Multiplate® Whole Blood Impedance Point of Care Aggregometry: Preliminary Reference Values in Healthy Infants, Children and Adolescents

Multiplate® – vorläufige Referenzwerte für Säuglinge und Kinder

Introduction

Primary haemostasis involves a controlled cascade of events where under flow conditions activated platelets play a key role. Vascular damage induces the interaction with collagen, and von Willebrand factor leading to platelet activation, which then through release of numerous activation propagates, i.e. platelet adhesion to the vascular tissue, platelet aggregation, and formation of a platelet plug. Apart from acquired platelet function disorders, which in the majority of cases are due to administration of antiplatelet agents or antibiotics, inherited disorders of platelets

Abstract

Background: The aim of the present study was to evaluate paediatric reference values for platelet function using a point-of-care whole blood impedance aggregometry.

Methods, Results & Conclusion: In 265 healthy infants and children aged ≤18 years aggregation was assessed on the Multiplate analyzer (Dynabyte®, Munich, Germany), using arachidonic acid (ASPI: 0.5 mM), TRAP-6 (TRAP: 32 uM), collagen (3.2 ug), ADP (6.5 uM) and Ristocetin (Risto: 0.77 mg/ml) in isotonic saline prediluted (0.3 ml) hirudin-anticoagulated (25 ug/ml) whole blood (1.3 or 5 ml tubes). Aggregation was continuously recorded over five minutes. The increase of impedance due to the attachment of platelets to the electrodes was detected and transformed separately to arbitrary aggregation units (AU) plotted against time. Aggregation measured was quantified as area under the curve (AUC) of arbitrary units [AU * min]. Median [95% confidence interval] values and cut-off values (≤5th and 10th age-dependent percentiles were shown. Following logarithmic transformation of baseline data variance analysis showed significant differences between age groups for induced aggregation with ASPI (p < 0.001) and TRAP (p = 0.002). Overall, infants aged 0.1–12 months showed the lowest values compared with the remaining age groups. No gender differences were detected.

Zusammenfassung

Hintergrund: Ziel der vorliegenden Arbeit war es, pädiatrische Referenzwerte für die Vollblutimpedanzaggregometrie mittels Multiplate® zu erstellen.

Methoden, Ergebnisse und Schlussfolgerung: Bei 265 gesunden Kindern (≤18 Jahre) wurde die Thrombozytenaggregation mittels Multiplate® Analyzer (Dynabyte®, München) mit den folgenden Reagenzien gemessen: Arachidonsäure (ASPI: 0,5 mM), TRAP-6 (TRAP: 32 uM), Kollegen (3,2 ug), ADP (6,5 uM) und Ristocetin (Risto: 0,77 mg/ml). Die Probenentnahmeröhrchen (1,3 oder 5 ml) für Vollblut waren mit Hirudin (25 ug/ml) zur Antikoagulation beschichtet. Die Phasen der Aggregation wurden kontinuierlich über einen Zeitraum von 5 min dokumentiert. Der durch die Anheftung der Plättchen an die Elektroden bedingte Anstieg des Widerstandes wurde in Einheiten (AU) pro Minute (AU/min) quantifiziert. Die gemessene Aggregation wurde als Fläche unter der Kurve als AU pro Minute (AU/min) quantifiziert. Median (95% Vertrauensbereich) und Schwellenwerte (≤5th und 10th altersabhängige Perzentile) wurden dargestellt. Nach logarithmischer Transformation der Daten konnte in der Varianzanalyse ein signifikanter Unterschied der induzierten Aggregation mit ASPI (p < 0.001) und TRAP (p = 0.002), nachgewiesen werden. Im Vergleich zu älteren Kindern hatten Kinder <12 Monate niedrigere Werte. Eine Geschlechtsabhängigkeit konnten wir nicht finden.

Key words
platelet function
reference values
infants
children
impedance
aggregometry

Schlüsselwörter
Thrombozytenfunktion
Referenzwerte
Säuglinge
Kinder
Impedanzaggregometrie

Bibliography
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Correspondence
Prof Dr. Ulrike Nowak-Göttl
UK Münster
Univ. Children’s Hospital
Pediatric Hematology/ Oncology
Chief pediatric coagulation laboratory
Albert-Schweitzer-Straße 33
48149 Münster
Germany
Tel.: +49/251/834 7783
Fax: +49/251/834 7828
leagottl@uni-muenster.de

Authors
S. Halimeh1, G. de Angelis2, A. Sander2, C. Edelbusch2, H. Rott1, S. Thedieck2, R. Mesters3, N. Schlegel4, U. Nowak-Göttl2

