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Schött, Ulf; Johansson, P I

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Bringing flow into haemostasis diagnostics.

Schött Ulf[1x], Johansson Pär I[2,,3]

[1]Department of Intensive Care and Perioperative Care, Skane University Hospital, Lund and Lund University, Sweden

[2]Section for Transfusion Medicine, Capital Region Blood Bank, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark, [3]Department of Surgery, Center for Translational Injury Research (CeTIR), University of Texas Medical School at Houston, TX, USA.

[x]E-mail: ulf.schott@skane.se

Virchow first defined the importance of blood flow in haemostasis and thrombosis, but first received acknowledgement 100 years after his publication 1856.[1] Blood is a non-Newtonian fluid and its viscosity is dependent on the flow, flow velocity, type of flow (laminar or non-laminar), concentration of different types of blood cells, plasma protein size/concentration and temperature.[2,3] At higher flow rates blood behaves as a Newtonian fluid with reduced viscosity. Flow transfers energy to the blood and the vascular wall and this is called shear stress. Shear rate is defined as the rate at which adjacent layers of fluid move with respect to each other. Shear rate/stress differs in the venous and arterial circulation, but also increases when the vascular lumen decreases, naturally when blood moves peripherally and with pathological constrictions like thrombus, plaques, mechanical valves, stents and vascular anastomoses. High shear forces leads to platelet activation through both von Willebrand factor (vWF) dependent and non-dependent mechanisms.[2] Blood flow and shear rates often change dynamically in perioperative patients due to changes in blood pressure or vascular tone, blood loss, and fluid replacements. Currently available coagulation assays are mostly performed under static condition, and therefore complex interactions among erythrocytes, platelets, white cells and coagulation factors under various shear rates cannot be appreciated.

In this issue of BJA, Ogawa and colleagues present a study of the effects of flow conditions on haemodilution-induced changes in coagulation.[4] The authors used a new commercialized automated microchip flow chamber technique called the *Total Thrombus-formation Analysis*

System (T-TAS®), assessing coagulation processes in recalcified/corn trypsin inhibited whole blood (350 µL) inside collagen/tissue factor-coated capillaries under arterial and venous flow conditions. Their flow data highlight some important differences in coagulation between flow and static conditions by comparing it with a conventional, static assay, rotational thromboelastometry (ROTEM®). The microscopic images of thrombus formation on the T-TAS® demonstrate that the initial thrombus deposition adjacent to the capillary wall with subsequent intraluminal thrombus growth against either arterial or venous flow. After *in vitro* haemodilution with normal saline, thrombus formation was delayed under both flow rates, but it was more extensively affected under the venous flow rate. The intraluminal thrombus growth in diluted whole blood was restored by adding vWF at the higher flow rate, whereas fibrinogen concentrate was effective in improving mural thrombus growth at the lower flow rate. Prothrombin complex concentrate (PCC) appeared to partially reverse dilutional coagulopathy at both flow rates. With ROTEM, fibrinogen was most effective in improving clot firmness, whereas procoagulant effects of vWF and PCC were not clearly demonstrated.

Flow/perfusion chambers have been used for several decades to assess haemorrhagic conditions *in vitro*, and evaluate antithrombotic agents under flow in research laboratories. The technique has been popularized with the introduction of commercial laboratory devices including the *Platelet function analyzer* (PFA-100®), and the IMPACT® Cone and Plate analyzer (CPA). Both systems are performed using small volumes of citrated whole blood (800 µL, and 200 µL, respectively). Although both systems have been utilized to assess platelet adhesion and aggregation in the setting of von Willebrand disease, thrombocytopenia, anaemia, and pharmacological platelet inhibition (e.g., aspirin), their results do not provide information beyond primary haemostasis because coagulation is inhibited by chelation of calcium ions.

