ORIGINAL ARTICLE

Platelet Aggregation Pattern on Light Transmission Aggregometry Among Malaysian Healthy Individuals

Azlina Muhsin[1], Eusni Rahayu Mohd. Tohit[2], Sabariah Md. Noor[2], Faraizah Abd. Karim[3]

[1] Clinical Laboratory Science Section, Institute of Medical Science Technology, Universiti Kuala Lumpur, Taman Kajang Sentral, 43000 Kajang, Selangor, Malaysia
[2] Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[3] Department of Pathology, Hospital Ampang, Jalan Mewah Utara, Pandan Mewah, 68000 Ampang, Selangor, Ministry of Health, Malaysia

ABSTRACT

Introduction: Platelet aggregation test using light transmission aggregometry (LTA) is considered as the gold standard for evaluation of platelet function. Variations of platelet aggregation had been reported in apparently healthy individuals whereby a normal cut–off value established locally is highly recommended. This study aims to determine the platelet aggregation pattern and the preliminary findings on reference values for multiple agonists–induced platelet aggregation among Malaysian healthy individuals in a single centre. **Method:** A total number of 63 informed consented healthy individuals consisted of Malay, Chinese and Indian were recruited among staff and blood donors at National Blood Centre, Kuala Lumpur. Platelet aggregation was measured using LTA against adenosine diphosphate 10 µM (ADP10), collagen 0.19 mg/mL (COL), ristocetin 1.5 mg/mL (RIS), arachidonic acid 1 mM (AA) and epinephrine 10 µM (EPI). Results were expressed as percent final aggregation (%FA). Reference values were calculated from mean±2SD. **Results:** Age, gender and ethnic groups had no significant effect on platelet aggregation. A variability of platelet aggregation response to EPI was observed among the healthy individuals. Ten of 33 respondents (30%) had impaired aggregation with <20% FA in response to EPI. The local population showed a slightly higher aggregation pattern in response to COL, RIS, AA and EPI (excluding non-responders) compared to manufacturer's reference values. **Conclusion:** This study has provided a glimpse of the aggregation pattern of the local nationality showing considerable differences in the reference values from manufacturer's; thus highlighting the need of establishing local reference values.

Keywords: Platelet aggregation, Platelet function test, Reference values, Light transmission aggregometry, Malaysian

Corresponding Author:
Eusni Rahayu Mohd. Tohit, MPath
Email: eusni@upm.edu.my
Tel: +603-9769 2379

INTRODUCTION

Light transmission aggregometry (LTA) is the gold standard for evaluation of platelet function. It measures platelet aggregation based on turbidity changes upon addition of agonists into platelet–rich plasma (PRP). The LTA allows detailed examinations of platelet receptors, signalling pathways and amplification pathways mediated by different agonists. Thus, LTA is valuable for detection of a wide range of platelet dysfunction associated with inherited platelet–based mild bleeding disorder (1).

Several factors affecting platelet aggregability such as age, gender, ethnicity and diet had been reported. Increased platelet aggregation in relation to increasing age was seen in healthy men where age was found to be an important determinant influencing platelet aggregability (2). Effects of different gender and ethnicity in healthy individuals towards platelet aggregation had also been discussed with conflicting findings (3,4). Variations of aggregation were observed among African–American, Hispanics and Caucasian (5), among Europeans, Americans, Koreans, Filipinos and West Africans (6) and among Asian Indians and Caucasians (7). Thus, a normal LTA cut–off value established locally is recommended (8) where the reference population should be as similar as possible to that for which the test will be applied except with the presence of disease (9).

The present study was undertaken to look into the pattern of agonist–induced platelet aggregation among Malaysian healthy individuals as local platelet research is quite scarce among the healthy population. Subsequent estimation of platelet aggregation reference values

of multiple agonists was also carried out to represent preliminary local values.

