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Regular article:

The role of platelets in blood coagulation - effects of platelet agonists and of GPIIb/IIIa inhibitors studied by free oscillation rheometry

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Abstract

We have studied the contribution of platelets to the coagulation of plasma and the effects of activation or inhibition of platelets on the coagulation process in unanticoagulated fresh whole blood (subsequently termed native blood). For this purpose we have used a free oscillating rheometer (FOR), the ReoRox4®, a new instrument enabling non-invasive viscoelastic measurements of clot formation in plasma and whole blood.

Platelets appear essential for the initiation of coagulation if no activating surface is present. We prepared platelet-free plasma by quick centrifugation and filtration of native blood, which did not coagulate if stored in plastic containers at 37°C but clotted if transferred to glass containers.

Addition of platelet agonists, such as collagen or the thrombin receptor agonist peptide SFLLRN, significantly accelerated the clotting of native blood and also changed the rheometer curve appearance, accelerating both onset and completion of clot formation (i.e. fibrin gel formation). Inhibition of platelet glycoprotein IIb/IIIa with the peptide-derived compound MK-852 or the antibody-derived abciximab (Reopro®) prevented clot retraction and prolonged the clotting time with SFLLRN. In collagen-stimulated samples MK-852 accelerated clotting but delayed completion of clotting while abciximab prolonged both clotting time and completion of clotting.

To our knowledge, this is the first report showing that activation of platelets in native whole blood shortens the coagulation time *ex vivo*. It also describes a new instrument, enabling studies of the viscoelastic properties of a forming whole blood clot.

Keywords: Haemostasis, Blood platelets, Blood coagulation tests, Platelet activation, Platelet procoagulant activity, Platelet aggregation inhibitors

Abbreviations:

APTT	Activated Partial Prothrombin Time
FMT	Frequency minimum time
FOR	Free oscillation rheometer
GP	Glycoprotein
INR	International normalised ratio
PRP	Platelet rich plasma
PS	Phosphatidylserine
PT	Prothrombin Time
SD	Standard deviation
SFLLRN	Peptide with the sequence Ser-Phe-Leu-Leu-Arg-Asp

Running title: Platelet effects on native whole blood coagulation

Introduction

The fact that blood cells affect the coagulation process has long been known. Already in 1905 Morawitz reported that cell-free plasma did not coagulate if stored in paraffin-lined containers [1]. It is also known that patients with severe thrombocytopenia ($<5 \times 10^9$/L) frequently experience serious bleeding episodes [2]. However, few laboratory tests of coagulation measure the contribution of platelets to clotting even though they are known to participate in all phases of hemostasis *in vivo*. Besides their role in platelet plug formation, they provide the surface on which the tenase and prothrombinase complexes form (for reviews see references [3, 4]). This entails the translocation of negatively charged phosphatidylserine (PS) residues to the platelet surface. Furthermore, platelets from healthy donors differ in their ability to support activation of factor Xa and thrombin, a difference not correlated to translocation of phosphatidylserine [5], thus indicating existence of additional platelet features important for assembly of these complexes. Another such example is the Stormorken syndrome, a disorder with spontaneous platelet exposure of PS [6], which is characterised by bleeding but no increase in thrombin formation [7].

Even though the tissue factor-factor VIIa complex is the initial physiological activator of factor X, the Xa so derived can not substitute for factor Xa formed by the tenase complex on the platelet surface since it is largely inactivated before it reaches the platelet surface [8]. This implies that normal amplification of the humoral coagulation cascade is critically dependent on normal platelet structure and activation.

Platelet surface receptors are also critical for platelet adhesion and aggregation. Binding of platelets to subendothelial structures at high shear stress is primarily mediated by interactions of the GPIb receptor and von Willebrand factor (vWF) [9]. Platelet

activation also induces a conformational change in the GPIIb/IIIa complex, making it able to bind vWF and fibrinogen, thereby strengthening the clot and inducing platelet aggregation [10]. GPIIb/IIIa antagonists have been used successfully to prevent re-thrombosis after coronary angioplasty and are of benefit in the treatment of acute coronary syndromes [11]. In addition, studies with GPIIb/IIIa antagonists suggest that this receptor is important for PS exposure and factor V binding to activated platelets [12].

In this work we have studied the contribution of platelets to the coagulation of plasma and to unanticoagulated fresh whole blood (native blood). For these studies we used a newly developed free oscillating rheometer (FOR), the ReoRox4® [13]. We have previously used this instrument in our laboratory to study the effect of adding endothelial cells to plasma in patients with thrombosis [14].

Materials and methods

Instruments and reagents

Four channel free oscillation rheometers (FOR), ReoRox4®, disposable polypropylene sample cups (prod. no. GHI 205) and softwares (ReoRox4®v2.00 and ReoRox4®Viewer v1.03, 2.0, 2.01) were obtained from GHI (Global Haemostasis Institute AB), Linköping, Sweden. In this instrument the cup containing the sample is placed on the sample cup holder, which is attached to the foundation by a perpendicular torsion wire [15] (figure 1). Oscillation is initiated by a forced turn of the sample cup around the longitudinal axis. After a brief hold time, the sample cup is released, allowing rotational oscillation at a characteristic amplitude and frequency (about 10 Hz for an empty sample cup). The mean wall shear rate is approximately 60 s^{-1}. Initiation of oscillation is repeated

