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Full Length Article

A ROTEM method using APTT reagent and tissue factor as the clotting activators may better define bleeding heterogeneity in moderate or severe haemophilia A (part I: Study in plasma samples)

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ABSTRACT

Bleeding heterogeneity observed in haemophilia A (HA) may attribute to that the available monitoring methods cannot appropriately reflect the coagulation profile. The present study aimed to develop a global approach by changing the clotting initiation way in rotational thromboelastometry (ROTEM) assay. ROTEM was run in Factor VIII (FVIII)-immune-depleted plasma to which different concentrations of recombinant VIII (rFVIII) had been added, and also in 31 patients with HA. The clotting activators were APTT reagent (1.2×10^{-3} of the dose used in the original APTT method) and recombinant tissue factor (0.02 pmol/L). In FVIII-immune-depleted plasma spiked with rFVIII, maximum velocity of coagulation reliably mirrored the rFVIII levels. This dose-response disappeared after the samples were pre-incubated with an antibody against TFPI, protein S, activated prothrombin complex concentrate or rFVIIa known to favour the extrinsic activation. In the HA patients with FVIII 0–0.21 IU/mL, APTT and ROTEM outcomes varied in significant correlations to FVIII activity; however, this correlation became non-significant when only samples with FVIII 0–0.05 IU/mL were included. Conclusions: The decreased coagulation in HA mostly result from deficiency/absence of FVIII; other pro-/anti-thrombotic proteins are also influential. The multiple effects may cause a mismatch between bleeding phenotype and FVIII concentrations. The ROTEM assay with the clotting activators i.e., tiny doses of APTT reagent and TF are more effective than the original APTT method as regards the assay sensitivity to influence by VIII activity and also to that by other pro-/anti-thrombotic proteins, showing the whole coagulation picture behind the phenotypic heterogeneity in HA.

1. Introduction

In patients with haemophilia A (HA), bleeding frequency is determined by Factor VIII (FVIII) deficiency/absence. Plasma levels of FVIII < 0.01 IU/mL, 0.01–0.05 IU/mL and 0.06–0.40 IU/mL are categorized as severe, moderate, and mild form of HA, respectively [1]. However, certain cases actually reveal incongruity in the bleeding phenotype, as the bleeding phenotype does not consistently correlate with the reported levels of FVIII activity. Data in the literature have reported that around 10% of patients with severe HA displays a mild bleeding pattern, while approximately 25% of patients with moderate HA suffer from frequent haemorrhage [2–5].

In the coagulation cascade [6], FVIII is converted into activated FVIII (FVIIIa) by activated Factor IX (FIXa) via the intrinsic activation, and also by trace amounts of thrombin generated from the extrinsic activation. Together with activated Factor V, FVIIIa aids FIXa to catalyse Factor X (FX); the activated FX is the energetic protease that contributes to thrombin burst in the presence of phospholipids and calcium. In patients with HA, FVIII deficiency/absence leads to a lack of or an undue delay in FX activation, which in turn decelerate thrombin generation and the consequent fibrin formation.

Numerous laboratory assays have contributed to diagnosis and monitoring of HA, among which activated partial thromboplastin time (APTT), Calibrated Automated Thrombogram (CAT) and rotational thromboelastometry (ROTEM) are most available. However, these approaches remain inadequate for defining the bleeding heterogeneity of

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HA, particularly in severe cases (FVIII levels < 0.01 IU/mL) or sometimes in moderate cases (FVIII levels 0.01–0.05 IU/mL) [2–4]. In the APTT method which employs a surface-stimulating substance to initiate clotting, only the rate of intrinsic activation could be detected. Indeed, abnormalities in both the intrinsic and extrinsic pathways can modulate the bleeding patterns of HA. For example, it has been suggested that some impairments in prothrombin, anti-thrombin, tissue factor inhibitor (TFPI), protein C or protein S may consistently mitigate the bleeding symptoms while factor VIII is deficient/absent [4,7]. In the CAT measurement [8] that uses of recombinant tissue factor (rTF) to elicit clotting, monitoring of many pro-thrombotic or haemorrhagic disorders has succeeded; but some cases suffering from HA have not fully benefited [9,10]. This drawback may account for the fact that activation of FX by FVIIIa:FIXa via the intrinsic pathway is 50-fold more efficient than by TF:FVIIa via the extrinsic pathway; but the intrinsic effects are essentially negligible in the TF-derived CAT method [11,12]. With regard to ROTEM assay used in HA management, its working mechanisms still need further improvements because the clotting activator is similar to APTT or CAT [13,14].

In a published study by Matsumoto et al., coagulation in the CAT assay was induced by ellagic acid (0.3 μM) or rTF (0.1 pM), or by a mixture containing both ellagic acid and rTF [15]. Use of the composite clotting activators - rather than either of the individual substances - yielded a greater dependence between the thrombin generation and FVIII levels.

Aiming to obtain a better laboratory tool to manage HA patient, we are interested in referring the design by Matsumoto et al. and developing a new compound of clotting activators for the ROTEM assay. Since some practical problems arose from the extremely low solubility of ellagic acid (data not shown) [16], ellagic acid was replaced with the APTT reagent which can be easily diluted with H_2O. Our working plan is divided into two parts: Part I is the present study using plasma samples, and Part II is being prepared in our group using whole blood samples.

