Procoagulant impact of the plasmapheresis procedure on coagulation state of collected plasma

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Introduction

Plasmapheresis is a group of extracorporeal techniques that involve removal of whole blood, separation of plasma from cellular elements and partial return of blood components (at least the red blood cells) into the circulation. It is widely used for therapy and plasma collection with the purpose of either further transfusion or manufacture of medicines. Plasmapheresis necessarily involves significant contact of blood with inner surfaces of the plasmapheresis kit, so the possibility of contact activation of coagulation is a concern with regards to both donor safety and quality of the product obtained.

Although initial[1] (and some recent[2]) studies did not detect any noteworthy coagulation changes in donors' blood, this view was challenged by other reports. Using a more sensitive global assay of thromboelastography, hypercoagulation was found following various apheresis procedures[3]. Some researchers found that there could be additional pro-coagulant mechanisms for donors who often undergo apheresis[4], while others did not detect any differences in intensive donors[5]. Dangerous or even fatal venous and arterial thromboses were reported in individual cases after large-volume donations, usually when donors had pre-disposing risk factors[6,7]. Nevertheless, existing screening processes that plasma donors undergo do not take into account blood coagulation.

There is even less information about the effect of plasmapheresis on the coagulation status of the collected plasma. Although the composition of such plasma with regard to coagulation factors and activation markers has been evaluated by some researchers[8-10], we are not aware of any attempts to systematically compare the coagulation system in collected plasma with that of the original donor plasma. Here we carried out such a comparison using three global assays of the coagulation system: thrombin generation, thromboelastography and thrombodynamics together with clotting assays and other methods. Global assays that are increasingly used these days aim to mimic coagulation in vivo better[11] and are particularly noteworthy for their ability to detect pro-coagulant changes, unlike traditional clotting assays[12-15]. Using these assays we found significant changes in coagulation occurred as a result of the plasmapheresis procedure.

Materials and methods

Donors and procedure

The study involved 54 regular donors (all male; aged 22-45 years, mean ± standard deviation (SD), 32.9±7.6, median 33 years) who had undergone prior plasmapheresis procedures at least four times. All donors fulfilled the requirements for plasma donation and gave informed consent to participation in the study. Plasmapheresis was performed using the PCS2 Haemonetics device (Haemonetics Corporation, Braintree, MA, USA) and the original expendables including the high separation bowl. The volume of plasma collected was 600 mL (250 mL of plasma per cycle). The anticoagulant used was sodium citrate 4% (Haemonetics Corporation, Braintree, MA, USA) with an anticoagulant:blood ratio of 1:16.

Samples

The samples for coagulation analysis were taken before and after the plasmapheresis procedure from two different sources: through the vacuum sample adapter in the donor blood line after venipuncture prior to beginning plasmapheresis using Vacuette plastic 9 mL 3.8% citrate tubes (Greiner Bio-One, Kremsmunster, Austria), and from the collecting bag after the end of the procedure. All plasma was handled using polypropylene tubes (Sarstedt AG&Co, Nuembrecht, Germany). Blood samples were centrifuged within 5 minutes after collection at 1,600 *g* for 15 minutes to obtain platelet-poor plasma (PPP), then at 10,000 *g* for 5 minutes to obtain platelet-free plasma (PFP). With the exception of the thrombodynamics assay performed on both fresh and frozen PPP and PFP before and after plasmapheresis, all other coagulation assays were performed on frozen samples. Pre-plasmapheresis samples were quickly

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frozen (within an hour after collection) and stored as aliquots at −80 ºC before testing; those obtained after plasmapheresis were frozen within 2 hours after plasma collection and stored at −38 ºC or lower until measurement.

