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Assessment of Multiplate® platelet aggregometry using Citrate, Heparin or Hirudin in Rhesus macaques

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Abstract

Electrical impedance aggregometry (EIA) has gained popularity for clinical and research applications. Nonhuman primates are used to study disease and drug-related mechanisms that affect hemostasis, therefore normal establishing normal EIA parameters are necessary. The anticoagulants sodium heparin, hirudin and sodium citrate and three agonists, ADP, ASPI, and collagen were evaluated. Whole blood from 12 adult male rhesus macaques was collected to evaluate anticoagulants, sodium heparin, hirudin and sodium citrate using three agonists (ADP, ASPI and collagen), on the Multiplate® 5.0 Analyzer. Platelet function was reported for three parameters: Area under the curve (AUC), aggregation, and aggregation velocity. There was a significant difference in mean AUC between citrate and heparin samples, and citrate and hirudin samples regardless of the agonist used. There was no difference in AUC between heparin and hirudin. ADP-activated samples showed an increase in impedance with hirudin samples compared to citrate. Furthermore heparin and hirudin out-perform citrate as the anticoagulant for EIA in the macaque. Finally, this study demonstrates the utility of the Multiplate® system in this model and provides important insight into anticoagulant choice when using EIA.

Keywords

citrate; heparin; hirudin; non-human primate; platelet aggregometry

Introduction

During the course of hemostasis, as well as in several diseases including thrombosis, atherosclerosis, diabetes [1], inflammation, and cancer metastasis [2], platelets undergo activation and aggregation. Life-threatening thrombocytopenia leading to hemorrhage comprises a significant aspect of the acute radiation hematopoietic syndrome; thus there is a

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critical need to develop medical countermeasures in order to mitigate the effects of acute radiation in the event of accidental or intentional radiation exposure. Non-human primates (NHPs) are phylogenetically similar to humans [3] and provide a translational platform for the development of therapeutics wherein platelet function might be altered, including the radiation induced hematopoietic syndrome seen in humans [4]. Although high doses of radiation seem to have little effect on blood cell function including erythrocytes, neutrophils, and platelets [4,5], the ability to assess off-target platelet function in the development of radiation countermeasures is nevertheless important.

Aggregation studies should be performed within four hours of sample collection to avoid a decline in agonist sensitivity [6] and the direct thrombin inhibitor hirudin should be used as the anticoagulant [7]. Several studies have now shown that EIA is a reliable assay in humans, dogs and rats [8], however, the literature is lacking in studies that determine whether EIA is applicable to NHP blood. Furthermore, there is a compelling need to determine which anticoagulants and platelet agonists function properly in the Multiplate[®] system using NHP blood. For this purpose we evaluated three different anticoagulants (sodium heparin, hirudin and sodium citrate) and employed three agonists (ADP, ASPI, and collagen).

Methods

Subjects

Twelve adult male rhesus macaques (*Macaca mulatta*) ranging from 10 to 13 years (median = 11.43 years) and weighing 9.3–27.9 kg (median = 14.9 kg) were used in this study. All animals were housed at the Wake Forest School of Medicine (WFSM) Center for Comparative Medicine Research and housed either in pairs or alone if socially incompatible. All animals were pre-screened to exclude simian retrovirus infection and tuberculosis, and were fed a custom diet modeling the Typical American Diet (TAD) (Lab Diet 50LP; calories provided by: 36.59% fat, 44.99% carbohydrates, 18.43% protein). All research procedures were approved by the (WFSM) Institute for Animal Care and Use Committee (IACUC) and performed in accordance with the *Guide for the Care and Use of Laboratory Animals* [9]. WFSM is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care AAALAC, and operates in compliance with the Animal Welfare Act.

Blood collection

On the day of blood collection, animals were sedated with ketamine (10–15 mg/kg body weight) via intramuscular injection (IM). Peripheral whole blood was collected from the femoral vein. Blood was drawn into blood collection tubes using 22 gauge needles in the following order: 2 ml no-additive tube, (BD Biosciences, San Jose, CA) for discard; 3 ml hirudin additive tube (DiaPharma, West Chester, OH); 2 ml sodium heparin tube (BD Biosciences) and a 1.85 ml sodium citrate tube (BD Biosciences). The no-additive tube was used to avoid the initial platelet activation that occurs due both to the act of drawing blood, or to tissue factor IV introduction into the blood tubes containing additive. A second blood draw was performed within two weeks of the first in order to assess the temporal stability of

the measures. The same technician collected all blood in a consistent manner between animals and sampling points.