2. Materials and methods

2.1. Subjects and plasma samples

The present study includes plasma samples from 31 patients with HA. Among them, 15 were the remained over from a published investigation on haemophilia A [17] and 16 from those after the routine laboratory investigations. The age range of patients was 2–59 years; median = 27. The study was approved by the regional Ethical Committee and all the patients have given informed consent. The FVIII levels in plasma were tested at the sampling time (not at the first bleeding). Because bleeding heterogeneity of HA particularly appears in cases with FVIII levels of 0–0.01 IU/mL and sometimes in those with 0.02–0.05 IU/mL as mentioned above [2–4,7], we divided the patients' samples into two groups: i) FVIII 0–0.05 IU/mL, n = 20; ii) FVIII > 0.05 IU/mL, n = 11.

The patients' whole blood was drawn from an antecubital vein into evacuated tubes containing 0.129 M citrate (1 part trisodium citrate to 9 parts of blood) and centrifuged within 30 min (2000 × *g*; 20 min; room temperature). The obtained platelet-poor plasma was immediately frozen in aliquots and stored at −80 °C until analysis.

A citrated platelet-poor plasma pool (NPP, lot 7133) was obtained from Precision Biologic, Dartmouth, Canada. The company reported that the sample contained fibrinogen 3.02 g/L (Clauss method), FVIII:C 0.96 IU/mL (one-stage clotting method), and other coagulation factors including FII, FV, VWF, FIX, FX, FXI, FXII and FXIII 0.91 to 1.20 IU/mL. In addition, we used the commercial COAMATIC Factor VIII kits (see the "Laboratory methods" reported later) to test the FVIII activity (chromogenic) that was 0.98 IU/mL.

FVIII immune-depleted plasma [platelet-poor; all the rest of the coagulation factor activities (other than VIII) were ≥50%] was obtained from Affinity Biologicals, Ancaster, Canada. Residual amounts of FVIII (< 0.005 IU/mL by a chromogenic method as mentioned later in this article) and fibrinogen (1.7 g/L by the Clauss method) were confirmed in the Coagulation Laboratory, Karolinska University Hospital, Stockholm, Sweden.

2.2. Reagents

Recombinant Factor VIII (rFVIII) was provided by CSL Behring, Marburg, Germany. APTT reagent (lot no. 112352) was provided by Diagnostica Stago, (Asnieres-sur-Seine, Cedex, France). Recombinant human tissue factor (TF) – Innovin® from Siemens Healthcare Diagnostics, Marburg, Germany was reconstituted to 6000 pmol/L with distilled water. The stock solution was stored in small portions at −20 °C until analysis. A purified phospholipid mixture (PPL) containing highly purified phosphatidylcholine, phosphatidylserine and sphingomyelin, was provided by Rossix, Mölndal, Sweden.

A murine antibody against human tissue factor pathway inhibitor (ab-TFPI) was obtained from American Diagnostica, Pfungstadt, Germany. A sheep antibody against human protein S (ab-protein S) - as a gift - was obtained from Enzyme Research laboratories, IN, USA. The activated prothrombin complex concentrate (FEIBA®; mainly containing prothrombin, Factor VII, Factor IX and Factor X) and recombinant activated Factor VII (rFVIIa) were obtained from the pharmacy at Karolinska University Hospital.

A washed-frozen platelet reagent was prepared from blood from a healthy platelet donor, as described earlier by our group [18]. This frozen platelet reagent was thawed at 37 °C and then centrifuged at 10,000 × *g* for 30 min. In the supernatant (plt-supernatant), FVIII activity (chromogenic assay, see below) was undetectable but residual FV activity was 10% of normal. After the plt-supernatant was incubated with SEKISUI-CD142-fitc (murine-ma-against human-tissue factor–fitc from Sekisui Diagnostics, MA, USA) for 20 min at room temperature, no tissue factor antigen was detected by using a flow cytometer (Becton Dickinson Immunocytometry Systems, CA, USA).

2.3. Laboratory methods

1) Preparation of FVIII immune-depleted plasma samples spiked with recombinant FVIII

Different doses of rFVIII diluted in a tris-buffer (50 mM Trizma base and 0.1 M NaCl in distilled water, adjusted to pH 7.4 with HCl) were added to the FVIII immune-depleted plasma, achieving FVIII 0, 0.005, 0.0075, 0.01, 0.02, 0.04, 0.05, 0.06, 0.10, 0.40 and 1.00 IU/mL

2) Test of FVIII activity in plasma

Commercial COAMATIC Factor VIII kits were obtained from Instrumentation Laboratory SpA, Milan, Italy. The reagent consists of bovine FIX, FX and thrombin lyophilized with $CaCl_2$ and phospholipid. The chromogenic substrate reagent is composed of FXa-sensitive substrate S 2761 and thrombin inhibitor I 2581 [19]. The assay was performed at the Coagulation Laboratory, Karolinska University Hospital: i) 50 μL substrate was added to 5 μL plasma which had been incubated with 50 μL reagent at 37 °C for 4 s; ii) the reaction was measured at 405 nm in a Siemens BCS XP instrument.

3) Clotting time

To NPP samples containing the platelet reagent (see Note 1) and PPL, $CaCl_2$ together with the APTT-reagent alone/rTF alone/both APTT reagent and rTF was added (see Note 2). Fibrin turbidity were registered (wavelength = 405 nm, temperature = 37 °C, reading interval = 30 s) at a TECAN Sunrise instrument (Tecan Austria GmbH, Austria), followed by defining clotting time (CT) using software earlier