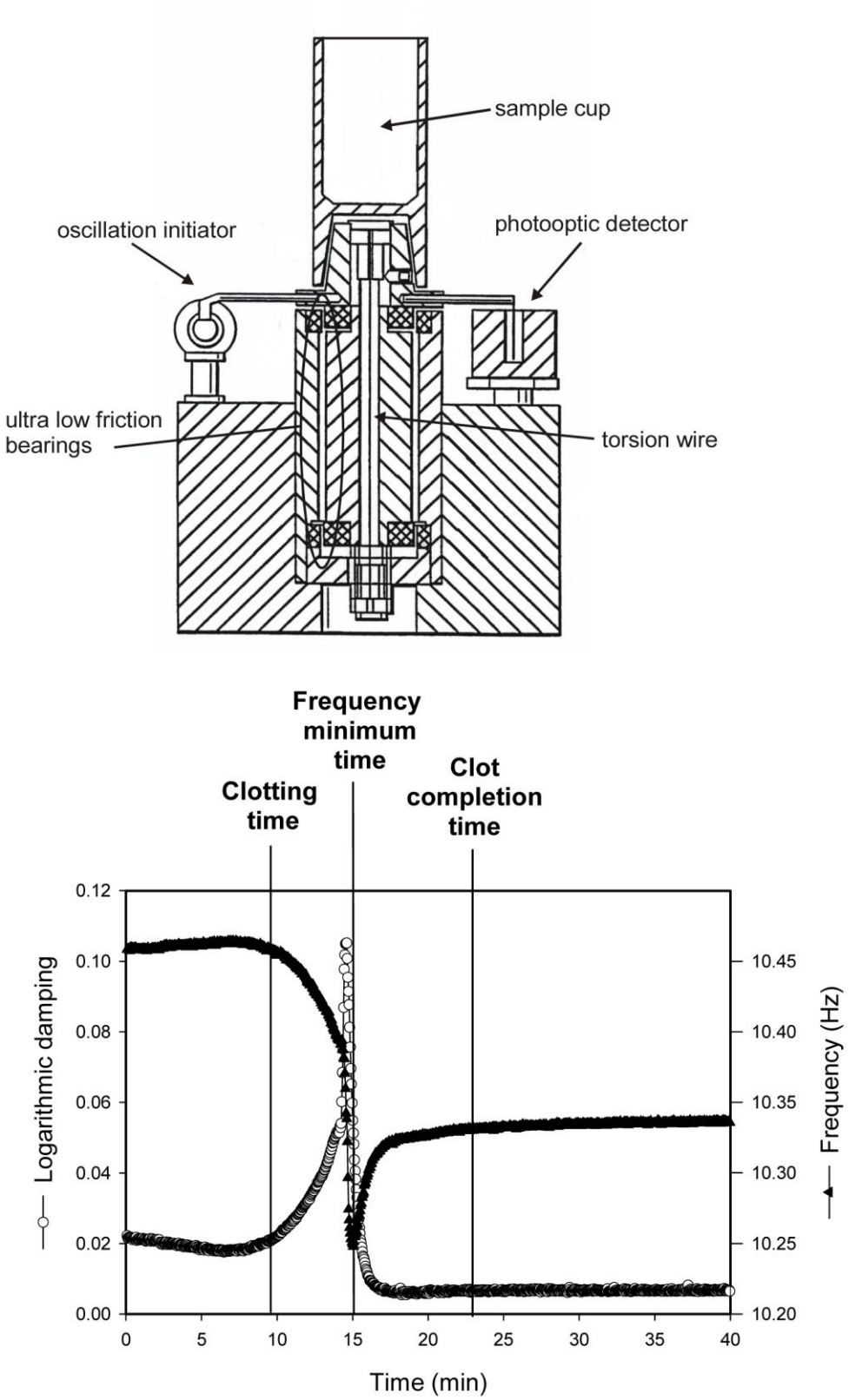

Figure 1: The measuring parts of the free oscillating rheometer ReoRox4® (figure modified from [15]), and a typical tracing from a coagulating blood sample.

every 2.5 seconds. During coagulation the rheological properties of the sample change, perturbing the oscillation. The software clot onset detector (called "state detector") determines the time when the sum of change in damping and frequency reaches a pre-set value. We have shown that this point correlates extremely well to the standard reference method of visual clot detection ($r^2 = 0.97$, submitted for publication in Biorheology). This state detector algorithm was set to high sensitivity in all experiments described here, and the related endpoint is referred to as "clotting time". To study further changes in the forming coagulum, the time for the frequency minimum (here referred to as "frequency minimum time", FMT) and the time when a plateau was reached after the frequency minimum (here called "clot completion time") (see figure 1) were also determined graphically.

APTT (Activated Partial Prothrombin Time) and PT (Prothrombin Time) were measured on an ACL Futura Plus™ (Instrumentation Laboratory, Lexington, MA, USA) with the reagents IL Test™ APTT-SP (Instrumentation Laboratory) and Owren PT 2× (product # GHI 131-20, Global Hemostasis Institute, Linköping, Sweden). The D-dimer analysis was performed on a Hitachi 911 (Roche Diagnostics Scandinavia AB, Bromma, Sweden) with the reagent Tinaquant D-dimer from the same company. Ca^{2+} activity in serum was determined potentiometrically with an ICA 2 Ionised Calcium Analyser (Radiometer, Copenhagen, Denmark).

