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Impaired Platelet Aggregation to Adenosine Diphosphate (ADP) Agonist in National Blood Centre, Malaysia

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ABSTRACT

Bleeding symptoms are common in population. However, it is difficult to assess the clinical relevance of mild bleeding disorder. One of the causes of mild bleeding disorder is due to platelet function defects either related to abnormality in the membrane receptor on platelet or its signal transduction pathway. Platelet adenosine diphosphate (ADP) receptor, namely $P2Y_{12}$ played a central role in platelet activation. The receptor is important as it is one of the therapeutic targets for antithrombotic drugs. The objective of this study was to determine causes of impaired platelet aggregation to ADP agonist, socio-demographic and clinical characteristics among patients in National Blood Centre. Data were recorded from 1st January 2009 to 31st May 2011. The results showed 32 (13%) out of 251 platelet aggregation test performed had impaired platelet aggregation to ADP (20 μM) using local cut off point of 65%. Out of those, 16 (50%) possibly had inherited ADP receptor dysfunction. Sixteen (50%) patients had secondary causes of impaired platelet aggregation to ADP either associated with von Willebrand disease (vWD), glycogen storage disease (GSD) or acquired platelet dysfunction with eosinophilia (APDE). Majority of patients were Malays (78.1%), with slight female preponderance (59.4%) and age ranged from 1-44 years old. Results obtained from this study could serve as a reference for a national registry on mild bleeding disorder related to platelet function defects. This knowledge is also important for future research related to platelets as biomarkers or potential therapeutic application in various diseases.

Key words: Platelet, impaired aggregation, ADP agonist, platelet function defects, mild bleeding, P2Y12 receptor

INTRODUCTION

Bleeding symptoms are frequent in the population; however there are inherent difficulties in diagnosing inherited mild bleeding disorders related to platelet function defects (PFD). Patients might present with mucocutaneous bleeding or asymptomatic. The type of bleeding in PFD is also indistinguishable from those patients with mild clotting deficiencies. An overwhelming majority of patients with PFD had platelet aggregation defects to collagen, ADP, arachidonic acid (AA) or epinephrine. The defect is either in combination or to a single agonist (Quiroga *et al*., 2007).

In PFD with impaired platelet aggregation to ADP agonist; patients have either lack of secondary wave aggregation, reduced reversible platelet aggregation or reduced irreversible platelet aggregation (Cattaneo, 2011a). The abnormality is similar to 'clopidogrel-like effect' seen in patients who are on anti-$P2Y_{12}$ drug therapy.

Platelet ADP receptors, namely $P2Y_1$ and $P2Y_{12}$ are responsible for the initiation of platelet aggregation in the primary haemostasis. $P2Y_1$ is responsible for the shape change of platelet whilst $P2Y_{12}$ is the main receptor responsible for a sustained, full aggregation response to ADP and formation of a stable platelet plug (Cattaneo and Gachet, 1999). Patients with reduced quantity or have a defect in the receptor will demonstrate impaired platelet aggregation to ADP. To date, defect in human $P2Y_1$ had not been identified whilst 13 cases were published describing mainly about mutation in the $P2Y_{12}$ receptor gene (P2Y12R) (Cattaneo, 2011b).

The $P2Y_{12}$ receptor is currently the sole receptor used as an antiplatelet targeted therapy in patients with cardiovascular disease. The use of anti $P2Y_{12}$ as one of the main therapy as a platelet inhibitor in preventing further coronary thrombosis has made a breakthrough in the management of cardiovascular disease. Despite that, there were patients who had impaired response to anti $P2Y_{12}$ (e.g., clopidogrel) and thus were not protected against subsequent coronary event (Cattaneo, 2010).

The prevalence of bleeding disorders related to PFD is unknown. Revel-Vilk and Rand (2010) reviewed that von Willebrand disease (vWD) is by far the most common inherited bleeding disorders, affecting around 1% of general population as reported by Werner *et al.* (1993). However, recent studies showed a lower prevalence of vWD (Bowman *et al.*, 2010) which indicated larger inherited bleeding disorders in PFD were probably due to defects in the receptor, secretion or signalling pathway (Hayward *et al.*, 2006).

National Blood Centre (NBC) is currently the national referral centre for laboratory tests related to platelet function defects. Thus, the main objective of this study was to gain information on patients with impaired platelet aggregation to ADP seen in the centre. The specific objectives of the study are to identify possible causes of impaired platelet aggregation to ADP agonist and to determine socio-demographic and clinical/laboratory characteristics among the subjects. The study would provide a baseline data on impaired platelet aggregation to ADP agonist among three main races in Malaysia.

