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Differences in the Composition of Activated Partial Thromboplastin Time (APTT) Reagents Affect Clot Waveform Analysis

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ABSTRACT

Background Clot waveform analysis (CWA) based on activated partial thromboplastin time (APTT) is a useful assay for hemostasis. However, the effects of activators and phospholipid conditions on CWA have not been adequately investigated. Therefore, we characterized CWA using four different APTT reagents.

Methods We used 39 archived plasma samples from patients with hemophilia A (HA), 16 samples from patients with HA under emicizumab treatment, and 10 samples from healthy individuals for CWA with four different types of APTT reagents (reagents A, B, C, and D). We then compared Ad|min1|, Ad|min2|, and Ad|max2| from the CWA, which reflect the maximum velocity, maximum acceleration, and maximum deceleration, respectively, among the four reagents.

Results Similar clot waveform shapes were observed for each reagent in the healthy donor group, HA group, and HA under emicizumab group, and the waveform was different for each target group. Significant changes were found in clotting time (CT) (s), Ad|min1| (%/s), Ad|min2| (%/s^2), and Ad|max2| (%/s^2). The waveform pattern for the coagulation reaction by reagent D, comprising silica and synthetic phospholipids, was the fastest among the reagents examined. Further, the difference in Ad|min1| (%/s) and Ad|min2| (%/s^2) was larger than that in CT depending on the reagent used(s), indicating that the measured value of CWA was affected by the reagent composition.

Conclusion Our results showed a significant difference among reagents with varying composition and concentration; this was found to affect the parameters obtained from CWA. Thus, the differences between reagents hinder standardization of quantitative evaluation using these parameters; further, this highlights the necessity of understanding the characteristics of APTT reagents and determining the reference range in individual facilities.

Key words activated partial thromboplastin time; clot waveform; emicizumab; hemophilia A

Clot waveform analysis (CWA) is an extension of the curve generated during the measurement of two routine coagulation assays, prothrombin time (PT) and activated partial thromboplastin time (APTT), using an optical detection system. The system detects light transmittance or absorbance. CWA is considered a global hemostatic assay that reflects the overall effects of all hemostatic factors.[1–3] Therefore, it provides additional information on coagulation processes while measuring PT and APTT, and can be conveniently obtained using special software in coagulation analyzers. The software program used in the CN-6000 analyzer for APTT using APTT-synthetic phospholipids facilitates display of the clot reaction curves as well as the associated first and second derivative curves (DCs). Thus, both qualitative (waveform pattern) and quantitative (various calculations derived from the curve) parameters can be generated and analyzed (Fig. 1).[1, 4–6] Applied assays such as modified CWA with a PT/APTT mixture reagent are useful for assessing coagulation potential in emicizumab-treated patients with hemophilia A (HA).[7]

However, the effects of activators such as silica and ellagic acid and of phospholipid conditions on CWA have not been adequately investigated. Further, there has been no comparative study of silica- and ellagic acid-based APTT reagents with the same composition and concentration of phospholipids in CWA. Therefore, the purpose of the present study was to compare differences in the parameters calculated from CWA among four types of APTT reagents prepared from silica and ellagic acid and/or the phospholipid conditions.[8]

MATERIALS AND METHODS

Subjects

Archived plasma samples were obtained from patients

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Abbreviations: Ad|max2|, Adjusted |max2|; Ad|min1|, Adjusted |min1|; Ad|min2|, Adjusted |min2|; APTT, activated partial thromboplastin time; bp, biphasic pattern; CT, clotting time; CWA, clot waveform analysis; DC, derivative curve; HA, hemophilia A; max2, maximum deceleration; min1, maximum velocity; min2, maximum acceleration; PT, prothrombin time; LA, lupus anticoagulant

