Thrombin generation, ProC®Global, prothrombin time and activated partial thromboplastin time in thawed plasma stored for seven days and after methylene blue/light pathogen inactivation

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Background. Methylene blue pathogen inactivation and storage of thawed plasma both lead to changes in the activity of several clotting factors. We investigated how this translates into a global loss of thrombin generation potential and alterations in the protein C pathway.

Materials and methods. Fifty apheresis plasma samples were thawed and each divided into three subunits. One subunit was stored for 7 days at 4 °C, one was stored for 7 days at 22 °C and one was stored at 4 °C after methylene blue/light treatment. Thrombin generation parameters, ProC®Global-NR, prothrombin time and activated partial thromboplastin time were assessed on days 0 and 7.

Results. The velocity of thrombin generation increased significantly after methylene blue treatment (increased thrombin generation rate; time to peak decreased) and decreased after storage (decreased thrombin generation rate and peak thrombin; increased lag time and time to peak). The endogenous thrombin generation potential remained stable after methylene blue treatment and storage at 4 °C. Methylene blue treatment and 7 days of storage at 4 °C activated the protein C pathway, whereas storage at room temperature and storage after methylene blue treatment decreased the functional capacity of the protein C pathway. Prothrombin time and activated partial thromboplastin time showed only modest alterations.

Discussion. The global clotting capacity of thawed plasma is maintained at 4 °C for 7 days and directly after methylene blue treatment of thawed plasma. Thrombin generation and ProC®Global are useful tools for investigating the impact of pathogen inactivation and storage on the clotting capacity of therapeutic plasma preparations.

Keywords: methylene blue, thawed plasma, thrombin generation, ProC®Global.

Introduction

Therapeutic fresh-frozen plasma from healthy donors is currently stored frozen between –18 °C (USA)[1] and –30 °C (Europe)[2]. Consequently, plasma must be thawed before usage, which can take up to 40 minutes. To enable fast issuing of plasma, it may be a useful strategy to store thawed plasma for several days in a thawed plasma bank.

It was previously demonstrated that thawed plasma retains most of its single clotting factor activities for up to 5-6 days at 1-6 °C[3-5]. Our group showed that the storage time of thawed plasma kept at 4 °C may be extended for up to 7 days, provided one accepts decreases of factor VIII and protein S activity[6]. We also found that application of methylene blue/light pathogen reduction (MB/light) to thawed plasma and storage for 7 days at 4 °C resulted in more pronounced decreases in most clotting factor activities. In contrast, MB-treated thawed plasma showed an increase of factor VII and factor X activities after storage, perhaps because tissue factor pathway inhibitor is affected[6]. This raises the question of whether these changes translate into a different thrombin generation capacity and/or lead to an alteration of the anticoagulant capacity in thawed plasma after different storage conditions.

The velocity and the peak of thrombin generation can be used to determine the endogenous thrombin generation potential (ETP) of plasma. The ETP has been applied to assess the risk of thrombosis in patients[7-10], to detect alterations in therapeutic plasma preparations[11,12], and to assess the impact of MB/light treatment[13-15]. The ProC®Global assay (Siemens Healthcare Diagnostics, Eschborn, Germany) is suitable for detecting defects in the protein C system, including protein S deficiency[16]; to date it has not been used to characterise therapeutic plasma preparations. Finally, the prothrombin time (PT) and activated partial thromboplastin time (aPTT)

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are standard coagulation tests used in routine practice. In combination, they are sensitive assays for detecting functional losses of the procoagulant clotting factors I-XII[17].

In this study, we used contemporary global coagulation tests to characterise thawed plasma after storage at 4 °C, 22 °C and after MB/light pathogen inactivation and storage at 4 °C.