Tri-sodium citrate and $CaCl_2 \times 2\ H_2O$ were from E. Merck, Darmstadt, Germany. Chemicals for the HEPES buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L $MgCl_2$, 5.6 mmol/L glucose, 1 g/L bovine serum albumin and 20 mmol/L HEPES, pH 7.40) were from Sigma Chemical Company (Saint Louis, MO, USA). The thrombin-receptor-agonist peptide SFLLRN [16] was purchased from the Biotechnology Centre of

Oslo, Oslo University, Oslo, Norway and was dissolved in DMSO to 30mg/mL and thereafter diluted to 1.50 mg/mL in 0.5 mol/L HEPES buffer, pH 7.4. Alternatively, the peptide was dissolved to 1.5 mg/mL in DMSO and thereafter diluted to 60 μg/mL in the 20 mmol/L HEPES buffer described above. Methylated collagen type I [17], kindly provided by Beate Kehrel, Münster, Germany, was dissolved in 0.05% HAc to 3.3 μg/mL. The GPIIb/IIIa antagonist MK-852 (143 μg/mL in the 20 mmol/l HEPES buffer described above) was generously provided by Merck, Sharp & Dohme (West Point, PA, USA). abciximab (c7E3, Reopro®, 2 mg/mL) was from Eli Lilly and Company (Indianapolis, IN, USA).

Blood collection

Venous blood was collected with minimum trauma via a 21-gauge needle (0.8×40 mm) in 4.5 mL plastic vacuum tubes (S-Monovette®, Sarstedt, Nümbrecht, Germany). Sterile empty tubes were used for native blood experiments. In experiments with platelet rich plasma, 500 μL of tri-sodium citrate (130 mmol/L) was added to the tubes before blood collection (yielding a final citrate concentration of 13 mmol/L). All blood donors gave informed consent. The blood collection protocol was approved by the Ethics Committee at Linköping University Hospital.

Preparation of cell free plasma without anticoagulant

Native blood (collected in empty plastic vacuum tubes) was collected as above and portioned in Eppendorff tubes, centrifuged (2500 g, 1 minute) immediately after blood collection and filtered through a 0.22 μm sterile filter (MILLEX®-GS, Millipore Corp., Bedford, MA, USA). Plastic materials and cautious pipetting and decanting was

employed to avoid activation of coagulation. The entire procedure was completed within five minutes from blood collection. Samples from 12 individuals were stored at 37°C for 24 hours to examine the stability of plasma factors. APTT and PT were measured on aliquots of the sample after addition of 0.129 mol/L sodium citrate in a sample-to-citrate ratio of 10:1. This was done immediately after plasma preparation and after 24 hours storage. D-dimer levels were also measured in samples stored for two months at room temperature (n=4) or at 37°C (n=6).

Effects of platelet agonists on coagulation

Native blood was collected in empty plastic vacuum tubes. Within 5 minutes of collection, 250 μL of blood was transferred to a sample cup pre-filled with either SFLLRN (6.7 μL 1.5 mg/mL) or collagen (25 μL 3.3 μg/mL) diluted in 20 mmol/l HEPES to a volume of 50 μL or with HEPES buffer alone (50 μL). The cup was then placed in the rheometer. Samples were analysed in duplicate.

Similar experiments were also performed on platelet rich plasma (PRP) prepared by centrifugation (200g, 20 minutes) of citrated whole blood. The monocyte count was below limit of detection in the analyser used in the clinical chemistry routine lab (CELL-DYN 4000, Abbott Diagnostics Division, Santa Clara, CA, USA) and was less than 0.05×10^9 monocytes/L as determined by flow cytometry using an antibody against CD-14 to identify the monocyte population. In these experiments, 250 μL of PRP was added to a rheometer cup containing 6.5-9 μL of 0.5 mol/L calcium chloride (calcium ion activity (measured in serum after the clotting experiments were completed) of 1.2-1.6 mmol/L) and either SFLLRN (11 μL 1.50 mg/mL + 31 μL HEPES buffer), collagen

(42 μL 3.3 μg/mL) both calculated to give a similar plasma concentration as in the whole blood experiments assuming a haematocrit of 40%, or HEPES buffer (42 μL). The final volume was 300 μL.

Clotting times were determined with the rheometer software. Duplicate PRP samples from 8 individuals were analysed.

Effects of platelet GPIIb/IIIa antagonists on coagulation

Blood was collected in empty plastic vacuum tubes. Within 5 minutes of collection, 250 μL of blood was transferred to a sample cup containing 25 μL of SFLLRN (60 μg/mL) or collagen (3.3 μg/mL) and 25 μL of HEPES buffer or a GPIIb/IIIa antagonist, MK-852 (43 μg/mL) or abciximab (3 μL 2 mg/mL + 22 μL buffer). The fibrinogen binding in the presence of these antagonist concentrations was shown be less than the binding of a control antibody in a flow cytometric assay [18].

Clotting times were determined by the instrument's software program. The frequency minimum and clot completion times were determined graphically from the curves. All samples were analysed in duplicate.

Statistics

Data analysis was performed using Statistica™ for Windows (StatSoft, Inc, Tulsa, USA). Comparisons between groups were made with the non-parametric Wilcoxon Matched Pairs test.

Results

Cell free plasma without anticoagulant

Cell free plasma with no anticoagulant, prepared as described, did not coagulate, even after lengthy storage at 37°C. If the plasma was transferred to glass tubes during the first day of storage, coagulation immediately occurred. After 24 hours storage, 12 plasma samples showed unchanged PT (INR 1.03 ± 0.11 (mean ± SD) at 0 h and 1.02 ± 0.09 at 24 h, $p = 0.92$) and slight prolongation of APTT (29.3 ± 3.2 s at 0h and 33.2 ± 6.5 s at 24h, $p = 0.02$). Small amounts of deposited fibrin were observed with time in some of the samples, but the coagulation did not seem to proceed further. In plasma stored at room temperature, however, gross fibrin formation was seen within 96 hours. This fibrin network dissolved with time and was not visible after two month's storage. D-dimer levels were high after storage for two months at room temperature (294.00 ± 64.63 mg/L, n=4). Samples stored at 37°C showed much lower levels (3.43 ± 2.98 mg/L, n=6) ($p< 0.01$), indicating that these samples had not been coagulated during storage.

