Laboratory QA

Aspirin Effects on Platelets Using Whole Blood Tested by Platelet Aggregometry: A Comparative Study for Test Validation in a Clinical Hemostasis Laboratory

Wan Zaidah Abdullah, MD, MPath,1* Sanada Abu Bakar, MBBS, MPath,1 Wan Soriany Wan Mohd Zain, BSc,1 Zurkurnai Yusof, MD, MMed,2 Rapiaah Mustaffa, MD, MMed,1 Rosline Hassan, MD, MMed,1

ABSTRACT

Objective: Assessment on platelet responsiveness to aspirin may be required in selected clinical conditions. So far, no standardization in laboratory practices for aspirin assessment using whole blood (WB) platelet aggregation based test. A study for method validation was performed to investigate the aspirin effects on platelets by comparing with a reference method.

Methods: Forty patients taking aspirin were analyzed using WB by conventional platelet aggregometer (Chrono-Log Model 500CA/560CA). Among these patients, nine of them had their platelet rich plasma (PRP) tested by the same analyzer (reference method). Another WB specimens from 25 patients were tested on both Chrono-Log and Multiplate® (Dynabyte GmbH), which is a newer generation platelet function analyzer.

Results: There were good and moderate agreements between WB on the Chronolog analyzer vs the reference method and WB on Chronolog vs WB on Multiplate® analyzers respectively.

Conclusions: There are agreements between PRP and WB aggregation (WBA) methods in detecting aspirin effects on platelets. It is recommended that the test validation for the assessment of platelet responsiveness to aspirin is interpreted and correlated with the reference method preferably PRP.

Keywords: aspirin, platelet aggregation, test validation, hemostasis laboratory

Aspirin is an antiplatelet agent used to prevent thromboembolic complications related to cardiovascular disease;1 it is the most cost-effective drug for the secondary prevention of acute coronary artery thrombosis. However, there are no universal guidelines or recommendations for the laboratory methods to measure the effect of aspirin on platelet function. Many newer-generation platelet function analyzers have been used to describe the aspirin effects on platelets; however, not all clinical laboratories have these facilities. Conventional aggregometers are usually available in clinical hemostasis laboratories for the investigation of bleeding disorders.

Assessment of platelet responsiveness to aspirin is not routine. However, there are clinical conditions in which knowledge of the effect of aspirin on platelet function may influence patient management. For example, failure of aspirin treatment may be suspected when patients develop recurrent acute coronary syndrome or post-stenting thrombosis. Failure of aspirin therapy to prevent thromboembolic events is relatively common; patient experiencing this have been described as being in an aspirin-resistant state.2 This condition has been used to describe not only an absence of the expected pharmacologic effects of aspirin on platelets but also related to poor clinical outcomes, such as recurrent acute vascular events and sudden death.3

The effects of aspirin on hemostasis can be harmful in patients undergoing surgery. It is important to assess residual
anticoagulant effects of aspirin before surgery, particularly when there is doubt of platelet recovery after cessation of aspirin intake. Unnecessary platelet transfusions may be avoided if there is no evidence of residual effects of aspirin on the patient’s platelets. Assessment of platelet function is clinically indicated when aspirin use has been discontinued for less than 7 days prior to surgery. Conventionally, platelet function is tested using the optical platelet aggregation method or light transmission aggregometry (LTA) in platelet-rich plasma (PRP) specimens. However, PRP preparation is laborious, requires a large volume of specimen, and is performed under nonphysiological milieu due to multiple centrifugation steps. This method, however, includes established criteria for classification of aspirin-sensitive and aspirin-resistance status and useful as a reference method. Using WB is less laborious and requires a smaller specimen volume suitable for testing in a clinical hemostasis laboratory.

When performing LTA using PRP specimens, aspirin resistance was defined as a mean aggregation of 70% or greater with 10 μM of adenosine diphosphate (ADP) and a mean aggregation of 20% or greater with 0.5 mg per mL of arachidonic acid (AA). Aspirin semiresponders were defined as meeting 1 but not both of these criteria.

