

GLOBAL TEST OF HAEMOSTASIS: THROMBIN GENERATION ASSAY

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Abstract: The coagulation system involves inter-related procoagulant and anticoagulant pathways. Given to its complexity, traditional coagulation tests, the prothrombin time and activated thromboplastin time do not assess the whole coagulation system and they correlate badly with the patients' clinical condition. Thrombin is the central enzyme in the coagulation cascade and the estimation of an individual's potential to generate thrombin may correlate better with the tendency of bleeding or thrombosis than traditional coagulation tests. Thrombin generation assays (TGA) measure the ability of a plasma sample to generate thrombin following *in vitro* activation of coagulation with tissue factor or another trigger. There is a growing interest in the use of thrombin generation testing, as a functional test, reflecting the clinical phenotype of patients with high risk of bleeding or thrombosis. This paper provides an overview of the thrombin generation assay measurement and its utility in clinical practice with an emphasis on Technothrombin® TGA reagents for Ceveron® alpha.

The coagulation system is complex and involves inter-related procoagulant and anticoagulant pathways. Thrombin is a key enzyme in the blood coagulation cascade and has pro- and anticoagulant functions in the coagulation cascade.(1) When the coagulation system is triggered, a burst of thrombin occurs and the clot appears at the very beginning of that burst. The great amount of thrombin is formed, through a positive feedback pathway, after activation of Factor (F) XI, F IX, F VIII and F V by minute thrombin generated.(2,3) The total amount of thrombin generated is determined by the concentrations of all clotting factors and inhibitors together with some plasma proteins.(4)

Widely performed for diagnosis, routine coagulation tests, prothrombin time (PT) and activated thromboplastin time (APTT) assays are imperfectly correlated to patients' clinical risk of bleeding or thrombosis. Both the PT and APTT use the formation of fibrin as the endpoint of the test, which occurs when only ~5% of the total amount of thrombin has been generated. In this case an individual's risk of bleeding or thrombosis may better correlate with their plasma thrombin generation in response to a procoagulant trigger.(5) Excessive thrombin formation always causes a thrombotic tendency but hardly affects any clotting times.

The aim of this paper is to provide an overview of the thrombin generation assays (TGA) measurement, and its utility in clinical practice, with an emphasis on Technothrombin® TGA reagents for Ceveron® alpha, used in the haemostasis research laboratory at the “Lucian Blaga” University in Sibiu.

Thrombin generation assay

Thrombin generation assay is one of the two best developed and tested global assays of haemostasis. The measurement of thrombin generation (TGA) has been possible since 1953 (6) but only recently, assays have been developed with which thrombin generation can be efficiently measured.(7)

Coenraad Hemker & colab., from Maastricht University, invented the present method available (8), that uses a thrombin-sensitive chromogenic or fluorogenic substrate. The

assay could be performed in platelet free plasma supplemented with phospholipids and in platelet-rich plasma. Triggering is done by picomolar tissue factor (TF) concentration, but other stimuli can be used too.

There are several thrombin generation tests commercially available, which rely on fluorogenic or chromogenic principles as: the Calibrated Automated Thrombogram developed by Hemker et al. and marketed by Thromboscop, Maastricht, The Netherlands, manual and automated fluorogenic assay by Technoclone, Vienna, Austria, chromogenic assay by Dade Behring and customized tests, such as the Novel Haemostasis Assay from the Radboud University Medical Centre, Nijmegen, The Netherlands.(9)

More recent, the whole blood thrombin generation test was developed, which allows the presence of erythrocytes and other blood cells. The advantage of the newly developed method may be the presence of the blood cells which contribute to coagulation *in vivo*, and which may be underestimated using plasma to test thrombin generation. This method is still a matter of research.(10)

Thrombin generation assay is a functional assay where we measure the amount of thrombin generated after the activation of coagulation, *in vitro*. The plasma biomarkers of haemostatic system activation, *in vivo*, are: thrombin-antithrombin complex, prothrombin fragments 1+2, and D-Dimeri.