Materials and methods

Study design and sample preparation

The design of the study was recently described in detail[6]. In brief, 50 healthy donors (15 blood group A, 24 blood group O, 10 blood group B, and 1 blood group AB) each donated 620 to 870 mL of plasma, which was collected on a PCS 2 device using PCS 2 sets (Haemonetics, Braintree, MA, USA). All plasma samples were frozen within 6 hours, and stored at –30 °C. After storage, plasma was thawed ("day 0") at 37 °C for 1 hour using a Plasmatherm thawing device (Barkey, Leopoldshoehe, Germany). Each thawed plasma sample was split into three subunits of approximately 220 mL each. Subunit I was kept at 4 °C and subunit II at room temperature (RT, 20-24 °C). Subunit III was treated with MB/light according to the manufacturer's instructions (Macotronic B2, MacoPharma, Mouvaux, France; peak wavelength 627±10 nm, 120 J/cm²) and kept at 4 °C. All three subunits were stored for 7 days. Aliquots for the coagulation studies were obtained by sterile docking of a sample bag at days 0 and 7. Plasma was immediately transferred into 3 mL Vacutainer tubes (Belliver Industrial Estate, Plymouth, UK) and stored at –80 °C until batch analysis. For batch analysis of all global coagulation parameters, the samples were thawed (10 min, 37 °C) and analysis initiated within 1 hour after thawing for all assays.

Thrombin generation

A calibrated automated thrombogram (CAT) was measured by a fluorometer (Fluorskan Ascent, ThermoLab Systems, Helsinki, Finland) as described elsewhere[18] using Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands) with excitation at 390 nm and emission at 460 nm. Either 20 μL of PPP-reagent (reference: TS 30.00; containing 5 pmol tissue factor and 4 μM phospholipids) or 20 μL thrombin calibrator (all reagents from Thrombinoscope BV) were added to clear, U-bottomed, 96-well microtitre plates (Immulon 2 HB, VWR International, Darmstadt, Germany). Then 80 μL of the respective sample were added to each well. After incubation in the pre-warmed plate reader (at 37 °C for 10 min), thrombin generation was initiated by automated dispensing of 20 μL pre-warmed Fluo-buffer into the microtitre wells. Fluo-substrate was added to the Fluo-buffer at a ratio of 1:40 and the mixture was immediately vortexed. The thrombin calibrator is required to correct for the activity of the α_2-macroglobulin-thrombin complex, substrate depletion, the inner filter effect, and the colour of the donor plasma. All reagents for the CAT assay were acquired from Stago (Asnière sur Seine, France) and the measurements were performed in triplicate. Raw fluorescence data were converted into thrombin activity (nmol) relative to the calibrator by the Thrombinoscope software. For each sample, the thrombin generation markers peak thrombin generation (maximum thrombin generation at a given time, nmol), endogenous thrombin potential (ETP, area under the thrombin generation curve, nmol), lag time (time until 10 nmol of thrombin are generated, min), and time to peak (time to peak thrombin generation, min) were automatically calculated by the software. The rate of thrombin generation (nmol/min) was calculated by the formula:

$$\frac{\text{peak thrombin generation}}{(\text{time to peak} - \text{lag time})}$$[19].

ProC®Global

ProC®Global is an aPTT-based assay, in which the clotting time is measured before (protein C activation time [PCAT]-0) and after activation of plasma protein C (PCAT) by Protac, a snake venom. For PCAT-0, 50 μL of aPTT reagents and 50 μL of buffer were added to 50 μL of plasma. For PCAT, 50 μL of aPTT reagents and 50 μL of Protac were added to 50 μL of plasma. After incubation (for 3 min at 37 °C) the measurement was started. Values for ProC®Global are given as normalised ratio (NR) which was calculated by the PCAT ratio (PCAT/PCAT-0) of the sample, the PCAT ratio of a lyophilised standard human plasma and a lot-specific sensitivity factor defined for each batch by the manufacturer. ProC®Global was measured on a BCS-XP analyser (Siemens, Marburg, Germany) using ProC®Global reagents.

Prothrombin time and activated partial thromboplastin time

PT and aPTT were determined by standard procedures using Dade Innovin and Dade Actin FS aPTT reagents (all obtained from Siemens), respectively, and measured on a BCS-XP-analyser. For the Innovin batch used, 8.2 s corresponded to a Quick-value of 100% and 9.8 s corresponded to a Quick-value of 70%. Each assay was monitored by two levels of quality control material according to the Guidelines of the German Federal Medical Council[2].

Data and statistical analysis

The baseline values of MB/light-treated and untreated plasma were compared on day 0 to identify changes attributable to the pathogen-inactivation

procedure. The baseline values obtained after thawing (day 0) were compared with those after MB- treatment and after 7 days storage at 4 °C and at RT. Furthermore, values of MB-treated plasma on day 0 were compared to the values of MB-treated plasma on day 7. The Wilcoxon signed-rank test was applied for the statistical analysis. Box plots show the median and the interquartile range with whiskers indicating minimum and maximum values. All other data are given as median values. P-values <0.05 are considered statistically significant.

