Preanalytical variables affect thrombomodulin-modified thrombin generation in healthy volunteers

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Calibrated automated thrombinography is widely used as a research tool to assess coagulation potential of a plasma sample, and is able to detect both hypo- and hypercoagulability. Traditionally, the test is performed by the addition of tissue factor, calcium chloride, and synthetic phospholipid vesicles or platelets to plasma. However, thrombomodulin-modified thrombin generation much better represents thrombin generating potential of plasma, as in this test the capacity of the anticoagulant protein C system to downregulate coagulation is also taken into account. Thrombomodulin-modified TGA has been used to assess hemostatic capacity of plasma from patients with complex hemostatic disorders including patients with liver disease [1]. Also the new automated thrombin generation device (ST Genesia) allows thrombin generation testing in the absence and presence of thrombomodulin and reports absolute values of total thrombi generation, peak, velocity, and lagtime in absence or presence of thrombomodulin [2].

Although calibrated automated thrombinography and other types of thrombin generation testing are widely used, there are significant issues with standardization. Although the variability of the test within one laboratory is acceptable, between laboratory variability is substantial which is at least in part due to significant variability in protocols, including preanalytical handling of the sample [3].

We have used thrombomodulin-modified thrombin generation extensively in hemostatic profiling of patients with complex hemostatic disorders, in particular patients with liver diseases [4]. Recently, we reported on thrombomodulin-modified thrombin generation testing in whole blood of patients with liver disease [5]. A striking finding is this study was the much larger concentration of thrombomodulin required to downregulate thrombin generation in whole blood as compared to plasma. Although the nature of the thrombomodulin resistance of whole blood compared to plasma has not yet been resolved, the fact that cellular components appear to reduce the anticoagulant effect of thrombomodulin made us wonder whether the method of preparation of platelet-poor plasma affects thrombomodulin-modified thrombin generation.

We drew blood from 10 healthy individuals into sodium citrate-containing vacutainer tubes (0.129 M) via the antecubital vein. Blood was processed to plasma via 4 different methods. 1) Blood was centrifuged within 15 min at 2.000*g* and 10.000*g* respectively for 10 min at ambient temperature (the procedure recommended for thrombin generation testing), 2) Blood was centrifuged within 15 min once at 2.000*g* for 10 min at ambient temperature, 3) Blood was centrifuged within 15 min twice at 2.000*g* for 10 min at ambient temperature, 4) Blood was left for 3 h at ambient temperature and the centrifuged at 2.000*g* and 10.000*g* respectively for 10 min at ambient temperature. Following centrifugation steps, plasma was gently pipetted off, without disrupting the buffy coat after the first centrifugation step. Plasma was stored at −80 °C until use.

We performed thrombin generation experiments using the fluorimetric method described by Hemker [6] (Calibrated Automated Thrombography) according to the instructions of the manufacturer on a Fluoroscan Ascent reader (Thermo Fisher, Breda, The Netherlands) using Immulon 2HB round bottom 96 well plates (Thermo Fisher), and Trombinoscope software version 05.0.0.385 (Thrombinoscope BV, Maastricht, The Netherlands). We used commercially available reagents composed of recombinant tissue factor at a concentration of 5 pM and phospholipids at a concentration of 4 μM in the absence or presence of a soluble form of thrombomodulin (the concentration of which is not revealed by the manufacturer), and a thrombin calibrator ((Thrombinoscope BV, Maastricht, The Netherlands). Plasma obtained by the 4 centrifugation methods was thawed at 37 °C and immediately used. In addition, an aliquot of the plasma sample that was centrifuged only once at 2.000*g*, was centrifuged again after thawing at 10.000*g* for 10 min.

We analysed the endogenous thrombin potential, peak thrombin, lag time, and velocity index in absence and presence of thrombomodulin, and calculated the % decrease in ETP and peak by thrombomodulin. Values of the 5 different groups were compared by repeated measures ANOVA followed by Tukey's post-test using GraphPad Prism (San Diego, CA, USA).

We measured thrombin generation in absence and pres
thrombomodulin in 10 healthy volunteers of which blood w
cessed in 5 different ways. Compared to the method that is

Abbreviations: ETP, endogenous thrombin potential; TM, thrombomodulin

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Table 1
Thrombin generation parameters in blood from 10 healthy volunteers processed according to various centrifugation protocols.

