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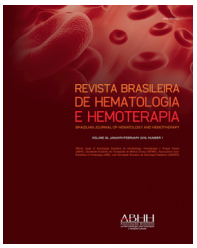

Review article

Thrombin generation assays for global evaluation of the hemostatic system: perspectives and limitations

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ABSTRACT

The existing techniques to evaluate hemostasis in clinical laboratories are not sensitive enough to detect hypercoagulable and mild hypocoagulable states. Under different experimental conditions, the thrombin generation test may meet these requirements. This technique evaluates the overall balance between procoagulant and anticoagulant forces and has provided new insights in our understanding of the coagulation cascade, as well as of the diagnosis of hypocoagulability and hypercoagulability conditions. Thrombin generated in the thrombin generation test can be quantified as platelet-rich or platelet-poor plasma using the calibrated automated thrombogram method, which monitors the cleavage of a fluorogenic substrate that is simultaneously compared to the known thrombin activity in a non-clotting plasma sample. The calibrated automated thrombogram method is an open system, in which different antibodies, proteins, enzymes and peptides can be introduced to answer specific questions regarding hemostatic processes. The thrombin generation test has great clinical potential, such as in monitoring patients taking anticoagulants and antiplatelet drugs, screening for genetic or acquired thrombotic disorders, and evaluating bleeding risk control in patients with hemophilia using bypass agents or replacement therapy. Different to conventional coagulation tests, the thrombin generation test can be used for an overall evaluation of hemostasis, the results of which can then be used to evaluate specific characteristics of hemostasis, such as prothrombin time, activated partial thromboplastin time, and levels of fibrinogen and other coagulation factors. The introduction of this method will contribute to a better understanding and evaluation of overall hemostatic processes; however, this method still requires standardization and clinical validation.

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Introduction

Different aspects of hemostasis can be studied using the methods that are currently available to researchers and clinical laboratories. These include coagulometry and chromogenic methods, which assess separate aspects of the hemostatic process. However, these methods do not provide an overall evaluation of hemostasis.[1] The method of evaluation is chosen depending on the patient's clinical data, and may separately provide important information about primary, secondary and tertiary phases of hemostasis, in addition to natural inhibitors such as protein C (PC) protein S (PS), antithrombin (AT) and tissue factor pathway inhibitor (TFPI). Molecular methods also contribute to the investigation of hemostatic disorders, in particular, in cases of genetic resistance to activated PC (aPC), which is primarily caused by factor V Leiden and other uncommon mutations, and in cases of hyperprothrombinemia caused by the G20210A prothrombin mutation.[2] Despite the diversity of laboratory methods used to evaluate hemostatic processes, none of the currently available methods is able to assess all the phases of hemostasis. Thus, the results obtained using these conventional methods are not always associated with clinical manifestations.[2]

Traditionally used methods have satisfactory sensitivity for moderate and severe hypocoagulability, but not for hypercoagulable or mild hypocoagulable states. Such methods only provide information about the beginning of the coagulation process, and therefore, the result of the test is not representative of the entire clot formation process, as measured using the total thrombin generation capacity.[2–4] The typical coagulometric measurements, such as prothrombin time (PT) and activated partial thromboplastin time (aPTT), measure only the clotting time corresponding to the initiation phase of the coagulation process. Furthermore, the end-point of these tests occurs after the formation of only 5% of total thrombin.[2,4] Therefore, PT and aPTT reflect only the initial coagulation process while the formation of thrombin and fibrin is still occurring.[2,5] A greater amount of thrombin is generated during the amplification and propagation phases, resulting in an exponential increase in thrombin, which becomes inactivated by physiological anticoagulants such as alpha-2-macroglobulin, AT, PC and PS.[1,2] Therefore, conventional tests do not provide information about the amplification and propagation phases of the hemostatic system.[1,2]

The introduction of a method able to evaluate the entire coagulation process is highly desirable, as this could better reflect bleeding and thrombotic risks. Many attempts have been made to achieve this goal, which would ideally accurately reflect all components and conditions of the hemostatic process, including platelets, coagulation factors, natural inhibitors, the endothelium and its interactions, as well as fibrinolysis and blood flow. Several authors have suggested the need to introduce *in vitro* methods representative of the main physiological aspects of hemostasis as a possible solution.[3,6–8] Attempts to develop a method to comprehensively evaluate hemostasis began several decades ago. In 1953, MacFarlane and Biggs[9] were the first to report thrombin generation in the blood using a laborious and time-consuming technique, which rendered it inapplicable for use in the clinical practice. In the same year, Pitney and Dacie[10] reported the measurement of thrombin generation in plasma. Many years later, convinced of the need for a comprehensive test for the broader assessment of hemostasis, the illustrious Professor Coenraad Hemker et al. at the University of Maastricht (Netherlands)[1,11] improved and semi-automated the thrombin generation technique, initially employing a chromogenic method, and later a fluorogenic method.[12] This improvement contributed greatly to the successful use of this technique in numerous studies.

Due to the lack of information about global tests of thrombin generation, we present a short discussion of this technique with emphasis on the calibrated automated thrombogram® (CAT) method. In this concise review, we present methodological aspects of the thrombin generation test (TGT), the evaluation of hemostatic components under some analytical conditions, the use of the test in experimental studies, potential clinical applications as a global coagulation test, as well as its limitations and future perspectives.

Thrombin generation assays and the calibrated automated thrombogram method

Thrombin is a key protein involved in the regulation of hemostatic processes; it has both procoagulant and anticoagulant properties.[13] While *in vivo* thrombin generation can be evaluated by measuring the thrombin-antithrombin complex (TAT) and prothrombin fragments 1 + 2 (F1 + 2), *ex vivo* TGT aims to evaluate the endogenous capacity of the overall hemostatic potential. Therefore, while high levels of TAT and F1 + 2 represent the pathological activation of *in vivo* coagulation, *ex vivo* thrombin generation reflects the endogenous capacity of the hemostatic system, and can be indicative of thrombotic or hemorrhagic risk. TGT continuously measures the proteolytic activity of thrombin formed in plasma using chromogenic or fluorogenic substrates following the activation of clotting using a triggering agent, as was comprehensively shown by Lecut et al.[1] The synthetic substrate, which is coupled to a chromogen or fluorophore, is selectively cleaved by thrombin, releasing the chromogen or fluorophore. The output signal is continuously measured, and is proportional to the amount of thrombin present in the reaction, the kinetics of which comprise two stages. The first, the initiation stage, corresponding to the coagulation time measured using tests such as PT and aPTT, can be inhibited by TFPI. The second, the amplification/propagation stage, is followed by a resolution phase resulting from the action of various inhibitors present in plasma, such as aPC, AT and alpha-2-macroglobulin.

The CAT method, developed by Hemker et al.[12] enables the quantification of thrombin concentrations in platelet-rich (PRP) or platelet-poor plasma (PPP) by monitoring the separation of a fluorogenic substrate, which is simultaneously compared to known thrombin activity in a non-clotting plasma sample.[12] This thrombin calibrator contains a known concentration of thrombin-like enzyme linked to alpha-2-macroglobulin. The thrombin-like enzyme is not inhibited by plasma components, and reacts only with the fluorogenic substrate. The color of the plasma may interfere with the results, and therefore, the thrombin calibrator is continuously measured for each plasma sample. The addition of tissue factor

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