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Methods for evaluation of platelet function

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Platelets play a pivotal role in haemostasis through adhesion to the injured vessel wall, aggregation, propagation of coagulation, and thrombus formation. Subsequently, platelets are also involved in fibrinolysis and the repair of the vessel wall, restoring blood flow and vascular integrity. Platelet may become activated through several different pathways, for example by collagen and von Willebrand factor exposed to the flowing blood upon vessel wall injury, by adenosine diphosphate (ADP) and adenosine triphosphate (ATP) released from activated platelets, and by thrombin – the key enzyme produced in the coagulation cascade. Upon activation platelet glycoprotein GIIb/IIIa ($\alpha_{IIb}\beta_3$) undergoes conformational changes and binds fibrinogen, which by bridging to other platelets leads to the formation of aggregates. Dense granule contents such as ADP are released, alpha-granule are released and P-selectin (CD62P) becomes exposed on the platelet surface. After the clot has been formed, the activated platelets incorporated in the clot, rearrange and contract their intracellular actin/myosin cytoskeleton. This mechanism is termed clot retraction and it is considered that its main physiological role is to clear the obstructed vessel for renewed blood flow.

There are many different methods available for measuring one or more of the many diverse events in platelet activation. However, no method covers all functions of the platelet and which method is the most useful depend on the specific clinical question.

Bleeding time

The oldest test is the *in vivo* bleeding time, described by Duke in 1910. However, the last decades it has become obvious that this method has severe draw-backs [1] and it is nowadays abandoned in many hospitals.

Swirling

Discoid platelets exposed to a light source reflect light and thus produces the “swirling” phenomenon. Swirling is routinely used to evaluate the quality of platelet concentrates (PC). Swirling determinations are performed by examining a PC against a light source while gently rotating the container or gently squeezing the PC. The presence of swirling indicate a pH value within the adequate range [2]. Thus, attenuated swirling indicates poor quality but perfect swirling does not guaranty good recovery or function.

Hypotonic shock response

Addition of a hypotonic solution to platelets results in initial swelling followed by a gradual decline as the platelets resume their baseline size. This can be measured with a

spectrophotometer. There are conflicting results on the correlation with platelet viability [3, 4],[5].

Aggregometry

The classical light-transmission aggregometry (LTA) invented by Gustav von Born is still regarded as the “golden standard” by many researchers [6]. Usually a panel of agonists is added to stirred platelets or citrated platelet-rich plasma and the change in light transmission caused by the aggregation is displayed, see Figure 1. Some instruments can also measure release of dense granular contents utilizing luminescence. LTA is not physiological; the stirring is low shear conditions, adhesion is not measured, relatively large volumes of blood are needed, whole blood cannot be used, and most instruments require a skilled technician. The most commonly used anticoagulant is citrate, thus the calcium ion concentration is far from physiological, which increases the response to many agonists. Recently more user-friendly instruments utilizing whole blood and impedance changes have been launched, for example Multiplate[®] (Dynabyte, Germany) [7] using single-use electrodes and an automated pipette and VerifyNow[®] (Accumetrics, USA) which is a fully automated cartridge-based instrument. The Plateletworks[®] (Helena Laboratories, USA) aggregation kits are based upon comparing platelet counts within a control EDTA tube and after aggregation with either ADP or collagen within citrated tubes. However, aggregation measurements on platelets from PCs do not appear to be able to predict platelet recovery since platelets stored at 4 °C have better aggregability than those stored at 22 °C despite low recovery and survival [8].

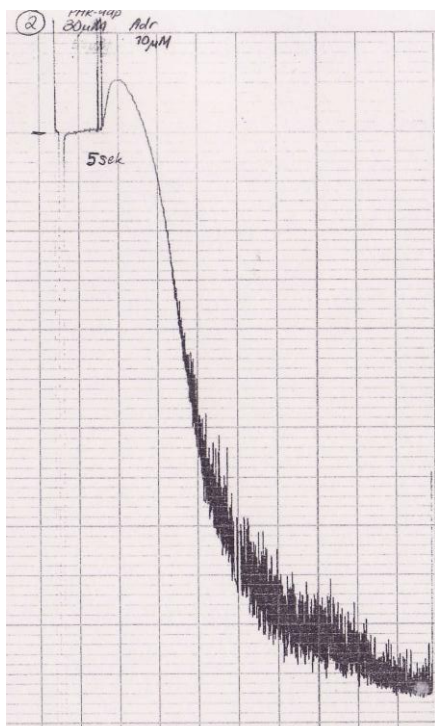


Figure 1. Typical tracing in light transmission aggregometry

Adhesion tests

The classical adhesion test is to count platelets before and after passage of heparinised blood through a column filled with glass beads. Commercially available instruments include the platelet function analyzer 100 (PFA-100[®], Siemens Healthcare Diagnostics Inc.), USA) and more recently the Impact-R[®] cone and plate(let) analyzer (DiaMed, Switzerland). Both of these tests measure platelet adhesion and aggregation under conditions of high shear and require anti-coagulated whole blood [9, 10]. The PFA-100[®] measures the time to occlusion of blood flow through a collagen coated membrane. It is used in many laboratories but the clinical usefulness is doubtful. The sample must be citrated blood and can thus not be used for quality control of PCs. The clinical experience of the Impact[®] instruments is still limited since the commercial version became available recently. See Figure 2 for a picture obtained with a cone and plate device constructed at the authors' lab.