The aims of this study were to determine the agreement between PRP and WB specimens using the same platelet aggregometer to assess the platelet response to aspirin and to compare the results between 2 different aggregometry methods (ie, different platelet function analyzers) for recognizing the effects of aspirin on platelet function. The findings from this study could be useful for assessment of platelet function in patients who have been treated with aspirin.

Materials and Methods

A prospective study was performed in the Hematology Laboratory at Hospital University Sains Malaysia (HUSM) in Kelantan. The study subjects were patients with stable coronary artery disease who had taken aspirin and were receiving regular checkups at the Cardiology Clinic at HUSM for period of 2 years. A platelet aggregation test was performed on WB from 40 patients by platelet aggregometer using an impedance method (Chrono-Log whole blood lumi-ionized calcium platelet aggregometer: Chrono-log Model 500CA/560CA; Chrono-Log Corporation, Havertown, PA). Platelets from 9 patients were analyzed using PRP (ie, an optical method) by the same analyzer. Platelets from another 25 patients were analyzed using WB via Multiplate analyzer (Dynabyte GmbH, Stockholm, Sweden). Reduced or no aggregation were expected among patients with aspirin effect. Platelets were measured on all patients to ensure that the counts were within normal range (150-400 × 10^9/L) using an automated multiparameter blood cell counter Sysmex KX-21 machine (Sysmex Corporation, Kobe, Japan).

Verbal and written consent were obtained from all the patients enrolled in this study. This study was approved by the ethical committee for clinical study in this institution. The study group included:

1. 29 males and 11 females, from 35 to 70 years old
2. Patients that were taking aspirin, with a minimum dose of 100 to 150 mg daily, for at least 1 month
3. Patients with hypertension, diabetes mellitus, and hyperlipidemia

Exclusion criteria for the study group were as follows:

1. Hospitalized patients
2. Patients taking aspirin in combination with other antiplatelet drugs, such as clopidogrel, ticlopidine, or dipyridamole
3. Suspected poor compliance with aspirin regimen
4. Abnormal platelet count (<150 × 10^9/L)

Blood samples were collected from the patients, avoiding trauma or hemostasis at the venipuncture site. For PRP and WB specimens analyzed using the Chrono-log aggregometer, 20 mL and 4.5 mL of blood, respectively, were collected in sterile tubes containing 3.2% buffered sodium citrate (ratio: 1 part anticoagulant to 9 parts blood). For WB specimens analyzed using the Multiplate analyzer, 2 mL of blood were collected in sterile lithium heparin tubes. The platelet aggregation samples were kept at room temperature and processed within 3 hours of blood collection.

The WB impedance method of aggregation is nonoptical (by electrical resistance). An electrode probe assembly is inserted into a cuvette containing a test sample. The instrument measures the impedance between the 2 immersed wires. During a brief period of equilibration, a monolayer of platelets forms on the exposed portions of the probes, produces a stable impedance, assigned a value of 0.

An aggregating agent (In this study: 10 μM of ADP, 0.5 mM of AA, 1.0 mg per mL of ristocetin and ATP release with AA were performed to detect platelet sensitivity to aspirin) was added to the cuvette to stimulate platelet aggregation on the immersed probes. The accumulation of platelets increases the electrical resistance; the change in impedance was measured in Ω.
Adenosine triphosphate (ATP) release was measured by luminescence. The principle of the lumi-aggregometer is to measure the luciferin-luciferase reaction as it consumes ATP. The photomultiplier detects luminescence produced when ATP is secreted by dense granules in WB, PRP, or washed platelets.

The amplitude of impedance aggregation results are measured 6 minutes after reagent addition. A tangent is drawn through the steepest part of the curve, and a right triangle is then constructed during an interval of 1 minute. The height of the triangle is the rate of change of aggregation in 1 minute, which is defined as the slope. Table 1 summarizes the interpretation of WB aggregation and ATP release by Chrono-log Model 500CA/560CA. The principle and procedure were adapted from Chrono-Par and Chrono-Lume reagents (Chrono-Log Corporation) for platelet function testing and secretion studies in WB and PRP.