Aggregation assay

Platelet aggregation was assessed using adenosine diphosphate (ADP, 6.5 μ M), arachidonic acid (ASPI, 0.5 mM), collagen (3.2 μ g/ml), and sodium chloride (NaCl) control as agonists. Each agonist was reconstituted, aliquoted, and stored according to the manufacturer's recommendations (DiaPharma, West Chester, OH). Briefly, following standardized setup of the Multiplate[®] 5.0 Analyzer, cuvettes were loaded with 300 μ l of NaCl or sodium chloride/calcium chloride (CaCl). Next, 300 μ l of whole blood was electronically pipetted into each cuvette followed by a three minute incubation while the sample was stirred automatically. After the incubation, 20 μ l of the agonist was added to each respective cuvette followed by a six-minute incubation period. At the end of six minutes, platelet aggregation was measured as electrical impedance. Measurements included each of the following parameters. Aggregation: measured in AU (aggregation units), which is the increase in impedance or change in electrical resistance between the electrodes. Area Under the Curve (AUC), measured in U=AU*min ([1U=10AU*min] which is determined by the height of the aggregation curve and the slope) is an expression of overall platelet activity and the preferred output parameter for the interpretation of platelet aggregation. Velocity, measured in AU/minute, is the maximum slope of aggregation. Figure 1 depicts a typical impedance graph obtained from the Multiplate[®] Analyzer for a single animal.

Statistical analyses

The values for each measure (aggregation, AUC, velocity), agonist (ADP, ASPI, collagen, NaCl control), and anticoagulant from sampling points were compared using paired *t*-tests to assess consistency between measures. None of the comparisons reached significance, indicating that the two time-points did not differ. Therefore, the values for both sampling points were averaged to create a mean value. For each agonist used in a given measure of platelet function, a repeated measures analysis of variance (RMANOVA) was employed to detect differences in the mean values for citrated, heparinized, and hirudin-mixed samples. To determine the degree of concordance between samples mixed with citrate, heparin, or hirudin, Pearson's correlations were calculated for the samples within each of the three agonists. This was done separately for the individual measures of platelet activation (i.e., aggregation, AUC, and velocity). Data were evaluated for normality using a one-sample Kolmogorov-Smirnov test and were inspected for homogeneity of variance. All statistical analyses were carried out using the Statistical Package for Social Sciences, version 17.0 (SPSS; IBM, Chicago, IL). Because of the relatively large number of statistical tests conducted in this report the criterion for significance was set at $P < 0.01$.

Results

Temporal stability

In addition to ADP, ASPI, and collagen as agonists, samples with NaCl used as a control were also evaluated for temporal consistency by comparing the parameters between two time-points. There were no differences detected between obtained values at the two

sampling points for any parameter ($P > 0.01$). Therefore, the time-points for all measures were averaged, with the resulting values employed in the subsequent analyses (Tables 1–3 for parameter means and standard errors). Control samples were withheld from further analysis.

Aggregation

Mean values and 95% confidence intervals are provided in Table 1. A significant main effect of anticoagulant was observed for ADP ($P < 0.001$; Figure 2). Specifically, platelet aggregation was higher for samples mixed with either hirudin or heparin than citrate ($P < 0.001$). There was no difference in aggregation between heparinized and hirudin-mixed samples ($P = 0.99$). Main effects of anticoagulant were also found for both ASPI and collagen-added samples ($P < 0.001$; Figure 2). Aggregation in blood samples collected in heparin and hirudin was strongly correlated when ADP was used as the agonist ($R = 0.74$, $P = 0.005$; Figure 3A), though neither were related to aggregation when blood was collected in citrate (heparin: $R = 0.03$, $P = 0.92$; hirudin: $R = 0.20$, $P = 0.54$). When ASPI was used as an agonist, differences between anticoagulants in degree of aggregation were similar to those observed with ADP ($P < 0.001$), though there was no association between aggregation in heparinized and hirudin-mixed samples ($R = 0.57$, $P = 0.05$; Figure 3B). Tests of simple effects in samples wherein collagen was used as an agonist yielded similar findings ($P < 0.001$). When stimulated with collagen, there was a positive association between aggregation in samples mixed with heparin and hirudin ($R = 0.74$, $P = 0.006$; Figure 3C). When ASPI was used as an agonist, there were no relationships detected between samples from any of the three anticoagulants (citrate and heparin: $R = -0.06$, $P = 0.87$; citrate and hirudin: $R = 0.27$, $P = 0.40$; heparin and hirudin: $R = 0.57$, $P = 0.05$). For collagen, there were no correlations between aggregation in citrate and hirudin ($R = 0.60$, $P = 0.04$), or citrate and heparinized samples ($R = 0.17$, $P = 0.61$).