MATERIALS AND METHODS

Ethical approval

The study was conducted at National Blood Centre (NBC), Kuala Lumpur. Institutional ethical approval was granted by Medical Research Ethical Committee from Universiti Putra Malaysia and Ministry of Health Malaysia (NMRR-11-871-10614).

Recruitment of respondents

Written informed consent were obtained from all respondents prior to participating in the study. A total number of 63 apparently healthy individuals consisted of 3 major ethnic groups in Malaysia with 40 Malays, 17 Chineses and 6 Indians were recruited among staff and blood donors at NBC. Inclusion criteria were regular blood donors or healthy individuals without known bleeding disorder and not on medication or consuming herbs supplements. Individuals with known bleeding disorders due to drugs or other underlying medical or surgical problems, on medication or taking herbs supplements and foreigners were not recruited.

From the total respondents, 49 were tested for platelet aggregation against adenosine diphosphate 10 µM (ADP10), 38 for collagen 0.19 mg/mL (COL), 53 for ristocetin 1.5 mg/mL (RIS), 52 for arachidonic acid 1 mM (AA) and 33 for epinephrine 10 µM (EPI). The concentration of agonists used (collagen and ristocetin) is as per guideline used in the National Blood Centre whereas adenosine diphosphate (ADP) concentration was adapted from Cattaneo (2011) (10) while arachidonic acid (AA) and epinephrine (EPI) concentration were adapted from Harrison et al. (2011) (8).

Sample processing

About 9 mL venous blood was drawn into a centrifuge tube containing 1 mL 3.2% sodium citrate and processed within 4 hours after sampling. Platelet aggregation test was carried out by light transmission aggregometry (LTA), PAP-8E (BioData Corp., USA), following the standard operating procedure of Haemostasis Laboratory, NBC. PRP was prepared by centrifuging the blood samples at 1000 rpm for 15 minutes (Kubota 2100, Kubota Corp., Japan). Approximately three-quarter of the PRP was carefully transferred to a polypropylene test tube by using a plastic Pasteur pipette. To obtain platelet–poor plasma (PPP), remaining of the blood sample was centrifuged at 3000 rpm for 10 minutes. PPP was then transferred to another polypropylene test tube. After that, PRP was standardized to platelet count ranged between $200x10^3$/µL to $300x10^3$/µL by addition of PPP. Platelet count was determined using automated haematology analyzer, Coulter LH 750 Analyzer (Beckman Coulter). 250 µL PPP ('Blank') and 225 µL PRP or diluted PRP ('Test') were transferred into a 7.25x55 mm flat bottom cuvette (BioData Corp. USA). The 'Test' samples were incubated and stirred at 1200 rpm with magnetic stirrer for 2 minutes followed with the addition of 25 µL agonist into the 'Test' cuvettes. Aggregation reaction was monitored for 6 minutes. Percent final aggregation (%FA) was taken for reporting. Test would be repeated twice for the respective agonist in the cases of aggregation result showed less than 65% as the value is the current laboratory cut-off value for impaired aggregation adapted from the manufacturer, BioData Corp., USA.

Statistical analysis

Data were statistically analysed with IBM SPSS Statistics 21. Results were expressed as mean (standard deviation, SD) following analysis of data distribution showing a normal distribution. Two-tailed statistical test with p-value<0.05 was considered significant. Reference values were calculated from mean±2SD.

RESULTS

Demographic characteristic of respondents

Details of demographic characteristics are summarized in Table I. The respondents' mean (SD) age is 35.7 (9.3) years with minimum age is 19 years and maximum age is 60 years.

Table I : Demographic characteristic of respondents

Characteristic	Frequency	Percent (%)
Race		
Malay	40	63.5
Chinese	17	27.0
Indian	6	9.5
Gender		
Male	45	71.4
Female	18	28.6
Age (years)		
Youth (15 – 24)	5	7.9
Adults (25 – 64)	58	92.1
TOTAL	63	100.0

Platelet aggregation pattern among healthy individuals

The platelet aggregation responses for all agonists did not show any association with age. Results also showed no significant difference between male and female platelet aggregation in response to all agonists (p>0.05). Table II shows the details of aggregation for each agonist in both genders. Similarly, there was no significant difference (p>0.05) of platelet aggregation between Malay, Chinese and Indian as shown in Table III.

