Thrombosis Research 194 (2020) 95–97

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Letter to the Editors-in-Chief

Determination of the reference range of platelet aggregation using a new automatic coagulation analyzer and visualization of platelet function data

ARTICLE INFO

Keywords:
Automated light transmission aggregometry
Coagulation analyzer
Platelet function
Platelet visualization

Light transmission aggregometry (LTA) is the gold standard to assess platelet function [1]. In 2013, the Platelet Physiology Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis announced recommendations for standardizing LTA [2]. However, reference intervals of platelet function were not provided. Possible reasons for the absence of these values include the fact that conventional LTA requires specialized skills, substantial blood sample volumes, and extended time periods [3]. Recently, an automated platelet aggregation method has been developed using a new coagulation analyzer (CS-2400; Sysmex, Kobe, Japan). Platton et al. [4] reported a reference interval for platelet aggregation using the CS-2400. However, the concentration ranges of agonists were relatively high, and platelet hyperfunction could not be assessed. We aimed to establish the concentration ranges of agonists to evaluate platelet hyper-and-hypofunction. Furthermore, using the CS-2400, we defined normal values for platelet aggregation for healthy Japanese subjects and evaluated platelet aggregation data using a novel method of data visualization.

Between July 2016 and September 2018, we evaluated 100 healthy individuals, who were aged > 20 years, and who were not currently undergoing medical treatment. The Committee on the Ethics of Research in Human Experimentation at the Iwate Medical University approved the study (H29–36). An abstract of the experiment, including detailed information regarding the study objectives, was provided to each study participant, and written informed consent was obtained from all the participants prior to the commencement of the study.

Using a 21-G needle, 22 mL of blood was collected from the antecubital vein at approximately 11:00 am. The first 2 mL was collected into ethylenediaminetetraacetic acid (EDTA)-2 K-containing tubes for complete blood count, and the remaining 20 mL was collected into a tube with 3.13% sodium citrate for LTA. In this study, blood sample was collected from each subject only once. Platelet-rich plasma (PRP) was separated by low-speed centrifugation and platelet-poor plasma (PPP) was obtained by high-speed centrifugation according to the standard protocol [5]. A previous report demonstrated that there was no dissociation in platelet counts of blood collected in EDTA-2 K and sodium citrate containing tubes, in healthy subjects [5]. Therefore, in this study, we measured the platelet counts of EDTA-2 K and sodium citrated blood samples from healthy subjects.

Platelet aggregation measurements were performed using fully automated aggregometry with the CS-2400. Maximum aggregation rates (MA%) were determined by allowing the reaction to proceed for 420 s after the addition of agonists. Based on a report by Sugawara et al. [5], we tested aggregation responses to agonists at various concentrations: adenosine diphosphate (ADP), collagen, and arachidonic acid (AA) agonists were obtained from Sysmex Corporation (Kobe, Japan). Thrombin receptor activator peptide (TRAP: SFLLRN-NH_2) was obtained from Bachem (Bubendorf, Switzerland). Protease-activated receptor 4 agonist peptide (PAR4-AP: AYPGKF-NH_2) was obtained from GenScript (Piscataway, NJ, USA). The statistical normality of the distribution in the platelet aggregation datasets has been often denied even in healthy subjects as shown in a previous study [4]. Therefore, we used the median and the 25th and 75th percentiles, to define the MA% reference range for all inducers.

We analyzed platelet aggregation using EZR (Easy R) statistical software (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which enabled us to show MA% of platelet aggregation. EZR is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria), which enables the application of statistical functions frequently used in clinical studies [6].

In the present study, 100 healthy subjects (age range, 22–69 years; median age, 36 years; 50 women) were included. Each mean ± standard deviation (SD) of white blood cells, red blood cells, and hemoglobin level was $57.46 \pm 18.94 \times 10^2$/μL, $513.22 \pm 71.48 \times 10^4$/μL, and 15.59 ± 2.16 g/dL. Platelet counts were $23.95 \pm 6.96 \times 10^4$/μL of EDTA-2 K and $21.52 \pm 5.56 \times 10^4$/μL of sodium citrated, respectively. No discrepancy was observed between EDTA blood and citrated blood platelet counts.