Results

Thrombin generation

Storage and MB treatment of thawed plasma affected the velocity of thrombin generation in different ways.

The thrombin generation rate decreased after storage at 4 °C (97 *vs* 63 nmol/min; $p<0.001$) and at 22 °C (92 *vs* 65 nmol/min; $p<0.001$), but increased after MB treatment (97 *vs* 132 nmol/min; $p=0.001$). Consecutive storage after MB treatment did not change the thrombin generation rate ($p=0.1$; Figure 1a). Correspondingly, the lag time increased significantly after storage at 4 °C (2.1 *vs* 2.7 min; $p=0.001$) and 22 °C (2.2 *vs* 2.8 min; $p=0.001$). MB treatment did not change the lag time. Storage after MB/light pathogen inactivation shortened the lag time (2.0 *vs* 1.7 min; $p=0.003$; Figure 1b). The time to peak responded similarly by increasing after storage at 4 °C (4.8 *vs* 6.1 min; $p=0.001$) and 22 °C (4.9 *vs* 5.8 min; $p=0.001$); and by decreasing after MB treatment (4.8 *vs* 4.3 min; $p=0.004$). Further storage after MB treatment did not influence the time to peak (Figure 1c).

In contrast to the different thrombin generation kinetics observed for different plasma preparations, the ETP was not altered by storage of thawed plasma at 4 °C or immediately after the MB treatment. Here, storage at 22 °C (1,266 *vs* 1,054 nmol; $p<0.001$) and storage of MB-treated thawed plasma (1,200 *vs* 1,197 nmol; $p<0.001$) similarly decreased the ETP (Figure 1d). The peak thrombin generation also decreased after storage at 4 °C (264 *vs* 208 nmol; $p<0.001$) and 22 °C (250 *vs*196 nmol; $p<0.001$). While it was not influenced by MB treatment alone ($p>0.05$), MB treatment and storage at 4°C mildly reduced peak thrombin generation (279 *vs* 271 nmol; $p=0.001$; Figure 1e).

The results indicate that the velocity of thrombin generation increases after MB treatment, whereas it decreases after storage. The overall potential to generate thrombin is preserved in untreated plasma stored at 4 °C, but decreases after storage at 22 °C and after storage of MB-treated plasma.

ProC®Global normalised ratio

The ProC®Global NR assay was applied to detect functional alterations in the protein C/S cascade to assess basic anticoagulant properties of thawed plasma throughout storage. A high PCAT ratio indicates good function of the protein C pathway, whereas a low ratio shows a less functional protein C pathway.

MB treatment of thawed plasma caused an elevation of the PCAT ratio (0.89 *vs* 0.94; $p<0.001$), which may indicate activation of the protein C cascade.

Seven days of storage at 4 °C also increased the PCAT-ratio (0.89 *vs* 0.99; $p<0.001$), whereas storage at 22 °C resulted in a decrease of the PCAT ratio (0.89 *vs* 0.76; $p<0.001$). Storage of MB-treated plasma at 4 °C did not alter the PCAT ratio ($p>0.05$; Figure 2).

Prothrombin time and activated partial thromboplastin time

The PT and aPTT are both classical tests for comprehensively assessing the global function of the clotting factors I-XII. Both assays use fibrin formation as the read out. Increases of PT and aPTT indicate reduced coagulation capacity. Compared to the values in thawed plasma, the PT increased slightly after MB treatment (8.7 s *vs* 9.5 s; $p<0.001$), after 7 days of storage at 4 °C (8.7 s *vs* 9.8 s; $p<0.001$) and markedly after storage at 22 °C (8.7 s *vs* 11.2 s; $p<0.001$). Storage for 7 days after MB treatment did not alter the PT ($p>0.05$).

The aPTT was significantly prolonged after MB treatment (30 s *vs* 33 s; $p<0.001$), after 7 days of storage at 4 °C (30 s *vs* 33 s; $p<0.001$), after 7 days of storage at 22 °C (30 s *vs* 36 s; $p<0.001$) and after storage of MB-treated plasma (33 s *vs* 36 s; $p<0.001$, Figure 3).

Discussion

Our study shows that a thrombin generation test, the ProC®Global assay and the classical clotting tests, PT and aPTT, were together able to detect changes in the pro- and anticoagulant properties of therapeutic plasma.