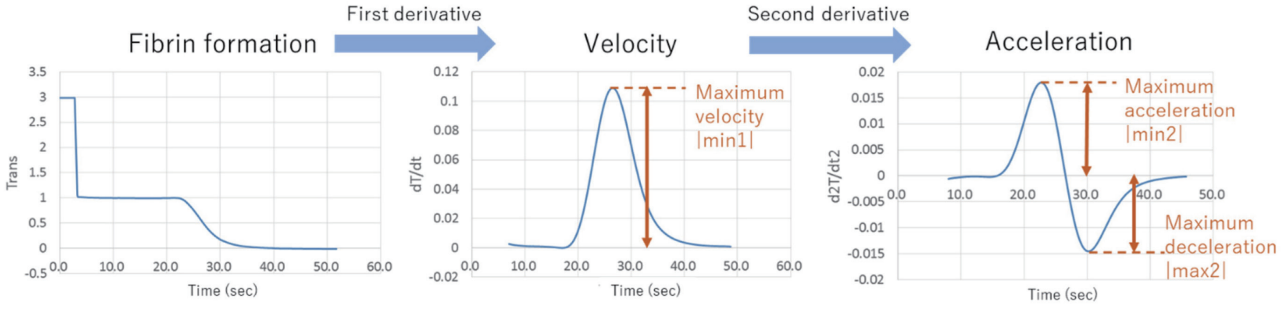

Fig. 1. Clot waveform analysis (CWA). The waveform shows the fibrin formation curve, first derivative curve, and second derivative curve. The first derivative reflects coagulation velocity, and the second derivative reflects coagulation acceleration. Clotting time (CT), maximum velocity (Ad|min1|), maximum acceleration (Ad|min2|), and maximum deceleration (Ad|max2|) were the basic parameters analyzed.

Table 1. Characteristics of APTT reagents

APTT reagents	Phospholipids	Activators	Vendor
Thrombocheck APTT-SLA	Synthetic phospholipids	Ellagic	Sysmex Corporation
Thrombocheck APTT	Rabbit brain-derived cephalin	Ellagic	
Data-Fi	Rabbit brain-derived cephalin	Ellagic	
SynthASil	Synthetic phospholipids	Silica	Werfen

with HA (n = 39) and patients with HA treated with emicizumab (n = 16). These patients (age range 0–76 years) were administered standard FVIII concentrates and emicizumab maintenance at the Tottori University Hospital. All samples from patients treated with emicizumab were obtained during the maintenance phase. Normal plasma samples were obtained from 10 healthy individuals. All samples were collected using standard venipuncture blood collection tubes containing one-tenth the volume of sodium citrate (3.2%/0.109 M) to provide a final citrate concentration of 0.32%/0.0109 M. Platelet-poor plasma was obtained after centrifugation of citrated whole blood for 15 min at 1500 × g. All plasma samples were stored at –80 °C and thawed at 37 °C immediately prior to the assays. All samples were analyzed within six months. This study was approved by the Ethics Committee of the Tottori University Faculty of Medicine (approval number: 19A056). Informed consent was obtained using an opt-out approach.

Measuring equipment and reagents

Four different types of silica-based reagents and an ellagic acid-based reagent were prepared in the laboratory. Thrombocheck APTT-SLA (reagent A; Sysmex Corporation, Kobe, Japan), Thrombocheck APTT (reagent B; Sysmex Corporation, Kobe, Japan), Data-Fi (reagent C; Sysmex Corporation, Kobe, Japan), and SynthASil (reagent D; Werfen, Bedford, MA) were used as commercial APTT reagents. The reagent characteristics are listed in Table 1. Plasma (0.05 mL) was incubated for 1 min at 37 °C in a cuvette, to which 0.05 mL APTT reagent was added, and incubated for 3 min at 37 °C. Finally, $CaCl_2$ (0.05 mL) was added, and clotting time was calculated. All APTT tests were performed using CN-6000 (Sysmex Corporation, Kobe, Japan), a real-time random access intelligent coagulation analyzer with both light scattering and absorbance detection capabilities.

