ORIGINAL ARTICLE

Flow Cytometric Analysis of Platelets Mepacrine-Labelled Dense Granules Among Individuals with Mild Bleeding Symptoms

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ABSTRACT

Introduction: Mild bleeding symptoms are commonly encountered in the general population & amongst individuals with platelet disorders. One of the possible causes is due to reduced number of dense granules synthesis in platelets and defective release of its contents. This study was aimed to evaluate platelets mepacrine-labelled dense granules storage and release using flow cytometry in healthy individuals and those presenting with mild bleeding symptoms. **Methods:** This study was conducted at the National Blood Centre (NBC) and Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM). Thirty- four individuals were recruited as controls (n=24) and patients (n=10). ADP-activated platelets and mepacrine-labelled dense granules was detected using flow cytometry. Results were expressed as mean fluorescent intensity (MFI) of mepacrine in resting and activated platelets; representing dense granules storage and release, respectively. Statistical analysis was considered significant if $p \leq 0.05$. **Results:** There was a significant difference of mean MFI between resting (1284.3 ± 91.8) and activated platelets (1233.8 ± 107.8) of overall respondents with mean difference of 50.5 ($p<0.01$). However, there was no significant difference of mean MFI in resting and activated platelets between controls and patients was observed. **Conclusion:** Results indicated there is no secretion defects in platelet dense granules among patients in comparison with controls. Flow cytometry provides alternative way of dense granule assessment in patients presented with mild bleeding symptoms.

Keywords: Dense granules, Mepacrine, Flowcytometric assay, Platelet disorders

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INTRODUCTION

Any defects affecting the haemostasis process can cause bleeding symptoms. However, mild-to-moderate bleeding disorders can be difficult to detect as symptoms like recurrent epistaxis or easy bruising are also common among healthy individuals (1). In a healthy population, the lowest values reported for bleeding symptoms were 25% in men and 46% in women (2).

These symptoms might be clinically insignificant yet pose an increased risk to develop severe haemorrhage after surgery or trauma (1,3,4). Mild bleeding disorders may be caused by abnormality of vWF and platelet defects (1). The prevalence of von Willebrand disease (vWD) with clinically significant symptoms is 1 in 1000 (5), whereas prevalence of platelet defects in the general population has not been established (6).

Inherited platelet secretion disorders (PSD) include defective release of dense granules contents in response to platelet activation and reduced number of dense granules synthesis. Granules secretion defect may be caused by abnormal platelet receptors, signaling pathway and/or granules biogenesis. A recent review reported that platelet secretion defects may be more prevalent than von Willebrand disease (vWD) which has been estimated to constitute more than 90 % of all inherited platelet disorders (7). In contrary, in a worldwide survey estimated suspected inherited platelet function disorders of approximately 1586 patients, 9.3% and 10.4% of families were diagnosed to have reduced dense granules storage (which include δ–SPD, Hermansky-Pudlak syndrome and Chediak-Higashi syndrome) and granules release defect, respectively (8), showing a low prevalence of the disorders globally.

Patients suspected of inherited mild bleeding disorder will undergo several laboratory investigations such

as platelet aggregation test to assess platelet function in response to specific agonists. Turbidimetric light transmission aggregometry (LTA) is considered the gold standard for platelet function testing (9). However, measurement of platelet granules specifically the δ-granules is also deemed important since PSD patients have been reported to display normal LTA responses (7).

Flow cytometric analysis of platelet dense granules using mepacrine as a fluorescent marker to evaluate both dense granules storage and release had been described (10,11). Here we reported dense granules evaluation among the local population comparing patients presented with mild bleeding symptoms suspected to have ADP receptor defect and normal controls using similar methods with some modifications.

MATERIALS AND METHODS

Ethical approval

This study was conducted at National Blood Centre (NBC), Kuala Lumpur and Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM), Serdang, Selangor. Institutional ethical approval was granted by UPM and Ministry of Health Medical Research Ethical Committee. Written informed consent was obtained from all respondents prior to participation in the study.