Effects of platelet agonists on clotting time

Addition of SFLLRN or collagen to native blood samples shortened the clotting time by over 40% as compared to untreated whole blood ($p=0.018$ for SFLLRN, $p<0.01$ for collagen). Results are shown in figure 2.

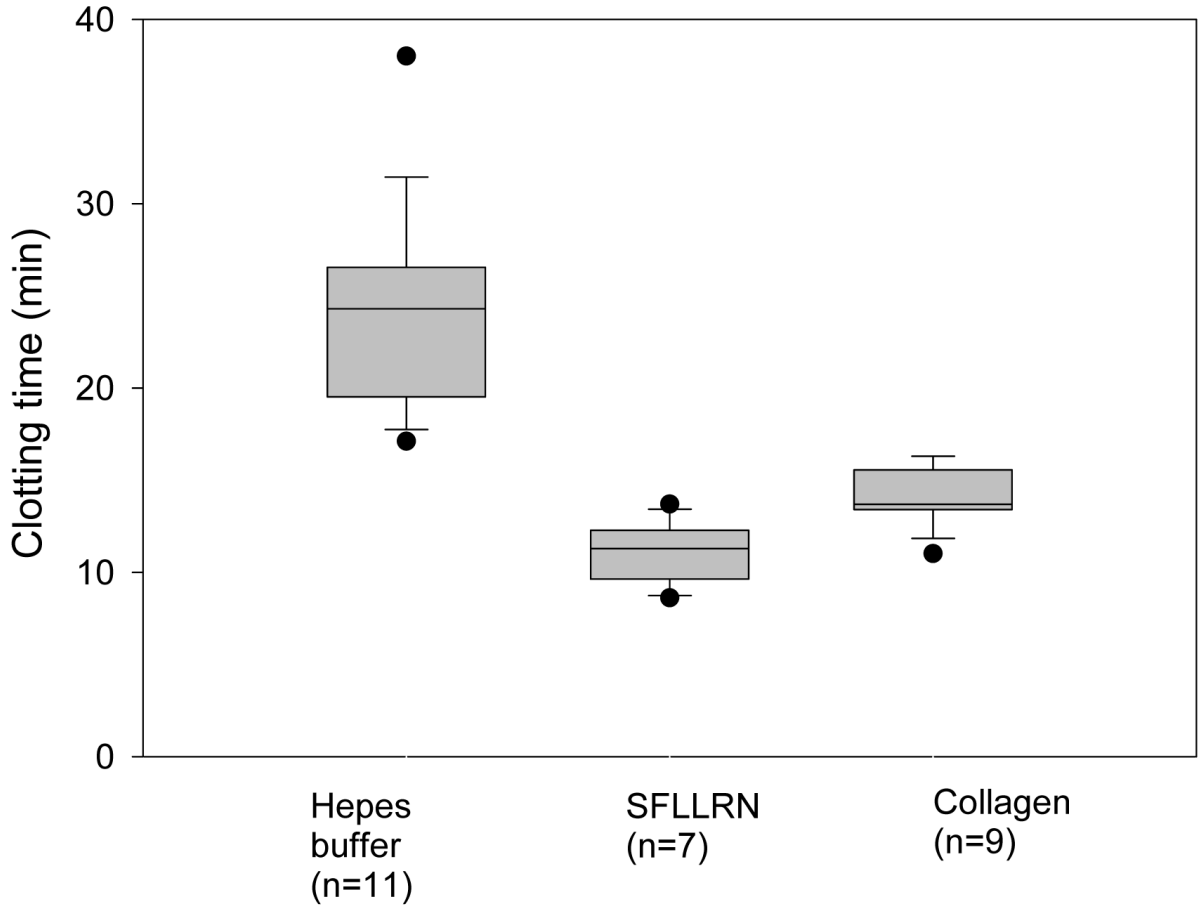

Figure 2: Clotting times for native blood stimulated with SFLLRN (33 μg/mL) or collagen (0.3 μg/mL). The box plot shows the median, the10th, 25th, 75th and 90th percentiles and all outliers.

To investigate whether the effects were caused by platelet activation, the same experiments were performed with citrated platelet-rich plasma (PRP). Results are shown in figure 3. Both collagen and SFLLRN addition decreased the clotting time by 50% (p=0.012).