MATERIALS AND METHODS

Study design: This is a descriptive study amongst patients and family members who were referred to National Blood Centre, Kuala Lumpur for platelet aggregation test. The sampling population was patients and family members who underwent platelet aggregation study for bleeding symptoms or screening, respectively from 1st January 2009 to 31st May 2011. The inclusion criteria were (1) patients and family members who underwent platelet aggregation study and (2) had impaired platelet aggregation to ADP agonist. Impaired platelet aggregation to ADP is defined locally as less than 65% of platelet aggregation (ADP 20 μM) in comparison with control. The exclusion criteria were (1) non Malaysia citizen (2) incomplete pro-forma.

Data collection: Secondary data were retrieved from patient's information sheet, platelet aggregation test result, full blood count and coagulation screening report. The recorded items were socio-demographic profile, haemoglobin level, eosinophil percentage, platelet count, mean platelet volume, bleeding time, prothrombin time (PT), activated partial thromboplastin time (APTT),

thrombin time (TT), fibrinogen assay, factor VIII levels, von Willebrand factor antigen (vWF:Ag), collagen binding assay (CBA), blood group, percentages of platelet aggregation with various agonists and pattern of platelet aggregation.

Statistical analysis: Statistical analysis was performed using PASW Statistics 18. Results were reported descriptively as frequency, percentage, median value and interquartile range (IQR). Kruskal-Wallis followed by Mann-Whitney test was computed to look for differences in socio-demographic characteristics between groups. To look for differences in clinical manifestations and laboratory characteristics between groups, chi-square test was used. All tests were taken as significant if $p<0.05$.

RESULTS

There were 251 patients and their family members referred for platelet aggregation tests at NBC from 1st January 2009 till 31st May 2011. A total of 32 (13%) patients and family members were found to have impaired platelet aggregation to ADP agonist (Fig. 1a). Based on their clinical presentation and laboratory results, patients with impaired aggregation to ADP were grouped into four different causes. The groups were possible inherited (familial) ADP dysfunction and acquired ADP dysfunction related to vWD, glycogen storage disease (GSD) and acquired platelet dysfunction with eosinophilia (APDE) (Fig. 1b). Patients with inherited ADP dysfunction were the

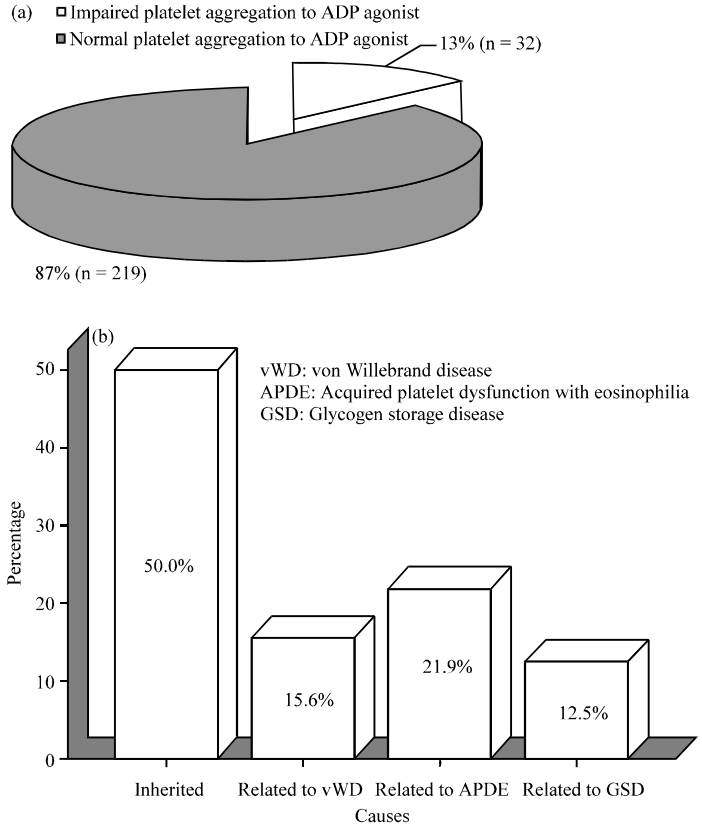

Fig. 1(a-b): (a) Prevalence of impaired platelet aggregation to ADP agonist (b) Distribution of subjects according to causes of impaired platelet aggregation to ADP agonist (n = 32), n: Number of patients

Table 1: Socio-demographic data of patients with impaired platelet aggregation to ADP (20 μM) according to possible causes (n = 32)