Study population and sampling

Patients who presented with bleeding symptoms and had platelet aggregation of <65% final aggregation in response to ADP 20 µM suggestive of familial inheritance and/or their family members were recruited. Less than 65% final aggregation is the local cut-off value for impaired aggregation in response to ADP agonist. They were identified from records of patients who were referred for platelet aggregation test at the NBC from January 2009 to May 2011. Controls were recruited from blood donors at NBC or healthy individuals without known bleeding disorders. Platelet light transmission aggregometry (LTA) was performed concurrently in all controls showing results within reference range. Individuals with known bleeding disorders due to drugs or other underlying medical/surgical problems or foreigners were excluded from the study.

Mepacrine release assay was part of the tests performed in this selected population of suspected ADP receptor defects in the center. The demographic and relevant findings which include LTA and coagulation results of the population were published earlier (12)

Blood sampling

Approximately 1.8 mL venous blood drawn into 2 mL 3.2% sodium citrate tube (BD Vacutainer®, USA).

Mepacrine solution preparation

Mepacrine staining solution was prepared using quinacrine dihydrochloride (Sigma Life Science, U.S.A) at 8.5 mM in Hank's Balanced Salt Solution (HBSS) without Ca2+ and Mg2+. Prior to addition of HBSS, the powder was dissolved in dimethyl sulfoxide (DMSO), EMSURE® ACS (Merck, USA). For 0.08 g mepacrine powder, approximately 500 µL DMSO was added. The solution was incubated at 37oC in water bath until it became transparent. The pH was adjusted to pH 7.4. The solution was kept at 4°C and stable for up to 4 months (11).

HEPES buffer preparation

HEPES buffer was prepared to maintain an optimal condition for the platelets. Components of the buffer include 20 mM HEPES solution, 137 mM NaCl, 2.7 mM KCl, 1mM MgCl2, 5.6 mM glucose and 1 g/L bovine serum albumin (BSA). pH was adjusted to 7.4 and kept at 4°C.

Sample preparation

Collected venous blood collected were processed within 4 hours after collection. Then 60 µL of blood were diluted 1:40 in HEPES buffer. Then, 123 µL of the diluted sample were transferred into plastic tubes; labeled as TA–activated platelets, TR–resting platelets. For identification of platelets population, 5 µL antibody PE-CD42b (BD Pharmingen™, USA) was added into both tubes and samples were gently mixed. This was followed by adding 20 µL ADP (BioData Corp. USA) to get a 10 µM final concentration to induce platelet activation (TA) and 20 µL HEPES buffer was added to TR. After 10 minutes, mepacrine was added into both tubes to get a 5.6 mM final concentration. ADP was used as platelet activator as the cohort of interest is those with suspected ADP defects; hence the research focuses on how exogenous ADP activates the receptors and its signaling pathway, thus influencing granules secretion. Samples were mixed and incubated in the dark at 37°C for 30 minutes. Later, the samples were stirred again and 50 µL samples were aliquoted into 5 mL (12x75 mm) polystyrene round–bottom tubes (BD Falcon™, New Jersey, USA) and diluted 1:20 in HEPES buffer. The aliquoted sample was kept in the dark at 37 °C until analysis within 24 hours. Unstained sample, PE-CD42b-stained only samples and sample stained with mepacrine-stained only samples were prepared as controls and diluted 1:20 in HEPES buffer.

Flow cytometric analysis

Analysis of prepared samples was done using BD FACSCanto™ II Flow Cytometer (BD Biosciences, San Jose, USA) and BD FACSDiva™ software (BD Biosciences, San Jose, USA). Detection of mepacrine was performed with FITC emission configuration which was set at 530 nm. Ten thousand platelets events were acquired for analysis. Platelet population was identified and gated based on CD42b expression (CD42b vs SSC plot). Prior to sample analysis, the flowcytometer would be calibrated using BD Cytometer Setup & Tracking

Beads (BD Biosciences, San Jose, USA).

Interpretation

Content of dense granules is presented as percentage of cells positive for mepacrine and intensity of mepacrine fluorescence. Difference in the percentage of positive cells and mean fluorescent intensity (MFI) of mepacrine was compared between resting platelets and after activation with ADP in patients and controls. Percentage of positive cells and MFI in the resting platelets represented the dense granules storage; whilst percentage of positive cells and MFI in the activated platelets indicated the dense granules were secreted or not.

Statistical Analysis

All results were statistically analysed with IBM SPSS Statistics 21 where one-way ANOVA, paired T-test and independent T-test were performed accordingly and reported as mean (standard deviation, SD). P ≤0.05 was considered significant.