Storage of thawed plasma in a plasma bank reduces the time to provide therapeutic plasma in emergencies and is a useful strategy to bridge the time interval of ~40 minutes, until freshly thawed plasma becomes available. Inactivation of any pathogens in the plasma reduces the risk of pathogen transmission. However, these treatments may alter the clotting capacity.

At present, the quality of therapeutic plasma is primarily determined by assessing the activities of vulnerable clotting factors or inhibitors such as factor VIII or protein S. However, these selective tests are not able to address the overall clotting capacity of therapeutic plasma, which is a result of the overall pro- and anticoagulant properties of all relevant factors. We utilised thrombin generation, the ProC®Global assay, PT and aPTT to assess the impact of three different treatments on the coagulant potential of thawed plasma.

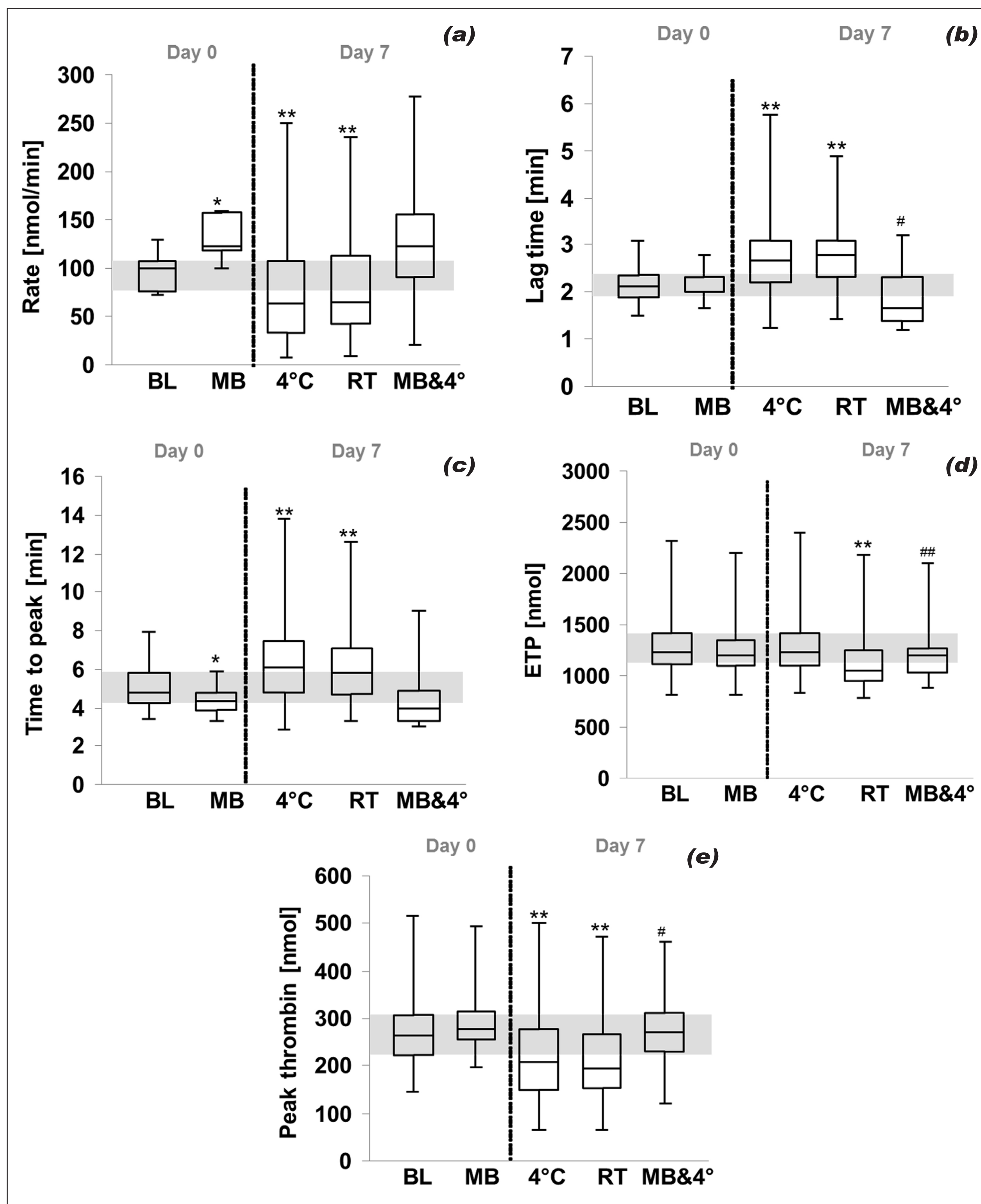

Figure 1 - Profiles of thrombin generation markers.
(a) Rate of thrombin generation, (b) lag time, (c) time to peak, (d) endogenous thrombin generation potential (ETP) and (e) peak thrombin generation at day 0 and at day 7. Grey shaded areas indicate the interquartile range at baseline.