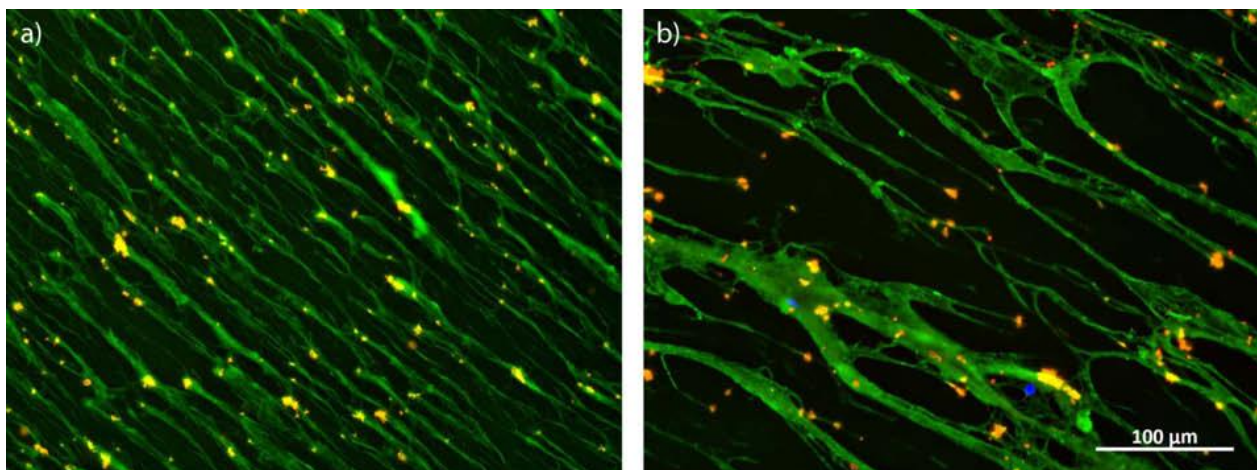


Figure 2. Platelets adhered to fibrinogen-coated glass after subjecting whole blood to shear stress in a cone and plate device.

Flow cytometry

In the last 20 years, flow cytometric analysis of platelets has also developed into a popular means to study many aspects of platelet biology and function. Preferred modern methods now utilize diluted anticoagulated whole blood incubated with a variety of reagents including antibodies and dyes that bind specifically to individual platelet proteins, granules and lipid membranes [11-13]. Platelets become activated during preparation and storage of PCs for transfusion. Platelet activation in PCs can be measured by release of P-selectin [14] (soluble or surface-bound), the active conformation of GPIIb/IIIa and GPIb expression. Surface expression of GPIIb/IIIa, P-selectin and GPIb can be measured by flow cytometry. Increased P-selectin expression during storage has been reported by several authors [15-17] whereas GPIb has been shown to decrease during storage [15, 17]. It is however unclear whether the level of *in vitro* platelet activation in stored PCs correlates with *in vivo* survival and haemostatic function of platelets after transfusion [18]. A special use is to assess the inhibition of the ADP-receptor P2Y₁₂ by clopidogrel in patient blood samples by measuring the degree of phosphorylation of the intracellular protein VASP, which recently has been used to guide dosage of clopidogrel and improved outcome after percutaneous coronary intervention [19].

Instruments measuring clotting and clot elasticity

When blood coagulates the blood viscosity increases as the fibrin network forms. The elasticity of the clot depends on several factors; the contractile force exerted by the platelets, platelet concentration, the hematocrit, the fibrinogen concentration and the thrombin generation during coagulation. **Thromboelastography** (TEG, Haemoscope, now part of Haemonetics Corp., USA) was first described by Hartert in 1948 [20]. An alternative instrumentation uses the term (rotational) thromboelastometry for the process and **ROTEM**[®] (Pentapharm, Switzerland) for the instrumentation. The measuring unit consists of a cylindrical cup, made of disposable plastic. A pin is suspended into the cup, and the pin is connected to a detector. The cup and the pin will oscillate relative each other through an angle of approximately 5°. The major difference between the instruments lies in the oscillation. In the TEG[®] instrument the cup oscillates and in the ROTEM[®] instrument the pin oscillates. The ROTEM[®] instrument has an electronic pipette connected to the instrument to simplify the pipetting of the different reagents provided, and has developed software with exact step-by-step instructions and automated pipetting to make the instrument easy to use. It is not possible to measure the blood viscosity, the first signals appear when the pin is connected via the first fibrin fibers spanning the whole distance to wall of the cup. The clot elasticity is expressed in mm in the tracing. See Figure 3.