The TG methods which are done in plasma combine 3 solutions: plasma sample, an activator reagent, and a combined fluorogenic substrate and calcium solution to start the reaction.(11)

TECHNOTHROMBIN® TGA reagents for Ceveron® alpha

TECHNOTHROMBIN® TGA reagents for Ceveron® alpha (Technoclone, Vienna, Austria) are an assay system for determination of thrombin generation. Test principle is based on monitoring the fluorescence generated by the cleavage of a fluorogenic substrate by thrombin over time upon activation of

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the coagulation cascade by different concentrations of tissue factor (TF) and negatively charged phospholipids in plasma. From the changes in fluorescence over time, the concentration of thrombin (nM) in the sample can be calculated using the respective thrombin calibration curve. The rate of thrombin generation is monitored over time resulting in a thrombin formation curve. The results are automatically calculated by the Ceveron® alpha software and displayed for five parameters: lag time, peak height of thrombin formation, time to peak (tpeak), velocity index (VI) and area under the curve (AUC) known as endogenous thrombin potential (ETP). All samples are analyzed in duplicates.

There are different trigger reagents with different concentration of TF and/or phospholipids (TGA RA, RB, RC low and high, RD) according to the purpose. The test can measure the thrombotic or bleeding tendency, can monitor bypass therapy with rFVII, anticoagulant therapy, heparin and NOAC therapy and can monitor the activity and thrombogenicity of microparticles.

New generation of the device for thrombin generation measurement, Ceveron Alpha, owns 4 channels with special fluorometric TGA modules consisting of an UV LED (365nm) for excitation and a photodiode for measurement of the emitted signal that are placed in the tempered cuvette rotor.

Blood preparation:

The pre-analytic phase includes every step from specimen collection up to the point of actual testing. According to the literature, it is the phase in which most laboratory errors occur. Because of activation of the haemostatic system in the pre-analytical phase, coagulation assays are probably most susceptible to outcome variations related to inadequate sample quality. Up to now, however, there has been substantial heterogeneity concerning the pre-analytical variables and the experimental conditions for the assessment of thrombin generation.(5,12)

The impact of pre-analytical variables on thrombin generation results is dependent on the concentration of tissue factor in the trigger reagent use.(13) A standardized protocol that reduces the pre-analytical variability is needed in order to reduce the analytic variability, leading to an acceptable validation criteria.(14)

All blood samples should be collected in the morning, after overnight fasting, from the antecubital vein, with the help of a light tourniquet, into vacuum tubes containing sodium citrate 3.2% 0.109M or citrate-theophyllin-adenosine-dipyridamole (CTAD) tubes.

The blood must be centrifuged right after collection at different speed, according to Technoclone recommendations, in order to obtain platelet poor plasma (PPP), platelet rich plasma (PRP) or platelet-and microparticles free plasma (PFP). The plasma will be carefully pipetted off and analyzed or should be frozen immediately after centrifugation.

The thrombin generation curve and its parameters:

- *Lag time (min)*, from the time point when the TGA reagents are added until the first burst in thrombin formation;
- *Thrombin Peak (nM)*, height of thrombin generation, maximal concentration of thrombin formed; more sensitive;
- *Time to peak (min)*, peak height time;
- *Velocity index or peak rate of thrombin generation or slope* = the steepest rate of thrombin formation per minute calculated by software as velocity index (VI);
- *Area under the curve (AUC)*, sum of thrombin concentration from 1 to 60 min (nM/L x min), endogenous thrombin potential (ETP); the most robust.

TGA curve reflects and integrates all pro and anticoagulant reactions that regulate the formation and inhibition

of thrombin (initiation, propagation and termination phases of coagulation cascade).

