Comparison between a new platelet count drop method PL-11, light transmission aggregometry, VerifyNow aspirin system and thromboelastography for monitoring short-term aspirin effects in healthy individuals

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Abstract

Platelet function has been described by many laboratory assays, and PL-11 is a new point-of-care platelet function analyzer based on platelet count drop method, which counts platelet before and after the addition of agonists in the citrated whole blood samples. The present study sought to compare PL-11 with other three major more established assays, light transmission aggregometry (LTA), VerifyNow™ aspirin system and thromboelastography (TEG), for monitoring the short-term aspirin responses in healthy individuals. Ten healthy young men took 100 mg/d aspirin for 3-day treatment. Platelet function was measured via PL-11, LTA, VerifyNow and TEG, respectively. The blood samples were collected at baseline, 2 hour, 1 day during the aspirin treatment and 1 day, 5 ± 1 days, 8 ± 1 days after the aspirin withdrawal. Moreover, 90 additional healthy subjects were recruited to establish a reference range for PL-11. Platelet function of healthy subjects decreased significantly 2 hours after 100 mg/d aspirin intake and began to recover during 4–6 days after the aspirin withdrawal. Correlations between methods were PL-11 vs. LTA (r = 0.614, p < 0.01); PL-11 vs. VerifyNow (r = 0.629, p < 0.01); PL-11 vs. TEG (r = 0.697, p < 0.001). There was no significant bias between PL-11 and LTA at baseline (bias = 1.94%, p = 0.829) using Bland-Altman analysis, while the data of PL-11 were significantly higher than TEG (bias = 42.03%, p < 0.001) during the aspirin therapy. The reference range for PL-11 in healthy young individuals was from 66.8 to 90.5% (95%CI). When aspirin low-responsiveness was defined as LTA > 20%, the cut-off values for each method were, respectively: PL-11 > 50%, VerifyNow > 533 ARU, TEG > 60.2%. The results of different platelet function assays were uninterchangeable for monitoring aspirin response and correlations among them were also varied. Correlations among PL-11 and other three major assays suggested the ability of PL-11 to assess the treatment effects of aspirin. But a large cohort study is needed to confirm the cut-off value of aspirin response detected by PL-11.

Introduction

Platelet function has been described by many clinical laboratory assays, and each of them has several advantages on the working principal [1, 2]. Though various methods for platelet function testing have been developed, such as light transmittance aggregation (LTA), VerifyNow and thromboelastography (TEG), technical and other limitations puzzle the clinicians and laboratory technologists since it is difficult to assess the uninterchangeable testing results. The gold standard LTA, which is widely used to evaluate platelet function and correlated with clinical outcomes reported in some documents [3–6], however, was time consuming, labour intensive, and less standardized. PL-11 is a new automated point-of-care platelet function analyzer based on platelet count drop method, which is measured as the single platelet loss after the addition of agonists into the whole citrated blood, using a standard electrical impedance cell counter (Figure 1). Considering PL-11 is different from other existent platelet function devices, in this study, we aimed to compare its ability to monitor the short-term aspirin response in healthy young individuals by comparing PL-11 with other three different platelet function assays (LTA, VerifyNow™ system, TEG), which have been widely used and well established in vitro.

Materials and methods

Study population

Healthy subjects were eligible if they were above 18-year-old. Those who were smokers, intolerant to aspirin, being treated with other medications known to affect platelet function (e.g. NSAIDs, clopidogrel), have a platelet count less than 100 × 10⁹/l or any evidence of chronic or acute disease within the period of the study were excluded. This study was in accordance with the Declaration of Helsinki and all the subjects provided informed consents prior to the study.
Study protocol

Ten healthy men were assigned to receive a dose of 100 mg/d enteric-coated aspirin (Bayaspirin®, Bayer) for 3 days and platelet function was measured in parallel using four different assays (PL-11, LTA, VerifyNow, TEG) prior to aspirin treatment, 2 hour (day 1), 1 day (day 2) during aspirin treatment, and 1 day (day 4), 5 ± 1 days (day 8 ± 1), 10 ± 1 days (day 13 ± 1) after aspirin treatment was discontinued. We enrolled additional 90 untreated healthy subjects and tested their platelet function by LTA and PL-11.

Blood sampling

Antecubital vein blood samples were collected for monitoring platelet function in all subjects at baseline. Five tubes of blood for aspirin-treated individuals were needed: three 3.2% sodium citrated tubes (Becton-Dickinson, Franklin Lakes, NJ) for LTA, PL-11 and TEG tests, respectively, one 3.2% sodium citrated tube (Greiner Bio-One Vacuette North America, Inc., Monroe, NC) for VerifyNow analysis and 1 heparin tube (Becton-Dickinson, Franklin Lakes, NJ) for TEG test. Two tubes of 3.2% sodium citrated blood were enough for the additional 90 healthy subjects. First 2 ml of blood was discarded. The tubes were gently inverted 3 times to ensure complete mixing of the blood sample with anticoagulant. Blood samples should be stored at room temperature before being tested. The whole procedure required being performed within 2 hours after sampling.