Interestingly, a variability of platelet aggregation response to EPI was observed among the healthy individuals. Ten of 33 respondents (30%) had <20% aggregation in response to this agonist. Out of 33 respondents, there were 24 respondents where platelet aggregation for both EPI and ADP were obtained, whilst

Table II : Mean platelet aggregation in response to multiple agonists in male and female

Agonist	Mean (SD) of final aggregation, (%)	
	Male	Female
ADP	89.4 (15.3)	91.3 (13.3)
COL	91.5 (13.1)	91.6 (7.2)
RIS	100.7 (9.8)	100.3 (9.9)
AA	94.2 (12.8)	91.5 (11.3)
EPI	94.9 (18.9)	99.4 (11.5)

ADP, adenosine diphosphate 10 µM;
COL, collagen 0.19 mg/mL;
RIS, ristocetin 1.5 mg/mL;
AA, arachidonic acid 1 mM;
EPI, epinephrine 10 µM

Table III : Mean platelet aggregation in response to multiple agonists by ethnic groups

Agonist	Mean (SD) of final aggregation, (%)		
	Malay	Chinese	Indian
ADP	89.8 (14)	90.1 (18.8)	90.4 (9.3)
COL	90.2 (11.7)	92.8 (11.8)	95.7 (17.0)
RIS	99.6 (10.2)	101.4 (9.5)	104.0 (7.8)
AA	91.6 (10.5)	96.7 (13.4)	96.8 (20.1)
EPI	92.9 (16.4)	100.8 (18.1)	103 (12.3)

ADP, adenosine diphosphate 10 µM;
COL, collagen 0.19 mg/mL;
RIS, ristocetin 1.5 mg/mL;
AA, arachidonic acid 1 mM;
EPI, epinephrine 10 µM

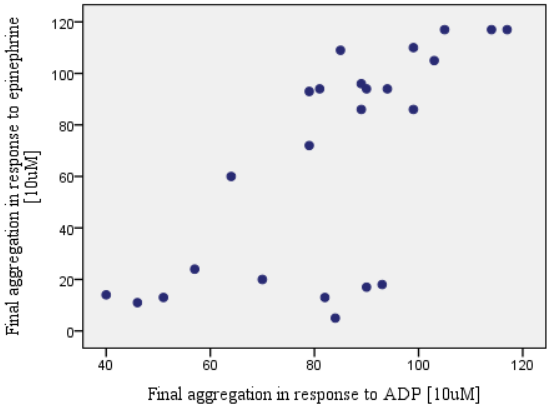

Fig. 1: Scatter plots of platelet aggregation in response to epinephrine 10 µM and adenosine diphosphate 10 µM.
The platelet aggregation shows a moderate positive association (rs=0.715, p<0.01, n=24) in response to these agonists.

for 9 other respondents, only platelet aggregation with EPI is obtained due to technical reason. Further analysis among the 24 respondents showed a significant moderate positive association between final aggregation in response to EPI and ADP (r_s=0.715, p<0.01), as illustrated in Fig. 1.

Reference values of platelet aggregation in response to multiple agonists

The estimation of platelet aggregation reference values was derived from all ethnic groups not by stratification of individual ethnic group. Results revealed that the local population showed a slightly higher aggregation pattern in response to COL, RIS, AA and EPI compared to manufacturer's reference values either at a similar or lower concentration of agonists used. To note, a lower aggregation response to ADP was expected as the concentration used in the present study was lower. Table IV illustrates the reference values derived from the current study as well as manufacturer's reference values. Eventhough in some of the agonists, concentration used were different i.e. higher in manufacturer's concentration but the platelet aggregation pattern obtained with local population were comparable, indirectly indicating some evidence of 'hyperreactivity' of platelets amongst local population.