The thrombin generation curve is obtained from the fluorescence that develops when plasma clots, in presence of a fluorogenic thrombin substrate. The calculation steps are routinely carried out by the software of the analyzer.(15)

Despite of a large number of studies reporting potential clinical interest for TGA, results obtained remain incomparable because of a lack of standardisation.(16)

The reference ranges are highly depended on the method used. Several studies found different reference values between adults and children for all parameters, but in adults the values do not differ significantly between women and men.(17,18) It is recommended that each laboratory establishes and controls its normal range.

Clinical application:

Global tests like TGA seem to better correlate the individual coagulation potential with phenotypic diagnostics and have been shown to be a useful tool in several clinical conditions: detection and quantification of bleeding tendency and monitoring of substitution therapy, detection of hypercoagulability, measurement of the effect of anticoagulant drugs and thrombogenicity of microparticles.

TG parameters were correlated with factors activity level and disease severity in haemophilia, (19) and may be useful for monitoring rFVII activity in inhibitor-positive haemophilia (20) or assess the effects of bypassing agents.(21) Increased risk of bleeding is observed when ETP drops below 20% of the average normal value.(22) Also, bleeding risk in patients with rare bleeding disorders can be predicted using TGA.(23)

Venous or arterial thrombotic complications occur in many cases: atherosclerosis, malignancies, trauma, surgery, sepsis, pregnancy etc. TG test showed useful in the detection and quantification of thrombotic tendencies.

Increased formation of thrombin in plasma always induces a risk of venous thrombosis, whether it is due to deficiency of anti-coagulant factors (antithrombin, protein C, protein S) presence of FV Leiden (APC resistance) or antiphospholipid syndrome (APS).

Thrombin generation is elevated in almost any thrombophilia including AT deficiency (24), Protein S deficiency, (25), Protein C deficiency (26), in carriers of FV Leiden (27), prothrombin G20210A mutation.(28) Is associated with the presence of antiphospholipid syndrome (29) and increased levels of factors VIII, IX, XI.(30)

A relationship between thrombin generation and risk of venous thromboembolism (VTE) was well documented in several studies. Increased TG was associated with an increased risk of recurrent VTE (31) and below a certain cut off value of TG the thrombosis risk was low.(32) Elevated thrombin peak values were associated with an increased risk of VTE in patients with malignancies (33) and may predict an increased risk of VTE in cancer patients during chemotherapy.(34)

Thrombin plays a role in arterial thrombosis but less evident than in venous diseases. High level of thrombin generation (ETP and peak) were associated with an increased risk of acute ischemic stroke particularly in women (35) and with acute myocardial infarction (MI), that could persist for at least 6 month.(36,37)

In severe sepsis, thrombin peak shows a positive correlation with survival (38), and reflects the severity of DIC along with ETP.(39)

TG is valuable to quantify the combined effect of heparin and vitamin K antagonists or other anticoagulants.

Low molecular weight heparins (LMWHs) have a

variable inhibitory effect on thrombin generation, *in vitro*, when compared by anti-FXa activity, but are similar when compared by their anti-FIIa activities.(40)

Herpers & colab. found in their study a positive correlation between TG parameters (ETP and thrombin peak) and prothrombin complex concentrate dose required to obtain parameter normalization.(41)

New oral anticoagulants (NOAC) inhibit TGA parameters in a dose dependent manner. Apixaban affect all the parameters of thrombin generation (lag-time, ETP, thrombin peak and VI) (42) and Dabigatran prolonged lag-time (43) and for Rivaroxaban, ETP could serve as a fine-tuned haemostatic balance indicator for patients.(44)

Conclusions:

The traditional coagulation screening tests, like PT and APTT, are imperfectly correlated to patients' clinical risk of bleeding or thrombosis. Global coagulation assays such as fluorescent thrombin generation measurements are automated, and proved to be worthwhile in thrombosis (venous and arterial), in inherited bleeding disorders, monitoring bypassing agent therapy and anticoagulation therapy. Even though the results of the studies suggest possible applications in routine clinical practice, the test needs to be standardized to reduce inter-laboratory variation.

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