Affiliations
Affiliation addresses are listed at the end of the article.
constitute a group of rare diseases that give rise to bleeding syndromes of variety severity also observed during infancy and childhood [2,12]. Monitoring antplatelet therapy or diagnosis of a platelet function disorder is based on the observation of abnormalities of the patient’s platelets as compared to normal platelets [2,4,5,12,13]. In the past, platelet function was assessed mainly for platelet haemostatic capacity to control bleeding. The physiologically closest way to test platelet function is the bleeding time by inducing a lesion and to measure the time required to stop bleeding. A standardized method to test the bleeding time has been developed using a template for the injury of the microvasculature tissue with a defined length and depth of the cut [9]. This method was widely used in the last decade but is not very popular today and in children because it hurts, leaves scars and is not very sensitive and specific. Another in vitro method, the light transmission aggregometry, in which agonists can be added to platelet rich plasma and then increase the light transmission when platelets start to aggregate was first described by Born in the early 60th [3]. This method still known as the gold standard, has also a variety of limitations such as the need to separate platelets from other cell components. However, none of the tests reported including the “gold standard” are suitable for clinical practice, where a simple instrument enabling quick measurement of platelet function with minimal volume of blood is needed. Thus, to appropriate diagnose a platelet function disorder in a point-of-care setting during early childhood or to monitor antplatelet agents in children the knowledge of the physiological characteristics of platelets in the paediatric population is mandatory [5–8,10–12]. The newly developed point-of-care instrument [14] permits platelet aggregation to be measured after adding commonly used agonists by detecting changes in electrical resistance in small volumes of whole blood: Since paediatric reference values for this promising method are missing, the aim of the present study was to establish reference values in healthy infants and children.

Methods

Ethics, patients and control subjects
The present study was performed in accordance with the ethical standards laid down in the updated version of the 1964 Declaration of Helsinki and was approved by the medical ethics committee of the University of Münster, Germany. With written parental consent, 265 healthy children were enrolled. The control children had no history of chronic diseases and were not on medication at the time of recruitment. They presented as outpatients for evaluation prior to minor surgery (planned circumcisions and hernias >90%) or potential bone marrow donation (~10%). Blood cell counts of the healthy children enrolled were within the normal age-dependent reference ranges reported for children in our institutions (150000/ul–350000/ul for platelet count). In addition, with parental consent aggregation curves of two paediatric patients with known Glanzmann’s thrombasthenia are shown.

Exclusion criteria
Children with underlying medical conditions including obesity (defined as body mass index >30 kgm⁻²) or a personal resp. family bleeding history or new laboratory evidence of an inherited or acquired plasmatic coagulation disorder were not enrolled in the present study to establish paediatric reference values.

To demonstrate the differences to patients with inherited platelet disorders the Multiplate aggregation curves of 2 children with Glanzmann’s thrombasthenia will be shown in addition.

Blood sample collection
Blood sample collection was done in the morning after a 12 h fasting period (infants 4–6 h); whole blood samples were drawn by peripheral venipuncture into 1.3 or 5 ml plastic tubes, containing hirudin (25 µg/ml) as anticoagulant. The blood was kept at room temperature during the experiment.

Measuring platelet aggregation
Aggregation was assessed on the Multiplate analyzer (Dynabyte®, Munich, Germany), using adenosine diphosphate (ADP:6.5 µM), collagen (3.2 µg), thrombin receptor-activating peptide [TRAP:6: 32 µM], arachidonic acid (ASPI: 0.5 mM), and ristocetin (Risto: 0.77 mg/ml) in isotonic saline prediluted (0.3 ml) hirudin-anticoagulated whole blood (0.3 ml per test) as recently described [14]. Measurements were performed between 0.75 and 1.5 h after venipuncture (time window given by the manufacturer: 30–240 min [14]). Aggregation was continuously recorded over five minutes in two independent measuring units (four silver-coated conductive copper wires) per test. The increase of impedance due to the attachment of platelets to the electrodes was detected and transformed separately to arbitrary aggregation units (AU) plotted against time (AU * min). Aggregation was quantified as area under the curve (AUC) calculated from the mean values from the two curves (internal control: accepted differences between curves: <20%). Method reproducibility was 6.5%.

Statistical analysis
Normal data distribution was tested with Levene’s test for equality of variance and D’Agostino-Pearson test for normality (MedCalc® software, version 11.1.1.0 for windows XP). Since normal distribution of baseline data was rejected, descriptive results were reported as median values and 95% confidence intervals (CI). Based on literature data controls were divided into four age groups: 0.1–12 months, 1.1–4 years, 5–9 years, and 10 – < 18 years. Differences in baseline data between age groups were analysed after log-transformed data using one-way analysis of variance and Student-Newman-Keuls post hoc test for all pairwise comparisons. A p-value < 0.05 was considered statistically significant.

Results

Following logarithmic transformation (log-normal data) of baseline aggregation data the ANOVA one way analysis of variance showed significant differences between age groups for induced aggregation with ASPI (aggregation: p < 0.001; AUC: p < 0.001) and TRAP (velocity: p = 0.001; aggregation: p < 0.001; AUC: p = 0.002). In contrast, no significant differences in baseline aggregations between age-groups were found for collagen-(velocity: p = 0.08; aggregation: p = 0.1; AUC: p = 0.07), ADP-(velocity: p = 0.6; aggregation: p = 0.3; AUC: p = 0.37) and ristocetin-(velocity: p = 0.45; aggregation: p = 0.5; AUC: p = 0.1) induced platelet aggregation respectively. Overall, the youngest children aged 0.1–12 months showed the lowest aggregation data compared with the remaining age groups. No gender differences were detected. Mean [min-max] body mass index [kgm⁻²] was within
the normal range [15.8 [7.9 – 25.0]]. Back-transformed from logarithmic transformation reference values for healthy infants and children are shown in Table 1. For each parameter median values, corresponding 95% CIs, and 10th and 5th lower percentiles are shown.