Table IV : Reference values of platelet aggregation in response to multiple agonists

Agonist	Reference values	
	Local	BioData Corp. (2012)
ADP	61 – 119% (10 µM)	63 – 89% (20 µM)
COL	68 – 115%	61 – 99%
RIS	82 – 120%	68 – 106%
AA	69 – 118% (1.0 mM)	65 – 90% (1.6 mM)
EPI	65 – 128% (10 µM)	54 – 101% (100 µM)

ADP, adenosine diphosphate;
COL, collagen 0.19 mg/mL;
RIS, ristocetin 1.5 mg/mL;
AA, arachidonic acid;
EPI, epinephrine

DISCUSSION

Results from this study demonstrated no significant variation of platelet aggregation response to multiple agonists in healthy individuals by gender and ethnic groups namely Malay, Chinese and Indian ethnicity. The aggregation response was also not influenced by age. Platelet aggregation responses showed a slightly higher aggregation pattern towards collagen, ristocetin, arachidonic acid and epinephrine.

A demonstrable increase in platelet aggregation responses to multiple agonists during middle age (25–65 years of age) were reported in several studies (11). Platelets may have a state of hyperactivity during aging due to altered NO synthase activity resulting in reduced endothelial capacity to produce nitric oxide (NO), a potent vasodilator and platelet activation inhibitor (12). However, hyperaggregation associated with increasing age was not observed in the present study since there were only 6 respondents aged ≥50 years.

Effects of different gender and ethnicity towards platelet aggregation had been discussed with conflicting findings. Results from the current study supported that gender did not influence platelet aggregation (3). On the contrary, gender was considered as a determinant of platelet aggregability where it had been reported that females showed higher platelet aggregation than males (4). High platelet aggregation in response to all agonists were consistently observed in

females, with response to collagen (0.19 mg/mL) was the most significantly higher than in males regardless of the ethnicity (5).

Different response of platelet aggregation between both genders might possibly due to the absence of testosterone in females (4). Production of endothelial NO was significantly increased in rats after *in vitro* treatment with testosterone. This condition resulted in impaired platelet aggregation in the male animals (13). These may likely explain the lower platelet aggregation observed among males compared to females. However, the study was limited to *in vitro* animal study as no human *ex vivo* study demonstrating increased NO upon treatment with testosterone was retrieved.

With regards to ethnicity, several studies highlighted significant difference of platelet aggregation response among different ethnic groups. Caucasian females were most prone to aggregation in response to arachidonic acid compared to Hispanics and African–American females. A lower platelet aggregation response to epinephrine was observed among African–American males in comparison with Caucasian males, whereby response to ADP and collagen were similar among those ethnic groups (5). Earlier studies also demonstrated variations in aggregation response in different ethnicities. Europeans and Americans (Westerners) showed better aggregation response towards ADP compared to Koreans and Filipinos (Asians) and West Africans (6). Contradicting to these works, aggregation observed among Malays, Chineses and Indians of current study showed no significant difference of aggregation in response to ADP, collagen, ristocetin, arachidonic acid or epinephrine. This might be due to the fact that these ethnic groups share a common ancestry as Asian populations with some genetic similarity (14). Intriguingly, no significant variation in aggregation response towards ADP and arachidonic acid was reported between Asian Indians and Caucasians (7).

As different aggregation response between ethnicity is often linked with genetic profile, it was established that polymorphisms of cyclooxygenase–1 (COX1) gene evidently present among African–American and Caucasian populations (15). COX1 is part of the prostaglandin–synthesis pathway. Apart from COX1, polymorphism of thromboxane synthase (TBXAS1) also confirmed in African–American and Caucasians could be another contributing factor of variation response of platelet aggregation towards arachidonic acid in these 2 populations and possibly in other ethnic groups as well. TBXAS1 converts prostaglandin H2 to TXA2, a potent platelet aggregation inducer (16). Polymorphism in these genes resulting in amino acid substitutions was predicted to affect function of the enzymes and subsequent aggregation (15,16).