	ETP (nM IIa * min)	ETP/TM (nM IIa * min)	% ETP inhibition	Peak IIa (nM)	Peak IIa/TM (nM)	% peak inhibition
1) Double 2.000*g*/10.000*g*	924 ± 168	495 ± 210	48 ± 15	141 ± 31	103 ± 42	30 ± 15
2) Single 2.000*g*	986 ± 193	689 ± 224	31 ± 12	172 ± 42	141 ± 52	20 ± 11
	P < 0.05 vs 1,3,5	P < 0.001 vs 1,3,4,5	P < 0.001 vs 1,3,4,5	P < 0.01 vs 1,3,4,5	P < 0.001 vs 1,3,4,5	P < 0.05 vs 1,3,4,5
3) Double 2.000*g*	909 ± 160	444 ± 208	53 ± 14	143 ± 33	92 ± 45	39 ± 15
4) Double 2.000*g*/10.000*g* after 3 h at RT	919 ± 167	464 ± 202	51 ± 12	141 ± 33	97 ± 44	34 ± 15
5) Single 2.000*g*, 10.000*g* after thawing	908 ± 194	445 ± 210	53 ± 14	137 ± 38	89 ± 44	38 ± 15

ETP = endogenous thrombin potential, TM = thrombomodulin, RT = room temperature.