Platelet function tests

Light transmittance aggregometry (LTA)

Light transmission aggregometry was performed with a CRONOLOG Model 700 Aggregometer (Havertown, PA). Platelet-rich plasma (PRP) was obtained by centrifuging at 800 rpm (120 x g) for 5 min. Platelet-poor plasma (PPP) was centrifuged at the 4000 rpm (850 x g) for 8 min using the remaining blood (PRP was not adjusted). PPP and PRP should be tested within 30 min at room temperature. During the analysis procedure, PRP was stirred at a fixed speed of 1000 rpm. Light transmittance changed in PRP while platelets aggregated after stimulating with 5 μl 50 mM arachidonic acid (AA). Final AA concentration in the PRP was 0.5 mM. Finally, the result was calculated and reported as a percentage of maximal light transmission in PRP, with PPP used as a reference.

VerifyNow analysis

VerifyNow system (Accumetrics, San Diego, CA) is an aggregation-based point-of-care platelet function test depended on the optical change in whole blood samples. The VerifyNow aspirin system contains micro fibrinogen-coated beads and agonists (AA) in the cartridges. When inserted into the apparatus, blood was drawn into the reaction cartridges from the sodium citrate tube. Optical turbidity changed in whole blood when platelets were activated by AA and bind to micro beads. Results would be calculated by the system with ARU which is short for Aspirin Reaction Units.

Thromboelastography platelet mapping™ assay

Thromboelastography™ (TEG) (Heamoscope Corporation, Niles, IL) is a point-of-care-testing that quantitatively analyses platelet function. Special cuvettes were placed into different anticoagulant venous blood samples and agonists. The whole blood was activated in a cuvette with a gently rotated tension wire within the sample (through 4°45′). Since platelets aggregated and clot formed, resistance to the movement of the wire increased and yielded the maximal clot strength named maximum amplitude (MA), which was displayed graphically on the TEG trace. Three channels were needed to complete this assay, including, kaolin- and calcium-activated coagulation MA thrombin- Activator™-induced MA fibrin, AA-induced MA AA, respectively. Sample volume in first channel was 360 μl heparinized whole blood and 340 μl citrated blood in each of the rest channel. Finally, the system calculated the result of %MA representing the relative strength of AA induced MA AA to MA thrombin from the following formula:

\[
\frac{\text{Percentage of } MA_{AA}}{\text{Percentage of } MA_{fibrin}} = \frac{(MA_{AA} - MA_{fibrin})}{(MA_{thrombin} - MA_{fibrin})} \times 100.
\]

PL-11 analysis

PL-11 platelet function analyzer (SINNOWA Medical Science & Technology Co., Nanjing, China) is a new point-of-care apparatus for platelet function analysis via automated impedance technique. PL-11 counts single platelets and the platelet aggregation is measured as the loss of single platelets. It contains an automated impedance-based hematology analyzer and agonist kits. The whole procedure was automatically done after transferring 500 μl citrated blood sample into a polycarbonate tube and inserting it into the detecting position. Blood sample in the polycarbonate tube was mixed gently during the whole testing process. Platelet count was detected in duplicate at the start and the mean value of platelet count was set as the baseline. There was a short interval between each test point for system cleaning. When 25 μl 6.13 mM (0.31 mM, final concentration) AA was automatically trickled into blood sample after second detecting time. The single platelet counting dropped when aggregates formed became too large to be counted as single platelets. PL-11 counted platelet several times till it detected the lowest level. The whole process was finished within 15 min (six detecting times). All the samples were tested within 1 hour after sampling. The system calculated the maximal platelet aggregation ratio according to the following formula:

\[
\text{MAR} = 100 - \left\{ \frac{(1\text{st platelet count} + 2\text{nd platelet count})}{2} - \text{lowest platelet count} \right\} \times 100%.
\]
Statistical analysis

Continuous variables were presented as mean ± SD. Repeated-measure analysis of variance (ANOVA) was used to compare platelet function changes in the study. Pearson’s correlation coefficient was adopted to evaluate associations among methods when data were in normal distribution, otherwise a non-parametric Spearman correlation will be used. We used the Bland-Altman test to check agreement between the MAR values of LTA and PL-11. Receiver-operating characteristics (ROC) curve analysis was used to get the cut-off values of each method using documented aspirin low response cut-off value LTA >20%. Reference limits for LTA and PL-11 were calculated according to the equation: mean ± 1.96 × SD. Statistical difference was specified as a p value less than 0.05 (two tailed). Data were analyzed using SPSS software (version 19.0, Chicago, IL) and GraphPad Prism 5 (GraphPad Software, CA).

