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Effect of *Rhipicephalus* (*Boophilus*) *microplus* ticks salivary gland proteins/peptides on mobilization of intracellular calcium ion

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Abstract
Mobilization of intracellular calcium ion plays a very important role in platelet aggregation. It occurs through P2Y1 receptor present on platelets surface. This mechanism of mobilization of intracellular pools of calcium is manipulated by ticks while feeding on their host's blood. So this study was conducted to know the effects of *Rhipicephalus* (*Boophilus*) *microplus* salivary gland proteins/peptides on release of intracellular calcium ion. Hundred pairs of salivary glands were dissected out and extract was prepared for fractionation of proteins/peptides by gel filtration chromatography. Fractions showing inhibitory activity were further tested for their effect on mobilization of intracellular calcium ion. For this fura-2 AM labelled bovine platelets were incubated with the inhibitory proteins/peptides fractions and then aggregation was stimulated with thrombin. Mobilization of intracellular calcium ion was studied by measuring the change in fluorescence at two excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. 8 fractions showed significantly higher inhibitory activity than antagonist and one fraction showed similar inhibitory activity on calcium ion release than that of antagonist. The present study suggested that possible mechanism of action of inhibition of platelet aggregation by proteins/peptides present in fractions could be through the inhibition of intracellular calcium ion mobilization.

Keywords: *Rhipicephalus* (*Boophilus*) *microplus* salivary gland, gel filtration chromatography, thrombin, anti-platelet aggregating proteins/peptides, intracellular calcium ion mobilization

Introduction
Ticks are of vast importance due to their ability to transmit a wide variety of infectious agents through the salivary secretions during feeding. They manage to remain attached to the host and actively engorge despite the hosts' rejection mechanisms like anti-haemostatic, anti-inflammatory and anti-fibrinoginolytic. Thus ticks can cause direct injury by piercing host skin resulting into production losses. Successful feeding of ticks relies on a sialome of bioactive chemicals located in their complex salivary glands and secreted in their saliva which they inoculate into the host during feeding. These chemicals in the salivary glands of the ticks could inhibit platelet aggregation and thus prevent the platelet plug formation. Platelet aggregation is important to stop bleeding from injured small blood vessels which is activated by multiple agonists including thrombin, ADP, collagen, and cathepsin G [17, 14, 3]. Mobilization of intracellular calcium ion also plays a very important role in platelet aggregation. It occurs when ADP binds to the P2Y1 receptor present on platelets surface which through the G protein-coupled mechanism mobilizes intracellular calcium ion resulting into activation of integrin αIIbβ3 receptor and thus subsequent binding of platelet with fibrinogen [15]. This mechanism of mobilization of intracellular pools of calcium can be prevented by ticks to feed on their host's blood.
Normally when platelet aggregation takes place intracellular free calcium ion play a critical role in platelet activation and thereby aggregation. So the mobilization of intracellular calcium ion is studied by measuring the change in fluorescence of Furs-2 AM labeled platelets.
Thrombin is the major serine proteinase in blood coagulation. It hydrolyzes fibrinogen to form the fibrin-clot, induces platelet aggregation and activates other coagulation factors to reinforce the coagulation cascade. Thrombin has high substrate specificity, determined by the selectivity of its deep active site and by a highly positively charged region in its surface, called exosite I, which participates in the interaction between the enzyme and substrates [16].
Several researchers have identified *Rhodnius prolixus* aggregation inhibitor 1 [4], pallidipin [21], variabilin [22] etc.

molecules from saliva of other haematophagus arthropods which inhibits platelet aggregation Our previous study suggested that platelets aggregation was inhibited by *Rhipicephalus* (*Boophilus*) *microplus* ticks salivary gland proteins/peptides *in vitro* [19] but the mechanism of action was still obscured. So, the present study was planned to know the effects of platelet aggregation inhibitory *R. microplus* salivary gland proteins/peptides on mobilization of intracellular calcium ion.

Material and Method

Preparation of ticks extract and pooling of bovine platelets

Adult female ticks of *R. microplus* were collected from villages around Hisar district of Haryana. Ticks were washed with normal saline and salivary glands were dissected out, preserved in liquid nitrogen followed by extract preparation and gel filtration chromatography [19]. Platelet aggregation assay was performed using bovine platelets as described by Francischetti *et al*. [4] with little modifications.