In aspirin resistance, platelet aggregation results should be normal whereas in the aspirin-sensitive state, the aggregation should be reduced or absent in the presence of AA and ADP. Qualitative changes in ristocetin-induced platelet aggregation has been described in patients treated with aspirin (for example, a saw-tooth appearance). The reference values were derived from the Chrono-Log WB lumi-aggregometer manual (Chrono-Log Corporation).

PRP Platelet Aggregation Using ADP and AA Agonists

The samples were centrifuged at 100g for 15 minutes. PRP was prepared by centrifugation in a polypropylene test tubes at 2400 g for 20 minutes.

The aggregometer setting was adjusted to the optical setting for PRP. The baseline aggregation (light transmission) was adjusted for 0%. Once the baseline was stable, the test was run. The aggregating agents were added to the PRP sample (5 μL of ADP or 5 μL of AA), and the light transmission was monitored for 4 minutes. The percent aggregation was then calculated by the instrument.

Interpretation of Results

Amplitude optical aggregation results are expressed as a percentage of aggregation at a given time interval from reagent addition; 100% aggregation is defined as the difference between the 0% PRP and the 100% PPP. The measurement is similar to the impedance method described above. Table 1 summarizes the interpretation of PRP aggregation by Chrono-log model 500CA/560CA (Chrono-Log Corporation) (Table 1).

### Table 1. Interpretation of PRP and WB Platelet Aggregation Including ATP Release by Chrono-Log Model 500CA/560CA

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Aggregation (Ω)</th>
<th>ATP (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>10 μM</td>
<td>6-24 (5-15)</td>
<td>NA</td>
</tr>
<tr>
<td>Ristocetin</td>
<td>1.0 mg/mL</td>
<td>&gt;5 (7-27)</td>
<td>NA</td>
</tr>
<tr>
<td>AA</td>
<td>0.5 mM</td>
<td>5-17 (4-22)</td>
<td>0.6-1.4</td>
</tr>
</tbody>
</table>

**Normal Ranges in WB**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Aggregation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>10 μM</td>
<td>71-88</td>
</tr>
<tr>
<td>AA</td>
<td>0.5 mM</td>
<td>74-99</td>
</tr>
</tbody>
</table>

**Abbreviations:** PRP, platelet-rich plasma; WB, whole blood; ATP, adenosine triphosphate; ADP, adenosine diphosphate; NA, not applicable; AA, arachidonic acid.

### WB Platelet Aggregation by Multiplate Analyzer

The principle of Multiplate analysis (F. Hoffman-La Roche, Basel, Switzerland) is based on the fact that platelets get sticky on activation and tend to aggregate on sensor wires in the Multiplate test cell. When platelets adhere to the Multiplate sensor wires, they increase the electrical resistance between them, which is continuously recorded. The instrument detects the change in impedance at each sensor, and the results are expressed in arbitrary aggregation units (AU). The Pearson correlation coefficient of the data points detected in each of the channels is calculated. If the correlation coefficient is less than 0.98, a measurement error is detected and the test has to be repeated. The areas under the aggregation curves detected in each channel are compared; if the difference is greater than 20% (vs the mean curve), the user is prompted to repeat the test by the Multiplate software.

In applied signal processing and implementation (ASPI) testing, platelet aggregation is activated by AA, the substrate of the cyclooxygenase (COX) enzyme. Aspirin inhibits the conversion of AA to prostaglandin H2, which is one step upstream of the thromboxane synthase reaction requires for platelet aggregation. The change in impedance is expressed in aggregation units (AU).
Interpretation of Results

The increase in impedance that results from the attachment of platelets to the Multiplate sensors is plotted over time. Three parameters are calculated, the most important of which is the area under the aggregation curve (AUC) which is affected by the total height of the aggregation curve and its slope.