Assays

Thrombodynamics assay was performed using the Thrombodynamics Analyzer (HemaCore LLC, Moscow, Russia) according to the previously described technique[15,16]. This assay is based on videomicroscopic observation of fibrin clot propagation in a non-stirred layer of plasma activated by immobilised tissue factor. The assay was performed in fresh and frozen PPP and PFP, before and after plasmapheresis. A sample of 120 µL of plasma was supplemented with lyophilised corn trypsin inhibitor solution (4.8 µL of 10 mg/mL corn trypsin inhibitor per 120 µL of plasma) and incubated at 37 ºC for 15 minutes. Plasma was recalcified and placed into the experimental chamber, where the activator for clotting initiation was also placed. Fibrin clot growth was detected by imaging light scattering every 15 seconds for 45 minutes. The following parameters of clot growth were determined on the basis of the image series: lag time (normal 0.3-1.4 min), initial clot growth velocity (Vin, normal 36-54 µm/min), stationary clot growth velocity (Vst, normal 19-30 µm/min) and presence of activator-independent clotting (characteristic of hypercoagulation)[13].

A thrombin generation assay was performed on frozen PPP and PFP samples before and after plasmapheresis using a technique described previously[16]. Thrombin generation induced by tissue factor (RabTF, Renam, Moscow, Russia; 5 pM final concentration) in plasma was monitored by measuring the rate of hydrolysis of the fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland). Plasma was also supplemented with lipid vesicles at a final concentration of 4 µM (phosphatidylserine/phosphatidylcholine at a 20:80 molar ratio). Plasma was re-calcified and coagulation was initiated by adding 20 µL of a 100 mM $CaCl_2$ solution containing RabTF to 100 µL of a mixture of plasma-substrate-phospholipids. Along with thrombin generation in "coagulation" wells, background fluorescence level and calibration of fluorescence signal in "non-clotting" wells were measured. After data were analysed using Origin software (OriginLab Corporation, Northampton, MA, USA), the standard parameters of thrombin generation tests were calculated: lag time, thrombin peak, time to peak and endogenous thrombin potential.

Thromboelastography was performed using the TEG 5000 Thromboelastograph Hemostasis Analyser System and disposable cups and pins (Haemonetics Corporation, Braintree, MA, USA). For this assay, thawed plasma samples (330 µL) were recalcified with 30 µL of 0.2 M $CaCl_2$. Reaction time, clot formation time, alpha angle and maximum amplitude were calculated.

Fibrinogen level (normal 2.20-4.76 g/L), prothrombin time (normal 9.0-12.6 s), international normalised ratio (normal 0.80-1.14), activated partial thromboplastin time (normal 24.3-35.0 s), antithrombin activity (normal 80-120%), protein C activity (normal 70-130%), factor VIII activity (normal 50-150%), and D-dimer levels (normal up to 255 µg/L) were determined on the ACL 9000 coagulation analyser (Instrumentation Laboratory, Bedford, MA, USA) using the corresponding standard reagents (Instrumentation Laboratory; Renam).

Statistical analysis

The analyses of statistical differences were performed using a paired Student's *t*-test with the significance level $p<0.05$. For the correlation analysis, Spearman's correlation coefficient was calculated.

Results

The coagulation status of plasma from 54 donors obtained by whole blood centrifugation was compared with that of plasma collected by plasmapheresis. Results of clotting assays, coagulation factor levels and activation markers are listed in Table I. Results of the global assays are illustrated in Figure 1.

Prothrombin time and related assays, activated partial thromboplastin time, as well as levels of fibrinogen, antithrombin, protein C, factor VIII, and D-dimer were normal before the plasmapheresis (Table I). In the collected product, there was significant shortening of prothrombin time indicating a possible shift towards hypercoagulation; all other parameters remained unchanged.

Among global assays, thrombodynamics revealed four cases of markedly increased hypercoagulation accompanied by activator-independent clotting in the collected plasma product, although all donors showed normo- or even hypocoagulation before the procedure. Images of clots for one of these donors are shown in Figure 1A; stationary clot growth velocity (Vst) increased from 26 to 52 µm/min. Spontaneous clotting[13] was also noted.

In general, both initial and stationary clot growth velocity increased in a statistically significantly increased manner in the collected plasma as compared with pre-plasmapheresis samples from the donor (Figure 1B); clot formation lag time did not change. There were no cases of hypercoagulation before plasmapheresis detected by means of thrombodynamics, but a few (n=3) cases of donors with hypocoagulation before the procedure were found, and two of them were among

Table I - Values of routine coagulation assay parameters before plasmapheresis (marked as donor) and in the collected plasma (marked as product), thawed samples. Results are expressed as mean ± standard error of mean.

