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Regular Article

Optimizing thrombelastography (TEG) assay conditions to monitor rFVIIa (NovoSeven®) therapy in haemophilia a patients

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ABSTRACT

Introduction: There is no established laboratory method that can predict the most optimal dose of bypassing agents for treatment of haemophilia A. The objectives of the study was to develop an assay that can a) differentiate between the haemostatic capacity in blood from healthy individuals and severe and moderate haemophilia patients; b) show a dose-response correlation to rFVIIa addition; and c) show dose response differences of rFVIIa addition to plasma samples from non-inhibitor patients of different severity.
Materials and Methods: Citrated whole blood from 25 haemophilia A patients was used in four thrombelastography (TEG) assays initiated with: 1) kaolin, 2) Tissue Factor (TF, Innovin 1:42,500), 3) TF 1:42,500 + 1.2nM tPA (tissue plasminogen activator) or 4) TF 1:200,000. rFVIIa was added to give a final concentration in the range of 0.02-4.8 μg/ml.
Results: The TEG assays showed large differences in clot formation demonstrated by prolonged clotting time (R-time), decreased maximum thrombus generation (MTG) between severe and moderate haemophilia A patients and between haemophilia patients and healthy males. The maximal amplitudes (MA) of the clot and resistance against fibrinolysis were only compromised when TF with tPA was added.
Conclusion: In vitro addition of rFVIIa improved all TEG profiles significantly in a dose-dependent manner; but only the TEG assay containing kaolin could differentiate between the rFVIIa doses, showing that blood from severe patients need higher doses of rFVIIa to normalize the clot formation profile compared to blood from moderate patients. Kaolin seems to be the most useful TEG assay for monitoring rFVIIa treatment.

Introduction

In haemophilia A patients with high-titre inhibitory antibodies, FVIII substitution therapy cannot be used. Instead, treatment of bleeding episodes is based on bypassing agents such as recombinant activated FVII (rFVIIa) and activated plasma derived prothrombin complex concentrates (pd-aPCC) [1,2]. There is, however, no established laboratory method for monitoring such treatment. This is highly required, as there seems to be very large inter-individual difference in dose-response to bypassing agents.

Global tests involving both platelets and coagulation factors, with which both rFVIIa and FVIII interact may be advantageous. Thrombelastography (TEG), which has been used to monitor pro-coagulant interventions [3–8], is a global test that provides a profile of the entire coagulation process - initiation, propagation and final clot strength measurements and apart from the possible influence of shear forces in flowing blood and the vasculature, TEG mimics the *in vivo* conditions of coagulation as the method measures the visco elastic properties of clotting whole blood There is, however, extensive diversity in the published TEG assays [9–11], and the correlation to the clinic response is inconclusive, indicating that there is still a need for improvement. In addition, there is a clinical requirement to design a TEG assay, which shows satisfying agreement between *in vitro* analysis and *in vivo* effect. Such an assay would be essential for predicting the most suitable treatment dose for a given patient and would provide knowledge that is not available today. The aim of this study was to develop an *in vitro* assay that can 1) distinguish between patients with moderate and severe haemophilia, and normal individuals, respectively 2) demonstrate a dose-dependent rFVIIa response, 3) show

Abbreviations: TEG, Thromboelastography; rFVIIa, recombinant Factor VIIa; FVIII, Factor VIII; FIX, Factor IX; FX, Factor X; tPA, tissue plaminogen activator; TF, tissue factor; R-time, Reaction time; MTG, maximum thrombus generation; MA, maximum amplitude; pd-aPCC, activated plasma derived prothrombin complex concentrate; EDTA, ethylenediaminetetraacetic acid; FVIII:C, Factor VIII concentration; HEPES, (2-hydroxyethyl)-1-piperazineethanesulfonic acid); NaCl, Sodium chloride; BSA, Bovine serum albumin; sTF, soluble tissue factor; Vmax, Maximum velocity; ANOVA, analysis of variance; CRP, c-reactive protein; INR, international normalized ratio; aPTT, activated partial thromboplastin time; CV%, Coefficient percentage.

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dose response differences of rFVIIa addition to blood samples from non-inhibitor patients of different severities, and 4) is simple, inexpensive and reproducible.

Materials and methods

Haemophilia A patients

Blood samples were obtained from 25 non-inhibitor haemophilia A patients at the Copenhagen University Hospital, Rigshospitalet, Denmark. One patient with severe haemophilia A later developed a high-titre inhibitor to FVIII, and may in connection with this study have had a low titre. below the detection limit of the laboratory's inhibitor assay. All patients were in a non-bleeding state. Thrombelastographic profiles were attained at baseline and following *in vitro* addition of rFVIIa (NovoSeven®, Novo Nordisk, Bagsværd, Denmark). In addition, blood was obtained from 20 healthy male volunteers. The study was conducted in accordance with the Declaration of Helsinki and ICH Good Clinical Practice, and was approved by the local Independent Ethics Committee in Denmark. Written informed consent was obtained from each patient and healthy volunteer prior to trial entry. One patient was 16 years old and his mother also signed the informed consent. All other patients and all healthy volunteers were >18 years of age.