Socio-demographic characteristic	Inherited (familial) ADP dysfunction n (%)	Acquired ADP dysfunction (%)			
		vWD	APDE	GSD	Total
Age (year)					
Median (IQR)	31.5 (26)[a]	27 (29)	6 (7)[a]	5.5 (9.8)	11 (31)
Min-max	2-44	5-37	2-10	1-11	1-44
Race (%)					
Malay	12 (75.0)[b]	4 (80.0)	5 (71.4)	4 (100)	25 (78.1)
Chinese	3 (18.8)	1 (20.0)	1 (14.3)	0	5 (15.6)
Indian	1 (6.3)	0	1 (14.3)	0	2 (6.3)
Gender (%)[c]					
Male	7 (43.8)	3 (60.0)	3 (42.9)	0	13 (40.6)
Female	9 (56.3)	2 (40.0)	4 (57.1)	4 (100)	19 (59.4)
TOTAL	16 (50)	5 (15.6)	7 (21.9)	4 (12.5)	32 (100)

[a]There was significant difference of age between four groups (p = 0.014). Further analysis showed that age was significantly different between subjects with inherited ADP dysfunction and acquired ADP dysfunction related to APDE (p = 0.013), [b]There was significant difference of races among subjects with inherited ADP dysfunction (p = 0.002), Further analysis revealed that inherited ADP dysfunction was significantly higher in Malay than Indian and Chinese (p<0.001), Analysis among subjects with vWD and APDE groups showed no significant difference in races distribution, [c]Analysis on gender showed no significant difference between male and female in all groups.

highest (50.0%). Patients with acquired ADP dysfunction related to GSD contributed only 12.5% of the total patients. Table 1 showed socio-demographic characteristics of subjects with impaired platelet aggregation to ADP. Majority of patients were Malays (78.1%) with slight female preponderance (59.4%). Age of patients ranged from 1 year to 44 years old. Further details of the socio-demographic characteristics among subjects were also shown in Table 1.

Referring to Table 1, 16 (50%) out of the 32 patients were in the possible inherited (familial) ADP dysfunction group. As shown in Table 2, majority (n = 11) of the patients were asymptomatic (as they were the family members who were screened for bleeding disorders). Among patients who were symptomatic, they did not require any blood product infusion except for one patient. Family members involved were either asymptomatic or had previous history or family history of trivial bleeding. The laboratory investigations showed that majority (n = 12) had normal bleeding time, PT and APTT as shown in Table 2. Percentage of platelet aggregation ranges from 7-61% aggregation and majority of platelet aggregation pattern were of reduced reversible aggregation (Table 2). Their haemoglobin, platelet count, mean platelet volume were within the reference range and platelet morphology was normal.

As shown in Table 1, there were 6 (15.6%) patients suffered from acquired impaired platelet aggregation to ADP associated with vWD. Table 2 showed that all of them had mucocutaneous bleeding but with normal bleeding time, yet with slightly prolonged APTT. Platelet aggregation percentages of three patients ranged from 6-35% and two patients had 56-64% with reduced reversible aggregation pattern. Two patients were diagnosed as type II vWD whilst the rest of patients were labeled as vWD type I although the levels of vWF antigen were within normal range, possibly they were diagnosed as vWD type I in other laboratories (Table 3). Other laboratory characteristics which were important in vWD screening were also illustrated in Table 3.

The number of patients with impaired platelet aggregation to ADP with GSD and APDE, was 4 (12.5%) and 7 (21.9%), respectively (Table 1). Majority of patients (n = 5) with APDE presented with mucocutaneous bleeding for certain duration i.e., weeks to maximum of two months. Some were asymptomatic. Bleeding time and PT were within the reference ranges. Eosinophils

Table 2: Frequency of clinical and laboratory characteristics of patients with different possible causes of impaired platelet aggregation to ADP (20 µM)

		Acquired ADP dysfunction related to		
Characteristic	Inherited ADP dysfunction	vWD	APDE	GSD
Clinical manifestations#				
Symptomatic^	5	5	5	2
Asymptomatic	11	0	2	2
Bleeding time (BT)				
Normal	14	5	5	2
Prolonged	2	0	2	2
Median (IQR): 2.0 min (1.99)				
Prothrombin time (PT)				
Normal	14	3	6	3
Prolonged	2	2	1	1
Median (IQR): 10.55 sec (1.2)				
Activated partial thromboplastin time (APTT)				
Normal	6	0	3	2
Prolonged	10	5	4	2
Median (IQR): 33.2 sec (5.2)				
Percentage of platelet aggregation to ADP 20 µM				
6-35%	8	3	0	1
36-55%	7	0	5	2
56-64%	1	2	2	1
Pattern of aggregation				
Reduced reversible	11	1	2	3
Reduced irreversible	5	3	5	0