Results

One hundred young healthy individuals were recruited to this study and the characteristics were shown in Table I. There were more males and non-smokers in the study young population.

Platelet function results

Ten young men underwent all platelet function tests during the 2-week period. The results from all four methods showed that platelet function was inhibited significantly at 2 hours after aspirin intake, and the repeated-measure ANOVA results were labeled in Figure 2. Platelet function values returned to the baseline level after 4–6 days when the subjects stopped to take aspirin. There was no overlap between pre- and post- aspirin intake.

PL-11 and LTA

Reference limits for AA induced LTA at baseline (n = 100) was 64.0–92.1% (95% CI) which was similar with PL-11 (66.8–90.5%). The results during aspirin treatment were 0–4% for LTA and 15.4–34.3% for PL-11. There was a relative reduction of 96.9% with LTA and 65.9% with PL-11 after exposure of aspirin, respectively. Both LTA and PL-11 were 100% sensitive to aspirin treatment according to the cut-off value defined previously. ROC analysis showed the cut-off value for PL-11 was 33.3% (sensitivity 100%, specificity 95%) (Table II). When increased to 50%, the sensitivity and specificity were both 100%.

PL-11 and LTA got a moderate correlation (r = 0.641, p < 0.001) (Table III). There was a small bias between PL-11 and LTA at baseline (bias = 0.194, p = 0.804) (Figure 3A). However PL-11 results were absolutely higher than LTA during treatment (bias = 24.0%; Figure 3B).

PL-11 and VerifyNow

A relative reduction of 32.9% was found with VerifyNow 2 hours after aspirin intake. Correlation between PL-11 and VerifyNow was highest in our study (r = 0.829, p < 0.001). There was also a moderate correlation during treatment (day 1 and day 2 during aspirin therapy, r = 0.547, p = 0.013). The ROC analysis suggested that 533 ARU should be the cut-off value for VerifyNow, with both 100% sensitivity and specificity.

PL-11 and TEG

TEG data decreased by 65.2% compared with the baseline after the exposure of aspirin, which was similar with PL-11 (65.9%). Correlation between TEG and PL-11 was moderate (r = 0.697, p < 0.001) and a cut-off value of 60.2% for TEG was obtained with ROC analysis (both sensitivity and specificity were 100%).

Discussion

We compared three platelet function assays (LTA, VerifyNow and TEG) with a new point-of-care device PL-11 to assess the short-term aspirin response in healthy individuals. A uniform and significant reduction of platelet function occurred two hours after 100 mg aspirin-treatment, demonstrating the sufficient ability of low dose of aspirin to inhibit platelet cyclooxygenase-1 (COX-1) in healthy individuals. It was consistent with previous reports [7–10]. The significant decrease with no overlap tested by LTA, VerifyNow, PL-11 and TEG after aspirin intake showed their high ability to distinguish pre-treatment from post-treatment values. It may be due to the character of AA induced all-or-nothing response [11]. On the contrary, though days of platelet function recovery varied among subjects, platelet function in our study returned to baseline level 5 days after platelet withdrawal. It was reported that platelet function began to recover less than 2 days and completely recovered 5 days after aspirin cessation [12]. In another study, aspirin function tested by LTA started to return 3 days after stopping aspirin [13]. These results were consistent with that in our study.

LTA was reported as a sensitive marker of aspirin response and considered as the gold standard with its prognostic value [14, 15]. LTA >20% was commonly used as a definition of aspirin low response [16–18]. The upper limit of aspirin response by LTA in our study was 4%, suggesting that there was no aspirin low response. In fact, it was rare to find low aspirin response by LTA in healthy individuals. Normand et al. found no aspirin low response in 45 healthy subjects with AA induced LTA [19]. Fontana et al. only found 1 in 96 healthy individuals treated with aspirin [20].

PL-11 is a new automated point-of-care platelet function analyzer based on platelet count drop method. It counts platelet twice before and more than twice after addition of an agonist in the same citrated whole blood samples. It is different from the original platelet count drop method or a FDA-approved Plateletworks [21], which counts platelets in EDTA and AA induced citrated whole blood samples respectively to obtain an...
Figure 2. Comparison of Aspirin response at baseline, during aspirin treatment and after aspirin withdrawal tested by four different platelet function tests. Boxes: 25th percentile; median, 75th. Whiskers: 5th and 95th percentiles.

Table III. Correlations between methods (baseline up to day 2, n = 30).