Effects of platelet agonists and GPIIb/IIIa antagonists on coagulation

Rheometer tracings of whole blood coagulation in the presence and absence of platelet agonists were distinctly different. Samples without platelet agonists often showed a small, broad peak in logarithmic damping accompanied by a slow decrease in frequency,

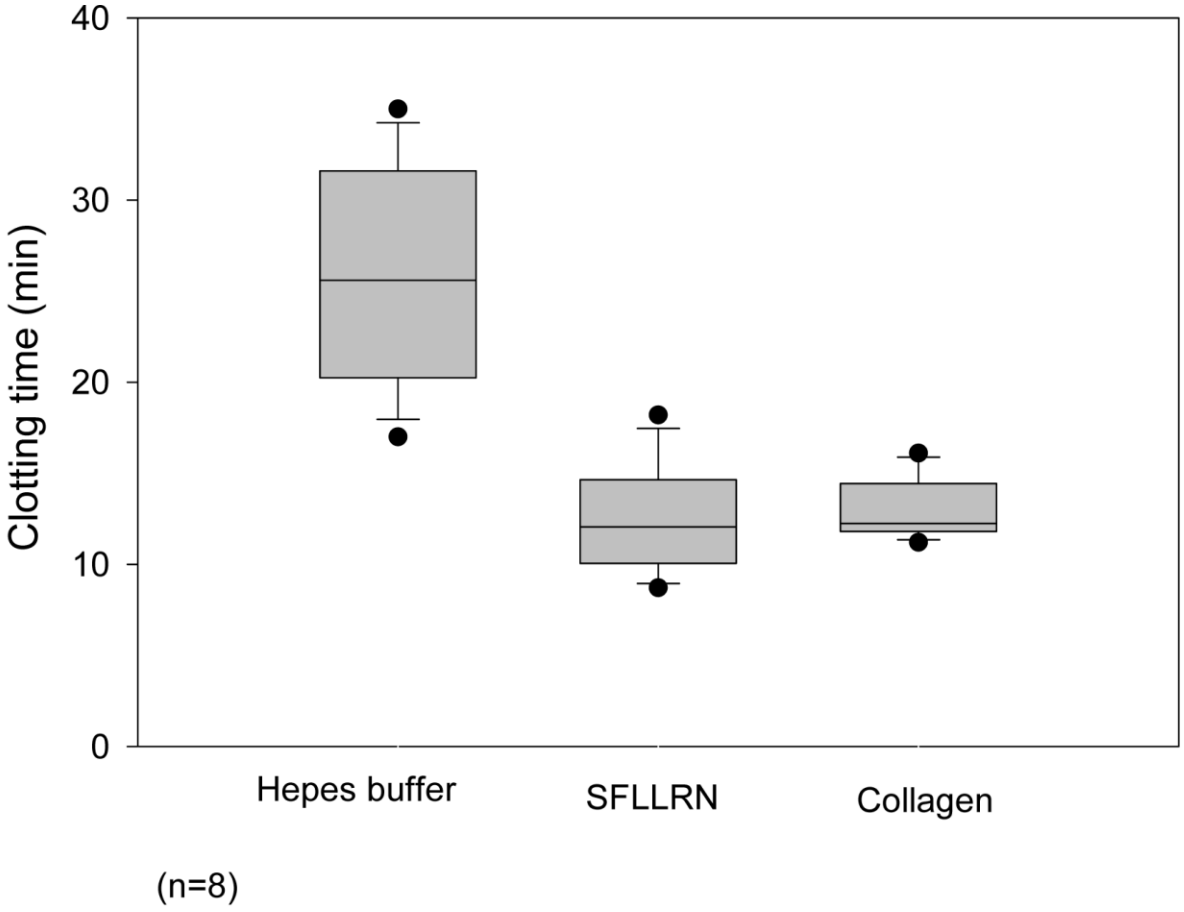

Figure 3: Clotting times for platelet rich plasma stimulated with SFLLRN (55 μg/mL) or collagen (0.5 μg/mL). The box plot shows the median, the10th, 25th, 75th and 90th percentiles and all outliers.

as compared to the fast and distinct changes when platelet agonists were added (figure 4). Clot retraction caused the clot to detach from the wall of the cup (usually within 5-30 minutes after clot completion), which led to increased damping and frequency. This phenomenon was not seen in samples containing GPIIb/IIIa antagonists.

Due to poor agreement between duplicate samples when native blood was allowed to clot spontaneously, all experiments with GPIIb/IIIa antagonists were done on samples activated with SFLLRN or collagen. In comparison to SFLLRN alone, MK-852 prolonged the clotting time by 20% ($p=0.015$). By contrast, MK-852 shortened the collagen clotting time by 20% ($p<0.01$). Results are shown in figure 5. This surprising