^Symptomatic-patients showed symptoms such as gum bleeding, bruises (including spontaneous bruises), per rectal bleeding, recurrent mucocutaneous bleeding, menorrhagia or epistaxis, #Asymptomatic patients was significantly higher in inherited ADP dysfunction than acquired ADP dysfunction ($p<0.05$)

Table 3: Distribution of patients by specific laboratory characteristics in acquired ADP dysfunction related to vWD (n = 5)

Characteristic	No. of patients
von Willebrand disease (vWD)	
Type I	3
Type II	2
Factor VIII Ag	
Normal	2
Reduced	3
Median (IQR): 48.5% (29.2%)	
Von Willebrand factor (vWF) Ag	
Normal	4
Reduced	1
Median (IQR): 58.3% (16.5%)	
CBA	
Normal	3
Reduced	2
Median (IQR): 51.1% (58.8%)	
Blood group (Rh+ve)	
A	-
B	2
AB	-
O	3

percentages ranged from 4.4-to 8.3% with median 6.5% (IQR = 2.7%). Among patients in GSD group, one patient had intraoperative bleeding during liver biopsy and needed four units of platelet transfusion The rest were asymptomatic and another patient probably had impaired ADP dysfunction too as both parents had impaired platelet aggregation to ADP. Comparing some of the laboratory characteristics between inherited and acquired ADP dysfunction showed that the characteristics were independent of the causes of the bleeding disorders. For clinical manifestations, patients with inherited ADP dysfunction were significantly asymptomatic. Further descriptions of results were shown in Table 2.

DISCUSSION

Platelet aggregation test was done using ADP concentration of 20 μM and the reduced aggregation response was taken as below 65% of aggregation. There is no standardized definition of reduced or impaired platelet aggregation response and it is mainly based on the local laboratory performing the test (Harrison *et al*., 2011). The prevalence of impaired platelet aggregation to ADP in National Blood Centre is 13% which is higher than in findings reported by Zhou and Schmaier (2005) whereby their prevalence was 6.5%. Their study considered aggregation response to ADP of below 55% as a reduced response. If we used similar cut off point as indicated in the study, the prevalence is reduced to 10.8% (n = 27). Median age of inherited ADP dysfunction was 31.5 years and APDE was 6 years. Acquired platelet dysfunction with eosinophilia (APDE) was known to mainly affecting the paediatric age group where parasitic infestation was common (Lee, 2012; Zaki, 2011). Subjects with inherited ADP dysfunction displayed no specific age group distribution as some patients were asymptomatic. They were the parents whom only being identified to have impaired aggregation to ADP after went for platelet function test screening with their symptomatic child.

The highest numbers of patients in inherited ADP dysfunction group were Malays (n = 12) compared to Chinese and Indians. Malaysia population and housing census report showed that Malays (63.1%) were the predominant ethnic group from 67.4% of bumiputera. While Chinese and Indians constituted 24.6 and 7.3%, respectively of total Malaysia population (DSM, 2010). There was possibility that genetic differences among the three main ethnic groups contributed to such ethnic distribution of the patients.

As for the possible inherited ADP dysfunction, the prevalence is estimated to be 6.4%. However, further platelet function test and secretion assay together with molecular characterization need to be elucidated from the family involved. The haemoglobin, eosinophil percentage and platelet levels were within the reference ranges provided in the National Blood Centre (results not shown), as to show that the symptom experienced by these patients did not give rise to bleeding which necessitate blood product infusion.

Inherited or familial ADP dysfunction was first published around two decades ago with a cumulative incidence of 13 cases published. The main defects were related to mutation such as frame shift, deletion or single nucleotide mutation in the $P2Y_{12}$ receptor gene (Cattaneo, 2011b). In this present study, most patients presented with mucocutaneous bleeding such as prolonged recurrent bleed from mucocutaneous tissue either spontaneous or post-invasive procedure such as dental extraction or circumcision. However, the family members were asymptomatic or had previous history of mucocutaneous bleeding. This showed that there is a spectrum of bleeding which is considered as very mild and blood product infusion is unnecessary in most of these patients. Only one patient had repeated admission for bleeding symptoms which necessitates treatment with

DDAVP and platelet transfusion. Both his parents had reduced platelet aggregation to ADP agonist. His ADP receptor dysfunction needs further identification and characterization using molecular technique.