The inclusion criteria were 1) a confirmed diagnosis of congenital haemophilia A with a FVIII:C at less than 5% (<0.05 kIU/L) as based on medical records and 2) age 16 years or older. Patients were excluded if they had received 1) any investigational drug within 30 days prior to this study, 2) factor replacement therapy within the last 48 hours prior to blood sampling, 3) any haemostatic treatment (e.g. pd-aPCC) within the last 7 days prior to blood sampling, except for rFVIIa, 4) rFVIIa within the last 48 hours prior to blood sampling. In addition, patients could not have had septicaemia or severe febrile illness, within 5 days prior to blood sampling.

Blood sampling

Blood samples were drawn from a smooth venipuncture site, employing minimal stasis and a 21-gauge needle, into vacutainer tubes, the first containing EDTA (1.8 mg/ml) for assay of the number of platelets and total platelet volume, followed by two citrated (3.2%) tubes for assessment of number of platelets and other general haematological measurements (including aPTT and fibrinogen level). Plasma samples obtained by centrifugation (2500 *g* at 15–25 °C for 10–15 min) were stored at –70 °C for subsequent local analysis of general haematological measurements and central analysis of FVIII:C (Coatest SP, Chromogenix, Mölndal, Sweden) with a detection limit of 0.008 U/ml corresponding to 0.8%.

Whole blood samples collected into a fourth citrated tube were stored upright at room temperature and sent to Novo Nordisk, Biopharmaceutical Research Unit at Måløv for *in vitro* titration with rFVIIa and coagulation analysis by TEG® at approximately 90 min after sampling.

Study design

In vitro effects of rFVIIa

Various concentrations of rFVIIa were added to whole blood samples (*in vitro* spiking) prior to thrombelastography in two separate experiments. In the first experiment, three different final concentrations of rFVIIa (0.5, 1.2, 4.8 μg/ml), corresponding approximately to doses of 36, 90, 360 μg/kg, respectively, were reached. In the second experiment, six lower concentrations of rFVIIa (0.02, 0.05, 0.1, 0.2, 0.4, 0.5 μg/ml), corresponding approximately to doses of 1.8, 3.6, 9, 18, 27, 36 μg/kg, respectively, were reached, in order to test the sensitivity of this method for low plasma levels of rFVIIa.

Thrombelastographic coagulation analysis (TEG)

Citrate stabilized fresh whole blood was used and the response was tested in 4 thrombelastographic assays: A) kaolin, B) tissue factor (TF, Innovin®, 0.22 μg/mL, Dade Behring, Deerfield, Ill, USA) diluted 1:42,500), C)TF 1:42,500 + 1.2nM tissue plasminogen activator (tPA, American Diagnostica) or D)TF 1:200,000. Whole blood was re-calcified with 15 mM calcium chloride (final free [$CaCl_2$] ~ 2–3 mM). Kaolin, TF and/or tPA, and rFVIIa, rFVIII or buffer (HEPES 20 mM, 150 mM NaCl, and BSA 2%) were carefully mixed with whole blood by inverting the tube 5 times before adding to the TEG cup containing $CaCl_2$ which was preloaded into the TEG cup. The haemostatic process was recorded by a TEG coagulation analyzer (5000 series TEG analyzer, Haemoscope Corporation, Chicago, USA). The TEG clotting time (R, denote the latency time from placing blood in the sample cup until the clot starts to form (2 mm amplitude), velocity of clot formation (MTG, maximum thrombus generation), and maximum strength of the clot (maximum amplitude, MA) were recorded.

Baseline samples were analyzed in duplicates and *in vitro* spiking was performed as single samples. References were generated for each TEG assay including 20 healthy males.

Data analysis was processed in Haemoscope Software 1.0.16 to obtain the clot formation and fibrinolysis parameters.

Determination of TF concentration in Innovin

Soluble TF (sTF, TF1-219) or serial dilutions of Innovin was incubated with FVIIa (final 2 nM) and the chromogenic substrate S2288 (final 500 uM). Absorbance was measured at 405 nm on a Spectramax 340 plate reader (Molecular Devices, Sunnyvale, CA, USA) and Vmax was calculated. For determination of the concentration in Innovin, sTF (final 50 nM) was used as standard.

Statistical analysis

Data from TEG experiment 1 were logarithmic transformed and estimated differences on the logarithmic scale were back transformed to the original scale by the exponential function and interpreted as ratios or percentages (100 times ratio). Buffer data were not included in the statistical analysis.

To investigate the dose effect data were analysed separately for each combination of parameter, assay, treatment (rFVIIa), and haemophilia classification (moderate or severe). The ratios high dose/low dose, high dose/middle dose and middle dose/low dose were estimated. The applied model was a two-way ANOVA with dose (3 levels) as a fixed effect and patient as a random effect.

For comparison of moderate and severe patients, data were analysed separately for each combination of parameter, assay and treatment. The applied model was a three-way ANOVA with dose and disease classification within dose as fixed effects and patient as a random effect.

Data from TEG experiment 2 were logarithmic transformed and analysed separately for each combination of parameter, assay, treatment and haemophilia classification. Mean values were estimated at each dose level from a two-way ANOVA with dose as a fixed effect and patient as a random effect. Estimated means were back transformed to the original scale and interpreted as medians.

Results

Characterization of haemophilia A patients

The average age of the haemophilia A patients was 40 years (range 16-64 years). Fourteen had severe disease (FVIII:C<0.01 kIU/L) and 11 had moderate (FVIII:C 0.01-0.03 kIU/L) as measured by a FXa activation assay (CoA test) on the blood sampling day prior to TEG