Assay	PFP_{donor} (n=42)	$PFP_{product}$ (n=51)	PPP_{donor} (n=51)	$PPP_{product}$ (n=31)	p -value	
					PFP_{donor} vs $PFP_{product}$	PPP_{donor} vs $PPP_{product}$
Fg, g/L	2.8±0.6	2.7±0.6	2.8±0.6	2.6±0.6	NS	NS
PT, sec	13.6±1.3	12.0±1.2	13.6±1.4	12.4±1.2	<0.01†	0.01†
PI, %	102±16	124±17	102±15	118±18	<0.01†	<0.01†
INR	1.05±0.15	0.9±0.1	1.05±0.13	0.93±0.11	<0.01†	0.01†
aPTT, sec	32.1±4.4	31.6±4.1	31.1±4.4	33.5±5.6	NS	NS
AT, %	94±10	95±12	94±10	98±12	NS	NS
PC, %	104±29	102±32	106±34	92±27	NS	NS
FVIII, %	77±22	82±25	78±23	80±26	NS	NS
D-dimer, µg/L	109±57	103±59	107±59	88±23	NS	NS

† Statistically significant; NS: not significant at p<0.05.
PFP: platelet-free plasma; PPP: platelet-poor plasma; Fg: fibrinogen; PT: prothrombin time; PI: prothrombin index; INR: international normalised ratio; aPTT: activated partial thromboplastin time; AT: antithrombin activity; PC: protein C activity; FVIII: factor VIII.