Most patients presented at childhood with minimum age of 2 years old. Most patient had a normal bleeding time (<3 min) except for one patient who had a bleeding time of 6 min. This showed that bleeding time may not be a sensitive test for detection of platelet dysfunction related to ADP defects. Furthermore, it is rather invasive which may cause inconvenience especially to young patient and has poor reproducibility. The basic coagulation screening such as PT, APTT, thrombin time (TT) and fibrinogen were within the local reference range except for one patient who had a prolonged APTT with his specific factor assays are within reference range (results not shown) together with his vWF antigen and activity assays.

For the acquired ADP dysfunction, five individuals (two from a family) were noted to have ADP dysfunction apart from their von Willebrand disease. Three patients had vWD type I with type O blood group. It was widely accepted for decades that patients with O blood group had lower vWF level than other blood groups which demonstrated the most in patients with vWD type I (Gill *et al*., 1987). But vWF of the three patients were within reference range. One patient was postulated to have compound heterozygotes of vWD type II-ADP dysfunction inherited from his father and mother, respectively. All patients had their bleeding time within reference range but prolonged APTT. The vWF antigen and CBA assays in two patients who had Type II vWD were reduced. The platelet aggregation test performed showed reduction of platelet aggregation to ADP varied in the range of 11-59% and most patterns seen were of reduced reversible aggregation. In National Blood Centre, CBA and low dose ristocetin induced platelet aggregation (RIPA) are currently the activity assays performed for diagnosis of vWD.

Acquired platelet dysfunction with eosinophilia (APDE) is a well known entity amongst population in tropical area especially South East Asia where parasitic infestation was endemic. Prevalence of parasitic infestation in Malaysia ranges from 40.4-57.8% for enterobiasis, 1-14% for amoebiasis and 2-19.4% for giardiasis (Norhayati *et al*., 2003). However, the prevalence of APDE seen amongst samples for platelet aggregation test was as low as 2.8%. This may not reflect the true incidence faced in the population as in National Blood Centre, the request for platelet aggregation test was screened. If there is evidence of parasitic infestation, anti parasitic measures is given and platelet aggregation test would not be performed if bleeding symptoms subside, as platelet aggregation test is costly. Thus, a true incidence may not be captured based on the above reason. The levels of eosinophils were between 4.4-8.3%. In this study, some had normal eosinophils count as disease process has resolved. Bleeding time was within normal range in most patients.

According to Zaki (2011), APDE might have association with genetic susceptibility. The bleeding tendency is postulated to be due to *in vivo* platelet activation. The activation is most likely to be mediated by platelet activating factor (PAF) released during mast cell degranulation. The expected result of platelet release and aggregation is the occurrence of an acquired storage pool defect with variable thrombocytopaenia. Lucas (2002) observed that platelet dysfunction among children with eosinophilia was highlighted by minimal or absence of collagen-induced platelet aggregation. However, in this study, the platelet aggregation in response to collagen were comparable to controls (result not shown) together with impaired endogenous release of ADP.

The least numbers of patients were of acquired ADP dysfunction associated with GSD. Glycogen storage disease type I is a metabolic disease caused by a defect in the glucose-6-phosphatase system which has a key role in glucose homeostasis. Deficiency of glucose-6-

phosphatase activity in the liver, kidney and intestine, results in accumulation of glycogen in these organs (Rake *et al.*, 2002). Patients with GSD are known to have hepatomegaly, hypoglycaemia and bleeding disorders. Bleeding is manifested by mucocutaneous bleeding or bleeding post invasive maneuver (Marti *et al.*, 1986), as seen in one patient in our study who required four units of platelet transfusion during liver biopsy. The bleeding in these patients is related to platelet function defect due to systemic metabolic abnormalities from deficiency of glucose-6-phosphatase enzyme. Although such changes have been found, no definitive explanation addresses how these alterations actually cause defective platelet aggregation (Roth, 2009).

CONCLUSIONS

Possible inherited impaired platelet aggregation to ADP constituted the highest percentage of impaired platelet aggregation to ADP. Most subjects were significantly asymptomatic or present with a mild bleeding which do not require any medical attention. Difference in ethnic distribution is suggestive of different susceptibility resulting from varied genetics make up. Results from this study could serve as a reference for a national registry on mild bleeding disorder related to platelet function defects.

LIMITATION AND RECOMMENDATION

As this is a descriptive retrospective study, information collected was limited. It is recommended to establish local reference range for all agonist use in platelet aggregation test to objectively assess the reduction in platelet aggregation. Secretion assay should also be included in the assessment of platelet function test to screen for the granule function in the platelet. Future study in relation to this baseline study is to look into the molecular characteristics underlying impaired platelet aggregation to ADP. The postulated 'cardioprotective effect' that patient with $P2Y_{12}$ receptor dysfunction may encounter is also a stimulating research to be conducted.

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