AUC

Descriptive statistics are reported for AUC in Table 2. There was a significant main effect of anticoagulant for samples in which platelets were stimulated with ADP ($P < 0.001$; Figure 4). As was the case with aggregation, activation was greater in hirudin- or heparin-mixed samples than for citrated blood ($P < 0.001$). There were no differences in activation for heparin and hirudin ($P = 0.03$). However, values from samples with the two anticoagulants were positively correlated ($R = 0.83$, $P = 0.001$; Figure 3), though neither was associated with activation in samples mixed with citrated blood (heparin: $R = -0.05$, $P = 0.89$; hirudin: $R = 0.28$, $P = 0.39$). Main effects of anticoagulant were also detected for ASPI and collagen ($P < 0.001$; Figure 4). When ASPI and collagen were employed as agonists, differences in platelet activation between anticoagulants were similar to those observed with ADP ($P < 0.001$). No differences were found between heparin and hirudin when either ASPI ($P = 0.46$) or collagen ($P = 0.99$) were used to facilitate activation. Degree of platelet activation in samples mixed with hirudin or heparin was positively correlated when collagen was used as an agonist ($R = 0.80$, $P = 0.002$; Figure 3), but not ASPI ($R = 0.67$, $P = 0.02$). Activation in citrated samples was not associated with that in heparinized or hirudin-mixed samples for ASPI (heparin: $R = 0.09$, $P = 0.79$; hirudin: $R = 0.41$, $P = 0.19$) or collagen (heparin: $R = 0.15$, $P = 0.65$; hirudin: $R = 0.52$, $P = 0.08$).

Velocity

Velocity measures are presented in Table 3. Significant main effects of anticoagulant were detected for blood samples treated with each of the three agonists ($P < 0.001$; Figure 5). Moreover, velocity of platelet aggregation differed significantly among blood samples combined with citrate, heparin, or hirudin in tests using any of the three agonists evaluated in the present study (ADP: $P < 0.001$; ASPI: $P < 0.001$; collagen: $P < 0.001$). When ADP was used as an agonist, there were no associations between the velocities of the samples of any of the three anticoagulants (citrate and heparin: $R = 0.18$, $P = 0.57$; citrate and hirudin: $R = 0.68$, $P = 0.01$; heparin and hirudin: $R = 0.70$, $P = 0.01$; Figure 3). In samples wherein platelet activation was facilitated using ASPI, there was a positive correlation between the velocities in heparinized and hirudin-mixed samples ($R = 0.78$, $P = 0.003$). No correlations between either anticoagulant with citrated samples were detected (heparin: $R = 0.34$, $P = 0.28$; hirudin: $R = 0.66$, $P = 0.02$). Similarly, the velocities of heparinized and hirudin-mixed samples were positively associated in collagen-facilitated samples ($R = 0.88$, $P < 0.001$), but neither were correlated with velocity in citrated samples (heparin: $R = 0.19$, $P = 0.56$, hirudin: $R = 0.42$, $P = 0.18$).

Discussion

In this study, we evaluated platelet aggregation using impedance aggregometry in rhesus macaques. In doing so, we assessed three different anticoagulants in samples that were activated with three separate agonists. Impaired platelet response was noted consistently with citrate samples, shown by a reduction in aggregation relative to heparin and hirudin as measured by each parameter (aggregation, AUC, and velocity). These differences were independent of the agonist used. Thus, the choice of anticoagulant used to assess platelet functioning should be carefully considered.

