In vitro Assessment of Haemocyte and Thrombocyte Count from the Blood Clam of Anadara inequivalvis

K. Suganthi, S. Bragadeeswaran, K. Prabhu, S. Sophia Rani, S. Vijayalakshmi and T. Balasubramanian

Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai-608 502, India

Abstract: The identification and characterization of blood components and clotting time of Anadara inequivalvis were studied, specific identification procedure were made on the mollusc blood which have haemocytes and platelets. In order to find out more informations from blood components, clotting time, haemocyte count, platelet count and platelets aggregation, studies were carried out briefly. Our results illustrate the WBC and platelet cells, with aggregation of RBC, was identified by binocular (Motic) microscope showed platelet cells with 1.77X10¹⁰ cu mm of blood. The whole blood component possesses rich chemical elements (Ca, Zn, K, Cl, Cu, P, S and Mg). The platelets are involved in the coagulation cascade. In this view, we tried with molluscs blood plasma with unfractionated heparin of the same individual showed prolonged clotting time. The present study suggests that the molluscs blood plasma play relevant role in the coagulation process.

Key words: Haemocytes • Thrombocytes • Prothrombin • Formed elements • Plasma • Anadara inequivalvis

INTRODUCTION

Over the last 15 years, a number of new antithrombolytic agents have been developed and introduced in clinical medicine to address limitations in existing some drugs. Hemostasis or blood clot formation involves a series of coordinated complete interactions of injured vessels, platelets coagulation factors and fibrinolysis platelets are involved in blood clotting [1]. The coagulation process, also known as coagulation cascade, is a series of enzymatic reactions involving the sequential activation of a number of circulation plasma proenzyme proteins.

There is a very close interaction between the coagulation cascade and activated platelets. Platelet activation results in the exposure of “procoagulant” anionic phospholipids on platelet membrane surfaces. This serves as a template to facilitate the surface assembly of coagulation factor enzyme complexes. Thrombin generation is 30,000 times more efficient by surface complex assembly than random circulating coagulation factor interactions [2].

Adhesion of platelets due to damage of vascular endothelium exposes subendothelial collagen and von Willebrands factor (vWF). Plasma vWF binding to collagen becomes a strong adhesive protein which binds or anchors circulating platelets via platelet glycoprotein surface receptor. Heparin induced thrombocytopenia, a severe clinical pathological syndrome. In this heparin induced thrombocytopenia is due to the development of IgG [3] observed after administration UFH complication was characterized by a delayed decreased platelet count (after 5th day of treatment).

The use of anticoagulant anti platelet agents and intravenous Unfractionated heparin sulfate, is now common treatment of several diseases [3].Patients with retro-peritoneal hemorrhage were taking antiplatelet agents alone, there were no significant differences found in clotting time (CT). Now a days anti platelet drugs are being used. The preoperative risk of bleeding with anti platelet acute hemorrhage agents varies depends upon the surgical procedure. The antiplatelet agents do not have a specific antidote, but their reversal effects are often relies on transfusion of platelets and other blood product [5]. Platelet plays a key role hemostasis and occurs by influence of fibrin with factor II. The present investigation, the platelet levels in the systematic circulation in bivalve mollusc, whole blood elements were also determined.

Corresponding Author: Suganthi, K., Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai-608 502, India
MATERIALS AND METHODS

Study Area: The blood clam of *A. inequivalvis* were collected from the Vellar estuary (lat. 29° N, 79° 46' E) the shells were opened and the blood was collected with syringe. The whole blood 0.5 µl in the RBC pipette with 101 level of platelet fluid were taken for counting platelets. 1cumm of blood was stained with leishmans stain to observe WBCs (granulocytes and agranulocytes). Blood plasma was separated for clotting time.