The details of MA% with each inducer are shown in Table 1. Each median and the range of MA% at each inducer concentration were calculated and used for the reference to assess the platelet aggregation. Platelet aggregation increased in a dose-dependent manner for all agonists. No platelet aggregation occurred with ADP 0.25 μM, collagen 0.05 μg/mL, AA 0.1 mM, TRAP ≤0.5 μM, or PAR4-AP 25 μM. Platelet aggregation with ADP 1, 2 μM, collagen 0.5 μg/mL, TRAP 1 μM, and PAR4-AP 50 μM exhibited the greatest variation among individuals. In addition, platelet aggregation occurred in most subjects with ADP ≥5 μM, collagen ≥1 μg/mL, AA ≥1 mM, TRAP ≥5 μM, and PAR4-AP

https://doi.org/10.1016/j.thromres.2020.06.001
Received 19 January 2020; Received in revised form 14 May 2020; Accepted 1 June 2020
Available online 18 June 2020

Table 1
Maximum aggregation rates of blood platelets under variable concentrations of agonists.

	Concentrations	Maximum aggregation rate (%) median (range)
ADP (μM)	0.25	0.2 (0.0–6.6)
	1	48.0 (31.3–83.2)
	2	79.8 (57.6–87.2)
	5	85.6 (81.5–89.0)
	10	86.2 (82.3–89.2)
	20	88.6 (85.7–91.8)
Collagen (μg/mL)	0.05	1.7 (0.8–6.0)
	0.5	81.7 (51.2–87.7)
	1	87.9 (84.6–90.2)
	2	91.0 (87.0–93.1)
	5	89.6 (86.5–92.2)
Arachidonic acid (mM)	0.1	1.5 (0.0–7.1)
	1	88.2 (85.4–90.8)
	1.2	91.4 (87.6–93.2)
TRAP (μM)	0.1	0.3 (0.0–1.2)
	0.5	0.0 (0.0–2.2)
	1	20.1 (9.3–86.1)
	5	90.9 (87.1–93.2)
	10	89.5 (85.7–90.8)
PAR4-AP (μM)	25	0.6 (0.2–1.4)
	50	14.4 (1.3–87.3)
	100	91.2 (88.3–94.3)
	200	89.0 (86.6–91.3)

ADP, adenosine diphosphate; TRAP, thrombin receptor activator peptide; PAR4-AP, protease-activated receptor 4 agonist peptide.

≥100 μM.

We used EZR to illustrate platelet function by overlaying each aggregation curve over the reference ranges. The results of healthy subjects are shown in a box plot (Fig. 1). Solid lines on the reference ranges show platelet aggregation of a 36-year-old man (Fig. 1). Overlaying these representations on the graphs enabled easy visualization of platelet function data and aided evaluation of platelet function.

We used the CS-2400 to establish the first reference intervals for platelet aggregation in healthy Japanese subjects. CS-2400 is automatic, and using it is less laborious and less time intensive compared with conventional manual methods [3]. This study demonstrates that increased platelet reactivity can be evaluated by assessing platelet aggregation with low concentrations of agonists and by evaluating platelet hypofunction or antiplatelet efficacy by assessing platelet response to high concentrations of agonists.

Until now, there was no visual method of evaluating platelet function; thus, we proposed this visual method described above. By overlaying sample data on the reference ranges using the EZR statistical software, it enabled us to evaluate platelet aggregation more clearly at the bench. Additionally, in a clinical practice, reports that provide medical information that is easy to understand are extremely valuable. Thus, such studies are important to enable more efficient laboratorial examinations.