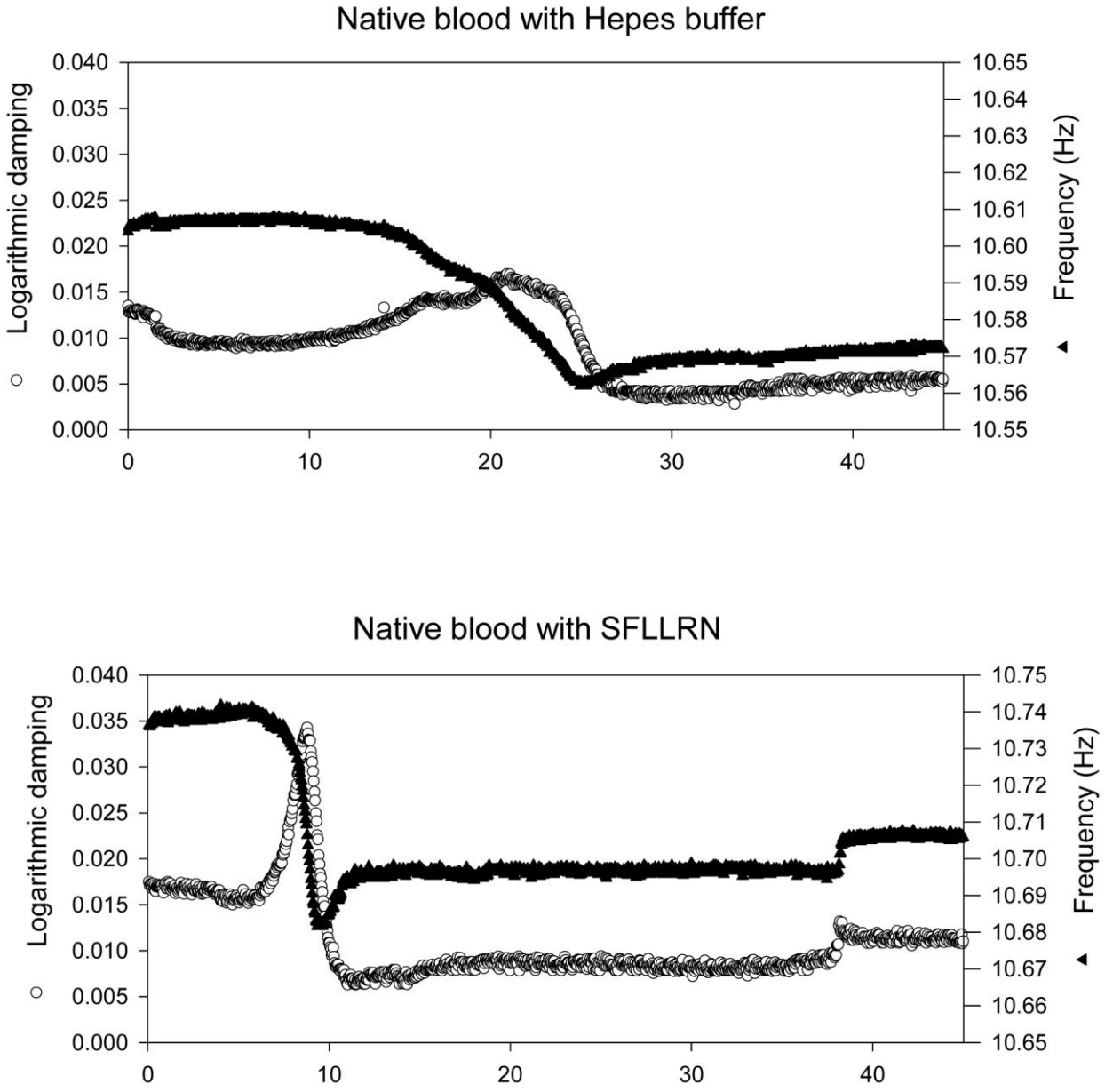

Figure 4: Typical tracings from native blood samples coagulating in the FOR instrument. To the samples were added Hepes buffer (upper panel) or SFLLRN (33 μ g/mL, lower panel).

result with MK-852 and collagen prompted us to evaluate another GPIIb/IIIa antagonist, abciximab. Unlike MK-852, abciximab increased collagen clotting time by 35% ($p<0.01$, figure 6). For SFLLRN, the results with abciximab were similar to the ones with MK-852 (clotting time 20.4 ± 2.6 and 19.7 ± 3.4, respectively, n=4).