Methods

Coagulation assays for APTT were performed according to the manufacturer's instructions. Additionally, we used the parameters Adjusted |min1| (Ad|min1|) (%/s), Adjusted |min2| (Ad|min2|) (%/s^2), and Adjusted |max2| (Ad|max2|) (%/s^2) obtained from the APTT clotting waveform, which was calculated using the blood coagulation analyzer CN-6000. With the modified CWA, the minimum transmittance (0%) was set in the immediate post coagulation phase, and Ad|min1| (%/s), Ad|min2| (%/s^2) and Ad|max2| (%/s^2) were accordingly defined as the maximum coagulation velocity, acceleration, and deceleration, respectively, and were obtained from the first or second derivative of this adjusted clot waveform.[7] The adjusted CWA data were generated based on the final plateau transmittance and were obtained from the analysis files by the Sysmex software. The adjusted CWA data eliminated the influence of fibrin clot density and fibrinogen levels.[7, 9] First, we performed CWA after

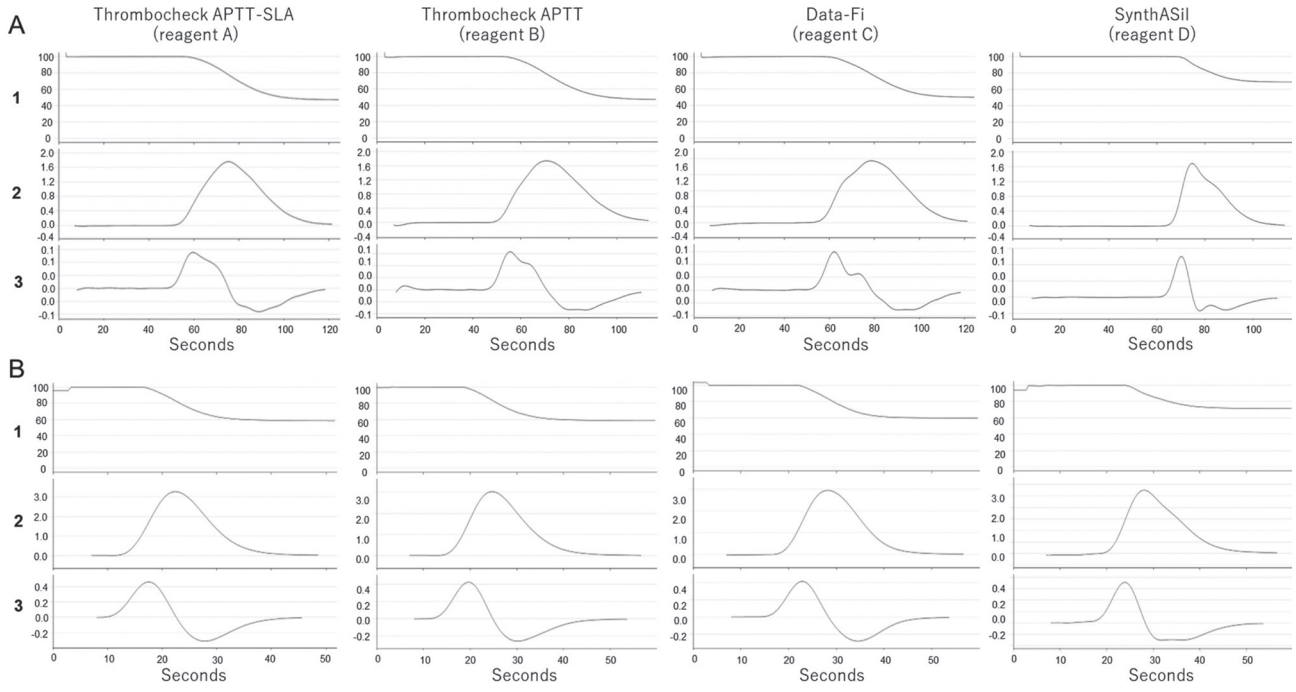

Fig. 2. Comparison of clot waveforms. **A**, Patients with hemophilia A (HA); **B**, patients with HA receiving emicizumab therapy; 1, coagulation; 2, 1st derivative; 3, 2nd derivative.

measuring APTT, and confirmed whether there was a difference in the waveform pattern depending on the reagent for each target group. Next, the CT (s), maximum velocity (Ad|min1|), maximum acceleration (Ad|min2|), and maximum deceleration (Ad|max2|) obtained by analyzing the clot waveform were compared and examined.