* $p<0.05$ ** $p<0.001$ for BL *vs* MB/4 °C/RT, respectively; # $p<0.05$, ## $p<0.001$ for MB *vs* MB&4 °C.
BL: baseline after thawing; MB: after thawing and methylene blue treatment; 4°C: storage at 4 °C; RT: storage at room temperature; MB&4°: storage at 4 °C after MB treatment.

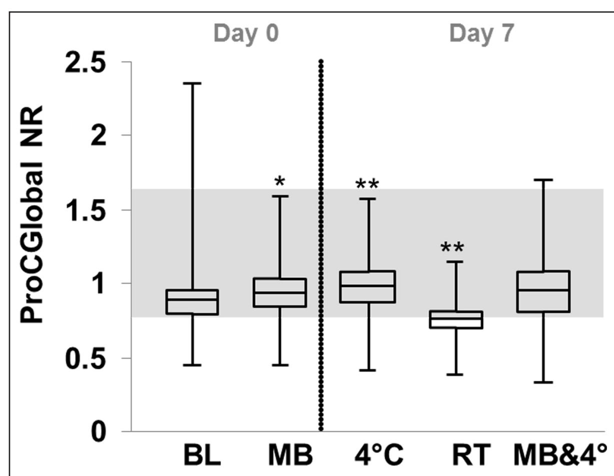

Figure 2 - Profile of the global protein C pathway.
ProC®Global normalised ratio (NR). High ratios indicate more procoagulant activity and low ratios indicate more anticoagulant activity. Abbreviations are identical to those in Figure 1. Grey shaded areas indicate the reference range and are specified by the respective test kits.
* $p<0.05$ and ** $p<0.001$ for BL *vs* MB and *vs* 4°C/RT, respectively.

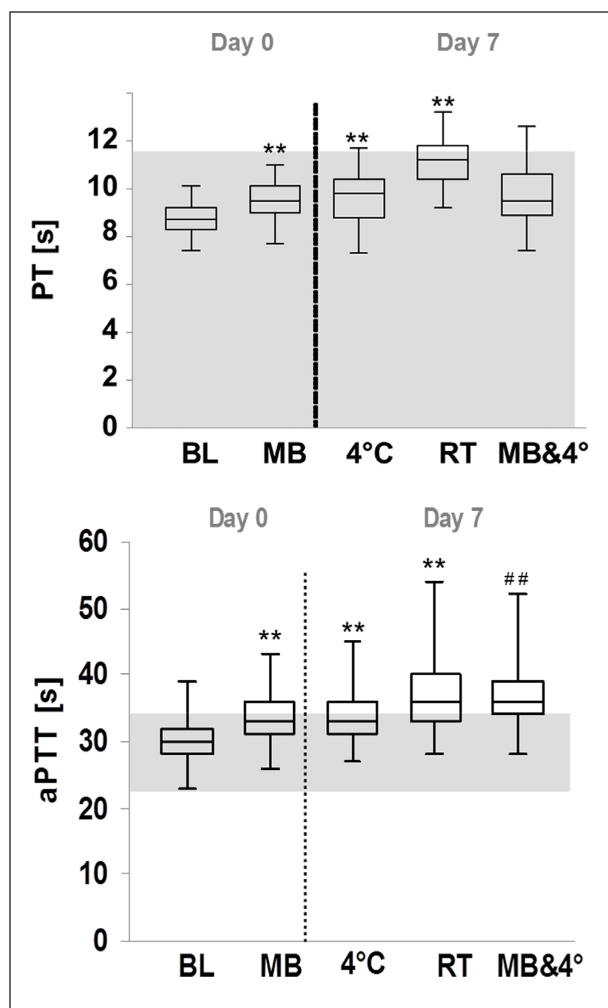

Figure 3 - Prothrombin times and activated partial thromboplastin times.
(a) PT and (b) aPTT in thawed plasma obtained directly after thawing on day 0 (baseline, BL), after MB/light-treatment on day 0 (MB); and after 7 days of storage at 4 °C (4°C), at room temperature (RT) and after MB/light treatment and storage at 4 °C (MB&4°). Grey shaded areas indicate reference ranges and are specified by the respective test kits.
** $p<0.001$ BL *vs* MB/4°C/RT; ## $p<0.001$ MB *vs* MB&4°.