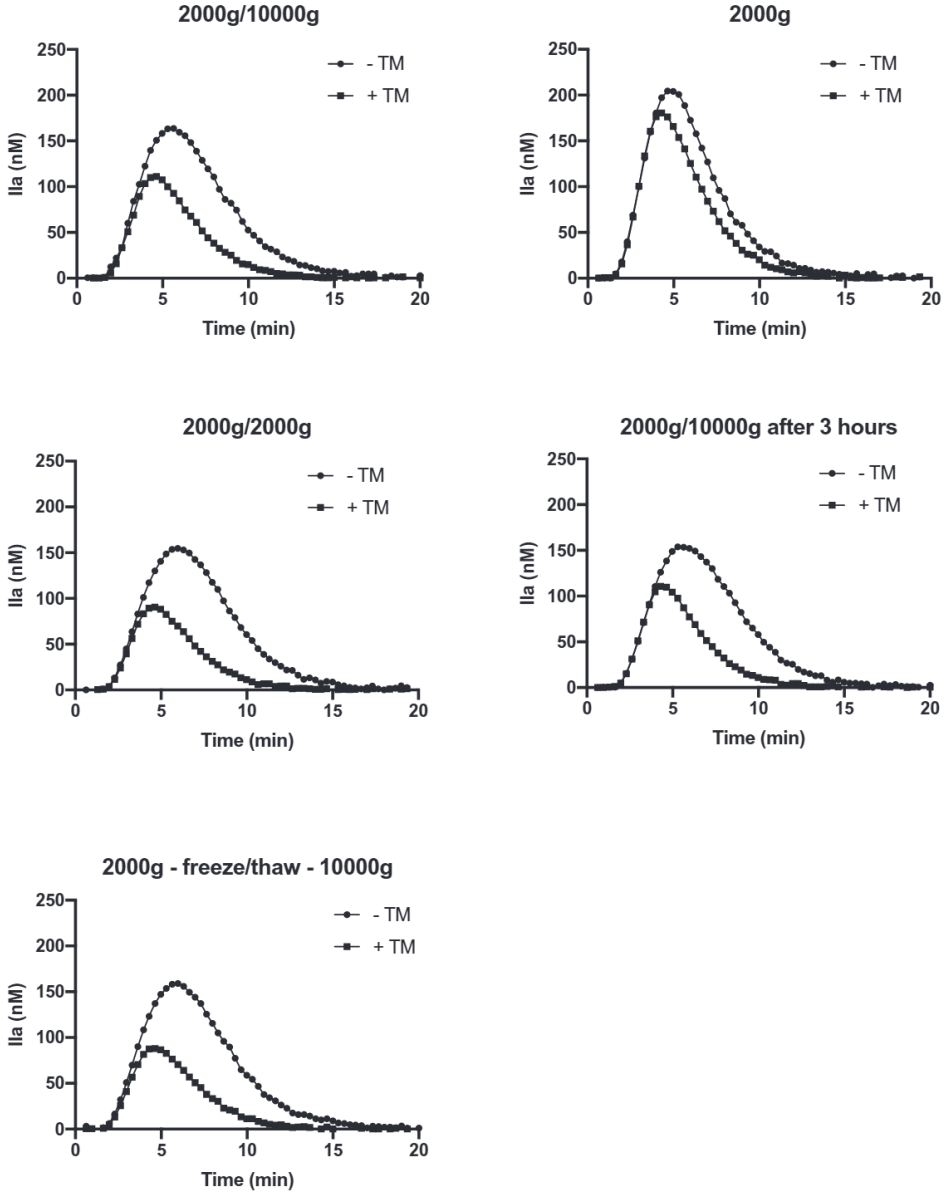

Fig. 1. Thrombin generation curves of one of the 10 healthy donors in this study. Each graph shows a thrombin generation curve in absence or presence of thrombomodulin (TM) for one of the 5 blood processing protocols tested in the study.

recommended by the manufacturer for thrombin generation (double centrifugation at 2.000 and 10.000*g*), single centrifugation gave higher thrombin generation in absence and presence of thrombomodulin, and a much less effective downregulation of thrombomodulin (31 vs 48% inhibition of the ETP, and 20 vs 30% inhibition of the peak thrombin, Table 1, Fig. 1). Lag time and velocity index were not different between the centrifugation methods (data not shown). Thrombin generation in absence or presence of thrombomodulin was similar between the

recommended method, a double centrifugation at 2.000*g*, or a double centrifugation at 2.000 and 10.000*g* after a 3-hour storage at room temperature. When single centrifuged plasma was centrifuged again after thawing, thrombin generation parameters were similar to the recommended protocol.

Here we demonstrate that a single centrifugation step for processing whole blood to plasma substantially decreases the anticoagulant capacity of thrombomodulin in thrombin generation assays. These results extend recent findings on a profound thrombomodulin resistance in whole blood compared to platelet-poor plasma [5]. Although its hasn't been established which cellular components reduce the anticoagulant activity of soluble thrombomodulin in thrombin generation tests, these results emphasize that special care should be taken in processing blood samples for thrombin generation tests, in particular when the thrombomodulin modified test is planned.

Although a double centrifugation step is recommended by the manufacturer of thrombin generation tests, in many hospital settings single centrifugation of citrated whole blood samples is still common, also when blood is collected for research purposes. As thrombin generation tests are increasingly used in research settings, also aided by the development of automated analyzers such as the ST Genesia, we recommend citrated blood for research purposes always to be processed using double centrifugation. However, when stored samples have been only centrifuged once, we recommend an additional centrifugation step at 10.000*g* after thawing of the sample, which we show in the present study to yield similar results compared to plasma that was double centrifuged immediately after the blood draw. We have also assessed the effects of a delayed plasma processing, which is common in clinical settings, and found no clear differences between immediate and delayed plasma processing.

Our results are in line with previous studies showing higher thrombin generation in single versus doubly centrifuged plasma [7], which is likely explained by the presence of higher levels of procoagulant phospholipids in single centrifuged plasma. Also, besides our recent study, one study has compared the inhibitory effect of thrombomodulin in platelet-poor versus platelet-rich plasma, and found a decreased anticoagulant capacity of soluble thrombomodulin in platelet-rich plasma [8].

It is unclear whether blood cells also affect the anticoagulant activity of cellular thrombomodulin in vivo, and this is an interesting topic for future study. Also, the modification of anticoagulant activity of soluble thrombomodulin in vivo requires further study, given the clinical use of recombinant soluble thrombomodulin (ART-123, Asahi Kasei Pharma) in sepsis with coagulopathy and disseminated intravascular coagulation [9,10]. Regardless, it has been well established that thrombomodulin-mediated activation of the protein C system is a key regulator of coagulation in vivo, and the potential modification of its anticoagulant activity by blood cells may be relevant in clinical conditions such as anemia or thrombocytopenia.

We studied a small number of healthy subjects, and additional studies with larger numbers of healthy individuals and patients are required to confirm our findings. Nevertheless, we feel it is important to warn the community using thrombin generation assays not to analyze single centrifuged plasma, particularly in studies using thrombomodulin-modified thrombin generation testing.

In conclusion, preanalytical conditions modify the anticoagulant activity of soluble thrombomodulin in thrombin generation assays. Our results stress the need for double centrifugation of plasma, preferably by the protocol recommended by the manufacturer of calibrated automated thrombinography.

Declaration of competing interest

None.

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