Clotting Assay: In platelet aggregation assay, Platelet Rich Plasma (PRP) was prepared as follows. Mollusks blood *A. inequivalvis* was centrifuged at 1000rpm for 10minutes. The supernatant was collected as PRP. After PRP was removed the residual plasma was then centrifuged at 4000rpm was 10 minutes again and the supernatant was collected as platelet poor plasma (PPP). The aggregometer was used with 100µl of PRP and 0.5µl crude fractionated heparin in cuvette containing magnetic stirrer. The cuvettes were placed in the chamber of aggregometer and prewarmed at 37° for 5 minutes. The aggregation was recorded (ADP solution may added µl).

RESULTS

Blood fluid with Leishman stain showed different haemocytes with granules (Table 1). Scanning electron micrograph (SEM) of the platelet shown in Fig. 3. The cell

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Formed elements</th>
<th>Percentage (%)</th>
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<tbody>
<tr>
<td>1.</td>
<td>RBC</td>
<td>33,000</td>
</tr>
<tr>
<td>2.</td>
<td>WBC</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Granulocytes</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Neutrophils</td>
<td>2-4</td>
</tr>
<tr>
<td>5.</td>
<td>Eosinophil</td>
<td>0</td>
</tr>
<tr>
<td>6.</td>
<td>Basophil</td>
<td>0</td>
</tr>
<tr>
<td>7.</td>
<td>Agranulocytes</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Lymphocites</td>
<td>3-5</td>
</tr>
<tr>
<td>9.</td>
<td>Monocytes</td>
<td>2-4</td>
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Fig. 1: Blood components of *Anadara inequivalvis* at different peak levels

Fig. 2: Blood components of *Anadara inequivalvis* with cytoplasmic fragments

Fig. 3: High power electron micrograph of the platelet with cell organelles.
RBC-Red Blood Corpuscles; SCS-Surface Connecting System; DTS-Dense Tubular system; VDG-Very Dense Granules; MT-Micro Tubules; M-Mitochondria and G-Glycogen Particle
coats does not show any advantage, but in the inclusion and cell organelles are revealed. They include Mitochondria (M), Microtubules (empty), a single profile of the surface connecting systems (SCS), profiles of the Dense Tubular System (DTS), a single Very Dense Granule, (VDG) and Glycogen particles (G). The EDX analysis of the *A. inequivalvis*, the number of peaks represents the different elements shown in Fig 1. Fig. 4 and 5 shows platelet and differential, count. (Neubauer improved haemocytometer, 0.0025mm Sq superior marienfeld, Germany). Platelets are about 2µm in diameter. Part of the platelets were non-nucleated. The platelet stained more intensely with the dyes used in a blood smear; that part called the chromomere or granulomere. In a blood smear the platelet may be seen as individual units but more often they adhere to each other, forming small clusters (Fig. 5).

Clotting time of the mollusc blood with crude heparin denoted prolonged (45 min. /1hr). The results firstly reported here indicate that the presence of crude heparin increases the clotting time of mollusk blood. Here noted that after treated with crude heparin coagulation process distressed. (Stage II) as an antithrombin in the presence of plasma protein.

**DISCUSSION**

Platelets are the main source in haemostasis. If platelet concentration is decreased, the risk of hemorrhage is increased [6]. Blood elements are the chemical elements, which are involved in the building of organisms and are necessary for its proper functioning. They play important role in metabolism, act as biocatalysts for enzymes, hormones, proteins, bone and blood formation etc. [7]. In the present study also same observation was noted from the blood of *A. inequivalvis*. Platelets are small cytoplasmatic fragments within the circulating blood. These cytoplasmatic fragments do not contain nucleus [8]. Platelets function is in blood clotting, clot retardation and clot dissolution etc.

Maria [9] demonstrated the structure and anticoagulant activity of sulfated galactan from red
algae *Gelidium crinale*, there was specific structural sulfated polysaccharide required for the anticoagulant activity [10]. Investigated on the morphology and function of the Zebra fish; thrombocyte has demonstrated that similar parallels to the mammalian platelets and also proved characterization of the Zebra fish. Coagulation system of zebra fish and mammalian coagulation pathways are similar. The significant degree of functional homology between the Zebra fish and human haemostatic system strongly suggests that the Zebra fish is the relevant genetic model for mammalian haemostasis.