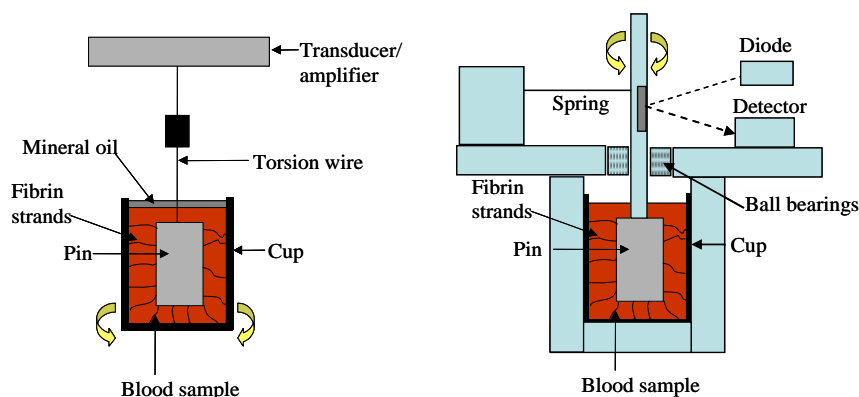


Figure 3. The measurement principle of TEG[®] (left) and ROTEM[®] (right). Reprinted by courtesy of Sofia Ramström, RCSI, Dublin, Ireland.

The coagulation might be modified, for example by the addition of kaolin in order to activate the intrinsic pathway of coagulation or by the addition of tissue factor to activate the extrinsic pathway. Also for the ROTEM[®] instrument, different commercial reagents are available, with tissue factor, with aprotinin to detect hyperfibrinolysis or with GPIIb/IIIa antagonists to evaluate the fibrin network contribution to the clot strength, with contact activator or with heparinase for the use during heparin treatment. TEG has been used to monitor blood component therapy during surgery [21-24]. **Free oscillation rheometry (FOR)** (MediRox AB, Sweden) is a new technology which makes it possible for the first time both to measure changes in viscosity and elasticity in clotting whole blood and in dissolving clots and to obtain results in real time in SI-units. In this instrument (ReoRox4[™]) oscillation is initiated by a forced turn of the sample cup every 2.5 seconds, see Figure 4. After a brief hold time, the sample cup is released, allowing rotational oscillation with very low friction around the longitudinal axis. An optic angular sensor records the frequency and damping of the oscillation as a function of time. To allow determination of

coagulum elasticity, gold-plated reaction chambers are used. The reaction chamber includes a cylindrical sample cup and an inner cylinder, a bob, attached to a hollow shaft and immersed from above into the centre of the sample cup. With the bob in a fixed position, the structure of the fibrin fibres coupling the cup to the bob, and the amount and activity of platelets bound to the fibrin network, will affect the frequency and damping of the sample cup oscillation. In our lab it was discovered that gold coating enables measurements in whole blood, since fibrinogen binds firmly to the gold surface. Platelets then bind to immobilised fibrinogen firmly enough to prevent the detachment as the platelets start to retract the clot. Algorithms are used to calculate the elasticity modulus from the frequency and damping data, results are obtained in SI-units in contrast to competing instruments. The instrument's accuracy in the detection of long clotting times has been validated [25], and also how the measurements are affected by changes in different blood components [26]. Other advantages are a wider measuring range for elasticity and the simultaneous measurement of blood viscosity [26]. Until now, the instrument has mainly been used for studies of the platelet contribution to whole blood coagulation and quality control of PCs [27-29]. The clinical studies published so far have been of limited size [30-33]. Commercial reagents have very recently been launched.

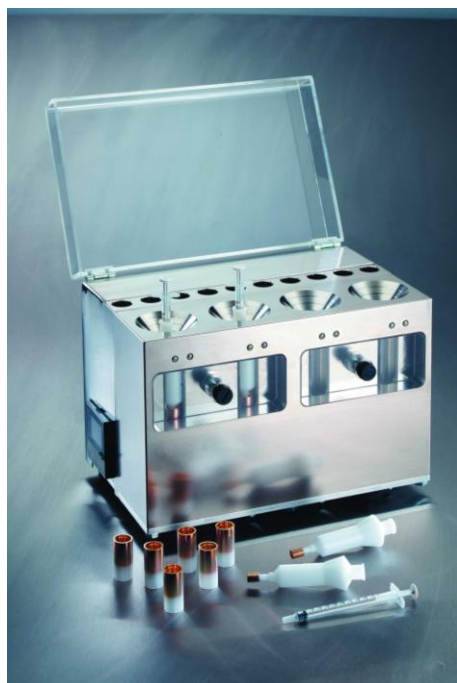


Figure 4. A ReoRox4™ instrument with shafts and cups.

Concluding remarks

Different methods have their pros and cons, the laboratory should choose the methods giving the most relevant information for the requesting clinician [34]. In general there is a lack of studies showing benefit for the patients by including point-of-care platelet function methods in the management.

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