Intracellular calcium release in fractions having platelet aggregation inhibitory activity

Intracellular free calcium (Ca ion) was estimated by using fluorimetric method of Lagrue *et al*. [9] and followed by Francischetti *et al*. [4] with little modification. Platelets reconstituted with tyrode buffer'B' having concentration of 2 x 10^8 cells/ml and OD of 0.15 at 650 nm were labelled with Fura 2-AM (10µM) by incubating for 1 hr with gentle mixing by rotating the platelets on rotomix. Fura 2-AM labelled platelets were taken in wells of microtitre plate. Then the platelets in each well were incubated Gly-Pro-Arg-Pro amide (1mM) as antagonist and fractions having platelet aggregation inhibitory activity in rest of the wells of microtitre plate for 10 min at 37°C. Then aggregation was initiated with thrombin (0.5nM). Change in fluorescence was measured using two excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm by using fluorimeter. Intracellular calcium release inhibition in presence of salivary inhibitory peptide and Gly-Pro-Arg-Pro amide antagonist as well as thrombin agonist was calculated on the basis of changes in the fluorescence emitted by the Fura-2 labelled platelets.

Statistical Analysis

The data was analyzed by applying one way ANOVA test using SPSS statistical software for statistical significance [18].

Result & Discussion

R. microplus ticks salivary gland extract was fractionated by gel filtration chromatography into 120 fractions of 1.5ml each. Nine fractions showed platelet aggregation inhibitory activities using thrombin as agonist and Gly-Pro-Arg-Pro amide as antagonist. Thrombin act through proteins platelet activating receptors-1 (PAR-1) and platelet activating receptors-4 (PAR-4) [13, 6] to initiate aggregation while Gly-Pro-Arg-Pro amide suppresses the early steps of fibrin polymerization. The amide derivative of this peptide prevents the degradation of fibrinogen and fragment D by plasmin [20].

Then these fractions were tested for their effect on intracellular calcium ion release. It was found that 8 fractions i.e. fraction nos. 22, 25, 27, 31, 36, 38, 39 and 51 showed significantly higher inhibitory activity (23296.7±399.6 to 25397.0±299.4) than antagonist (28256.7±1127.4) induced intracellular calcium release inhibition (Table-1) and fraction no. 23 (27297.0±2198.3) showed similar inhibitory activity on calcium ion release as that of antagonist. So the proteins/peptides present in salivary gland of *R. microplus* ticks inhibit platelet aggregation by inhibiting mobilization of intracellular calcium ion. Further studies are needed to purify these inhibitory proteins/peptides and to elucidate mechanism of platelet aggregation inhibition by *R. microplus* salivary gland proteins/peptides.

Similarly, other researchers also isolated platelet aggregation inhibitors from haematophagous animals. Jandrot-Perrus *et al*. [7] analyzed the interaction of Convulxin, isolated from the venom of *Crotalus durissus terrificus*, with human platelets which was a potent platelet agonist that induced an increase in the intracellular Ca^{2+} concentration, granule exocytosis and aggregation. Belisario *et al*. [2] isolated Echistatin, which was found to competitively inhibit platelet $\alpha_{IIb}\beta_3$ receptor binding to fibrinogen. Lin *et al*. [10] determined that GABA specifically inhibited collagen-induced platelet activation accompanied by Ca^{2+} ion mobilization, PLCγ2, PKC, and may be involved in an endogenous negative feedback mechanism for platelet activation.