One study on healthy blood donors showed strong aggregation in ASPI testing, with a median of almost 100 U. More than 90% had an aggregation of at least 75 U in ASPI test results. In inpatients not given aspirin treatment, more than 75% had an ASPI test result of at least 80 U. Another study examined patients undergoing aspirin treatment; most of them showed strongly decreased aggregations in ASPI testing to values lower than 30 U; therefore, we used this value as the cut-off point to classify aspirin resistance and aspirin sensitivity by this method (1 U = 10 AUC).

Statistical Analysis

All data were analyzed using SPSS, version 14.0 for Windows, (SPSS Inc, Chicago, IL). Categorical variables were presented as frequencies and percentages. For the categorical variables, patient demographics and clinical characteristics between the 2 groups were compared using Fisher’s exact test. Continuous variables are presented as mean (SD). Independent student’s t tests were used to compare continuous variables between the 2 groups. The \( \kappa \) statistic was used to determine the agreement between the different methods of platelet aggregation testing.

Results

Comparison Between PRP and WB Specimens Using the Chrono-Log Analyzer

Four patients were aspirin sensitive, 1 was aspirin resistant, and 4 were aspirin semiresponders based on the criteria outlined earlier for PRP method. Results of these 9 patients are summarized in Table 2. Correlating with PRP criteria, we observed that with impedance WB aggregation (WBA), the aspirin-sensitive group had no aggregation with 0.5 mM of AA and absent or reduced aggregation with 10 μM of ADP. The aspirin semiresponder group had no aggregation with 0.5 mM of AA and normal aggregation with 10 μM of ADP by the same method (Table 2).

Results for PRP and WB specimens analyzed by Chrono-Log aggregometer categorized into aspirin-sensitive, aspirin-resistant, and aspirin-semiresponder groups were in significant agreement (\( \kappa = 1.000, P < .001 \)).

Results of WB Specimen Using Chrono-Log Analyzer

Blood specimens from 40 patients were analyzed by WBA using the Chrono-Log aggregometer. The aspirin effect on platelet aggregation had been reported as showing a saw-tooth pattern with ristocetin and no ATP release with the AA agonist. Based on the PRP, our study allowed interpretation of WBA for aspirin resistance as a normal aggregation with 10 μM of ADP and 0.5 mM of AA agonists. Aspirin semiresponders were defined as having normal aggregation after activation with 10 μM ADP and absent or reduced aggregation with 0.5 mM of AA.

<table>
<thead>
<tr>
<th>Specimen and Agonist, Patient</th>
<th>PRP (%)</th>
<th>WB (Ω)</th>
<th>Interpretation by PRP Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen and Agonist, Patient</td>
<td>0.5 mg/mL AA</td>
<td>10 μM ADP</td>
<td>0.5 mg/mL AA</td>
</tr>
<tr>
<td>A</td>
<td>3</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>11</td>
<td>39</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>13</td>
<td>76</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>3</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>10</td>
<td>74</td>
<td>0</td>
</tr>
<tr>
<td>G</td>
<td>79</td>
<td>89</td>
<td>15</td>
</tr>
<tr>
<td>H</td>
<td>16</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>I</td>
<td>14</td>
<td>41</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviation: PRP, platelet-rich plasma, WB, whole blood; AA, arachidonic acid; ADP, adenosine diphosphate.
Patients with absent or reduced aggregation with both AA and ADP were classified as aspirin-sensitive. Thirteen patients (33%) were aspirin sensitive, 4 (10%) were aspirin resistant, and 23 (58%) were aspirin semiresponders.

