mass spectrometry. It prolonged APTT, PT and TCT in a dose and time dependent manner, suggesting that it interfered in the common pathway of blood coagulation. NN-PF3 exerts anticoagulant activity through the direct degradation of fibrinogen and fibrin but not by plasminogen activation nor by thrombin inhibition. NN-PF3 preferentially cleaves the Aα chains followed by the Bβ chains of fibrinogen [Jagadeesha et al, 2002]. Further, NN-PF3 preferentially cleaved the α-chains and α-polymers of fibrin. In in vivo studies, following i.v. injection of NN-PF3, incoagulability of blood was observed with a MDD of 2.3 μg/g body weight of mice and the fibrinogen content was not detectable and prolonged bleeding time at MDD was also observed. However, the clotting property of the blood, fibrinogen content and the bleeding time were restored to normal after 24 h. NN-PF3 inhibited completely the collagen and partially the ADP and epinephrine induced platelet aggregation with respective IC₅₀ of 75 ± 5, 185 ± 10, and 232 ± 12 nM, but no inhibition was found in thrombin, arachidonate and ristocetin induced platelet aggregation. Further, native NN-PF3 and EDTA inactivated NN-PF3 inhibited collagen induced aggregation of washed platelets with respective IC₅₀ of 75 ± 4 and 180 ± 6 nM. The higher inhibitory effect of native NN-PF3 as compared to the EDTA
inactivated NN-PF3 suggested the enzymatic and non-enzymatic mechanisms of inhibition.

The mechanism of platelet aggregation inhibition involves the interaction of NN-PF3 with the collagen receptors but not with the fibrinogen receptors. The non-enzymatic mechanism of platelet aggregation inhibition is by binding to $\alpha_2\beta_1$ integrin, the platelet adhesion collagen receptor. NN-PF3 physically binds to but not proteolytically cleave the $\alpha_2\beta_1$ integrin in the western blot study. However, GPVI is the major collagen receptor and this receptor mediated signal transduction accomplishes the involvement of several cytosolic proteins which are the substrates of tyrosine kinases. The reduction in the intensity of several intracellular signalling phospho-tyrosine protein bands when anti-phosphotyrosine monoclonal antibody was used suggested the inhibition of GPVI mediated pathway of platelet activation. Considering the partial inhibition of platelet aggregation by EDTA inactivated NN-PF3 suggests that, the NN-PF3 is inhibited aggregation of platelets by physical binding to $\alpha_2\beta_1$ integrin and proteolytic cleavage of GPVI. Further, the observed inhibition was not due to fibrinogen depletion or due to interference by fibrinogen degradation products. The $\alpha_2$-macroglobulin did not inhibit but the therapeutic polyvalent anti-venom inhibited the enzymatic activity of NN-PF3. NN-PF3 did not degrade extracellular matrix molecules, such as collagen type-I, collagen type-IV and fibronectin.

The NN-BMP is a low molecular mass basic metalloprotease and was purified from the bound fractions of CM-Sephadex C-25 column chromatography. Homogeneity of NN-BMP was assessed by SDS-PAGE and RP-HPLC. The molecular mass of NN-BMP was found to be 13.87 kDa by MALDI-TOF mass spectrometry. NN-BMP was purified to 9.54 folds with 1.2 % protein recovery and 11% enzyme activity recovery. The specific activity was found to be $2.1 \pm 0.08$ units/min/mg. NN-BMP was active in the broad pH range of 7–9 and temperature range of 15–45°C and optimally active at pH 8.0 and at temperature 35°C. NN-BMP prolonged APTT, PT and TCT in a dose and time dependent manner, suggesting that this also interfered in the common pathway of blood coagulation. The anticoagulant property exerted was by degrading both fibrinogen and fibrin but not by plasminogen activation nor by inhibition of thrombin. The NN-BMP cleaved only the $\alpha\alpha$ chains of fibrinogen and the $\alpha$-chains of fibrin. The fibrinogenolytic activity was inhibited by
EDTA and 1, 10-phenanthroline but insensitive to PMSF, IAA and Pepstatin A. The NN-BMP did not exhibit any pharmacological properties such as hemorrhage, edema, myotoxicity and cytotoxicity. The extracellular matrix molecules, such as collagen type-I, collagen type-IV are not degraded where as fibronectin was degraded. The NN-BMP inhibited only the collagen induced platelet aggregation but did not affect the ADP, epinephrine and arachidonate induced aggregation. The complete aggregation inhibition of washed platelets was seen at 576 ± 12 nM with an IC$_{50}$ of 147 ± 10 nM. Pretreatment of NN-BMP with EDTA abolished the platelet aggregation inhibitory effect suggesting that the enzymatic activity of NN-BMP contributes to this effect.

The NN-BMP and NN-PF3 are SVMPs belonging to P-I and P-III classes respectively. Both the proteases exhibited anticoagulant effect by fibrin(ogen)olytic activity but not by plasminogen activation or thrombin inhibition. The NN-PF3 preferentially cleaved the Aα chains followed by the Bβ chains [Jagadeesha et al, 2002] where as NN-BMP cleaved only the Aα chains of fibrinogen. The NN-PF3 degraded α chains and α polymers, in contrast NN-BMP degraded only α chains of fibrin. The enzymatic/fibrinogenolytic activity of both the proteases was inhibited by EDTA. The metal chelator 1, 10-phenanthroline did not inhibit NN-PF3 (Jagadeesha et al, 2002) but inhibited NN-BMP, suggesting the structural differences. However, both proteases were insensitive to PMSF, IAA and Pepstatin A. NN-PF3 but not NN-BMP exhibited defibrinogenating activity and reduced the fibrinogen content. Both the proteases prolonged the tail bleeding time following i.v injection. Both the proteases interfered in platelet function inhibiting completely the collagen induced platelet aggregation. The NN-PF3 exerts platelet aggregation inhibition both by enzymatic and non enzymatic mechanisms whereas NN-BMP by enzymatic mechanism only. The polyvalent anti-venom inhibited both the proteases whereas α2-macroglobulin inhibited only the NN-BMP activity. Collagen type-I and collagen type-IV were not degraded by both the protease, where as fibronectin was degraded by NN-BMP.

In conclusion, the data from this study clearly suggests that the proteases NN-PF3 and NN-BMP from *Naja naja* venom strongly interfere in the pathophysiology of cobra envenomation causing hemostatic alterations most likely by reducing the coagulability of blood that results in less viscous blood which will accelerate the
distribution of toxins from the site of bite to the target tissues/cells. Nevertheless, in most cases the specificity guided targeting provide insight for exploring them as therapeutic molecules or prototypes for designing better and effective new therapeutics/drugs. Thus, hemostasis inhibition property of both NN-PF3 and NN-BMP involving depletion of clottable fibrinogen, dissolution of the preformed blood clot and the inhibition of platelet aggregation properties finds immense value to explore these two proteases as therapeutic tools or lead molecules in the management and treatment of life threatening clinical conditions such as stroke and angina (cardiac pain).