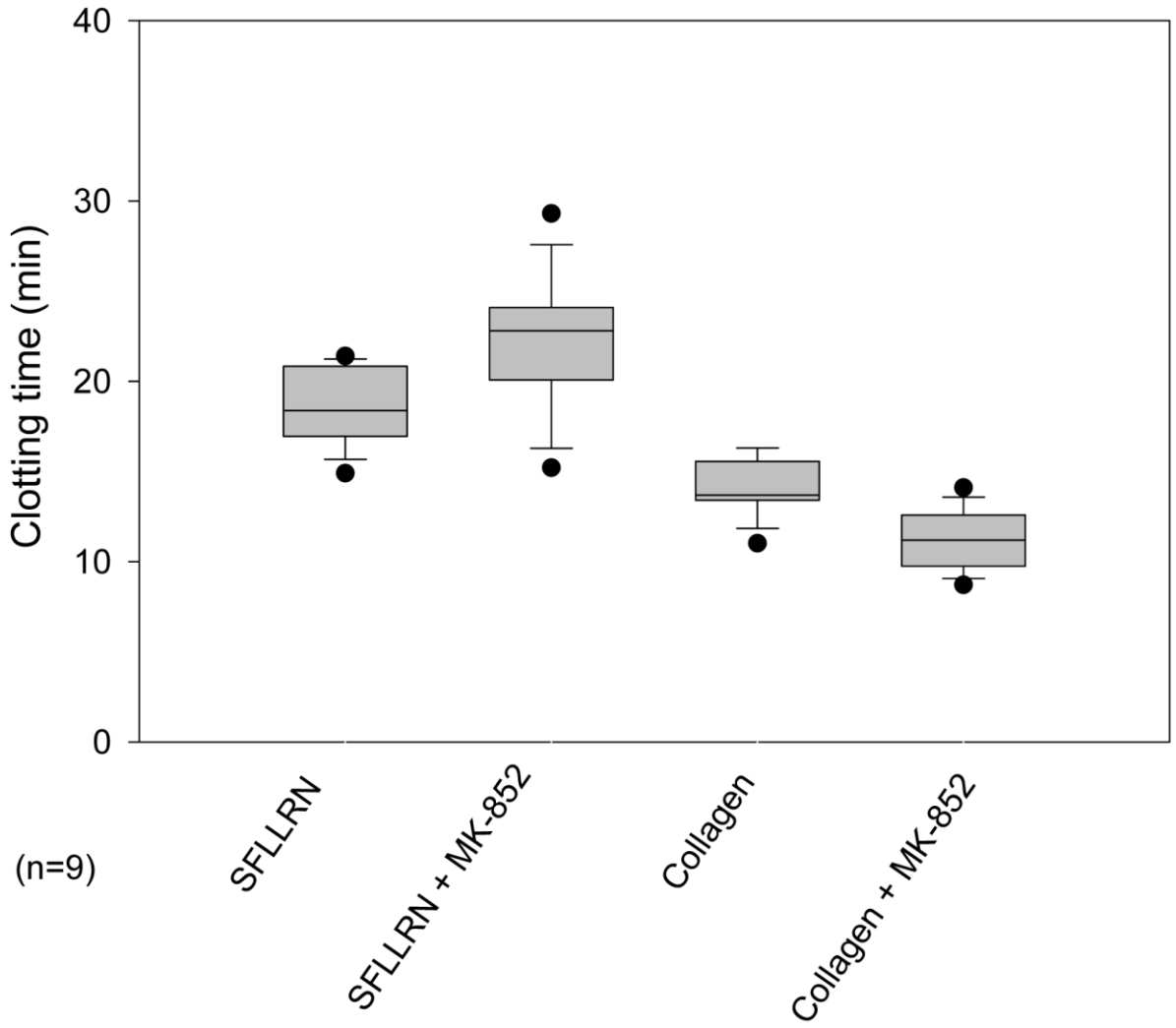

Figure 5: Effects of addition of the platelet GPIIb/IIIa inhibitor MK-852 (12 μg/mL) to native blood stimulated with SFLLRN (5 μg/mL) or collagen (0.3 μg/mL). The box plot shows the median, the10th, 25th, 75th and 90th percentiles and all outliers.

Apart from the clotting time and the absence of clot retraction, the GPIIb/IIIa antagonists affected other features of the rheometer tracings. The frequency minimum time (FMT) and clot completion time were therefore determined for all collagen stimulated samples. Results are shown in figure 6 and table 1. All curve features were delayed in samples with abciximab. For the samples with MK-852, the largest differences were seen for the clotting time and FMT ($p<0.01$), while no difference was

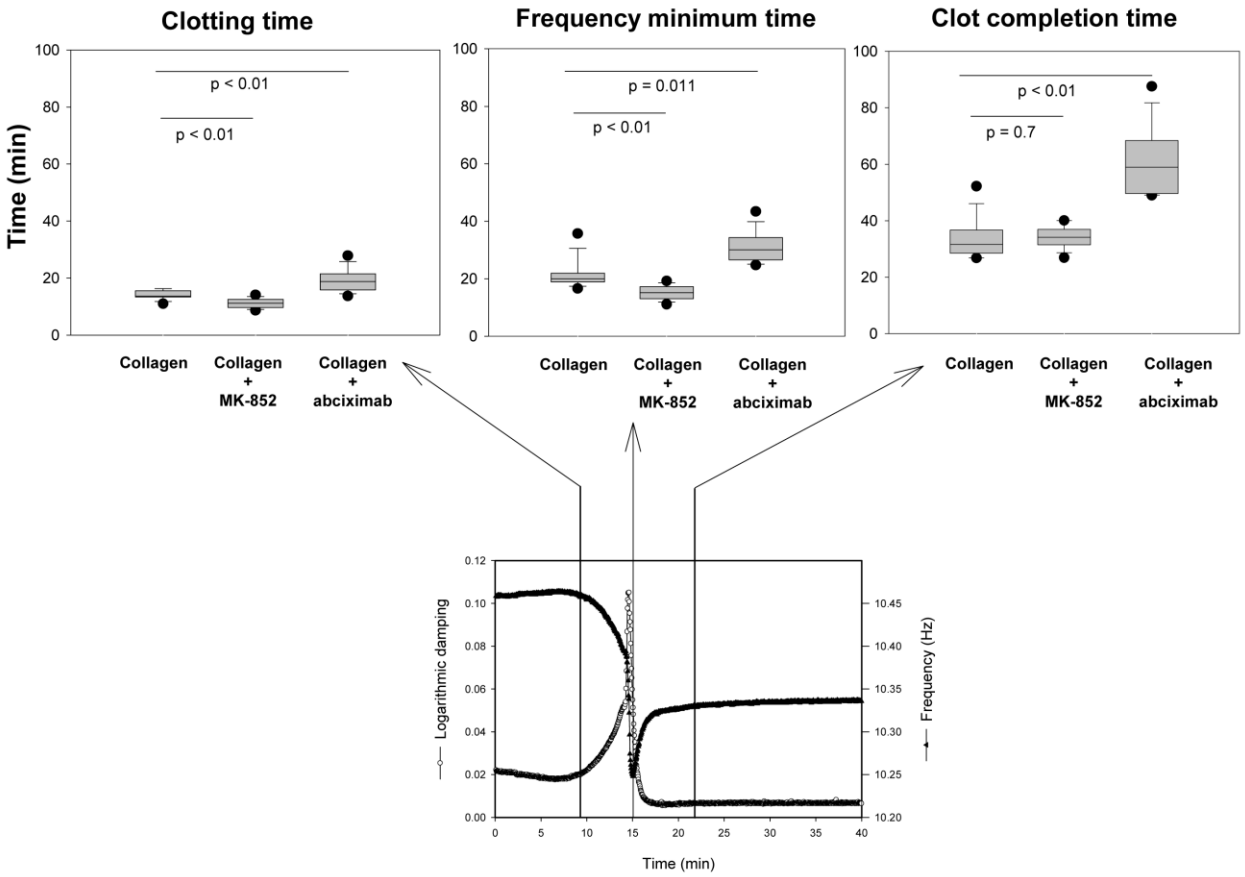

Figure 6: Comparison of different curve features (clotting time, frequency minimum time (FMT) and clot completion) for samples from 9 individuals with collagen (0.3 μ g/mL), and MK-852 (12 μg/mL) or abciximab (20 μg/mL). The box plots show the median, the10th, 25th, 75th and 90th percentiles and all outliers.