The overall potential to generate thrombin (ETP) remained stable after MB treatment and storage at 4 °C, whereas storage at 22 °C and storage after MB treatment reduced the ETP. Thrombin generation over time increased after MB treatment, as demonstrated by a shorter lag time, a shorter time to peak and a higher thrombin generation rate. These observations reflect the increased activities of factor VII and X rather than the loss of factor V, VIII and fibrinogen activity found in these plasma units[6]. In contrast, storage of plasma decreased the velocity of thrombin generation (Online Supplementary Figure).

Thrombin generation has been examined in earlier studies. Cardigan *et al*. found a more pronounced loss of the ETP in thawed MB-treated plasma[13]. Other groups also found a reduced time to peak after MB treatment of plasma before freezing, similar to our study[14,15]. Cookson and colleagues investigated storage-dependent changes in thawed plasma at 4 °C and detected no change in the lag time or ETP and a decrease in the peak thrombin generation between the time point immediately after thawing and day 4, which is comparable to our results. Interestingly, these changes returned to baseline values by day 6[11]. Gosselin *et al.* found a decrease in the ETP after refrigerated storage of thawed plasma[12]. Differences in the production process of the plasma, freeze-thaw effects, residual platelet content during the measurement, storage conditions of plasma before thawing, and the reagents used in the thrombin generation assays may account for the slightly different results in these studies[20,21].

To our knowledge, this study is the first to use the ProC®Global test for systematic assessment of therapeutic plasma. MB treatment of thawed plasma and 7 days of storage at 4 °C increased the PCAT ratio, which indicates activation of the protein C cascade. In contrast, storage at 22 °C resulted in a decrease of the PCAT ratio and storage of MB-treated plasma at 4 °C did not change the PCAT ratio. This is interesting because protein C activity only decreased after storage of MB-treated plasma, but remained unaffected after the other two treatments[6]. Hence, the ProC®Global assay may be more sensitive than the traditional protein C activity test at detecting alterations in the protein C pathway. Of

note, protein S activity was reduced in all treatment groups after storage with a marked decrease after storage at 22 °C[6]. While protein S is part of the protein C/S-pathway, this might explain the drop of the PCAT ratio observed after storage at 22 °C.

The increase of PT was most pronounced after storage at RT and after MB-treatment and storage. The aPTT increased after all three interventions, being most pronounced after storage at RT and after MB treatment and storage. Overall, these changes were rather small and reflected the decreases in clotting factors after storage at higher temperatures[6].

Combining all four assays, the changes in the pro- and anticoagulant potential were least pronounced in plasma directly after MB treatment and in untreated plasma stored at 4 °C. These data are helpful to judge the impact of different production and storage methods on the clotting capacity of therapeutic plasma, but the respective clinical impact can only be assessed in a clinical study.

Conclusions

In conclusion, our study shows that functional alterations during pathogen inactivation and storage of plasma can be detected using the thrombin generation and ProCglobal assays, and that storage of plasma in a thawed plasma bank alters its pro- and anticoagulant capacities. Accordingly, we recommend that only a small number of thawed plasma units (e.g. 4 per blood group) should be kept at 4 °C for 7 days to permit immediate issuing of plasma. Patients requiring more than a few units of thawed plasma will then receive freshly thawed plasma with maximal clotting factor activity. Clinical studies should address the *in vivo* effects of the alterations in coagulation measures observed in plasma products *in vitro*.

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Authorship contributions

TT, GH, SK and KS designed the study and provided the study material, GH, SK and CW performed the measurements; TT, GH, TEW, AG and KS analysed the results; AW performed the statistical analysis; TT, GH, TEW, AG and KS wrote the manuscript. All authors reviewed and approved the final version of the manuscript. TT and GH contributed equally.

Disclosure of conflicts of interest

AG has a consultant contract with MacoPharma, Germany. The other authors declare that they have no conflicts of interest relevant to the manuscript.

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