Consistent with the manufacturer's recommendation, previous studies suggest that hirudin is the standardized anticoagulant of choice for multiple electrode aggregometry [10–13]. However, some studies report that heparinized blood yields reliable results. Data from the present study indicate that higher levels of platelet activation were found in samples mixed with either heparin or hirudin. Hirudin elicits its anticoagulant effects by directly inhibiting thrombin, which does not seem to affect ionized calcium levels and thus influence platelet function as seen with citrate [14]. Heparin, on the other hand, elicits its anticoagulating action by activating anti-thrombin, thus indirectly inhibiting thrombin. Heparin induces microaggregates that may influence MEA results [15,16]. In contrast to its use in whole-blood platelet aggregometry, heparin should be avoided in LTA, as it may increase *in vitro* formation of microaggregates in platelet rich plasma which lead to opacity and an inaccurate, false positive [15]. Mean aggregation values for heparin and hirudin were significantly different from citrate, though they did not differ from one another in aggregation (Figure 2). Again, this could be due to the calcium-chelating effects of citrate [17], which are not observed with heparin or hirudin. This was further supported by the observation that mean AUC for heparin and hirudin were not significantly different from one another. They were statistically different from citrate (Figure 4). The mean velocities for each anticoagulant, however, were all significantly different from one another (Figure 5).

These differences may be due to the particular agonist used; however we also considered the possibility that microaggregates formed in the heparinized samples. Due to the similarity between mean values of heparinized and hirudin-mixed samples, and in an effort to determine whether there were correlations between citrate and the other employed anticoagulants, we performed a correlational analysis between anticoagulants for each of the agonists in all three measures of platelet activation. These data suggest that hirudin and heparin tend to be positively associated, especially when platelets are stimulated by collagen (Figure 3C, F, I). Citrate did not correlate with heparin or hirudin when any of the agonists were employed in any activation measures. Thus, for multiple electrode aggregometry, our data indicate that heparin and hirudin are the optimal anticoagulants. Hirudin, in particular, should be considered, as it is a highly selective inhibitor of thrombin and does not affect intracellular calcium levels [18].

Normative values for platelet aggregation using the multiple electrode aggregometry system have not been established for rhesus macaques. Our study provides essential data regarding anticoagulant choice and agonist usage in Multiplate® aggregometry. Finally, it provides a reference range for rhesus macaque whole blood impedance aggregometry, which will be useful in evaluating platelet function in laboratory and zoo-housed nonhuman primates.

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Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Table 1

Mean aggregation across three platelet activation agonists (ADP, ASPI, and collagen) and three anticoagulants (citrate, heparin, and hirudin) expressed in aggregation units (AU) in bold print. The 95% confidence interval (CI) about the mean is provided in normal typeface below the mean.

	ADP			ASPI			Collagen		
	Citrate	Heparin	Hirudin	Citrate	Heparin	Hirudin	Citrate	Heparin	Hirudin
Mean	58.70	127.61	130.55	61.67	139.30	135.31	57.85	134.15	126.91
95% CI	49.54–67.87	119.27–135.95	117.10–144.00	47.25–76.08	127.99–150.62	126.41–144.22	45.89–69.82	123.93–144.38	115.78–138.04

Table 2

Mean area under the curve (AUC) is depicted in bold for blood samples mixed with three anticoagulants (citrate, heparin, and hirudin) and agonists (ADP, ASPI, and collagen). Values are expressed as units (U), and the 95% confidence interval (CI) about the mean is provided in normal typeface below the mean.

	ADP			ASPI			Collagen		
	<u>Citrate</u>	<u>Heparin</u>	<u>Hirudin</u>	<u>Citrate</u>	<u>Heparin</u>	<u>Hirudin</u>	<u>Citrate</u>	<u>Heparin</u>	<u>Hirudin</u>
Mean	29.96	72.17	77.79	34.04	79.83	83.54	16.54	68.00	69.25
95% CI	25.27–34.65	67.16–77.17	70.74–84.84	23.88–44.21	72.88–86.79	77.52–89.56	12.40–20.68	62.98–73.02	62.40–76.10

Table 3

Mean velocity is presented in bold print across three platelet activation agonists (ADP, ASPI, and collagen) and three anticoagulants (citrate, heparin, and hirudin) expressed in aggregation units per minute (AU/min). The 95% confidence interval (CI) about the mean is provided in normal typeface below the mean.

	ADP			ASPI			Collagen		
	<u>Citrate</u>	<u>Heparin</u>	<u>Hirudin</u>	<u>Citrate</u>	<u>Heparin</u>	<u>Hirudin</u>	<u>Citrate</u>	<u>Heparin</u>	<u>Hirudin</u>
Mean	7.80	16.93	20.33	9.45	19.18	22.87	8.37	15.62	19.19
95% CI	6.72–8.88	15.74–18.12	18.71–21.96	6.90–12.00	16.99–21.37	20.59–25.15	6.63–10.10	14.46–16.78	17.25–21.13