*Fig. 1a–e* show typical Multiplate curves during measurement periods of 5–6 min in a five-year-old girl. The change in

<table>
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<tr>
<th>Table 1</th>
<th>Median and 95 % confidence interval M [95% CI] as well as 10th and 5th age dependent percentiles of platelet function according to age groups (back-transformed after logarithmic transformation).</th>
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<tbody>
<tr>
<td>Age group</td>
<td>Proband [n]</td>
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<td></td>
<td>M [95% CI]</td>
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* significant difference between age groups
NO: not determined
AU: arbitrary units
AUC: area under the curve
electrical impedance is calculated from the mean values of the two curves. In addition, in Fig. 2a–e Multiplate tracing curves in a 7.5-year-old male patient with Glanzmann’s thrombasthenia type I are shown: In accordance with the classical aggregometry (data not shown), none of the agonists added led to a change in electrical impedance over the measurement period. In this patient the genetic analysis confirmed the basic disease.

Discussion

Patients with hereditary platelet function defects may display in haemorrhagic patterns like thrombocytopenic bleeding. Bleeding manifestations, however, may be severe or mild depending on the underlying defect. Although exceptions are known, the more severe the specific defect, the worse the bleeding tendency is detected. Since the mid 1960s platelet aggregation testing has been the mainstay used to diagnose and classify hereditary platelet function disorders in patients in whom von Willebrand disease or further plasmatic bleeding disorders have been excluded [1, 16]. With careful attention to technical details, the aggregation curves produced by various agonists provide a sensitive method of detection of most the underlying patho-mechanisms: the patterns of abnormalities in the aggregation curves can be used to tentatively classify the underlying disease. Classical ag-
gregometry is performed in platelet rich plasma prepared by centrifugation of anticoagulated blood in specialized laboratories only. Apart from its poor standardization across laboratories, a major limitation of the classical aggregometric methods for routine use in a paediatric population is that these techniques are consuming much more plasma, i.e. up to 20 ml when performing the complete testing. Technically, whole blood systems are advantageous because they do not require preparation of platelets. Thus, any whole blood systems test not platelets alone, but also their interaction with the other cells in blood. This approach appears to be closer to physiological conditions, and is easier to handle. Therefore, the aim of the present study was to establish paediatric reference values as a prerequisite for future clinical studies for this whole blood point-of-care device. This is the first study evaluating the point-of-care Multiplate® whole blood aggregometry in healthy children. This method is independent on platelet concentration [when platelets are within the normal range], and had an analytical time window between 30 and 240 min after blood sample collection [14]. In addition, the anticoagulant hirudin used in the sample collection tubes has the advantage of preserving the physiological concentration of ionised calcium and magnesium, and thus prevents clotting by inhibiting the action of thrombin. In contrast, the commonly used sodium citrate induces platelet Ca++ flux. Therefore, blood anticoagulated with sodium citrate should rest after collection that platelets could recover [4]. A further advantage of this method allows the clinician to use small amounts of whole blood (1.3–5 ml) to discriminate between severe inherited platelet function disorders and normal platelet function, as shown exemplarily for the two patients with Glanzmann’s thrombasthenia. Although we have shown the usefulness of whole blood impedance aggregometry to discriminate between normal platelet function and Glanzmann’s thrombasthenia in children, we further have to clarify the role of this point-of-care instrument in diagnosing other inherited or acquired platelet function disorders like Bernard-Soulier-Syndrome, or aspirin therapy in children with stroke. In addition, due to the small number of infants enrolled further infants within the first year of life have to be enrolled to obtain robust references values in this specific age group. Especially for the paediatric stroke population this approach seems to be very promising: as shown and discussed before by Riess in 1986 and Velik-Salchner and coworkers results of this method were no different from those obtained with classical aggregometry for detecting the effects of aspirin and clopidogrel in adult preoperative patients scheduled for elective surgery [15,17]. Keeping in mind that there is no single best method currently available the correct diagnosis of inherited platelet function disorders cannot be performed alone by aggregation studies in platelet rich plasma or whole blood, and requires additional tools such as flow-cytometry and molecular diagnosis, further multicentre studies are mandatory to get a feasible consensus for the step by step diagnostic strategy on inherited and/or acquired platelet function disorders in children.

**Conflict of interest:** The authors have no conflict of interest to disclose.

**Affiliations**

1. Ambulatory Health Care Center for coagulation disorders and haemophilia, Duisburg, Germany
2. Univ.-Children’s Hospital, Pediatric Hematology/Oncology, UK Münster, Germany
3. Department of Internal Medicine, Univ.-Children’s Hospital, Pediatric Hematology/Oncology, UK Münster, Germany
4. CHU, Service d’Hematologie Biologique, Hopital Robert Debre, Paris, France

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