Ca^{2+} is a central and common second messenger downstream of most signaling pathways in platelets. Therefore, regulators of Ca^{2+} signaling might be interesting targets for platelet inhibition [1]. In present context, thrombin induce platelet aggregation was inhibited by proteins/peptides present in various fractions of *R. microplus* could be explained by less release of intracellular calcium ion [11, 12]. It is known that ADP (P_{2T}) receptors present on platelets plays an important role in platelet aggregation by intracellular calcium ion mobilization [8, 5]. In addition to the ADP (P_{2T}) receptor, ATP receptor also present on platelets surface. Platelets are a major source of extracellular ATP for hemostasis. The released ATP continuously activate newly recruited platelets which results into the production of platelet aggregates and could occlude the injured blood vessels. When platelets were stimulation with thrombin results in PIP_2 hydrolysis through the G protein-coupled mechanism, followed by production of IP_3 and DG. This in turn mobilizes intracellular calcium ion which activates $\alpha_{IIb}\beta_3$ receptor present on platelet surface. This activated $\alpha_{IIb}\beta_3$ receptor will binding to fibrinogen which works as a bridge between two activated platelets, leading to the formation of platelet aggregates. Molecules regulating Ca2+ entry in platelets a promising therapeutic target for the prevention and treatment of ischemic cardio- and cerebrovascular events.

Table 1: Variations in intracellular calcium (Mean±SE) release in response to various *R. microplus* salivary gland protein fractions.

Species	Agonist induced intracellular calcium release	Antagonist induced intracellular calcium release inhibition	Salivary gland Fraction no.								
			22	23	25	27	31	36	38	39	51
Rhipicephalus (Boophilus) microplus	36704.2± 1240.0[a]	28256.7± 1127.4[b]	24412.0 ± 629.7[cd]	27297.0 ± 2198.3[b c]	24424.7 ± 549.5[cd]	25397.0 ± 299.4[cd]	23296.7 ± 399.6[d]	23817.0 ± 467.7d[d]	24916.2 ± 359.9[cd]	25209.0 ± 211.8[cd]	25223.2 ± 457.9[cd]

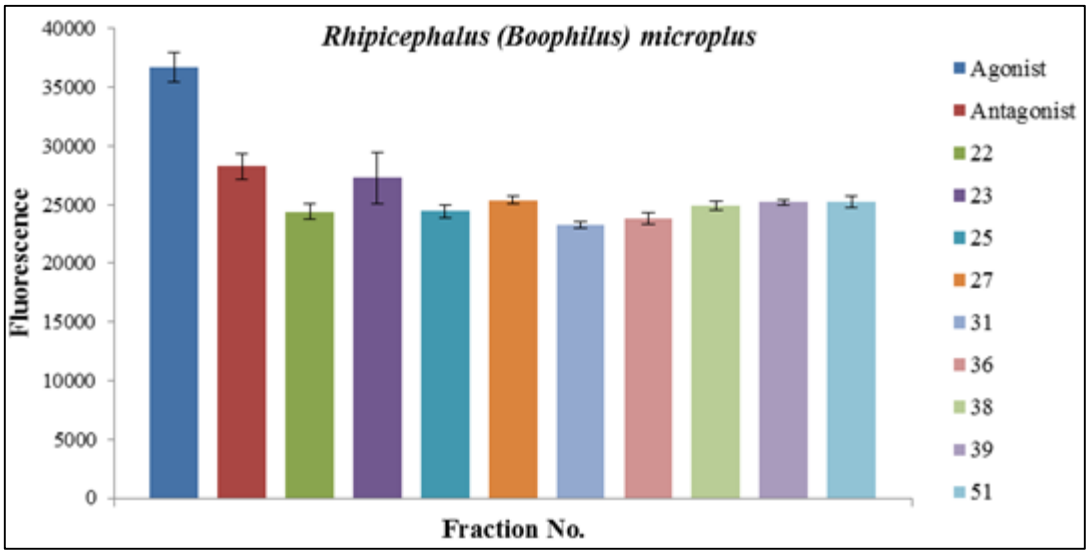

Fig 1: Effects of agonist, antagonist and *R. microplus* protein fractions on release of intracellular calcium

Conclusion

It is concluded that *R. microplus* ticks salivary gland fraction no. 22, 23, 25, 27, 31, 36, 38, 39 and 51 have intracellular calcium ion release inhibitory activities higher or equivalent (fraction 23) to antagonist. Calcium channels are widely found in living organism with varied functions. Deep insight into interaction between peptides/ proteins present in tick saliva with these calcium channels could help in preparation of certain drugs which can be used in prophylaxis and treatment of thrombus formation, hypertension, myocardial infarction and pulmonary embolism.

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