Comparisons of Other Laboratory Results Between the Aspirin-Sensitive and Aspirin-Resistant Groups (WBA with Chrono-Log Analyzer)

With 1.0 mg per mL of ristocetin and ATP release with AA, a response greater than 5 Ω and 0.6 to 1.4 nmol was considered to reflect normal aggregation and normal ATP release, respectively. Because semiresponder patients had absent or reduced aggregation with AA and normal aggregation with ADP, we grouped these patients into the aspirin-responder/sensitive group to examine the significance of ATP release with AA in detecting aspirin effects. Thirty-six patients (90%) were aspirin sensitive or aspirin semiresponders and 4 (10%) were aspirin-resistant. There was a significant difference in platelet aggregation induced with ristocetin between the aspirin-sensitive and aspirin-resistant groups \( (P < .05) \).

No significant difference was observed in the ‘saw tooth’ pattern of platelet aggregation induced with ristocetin when the aspirin-sensitive and aspirin-resistant groups were compared \( (P > .05) \). ATP release with AA was significantly different between the aspirin-sensitive and aspirin-resistant groups \( (P < .05) \). Table 3 summarizes these findings.

### Table 3. Ristocetin, Saw-Tooth Tracing With Ristocetin, ATP Release Results, and Aspirin Status

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Status, No. (%)</th>
<th>Aspirin Sensitive or Semiresponder</th>
<th>Aspirin Resistant</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ristocetin</td>
<td>Normal</td>
<td>7 (19)</td>
<td>4 (100)</td>
<td>.004</td>
</tr>
<tr>
<td></td>
<td>Abnormal</td>
<td>29 (81)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Saw-tooth with ristocetin</td>
<td>Yes</td>
<td>5 (14)</td>
<td>0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>31 (86)</td>
<td>4 (100)</td>
<td></td>
</tr>
<tr>
<td>ATP release with AA</td>
<td>Normal</td>
<td>1 (3)</td>
<td>4 (100)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td></td>
<td>Abnormal</td>
<td>35 (97)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ATP, adenosine triphosphate; AA, arachidonic acid. Aspirin sensitive or semiresponder: \( n = 36; \) Aspirin-Resistant: \( n = 4; \) \( P \) value determined by the Fisher exact test.

Agreement Between Results From the WB Chrono-Log and the Multiplate Analyzers in Detecting Platelet Response to Aspirin

Moderate agreement was observed between WB results in aspirin-sensitive and aspirin-resistant subjects using the Chrono-Log and Multiplate analyzer (\( \kappa = 0.706; P < .001 \)). The state of semiresponsiveness to aspirin, based on WB using Chrono-Log results was categorized as aspirin sensitive/responder in this analysis.

Discussion

No consensus was reached regarding the method of choice to detect the antplatelet effect of aspirin. LTA using PRP is the conventional method for measuring the antiaggregatory effect of aspirin (also regarded as the criterion standard/reference method). Aggregometry using PRP is subject to variation during preparation of PRP (ie, during centrifugation) and measurement imprecision. In this study, we used 10 μM of ADP and 0.5 mM of AA, as reported by Gum et al. LTA is also a labor-intensive and time-consuming method that requires technical expertise. The impedance WBA method has fewer of these problems. With impedance WBA, blood can be analyzed immediately after sampling without centrifugation and testing can be performed in a more physiologic state. WB remained the normal composition of blood cells and simulating a physiological state as platelets are tested in their natural environment.

In this study, out of the 9 patients whose samples were tested using the PRP LTA method, 4 patients were classified into 2 groups: aspirin sensitive (<300 AUC) or aspirin resistant (≥300 AUC). Twenty patients (80%) were aspirin sensitive and 5 patients (20%) aspirin resistant via the Multiplate analyzer.
aspirin sensitive, 1 was aspirin resistant, and 4 were aspirin semiresponders. In impedance WB aggregometry using Chrono-Log analyzer, aspirin sensitivity was defined as no aggregation with 0.5 mM of AA and absent or reduced aggregation with 10 μM of ADP; the aspirin semiresponder state was defined as no aggregation with 0.5 mM of AA but normal aggregation with 10 μM of ADP. Aspirin resistance was defined as normal aggregation with 10 μM of ADP and 0.5 mg per mL of AA. In this study, we found that strong agreement existed between PRP (ie, the reference method) and WB analyzed by the Chrono-Log aggregometer in detecting the platelet effect of aspirin, shown by the κ coefficient (κ = 1.000; P < .001). The normal reference ranges used for WBA in this study had already been established in our laboratory.