<table>
<thead>
<tr>
<th>PL-11</th>
<th>%MAAA, TEG</th>
<th>ARU, VerifyNow</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTA</td>
<td>$r = 0.614, p &lt; 0.001$</td>
<td>$r = 0.662, p &lt; 0.001$</td>
</tr>
<tr>
<td>ARU, VerifyNow</td>
<td>$r = 0.829, p &lt; 0.001$</td>
<td>$r = 0.684, p &lt; 0.001$</td>
</tr>
<tr>
<td>%MAAA, TEG</td>
<td>$r = 0.697, p &lt; 0.001$</td>
<td></td>
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Due to the abnormal distribution of PL-11, LTA, ARU and %MAAA (baseline up to day 2), Spearman correlation was used between the tests.

Figure 3. Bland-Altman analysis between LTA and PL-11 results at baseline (n = 100) and during treatment (n = 30). The solid lines represent bias while the dotted lines represent 5th and 95th limits of agreement.
aggregation ratio. Similar with our results, platelet count drop method was correlated moderately with LTA in previous studies [2]. Van et al. documented that results of Plateletworks were highly time dependent and could not determine the maximal aggregation time [22]. In their study, a decrease of aggregation ratio during 10–15 min after blood drawing were showed at three incubation times in the stimulated blood sample (5 min, 10 min and 15 min). But their study did not show whether there was a maximal aggregation ratio during 5–10 minutes. Plateletworks and platelet count drop method just set an arbitrary incubation time, which is one of the most important factors that might affect platelet aggregation ratio. However, PL-11 was able to give an exact result of maximal aggregation ratio rather than an equivocal result by testing several times intensively after the addition of agonists in the citrated blood sample, suggesting that PL-11 offers a standardized operation for platelet count drop method.

The small bias between LTA and PL-11 at baseline suggested that results of LTA and PL-11 might be interchangeable in healthy individuals without exposure of aspirin. However, the result of PL-11 was significantly higher than LTA during aspirin intake. It might be associated with the intercellular interactions between platelets with other haemocytes in whole blood such as monocytes, leukocytes or erythrocytes, which stimulate platelets to promote the release of 5HT (serotonin), resulting in a higher aggregation ratio than LTA [23, 24]. But it seemed to have little effect on its ability in monitoring aspirin response because there was still a significant difference pre- and post-aspirin treatment. Moreover, PL-11 is more sensitive than light transmittance because it detected single platelets rather than the macroaggregates by TEG because there was a wide overlap between pre- and post-aspirin treatment. PL-11 is more sensitive than light transmittance because it detected single platelets rather than the macroaggregates 5HT (serotonin), resulting in a higher aggregation ratio than LTA [23, 24]. But it seemed to have little effect on its ability in monitoring aspirin response because there was still a significant difference pre- and post-aspirin treatment. Moreover, PL-11 is more sensitive than light transmittance because it detected single platelets rather than the macroaggregates, which will not be counted as single platelets by PL-11 because they exceed the threshold limits for platelet size (<30 fl).

VerifyNow is a commonly used point-of-care platelet function assay. PL-11 correlated well with VerifyNow, suggesting that it was due to the whole blood samples they used. When using a cut-off value of <550 ARU as the presence of aspirin, VerifyNow was reported to be correlated with clinical outcomes [27, 28]. And the result in our study (533 ARU) was closed to such value.

TEG PlateletMapping assay in the present study distinguished pre-aspirin from post-aspirin fairly well. PL-11 had an obvious correlation with TEG. Norman et al., however, in a previous study found an insufficient ability of aspirin inhibition monitored by TEG because there was a wide overlap between pre- and post-aspirin treatment in 45 healthy individuals [19]. Nalyaka et al. estimated AA induced MA in 25 healthy subjects who were treated with aspirin but failed to distinguish a significant aspirin response in 4 (16%) either, who might be the so-called aspirin low responders [29]. Short-term aspirin response was various detected by the same method in different studies, so it is imprudent to compare such different results with ours since the small population in these studies. But it is clear that a wide overlap means a high false positive rate, making a platelet function device unreliable. These studies accompanied with ours, on the other hand, suggested that the difference of aspirin response with TEG might be due to the variation of subjects involved in each study, not the method itself and large cohort studies need to be done to solve the problem.

Limitations

Though 100 healthy individuals were enrolled, only 10 of them took aspirin for treatment and the results were limited by young subjects. So, the cut-off value for each assay was therefore arbitrary due to the small population. We did not detect thromboxane B2 to ensure compliance as it was reported correlated with LTA and was assessed as a suboptimal choice to detect the presence of aspirin [30]. Correlations between each assay for monitoring other antiplatelet medications were not involved in the present study. Therefore, further large cohort clinical and experimental studies are required.

Conclusions

Platelet function assays were not equally interchangeable for monitoring aspirin response and the correlations among them were varied. PL-11 is correlated with the other three major assays and would be a substitute for assessing aspirin response. The cut-off value <50% for PL-11 in our study suggested the presence of aspirin needs to be confirmed by large cohort studies.

Declaration of interest

The authors declare no conflicts of interests. The authors alone are responsible for the content and writing of this article.

References

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