RESULTS

Content of dense granules in platelet resting state and after activation by ADP 10 µM were evaluated using flow cytometry. Figure 1 illustrates the difference of cell count and fluorescence intensity in cells positive for mepacrine.

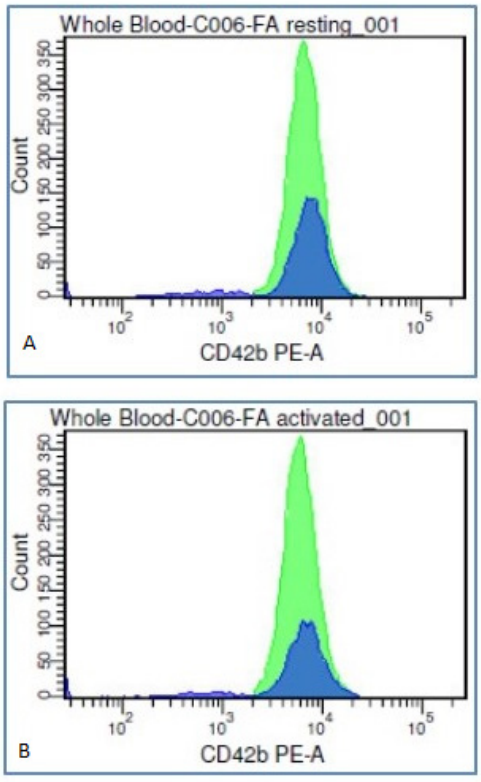

Figure 1: Cell count and fluorescence intensity in resting and activated platelets. A -resting platelet B-activated platelet.

MFI of mepacrine in resting platelet reflected platelet dense granule storage while reduced MFI in activated platelet reflected dense granule release, as illustrated in Figure 2. Referring to Table I, analysis of MFI in resting and activated platelet between genders showed no significant difference between both sexes. Same results were also obtained for analysis of MFI in resting and activated platelet across different races.

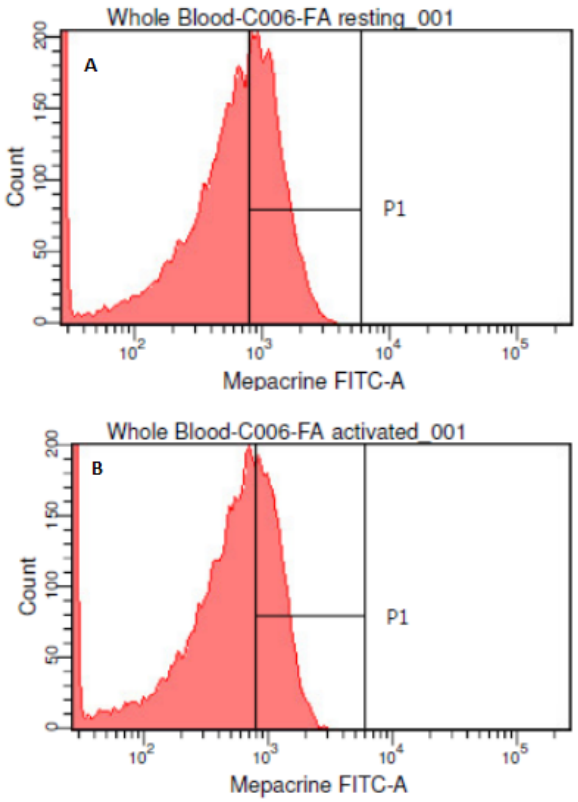

Figure 2: Dense granules content in resting & activated platelets. A -resting platelet B-activated platelet.

Table I: Mean MFI of mepacrine in resting and activated platelet by gender and race

Characteristic	Resting platelet		Activated platelet	
	Mean MFI (SD)	Coefficient of variation	Mean MFI (SD)	Coefficient of variation
Gender				
Male	1274.5 (77.5)	6.1%	1224.6 (100.2)	8.2%
Female	1300.1 (112.8)	8.7%	1248.6 (121.9)	9.8%
Race				
Malays	1286.3 (105.8)	8.2%	1229.5 (105.1)	8.5%
Chineses	1293.3 (79.7)	6.2%	1279.4 (138.2)	10.8%
Indians	1268.5 (70.5)	5.6%	1193.4 (62)	5.2%

(n = 34)

Referring to Table II, the result showed that there was significant difference between mean MFI in the resting (1284.3 ± 91.8) and activated platelets (1233.8 ± 107.8) of overall respondents with their mean difference was 50.5 ($p<0.01$). Reduction of mepacrine MFI in activated platelet indicate that released of dense granules contents had occurred. Despite that, no significant difference in mean MFI in resting and activated platelet between control and patient group was observed ($p>0.05$).