A small group of non-responders to epinephrine stimulation was noted in the current study. Such impaired responses occurred among apparently healthy Japanese individuals was shown to be associated with reduced numbers of platelet α2–adrenergic receptors. This had been linked to possible familial trait but the mode of inheritance was not investigated and determined (17). However, a separate molecular study revealed no mutation involved in the respective gene among those who did not response to epinephrine (18). Presence of platelet inhibitor in non–responders' plasma had been suggested based on the finding showing a reduced platelet aggregation of normal responders to epinephrine following their plasma incubation with plasma from non–responders (19). Other than that, the fact that both ADP and epinephrine mediate aggregation via Gi protein–coupled signalling pathway might explain the scenario of reduced response to ADP among these non–responders (18); consistent with our finding in the current study. Due to limited resources, no further test was conducted investigating the non-responders phenomenon in the current study.

A recent genome–wide association study (GWAS) of platelet aggregation conducted in European–Americans and African–Americans provide evidence of association between genetic variants and platelet aggregation (20,21). Single nucleotide polymorphism (SNP) at 7 loci associated with platelet aggregation had been identified. Three regions namely 1q23.1 (*PEAR1*), 11p15.4 (*MRVI1)* and 7q36.3 (*SHH*) were associated with ADP–induced aggregation; four regions namely 10q25.2 *(ADRA2A*), 1q23.1 (*PEAR1*), 7q22.3 (*PIK3CG*) and 10q21.2 (*JMJD1C*) were associated with epinephrine–induced aggregation and 19q13.42 (*GP6)* was associated with collagen–induced aggregation. MRV1 and SHH gene variants were related with increased aggregation in response to ADP. Decreased aggregation observed in response to epinephrine was associated with ADRA2A, PEAR1 and PIK3CG gene variants, while polymorphism of *MRV1* and *JMJD1C* were associated with increased aggregation. The association of *PEAR1* variant with reduced aggregation response towards epinephrine as well as ADP and collagen was corroborated in later work (20). Further work to demonstrate variations of these genes in addition to COX1 and TBXAS1 among Malay, Chinese and Indian of Malaysian population might provide explanation of the current observed platelet aggregation pattern among these healthy individuals.

CONCLUSION

In conclusion, platelet aggregation responses in the local population did not varied by age, gender and ethnicity. The present study provided a glimpse of the aggregation pattern of the local nationality and indirectly emphasize on the need of establishing local reference values. The preliminary reference values derived may serve as a rough guide for local laboratories conducting

LTA. Nevertheless, a slightly high aggregation pattern in response to collagen, ristocetin, arachidonic acid and epinephrine warrant further investigation and remain to be elucidated.

ACKNOWLEDGEMENT

We would like to thank the Director General of Health Malaysia for his permission to publish this article. Special thanks to staff at Haemophilia Clinic, Bleeding Room, Hematology Laboratory and Haemostasis Laboratory at National Blood Centre, Ministry of Health Malaysia and staff at Department of Pathology, Faculty of Medicine & Health Sciences, Universiti Putra Malaysia for their support and assistance in this study. This study was funded by Universiti Putra Malaysia Research University Grant Scheme (RUGS).