PFA-100® is an automated test system in which citrated whole blood is aspirated at high flows through a disposable cartridge containing an aperture coated with either collagen + epinephrine or collagen + ADP. These agonists trigger platelet adhesion, activation, and aggregation leading to a rapid occlusion of the aperture and cessation of blood flow. Thrombocytopenia (< 100 x 10^9 $liter^{-1}$), anaemia (< 20% haematocrit), vWF levels, and COX-1 inhibitors such as aspirin prolong time before occlusion. However a normal PFA result cannot be used to rule out platelet or coagulation defects.[5] The IMPACT®-CPA uses rotational shear stress, that could be varied to very high shear rates and an artificial surface that reacts similarly to activated (damaged) endothelium, but is laborious and has so far not been automated.[6] Both PFA-100 and the CPA are able to measure platelet aggregate formation under high-shear conditions. However, these devices give only endpoint values (closure time or final thrombus size, respectively), while flow chamber measurements provide real-time information on a whole panel of output parameters, such as platelet adhesion and activation, thrombus growth, and fibrin formation/coagulation, at well-predicted venous or arterial shear rates. In the T-TAS® system, coagulation processes are evaluated in recalcificated blood providing multiple parameters on thrombus growth patterns under venous and arterial shear rates (330 - 1100 s^{-1}).[7] Of note, a separate microchip (PL chip) is available for specific assessments of platelet adhesion and aggregation in hirudin-anticoagulated blood at higher shear rates.[8] **Table 1** summarizes differences between PFA-100, CPA, T-TAS and ROTEM.

T-TAS has chambers with either collagen or thrombin/tissue factor, but the in vivo activated endothelial cell (EC) lining is more complex with procoagulant, anticoagulant and fibrinolytic components. This also holds true for the denuded subendothelial matrix that interacts with

platelets in formation of a stable platelet plug. This is a complex process, not only collagen, tissue factor (TF) and vWF are involved, also smooth muscle cells, fibronectin, laminin, thrombospondin and deposited fibrinogen – and regulating proteins are involved.[1,9] On the endothelial surface there is also a protective glycocalyx layer with proteoglycans like heparan and dermatan sulphates. Glycocalyx can be damaged in many diseases increasing the risk for thromboses.[10,11] It has been difficult to study the EC, but in 1964 Weibel-Palade body (WPB) was discovered, a unique organelle, which proved to be a specific marker.[12] Injury/inflammation releases from WPB several preformed proteins. vWF is the predominant protein in WPB and diffuses into the subendothelial cell matrix (ECM) where it binds to different proteins. Within seconds after stimulation of WPB release, a carpet of rolling platelets and leukocytes forms along the damaged endothelial cell surface. This process is enhanced by platelet migration towards the vascular wall secondary to red cell rheology mechanisms, especially in arterioles.[2]

In vivo vascular damage contract the arterial media and aventitia with decreased intravascular lumen slowing blood flow and increasing the possibility for formation of platelet plugs that seal the vascular defect and stops bleeding, "primary hemostasis". The vasoconstriction is enhanced by endothelin released from WPB, thromboxane and platelet activating factor 4 (PF4) from platelets. In a later stage the renin-angiotensin system also contributes. The platelet plug is then stabilized with cross-linked fibrin, "secondary hemostasis". The reduced flow strengthens the latter process with higher local concentrations of various coagulation factors and prevents non-stabilized platelet plugs to be washed away. This sudden intravascular change of lumen is hard to copy for *in vitro* systems.

The flow chamber systems incorporating vascular endothelium and intravital microscopy in animal models have been previously reported. Recently this has been taken to new hights by the Furie group in Boston, using 3-D computerized image interpretation, flourescent antibodies and animal knock-out models of various coagulation factors.[13] These techniques are not currently applicable for clinical use. However, it is possible with confocal microscopy and directing lasers through human skin to study haemostasis in intradermal vessels.[14]

Many of the flow chamber systems introduced in the past had a limited perfusion time and needed a large blood sample. The *Xylum clot signature analyzer* used non-anticoagulated whole blood under physiological flow conditions and measured thrombus formation in a punch hole with a collagen surface with variable flows. Antiplatelet effects of anti-vWF, anti-GPIb, and anti-GPIIb/IIIa could be demonstrated on the Xylum analyzer. However, measurement variability of the device was large (coefficient of variation, 20-47%) even in normal subjects.[15]