All the patients who were semiresponders to aspirin had no platelet aggregation with AA and normal platelet aggregation with ADP. This finding indicates that AA-induced platelet aggregation is more sensitive to the effect of aspirin than ADP induced aggregation. We tested WB from 40 patients using the Chrono-Log platelet function analyzer; 13 (33%) were aspirin sensitive, 4 (10) were aspirin resistant, and 23 (58%) were aspirin semiresponders. Estimates of the prevalence of aspirin resistance in patients with stable coronary artery disease vary from 5.5% to 29.0%, depending on the method of assessing platelet function and the definition of aspirin resistance. Gum et al reported that the prevalence of aspirin resistance in 325 patients with stable coronary artery disease was 5.5% and 9.5% as measured by optical LTA and PFA-100 methods, respectively. Platelet aggregation induced by ristocetin may be affected by aspirin. In this study, we observed a significant difference in ristocetin-induced platelet aggregation between the aspirin-sensitive and aspirin-resistant groups (P < .05). Ristocetin-induced platelet aggregation was reduced in aspirin-sensitive subjects. A qualitative defect, such as a saw-tooth pattern with ristocetin, may be observed in the serum of subjects taking aspirin. Regarding this pattern, no significant difference was observed between the aspirin-sensitive and aspirin-resistant groups (P > .05) in this study.

ATP release after addition of AA is a useful test for detecting aspirin sensitivity: Lack of aggregation and lack of ATP release with AA indicates impaired thromboxane synthesis. In this study, ATP release with AA was significantly different between the aspirin-sensitive and aspirin-resistant groups (P < .05). In the aspirin-resistant group, ATP release was within normal limits, whereas it was suppressed in aspirin-sensitive subjects.

The Multiplate analyzer is a newer WB platelet-function analyzer in which platelets are activated by AA, which is converted by cyclooxygenase to the potent platelet aggregator thromboxane A2. In this study, moderate agreement was observed between WB test results per the Chrono-Log and Multiplate analyzers in detecting platelet response to aspirin (κ = 0.706; P < .001). A study of 6 major platelet function tests to determine the prevalence of aspirin resistance in patients with stable coronary artery disease has been reported; the researchers found that these tests showed poor correlation and agreement.

A study reported on the performance of the VerifyNow platelet function analyzer (Accumetrics, Inc, San Diego, CA) compared to the WB aggregation method. The researchers reported that VerifyNow was able to identify only aspirin effect and failed to detect aspirin nonresponsiveness, and suggested that the cut-off value needs to be re-adjusted compared with that of WB aggregation. Reduced aggregation with ristocetin using the WB method was also noted in this study, probably because the ristocetin concentration for WBA used in this study was 1.0 mg per mL.

The clinical implication of aspirin therapy is well-known. It is the task of the clinical laboratory to establish reliable methods for measuring the effect of aspirin on platelets. WBA using conventional platelet aggregometry can help distinguish between aspirin responsiveness and aspirin resistance, and the results agree with the reference method that uses PRP. A recent paper also discussed findings along these same lines; the researchers concluded that the prevalence of aspirin resistance depends on the type of test and agonist used.

In this study, aspirin effects on platelets using WB tested on conventional aggregometry (Chrono-Log aggregometer) could be determined by the absence of aggregation with AA, reduced ATP release with AA, and reduced aggregation with ADP and ristocetin. Although qualitative changes (ie, saw-tooth appearance) on ristocetin tracing did not yield significant findings, an association with platelet response to aspirin was observed.

A limitation of this study is the small sample size used for comparison. However, this study has yielded significant findings, which add to the literature on this subject.

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References