REFERENCES

1. Hayward CPM, Liu Y, Moffat KA. Diagnostic utility of light transmission platelet aggregometry: results from a prospective study of individuals referred for bleeding disorder assessments. Journal of Thrombosis and Haemostasis. 2009;7:676-684.
2. Terres W, Weber K, Kupper W, Bleifeld. Age, cardiovascular risk factors and coronary heart disease as determinants of platelet function in men. A multivariate approach. Thrombosis Research. 1991;62(6):649-661.
3. Beyan C, Kaptan K, Ifran A, Savasci S, Ozturk Y, Okmen B. Effect of sex different on platelet aggregation using an optical method in healthy subjects. Clin Lab Haematol. 2006;28(1):14-16.
4. Haque SF, Matsubayashi H, Izumi S, Sugi T, Arai T, Kondo A, et al. Sex difference in platelet aggregation detected by new aggregometry using light scattering. Endocrine Journal. 2000;48(1):33-41.
5. Otahbachi M, Simoni J, Simoni G, Moeller JF, Cevik C, Meyerrose GE, et al. Gender differences in platelet aggregation in healthy individuals. J Thromb Thrombolysis. 2010;30:184-191.
6. Gader AM, Bahakim HM, Malaika SS. Ethnic variations in platelet aggregation – comparison between Saudi Arabs, Westerners (Europeans and Americans), Asians and Africans. Platelets. 1991;2(4):197-201.
7. Patel RT, Lev EI, Vaduganathan M, Guthikonda S, Bergeron A, Maresh K, et al. Platelet reactivity among Asian Indians and Caucasians. Platelets. 2007;18(4):261-265.
8. Harrison P, Mackie I, Mumford A, Briggs C, Liesner R, Winter M, et al. Guidelines for the laboratory investigation of heritable disorders of platelet function. British Journal of Haematology. 2011;155(1):30-44.
9. Jones G, Barker A. Reference intervals. Clin Biochem Rev. 2008;29(Suppl (i)):S93-97.
10. Cattaneo M. Molecular defects of the platelet P2 receptors. Purinergic Signalling. 2011;7:333-339.
11. Jones CI. Platelet function and ageing. Mamm Genome. 2016;27:358-366.
12. Cau SBA, Carneiro FS, Tostes RC. Frontiers in Physiology. 2012;3(Article 218):1-11.
13. Campelo AE, Cutini PH, Massheimer VL. Testosterone modulates platelet aggregation and endothelial cell growth through nitroc oxide pathway. Journal of Endocrinology. 2012;213:77-87.
14. Ballinger SW, Schurr TG, Torroni A, Gan YY, Hodge JA, Hassan K, et al. Southeast Asian mitochondrial DNA analysis reveals genetic continuity of ancient mongoloid migrations. Genetics. 1992;130:139-152.
15. Ulrich CM, Bigler J, Sibert J, Greene EA, Sparks R, Carlson CS, et al. Cyclooxygenase 1 (COX1) polymorphisms in African-American and Caucasian populations. Human Mutation. 2002;20(5):409-410.
16. Ulrich CM, Carlson CS, Sibert J, Poole EM, Yu J, Wang L, et al. Thromboxane synthase (TBXAS1) polymorphisms in African-American and Caucasian populations: Evidence for selective pressure. Human Mutation. 2005;26(4):394-395.
17. Kambayashi J, Shinoki N, Nakamura T, Ariyoshi H, Kawasaki T, Sakon M, et al. Prevalence of impaired responsiveness to epinephrine in platelets among Japanese. Thrombosis Research. 1996;81(1):85-90.
18. Nakahashi TK, Kambayashi J, Nakamura T, Le SN, Yoshitake M, Tandon NN, et al. Platelets in nonresponders to epinephrine stimulation showed reduced response to ADP. Thrombosis Research. 2001;104(2):127-135.
19. Choi JW. Incidence of nonresponsiveness to epinephrine in platelets from healthy humans. Acta Haematologica. 2002;108(2):106-108.
20. Johnson AD, Yanek LR, Chen MH, Faraday N, Larson MG, Tofler G, et al. Genome-wide meta-analyses identifies 7 loci associated with platelet aggregation in response to agonists. Nature Genetics. 2010;42(7):608-613.
21. Qayyum R, Becker LC, Becker DM, Faraday N, Yanek LR, Leal SM, et al. Genome-wide association study of platelet aggregation in African Americans. BMC Genetics. 2015;16:58-68.