Haemodilution affects coagulation by dilution of coagulation factors and corpuscular elements in blood. Plasma coagulation factors can be diluted to <30% and some factors down to 10% and blood still clots – this extraordinary capacity of the coagulation system is probably linked to evolution.[16] Ogawa and colleagues used crystalloid dilution to induce a dilutional coagulopathy. It has long been known that synthetic colloids affect haemostasis more than through simple decrease of plasma concentrations of coagulation factors and platelets.[17] Dextran, gelatin and starches make clots more brittle and easily dissolved by the fibrinolysis system. This has been studied with electron, atomic force and confocal microscopy, but in clinics also extensively with the viscoelastic haemostatic tests (VHT) thrombelastography (TEG®) and ROTEM®.

Perioperative haemostatic interventions have been increasingly attempted using purified plasma-derived factor concentrates.[18] The primary replacement of fibrinogen guided by ROTEM® has been shown to be a simple and effective approach to dilutional coagulopathy[19], but the optimal dose and impact of combining PCC have not been fully elucidated. Indeed, Ogawa and collegues pointed out that *in vitro* fibrinogen addition (2 g liter^{-1}) was most effective in improving clot firmness on ROTEM®, but combining fibrinogen (1 g liter^{-1}) and PCC (0.3 IU mililiter^{-1}) was more potent than fibrinogen alone under flow conditions. However, *in vitr*o testing with PCC containing heparin can inhibit clotting and in ROTEM a diluted TF reagent is needed to detect PCC effects.[20]

A Previous T-TAS study has shown that heparin at different dosages prolonged clotting at both shear rates, whereas low molecular weight heparin only inhibited the growth at a low shear rate.[7] T-TAS has also been used to study warfarin reversal, and the onset of thrombus formation was shortened by PCC, but not by FFP.[21] T-TAS demonstrated that in haemophilia the reduced activity of the intrinsic tenase complex (FIXa-FVIIIa) is only detected under venous (low) shear rates.[22] T-TAS can also indicate inefficient platelet inhibition both in aspirin- and clopidogrel-treated patients.[8]

The flow chamber technique has been popularized during the last years with the introduction of commercial flow chambers (also custom adapted), such as Ibidi, Venaflux, Bioflux, and Glycotech. The Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) has recently reviewed this field.[23] The majority of laboratories use collagen coatings, but also endothelial cells, fibrinogen, vWF and other synthetic peptides.

Although additional clinical data are needed to validate the role of T-TAS, it may complement ROTEM or TEG in terms of assessing platelet adhesion/aggregation, and platelet procoagulant activity. When introducing flow chambers into clinics, the type of flow chamber, coating of surfaces, collection and storage of blood before start of analysis, recording of digital (fluorescence) images, and the method of image quantification should be addressed. Hopefully high profile laboratories will have the possibility to test different automated microchip flow chamber devices in the near future and correlate their data to clinical endpoints.

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Table 1. Differences in between static (ROTEM) and systems with higher shear rates.

	ROTEM / TEG	**T-TAS**	**Impact-CPA**	**PFA-100**
Blood	Recalcified. Whole Blood (WB)	Recalcified. WB or Hirudin. WB	Citrated WB	Citrated WB
Shear rate	0.1 s^{-1}	110 - 2400 s^{-1}	1300-1900 s^{-1}	~5000 s^{-1}
Agonists	Tissue factor(TF) Ellagic acid or Kaolin	TF/Collagen or Thrombin	Collagen	Collagen/Epinephrine or Collagen/Adenosine Diphosphate (ADP)
Endpoints	Viscoelasticity changes	Capillary occlusion (flow pressure change) Microscopic image	Microscopic image (surface coverage, thrombus size)	Capillary occlusion (flow pressure change)
In vivo correlations	Coagulation Clot strength	Coagulation, Platelet adhesion /aggregation	Platelet adhesion/ aggregation	Platelet adhesion/ aggregation