This study has three limitations. First, the number of subjects in the study was small. Second, the subjects were relatively young. As cerebrovascular disease is more common at an older age, the reference range in these age groups should be assessed in future studies. We plan to investigate this further using age-matched control samples. Third, the threshold concentrations of most agonists are quite variable and may require a tighter titration. A further detailed investigation is required to determine whether the provided reference ranges are applicable to older or diseased study groups.

In conclusion, we established reference ranges for platelet aggregation in a healthy Japanese population. Overlaying representation of platelet aggregation data on the reference ranges enabled us to evaluate platelet function visually and clearly. In the future, this method may be useful in the evaluation of platelet hyperaggregability and drug efficacy and can potentially contribute to the practical management of antiplatelet therapy.

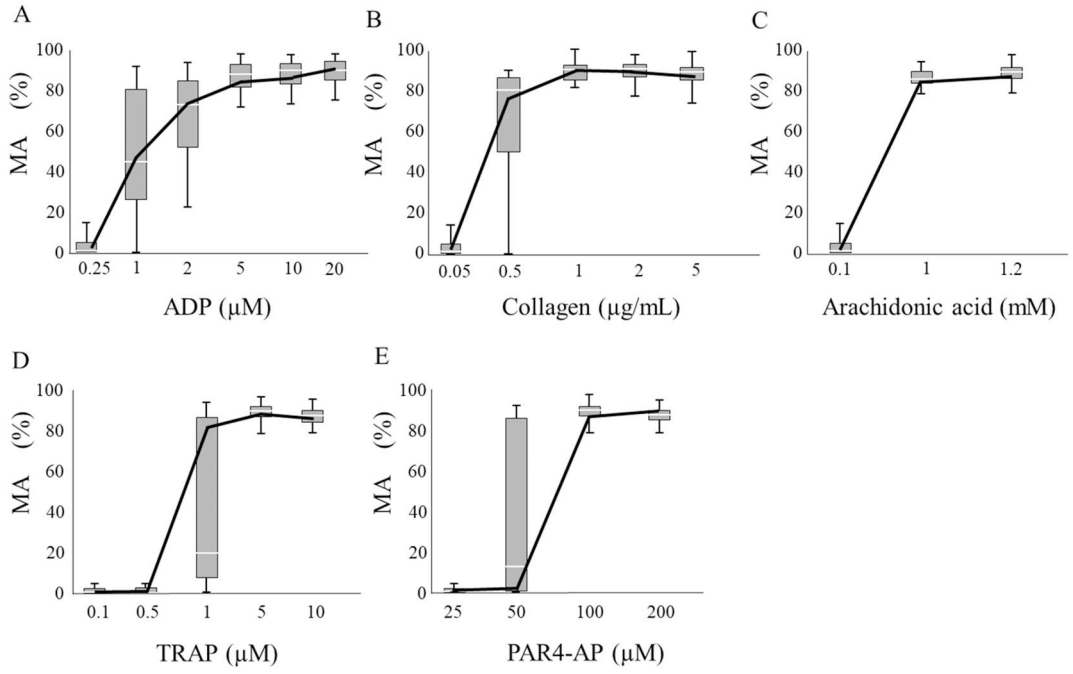

Fig. 1. Platelet aggregation induced with some agonists and visualization of platelet function in a sample subject
Platelet aggregation induced with adenosine diphosphate (ADP) (A), collagen (B), arachidonic acid (AA) (C), thrombin receptor activator peptide (TRAP) (D), protease-activated receptor 4 agonist peptide (PAR4-AP) (E) are shown. The results of healthy subjects are shown in a box plot (gray box) as reference ranges. Median values are represented by white lines. No aggregation was observed in any subject with ADP 0.25 μM, collagen 0.05 μg/mL, AA 0.1 mM, TRAP ≤0.5 μM, or PAR4-AP 25 μM. Solid lines (black) on the reference ranges show platelet aggregation in the blood sample of a 36-year-old man. The aggregate curve (black) is overlaid to aid the visualization of trends.

Acknowledgements

Funding

None.

Declaration of competing interests

None.

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