seen for the clot completion time (p= 0.7). Therefore we compared the times from clotting time to FMT, and the time from FMT to clot completion, to investigate the effects on the early and late stages of coagulation, respectively. Results are shown in table 1. The addition of MK-852 shortened the early phase of coagulation ($p<0.01$), while the addition of abciximab increased it slightly ($p=0.04$), but when comparing the late phase, both GPIIb/IIIa inhibitors resulted in a prolongation of the time ($p=0.015$ for MK-852, $p<0.01$ for abciximab).

Discussion

It is obvious that platelets are important in several aspects of the coagulation process. We showed in this study that in plasma contacted by a low-activating surface, a rapid removal of all cells was enough to keep the plasma anticoagulated. Stimulation of platelets via the thrombin receptor (with SFLLRN) or the collagen receptor shortened the clotting time for native whole blood, indicating an important role for the platelet in the amplification of the coagulation process. The same results were also obtained in platelet rich plasma, indicating that no other blood cell is responsible for this effect. Normally, the clot retracted from the wall of the sample cup after clot formation. In samples with GPIIb/IIIa inhibitors, however, no clot retraction was seen, corresponding to the situation in patients with Glanzmann thrombastenia [19].

The experiments with the GPIIb/IIIa antagonists MK-852 and abciximab indicate that important differences exists in the action of these two inhibitors, but that that this difference only shows when collagen is used as platelet agonist. This is in accordance with earlier reports about an increased procoagulant activity in platelets stimulated with collagen and thrombin in the presence of MK-852, but not with thrombin as single stimulus [20].

The activating effect of MK-852 in collagen stimulated samples was only affecting the early stages of clotting. The late phase of coagulation was prolonged as compared to collagen, just as in the samples with abciximab. In another study measuring only thrombin generation, the increase in thrombin generation with some GPIIb/IIIa antagonists was observed for all measured time points [21]. Some factors apart from the rate of thrombin generation therefore seem to affect the coagulum formed in the rheometer. One possible explanation is that the late phase is much dependent on forces

exerted by retracting platelets integrated in the fibrin network. In the presence of GPIIb/IIIa receptor antagonists, the integration of platelets are likely to be compromised whereby the effects of platelet retraction on the clot are diminished.

GPIIb/IIIa has been shown to bind prothrombin and to accelerate its conversion to thrombin [22]. GPIIb/IIIa antagonists have been shown to decrease both prothrombinase and tenase activity and PS exposure by thrombin stimulated platelets [23, 24], and to decrease the binding of factor V and the exposure of PS by activated platelets in a whole blood assay [12], but the efficiency varied between different antagonists. The addition of abciximab has been reported to decrease the thrombin generation by 25-30% [21]. This could explain the prolonged clotting times observed in our experiments. By contrast, addition of MK-852 to collagen appears to enhance the procoagulant properties of the platelet. MK-852 interferes with the RGD binding site of the GPIIb/IIIa receptor [25], just like the oral substances shown to display an even increased mortality in clinical trials in a recent meta-analysis [26].

The ability to detect changes in whole blood samples due to the treatment with platelet antagonists opens interesting possibilities in the diagnostic area. FOR analysis of patient samples could be used to monitor the effects of treatment with e.g. abciximab, without disturbing effects of citrate in the samples. This instrument measures the combined effect of these drugs, including both anti- and procoagulant effects. It has earlier been reported that citrate seems to enhance the potency of some GPIIb/IIIa antagonists [27], implicating that the analysis of citrated samples is unsuitable for the prediction of their efficiency *in vivo*.

From this study, we conclude that stimulation of platelets via their natural receptors is important for the amplification of coagulation *ex vivo*, since it significantly affects the

clotting times. This approach may be used for assays, giving a picture of the entire coagulation status of the patient, with all blood cells present and at physiological calcium levels. Such tests may have great potential both in intensive care or surgery and as a screening test in patients with suspected coagulation abnormalities. We think that coagulation activation by platelets has many advantages over the use of non-stimulated samples, where the coagulation will depend on activation of the intrinsic pathway by contact with foreign surfaces during sampling and testing, which is poorly reproducible and give abnormal values in patients with deficiencies not causing any bleeding problems. The new viscoelastic instrument described here enables global coagulation testing and offers a possibility to measure clotting times and to view changes in the forming coagulum.

Acknowledgements

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Table 1

Curve feature	Time for collagen (min) (mean ± SD)	Time for collagen +MK-852 (min) (mean ± SD)	Time for collagen + abciximab (min) (mean ± SD)
Early coagulation phase (time from clotting time to FMT)	7.4 ± 5.0	3.9 ± 1.1**	11.58 ± 2.6*
Late coagulation phase (time from FMT to clot completion)	12.1 ± 4.0	19.0 ± 4.1*	30.0 ± 9.9**

Table legends:

Table 1: Time for different curve features in native blood samples from 9 individuals with collagen (0.3 μg/mL) and either MK-852 (12 μg/mL) or abciximab (20 μg/mL) added. Asterisks show significant differences as compared to the samples with collagen alone from the same donor (*= $p<0.05$, **=$p<0.01$).

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