Statistical analysis

Data for various parameters of the four reagents were compared using the Friedman test with Bonferroni correction. Statistical analysis was performed using EZR software (ver. 1.54; Saitama Medical Center, Jichi Medical University, Saitama, Japan).[10]

RESULTS

Comparison of clot waveform patterns among four APTT reagents

The waveform patterns of four APTT reagents (A, B, C, and D) were compared. The APTT waveform of a patient with HA (Fig. 2A) showed a biphasic pattern (bp) of the second DC, a markedly prolonged peak time of the first and second DC, and fibrin formation. The waveform pattern of reagent D, comprising silica and synthetic phospholipids, was the fastest among the reagents examined. Further, a bp was observed in cases with severely prolonged APTT among patients with HA in all the reagents, but none of the reagents showed a bp in cases showing mild APTT prolongation among patients with HA. The bp observed in patients with HA was not observed in healthy donors or in patients with HA treated with emicizumab (Fig. 2B).

CT was compared among reagents A, B, C, and D in healthy controls, patients with HA, and patients with HA under emicizumab (Fig. 3A). Similarly, the Ad|min1| (%/s), Ad|min2| (%/s^2), and Ad|max2| (%/s^2) values were assessed and the results are shown in Figs. 3B–D. Further, the difference depending on the reagent in Ad|min1| (%/s) and Ad|min2| (%/s^2) was larger than that in CT (s) from the comparison of *P* values using the Friedman test with Bonferroni correction and indicated that the reagent composition affected the measured value of CWA.

DISCUSSION

Qualitative evaluation of the clot waveform is reported to be useful for detecting hemostatic abnormalities and diagnostic assistance. Further, the bp observed in cases of hemophilia, hemophilia inhibitor use, and lupus anticoagulant (LA) is interesting and useful in CWA[11]; however, in recent years, the parameters obtained from CWA are considered important, and are useful for distinguishing coagulation disorders.[12, 13] Clot waveform shapes were observed to be similar among the reagents in the healthy donor, patients with HA, and patients with HA under emicizumab groups. Our study also indicates that in cases showing a bp, the bp was observed with all