Table II: Mean MFI of mepacrine in resting and activated platelets between controls and patients

MFI	Mean (SD)	Coefficient of variation	95% CI for Mean	Mean difference of MFI between resting & activated platelets
Resting				
Overall	1284.3 (91.8)		1252.2 – 1316.3	
Control	1283.9 (96.3)	7.5%	1243.3 – 1324.6	Overall: 50.5 *
Patient	1285.1 (85.1)	6.6%	1224.3 – 1346.0	Control: 61.7
				Patient: 23.4
Activated				
Overall	1233.8 (107.8)		1196.2 – 1271.4	
Control	1222.2 (104.7)	8.6%	1178.0 – 1266.4	
Patient	1261.7 (115.9)	9.2%	1178.8 – 1344.6	

(n = 34) * p<0.01

Mean difference of MFI in resting platelet was 1.2 and activated was 39.5; whilst the mean difference of MFI in resting and activated platelets between control and patients group were 61.7 and 23.4 respectively.

DISCUSSION

Results from the current study showed that dense granules storage and release among patients with suspected ADP defects were normal in comparison with controls; indicating that their bleeding symptoms were not due to PSD related to ADP receptor or signaling pathways. Ironically, about 60% of patients suspected of platelet dysfunction does not have a defect. Apart from this, a specific platelet dysfunction was not diagnosed in approximately 34% of patients even after they had undergone all laboratory tests available at their respective regions (8). Despite of this, the utility of flow cytometry for analysis of dense granules seems promising. Reduction of platelet mepacrine staining was seen in patients with significant dense granules deficiency such as in Hermansky-Pudlak syndrome and δ–SPD when compared to controls and release of mepacrine–stained dense granules could be detected by flow cytometry (10,11,13).

Generally, to achieve a definitive diagnosis of PSD, a combination of different methods of assessment such as lumiaggregometry, serotonin uptake and release as well as direct visualization of dense granules by fluorescent or electron microscopy are recommended (10,13). Wall and colleagues (1995) highlighted several advantages of utilising flow cytometric analysis and mepacrine staining for assessing platelets dense granules as such that it could serve as a rapid diagnostic tool to determine granules storage and release compared to conventional granules enumeration using fluorescent microscopy (13); minimal blood volume is required making it convenient for paediatrics sampling and analysis could be performed even in individuals with low platelet counts.

It should be noted that mepacrine could interfere in the signal transduction pathway for dense granules release thus requiring the usage of a strong agonist to study its release, however in this study it was shown that even a weak agonist like ADP is able to cause granule release. Approximately 80% of dense granules were released upon activation with thrombin, a strong agonist (10). The present study demonstrated that a weak agonist such as ADP could activate platelets with subsequent release reaction of dense granules but the released granules from platelets were not as high as seen in the activation by thrombin with mean difference of MFI was 61.7 in controls and 23.4 in patients. ADP induces significant degranulation only if platelets are allowed to aggregate. Relatively little degranulation is observed in response to ADP as sample preparation for flow cytometric method was designed to prevent platelet to platelet aggregation. On the contrary, collagen and thrombin are able to induce maximal degranulation in the absence of platelet aggregation (14).

This study was limited to a small number of patients which may contribute to the non-significant difference in results. It is therefore recommended to increase the number of patients in any future studies. Utilization of CD62P as platelet activation marker and incorporation of CD63 as marker of degranulation of activated platelet would be useful.

CONCLUSION

Mepacrine–stained dense granules storage and release assessed by flow cytometry were similar among patients with suspected ADP defects and healthy individuals. The flow cytometric mepacrine–stained dense granules storage and release analysis method is potentially useful for specific assessment of individuals suspected with platelet secretion defect involving dense granules. For improvement of methodology protocol, use of strong agonist such as collagen is preferable in addition to the use of a different fluorochrome for identification of platelet population based on CD42b.

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