Whereas our UFH from *A. inequivalvis* crude sample with blood plasma showed prolonged clotting time, due to one of the coagulation factor (platelet factor III). Surprisingly, we observed marked differences in platelet or thrombocyte count. Raw heparin isolated from camel porcine intestine mucosa showed a somewhat reduced activity. In this investigation crude heparin from *A. inequivalvis* (UF), with platelet showed prolonged anticoagulant activity 45 minutes in 1mg/µl concentration showed more potential activity.

In vivo determination of anticoagulant activity are investigated and compared to UFH [11]. Platelet aggregation is induced by 10µm ADP. Reduction in platelets response was observed only for UFH. While there was no change in platelet aggregation on whole blood. An whole blood for any heparin derivatives nor for LMWH. Effect of UFH, its derivatives (HD) and LMWH on the platelet, aggregation induced in whole blood by ADP.

Treatment with UFH is known to affect screening test for Lupus Anticoagulant (LA). Therapy with plasma suggest significant result in LA patients [12]. Ana et al. [13] investigation made on anticoagulant and antithrombotic activities tested on *in vitro* and *in vivo* assay. Thrombin may induce smooth muscle cell proliferation both directly or by causing platelet to secrete, platelet-derived growth factor [14]. George et al. [15] effect of platelet and WBC, antiplatelet agent C.E (Reopro™) in a new test of PAF procoagulant activity on human blood with different amounts of C.E (0 to 6µg/ml) showed prolonged affect on platelet function. Their study also demonstrated clearly that to the addition of argatroban anticoagulant, which leads, prolonged platelet function [16]. In our study platelet aggregation of molluscs platelet rich plasma (PRP) may also induced by UFH.

David [17] PAF may influence not only platelet function but also leucocyte function. In addition to stimulation of aggregation and degranulation of neutrophils, PAF has been shown to mediated adhesion of neutrophils to thrombin [18]. Their findings demonstrate that changes in WBC may effect whole blood procoagulation action. Monocytes have been initiate coagulation through tissue factor dependant or independent mechanisms [12]. Lymphocytes are also influence PAF mediated acceleration of coagulation process. According to these studies, the role of specific WBC (leukocytes, monocytes and lymphocytes) population on whole blood *A. inequivalvis*. Our study assumed that blood platelets have its numerous effects in platelet aggregation and coagulation cascade. The present paper provides more details on the platelets found in the blood plasma of *A. inequivalvis*.

In the earlier study Machado et al. [19] demonstrated the presence of GAGs in the haemolymph and extrapallial fluid of *Anodonta cygnea*. The acetic properties of GAGs were suggested by Wheeler and Sikes (1989) as a relevant factor giving them great affinity for calcium binding. In our investigation blood component study showed Calcium peak level as maximum and also microphotograph clearly showed Ca spicules. Because Ca place a role in coagulation cascaded, stage II (prothrombin-thrombin) [20].

This is the first study to demonstrate that there are no comparative or identification studies on platelets molluscs and anticoagulant studies. The antiplatelet effect is prolonged in patients with renal impairment [21]. This antiplatelet agents do not have a specific antidote bleeding with ant platelet agents vary depends on the surgical procedure. In the event of acute platelet (ie ASA, Clopidogrel, ticlopidine) is often inadequate due to their irreversible effect on circulating platelets [22].

Anti platelet drugs being used due to its reversal effects (Schrader and Scoer, 1992) recover within 24-48 hrs. [23]. The quantification of total GAGs concentration was made in the haemolymph and extra pallial fluids in both summer and winter periods [24]. According to this statements *A. inequivalvis* blood component have blood platelet and UFH from the same animal having its clotting activity as maximum level. But we have not found any evidence from purified sample; have confirmed presence of blood platelet is necessary for the action of coagulation.

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