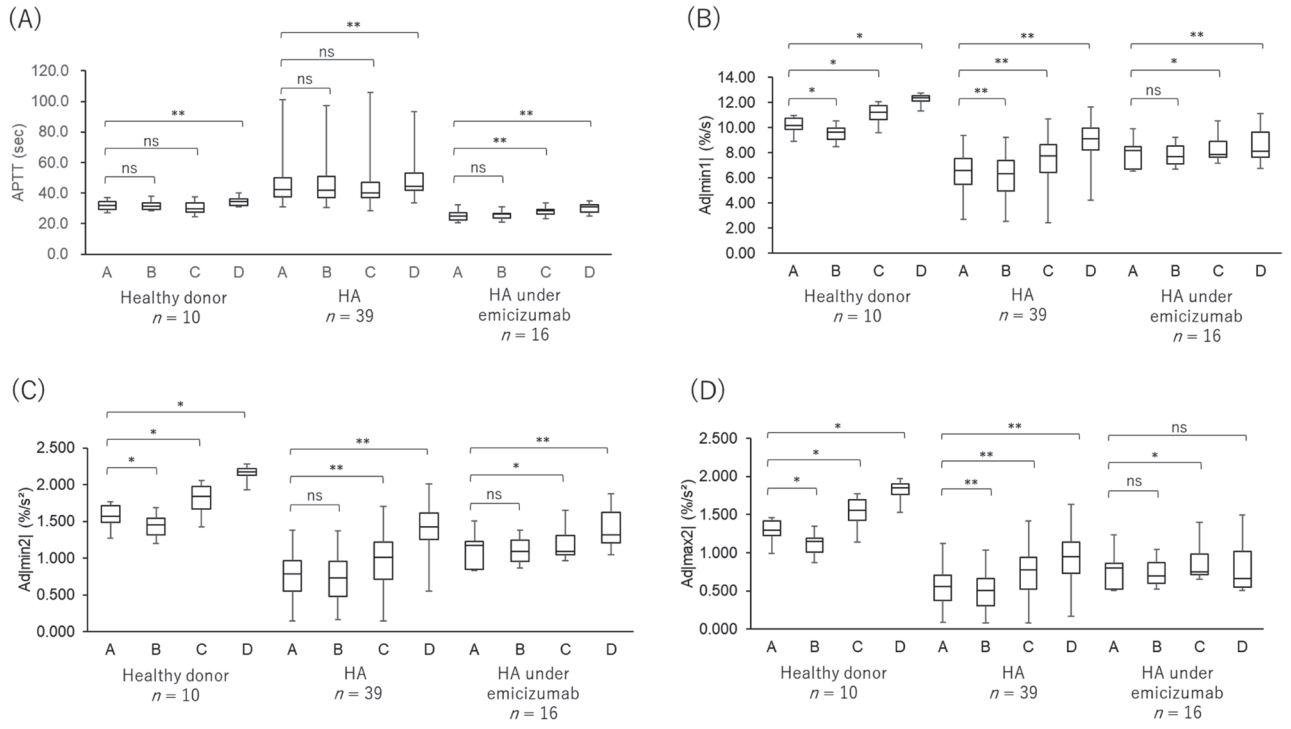

Fig. 3. Comparison of (**A**) Clotting time (CT), (**B**) Ad|min1|, (**C**) Ad|min2|, and (**D**) Ad|max2| obtained from the clot waveform analysis (CWA) of four reagents. Reagent A, Thrombocheck APTT-SLA; reagent B, Thrombocheck APTT; reagent C, Data-Fi; reagent D, SynthASil. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. ns, not significant.

four reagents, and there was no difference in its appearance; however, the bp was not observed in all cases of patients with HA, and was only observed in cases with highly prolonged APTT. For this reason, even though detection of the bp is useful as a diagnostic assistance in patients with HA, combining qualitative evaluation of the clot waveform and quantitative evaluation using the parameters is important to determine patients with HA showing mildly prolonged APTT. Further, the CWA needs to be modified for more accurate monitoring of hemostasis in HA under emicizumab treatment, and a new study on its modification is underway.[7, 14] Additionally, the use of a trigger reagent comprising a balanced mixture of ellagic acid and tissue factor has also provided promising data for the quantitative hemostatic assessment of bypassing therapy. This includes recombinant activated FVII and activated prothrombin complex concentrates in HA patients with inhibitors, reflecting intrinsic and extrinsic coagulation activities.[7, 15] The improvement of a reagent for CWA is required for more accurate monitoring of hemostasis in patients with HA treated with emicizumab.

APTT reagents are also reported to differ in the frequency of atypical derivative plots in FVIII- and FIX-deficient specimens depending on differences in the composition of activators and phospholipids.[16] Even in the normal range, there was a difference in the measured values at min1 (%/s), min2 (%/s^2), and max2 (%/s^2), and the composition of the reagent was found to affect the parameters obtained from CWA. Although reagent A (synthetic phospholipids) and reagent B (rabbit brain-derived cephalin) used different phospholipids, there was no significant difference in the CT (s) of all patient groups and all parameters of patients with HA with emicizumab group. In contrast, reagents B and C, both containing ellagic and rabbit brain-derived cephalin, showed a significant difference in coagulation rate. This suggests that even if the reagent composition is similar, differences in component concentration and activity. Therefore, it is difficult to standardize quantitative evaluation using the calculated parameters because of the difference between reagents; it is thus necessary to understand the characteristics of APTT reagents used in individual facilities and to set respective standards.

In recent years, CWA has been applied to the diagnosis of blood diseases and other diseases. Some trials have comprehensively used CWA to predict hemostatic effects in emicizumab prophylaxis.[7, 17, 18] Further, CWA can be used to obtain specific parameters even in infections without disseminated intravascular coagulation (DIC), to distinguish bacterial or viral infection and facilitate selection and early administration of optimal

antibiotics for each infection[19]; moreover, CWA has been associated with the diagnosis of COVID-19, pathological conditions, and prognosis prediction.[20, 21] CWA can reflect the entire process of clot formation and is expected to be clinically applied for the differential diagnosis of diseases, severity determination, prognosis prediction, and treatment monitoring in the future. A limitation of this study was its small sample size. Further, the effect of LA on the composition and phospholipids remains to be investigated.

In conclusion, we report the effects of the parameters obtained from CWA. The utility of these data is limited to the type of APTT reagent used for testing. It is difficult to standardize the quantitative evaluation using these parameters because of the difference between reagents, and it is necessary to understand the characteristics of APTT reagents and to determine the reference range in individual facilities.

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The authors declare no conflict of interest.

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