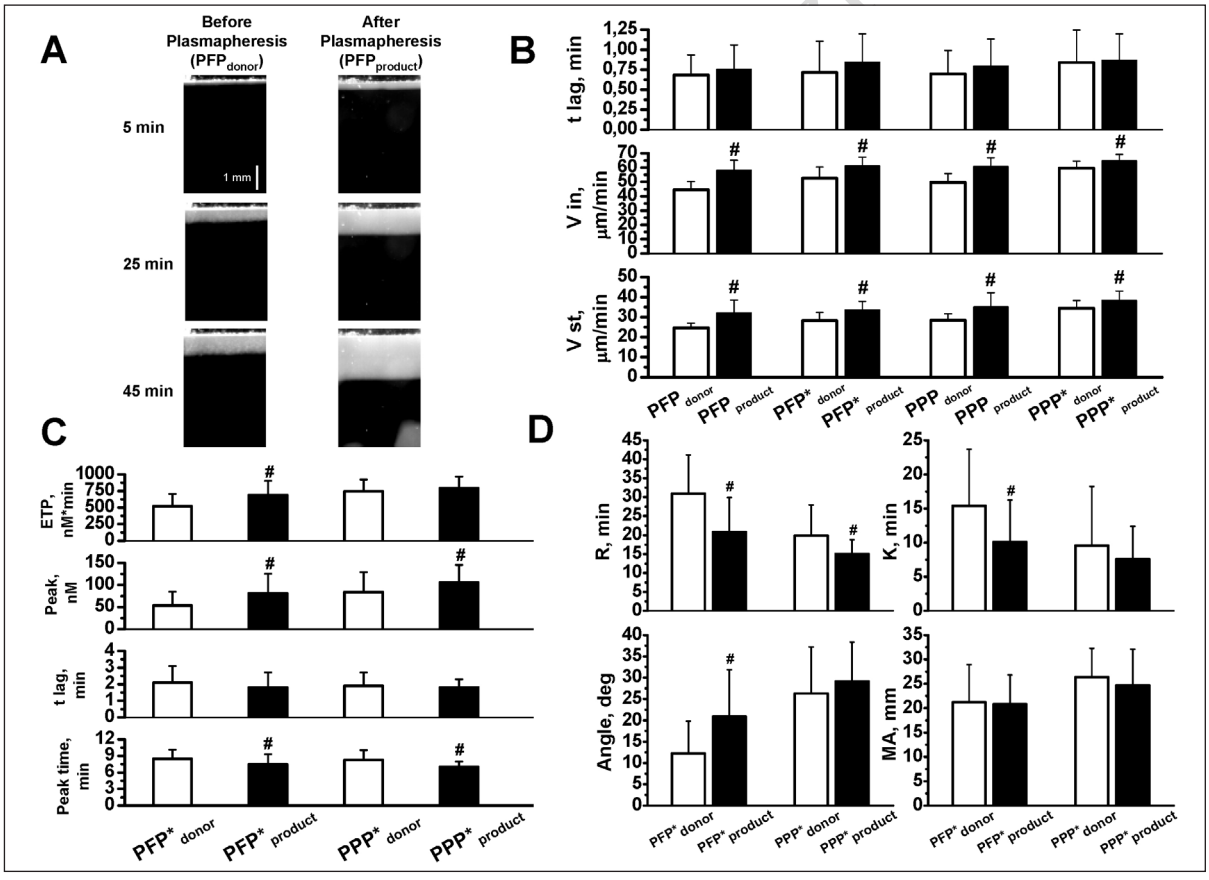

Figure 1 - Effect of plasmapheresis on the coagulation status in the global assays.
(A) Images of clot growth in the thrombodynamics assay for one of the four donors with marked effects. (B) Thrombodynamics parameters (n=39-54): lag time (t lag), initial clot growth velocity (Vin), stationary clot growth velocity (Vst); (C) Thrombin generation parameters (n=28-35): endogenous thrombin potential (ETP), thrombin peak (peak), lag time (t lag), time to peak (peak time); (D) Thromboelastography parameters (n=21-40): reaction time (R), alpha angle (angle), clot formation time (K) and maximum amplitude (MA) before plasmapheresis and in the collected PFP and PPP (thawed samples marked with *). Results are expressed as mean ± standard error of mean. # p<0.05. PFP: platelet-free plasma; PPP: platelet-poor plasma.

those with a particular tendency to hypercoagulation in the collected plasma product (online Supplementary Figure S1). Results obtained in fresh and frozen plasma samples, or in PFP *vs* PPP were in good agreement (Figure 1B).

In agreement with the thrombodynamics findings, thrombin generation demonstrated a statistically significant shift of endogenous thrombin potential and thrombin peak, as well as shortening of peak time upon plasmapheresis (Figure 1C). Lag time did not change. The results were qualitatively similar for PFP and PPP, though their magnitude was smaller in the latter case (Figure 1C).

Finally, thromboelastography showed the same shift towards hypercoagulation in plasma obtained by plasmapheresis for all parameters except for maximal amplitude (Figure 1D), also both in PFP and PPP. As in two other assays, the magnitude of the effects was 20-30%.

Correlation analysis of the global assay parameters showed an excellent correlation within the assays (e.g. Vst *vs* Vin with a Spearman's correlation coefficient $r=0.87$ and $p<0.0001$) (online Supplementary Figure S2). For some cases, there was also good correlation between the different assays (e.g. reaction time *vs* peak: $r=-0.76$ and $p=0.002$). These data indicate that, although the various parameters of the different assays are sensitive to different changes in the coagulation system, the shift towards hypercoagulation in the collected plasma is reproducible and was observed in the same donors' plasma by three very different assays.

Discussion

The main conclusion of the present study is that the plasmapheresis procedure significantly shifts coagulation parameters from normocoagulation in the donors' plasma before plasmapheresis to a hypercoagulable state in the collected plasma, as shown by thrombin generation, thromboelastography, thrombodynamics, and the prothrombin time test. In some donors, these changes were particularly pronounced, going far beyond the normal ranges, reaching values observed in plasma samples from thrombotic patients[12,13,15] and raising concern about the safety of transfusing a product with such parameters. Although these changes could have clinical significance, within the limits of this study we had no direct way to evaluate risks associated with transfusion of such plasma.

Quantitative changes between PFP and PPP observed in global assays could be explained by the different properties of the two types of plasma, particularly after freezing/thawing when cells get disrupted. It is likely that the presence of residual platelets and cell-derived vesicles in PPP could make the plasma slightly more procoagulant and mask other effects[13,14,17]. Nevertheless, the qualitative effects were reproduced between assays and plasma types.

The magnitude of the procoagulant changes in the global assay parameters (20-30% on the average, up to 100% in the minority of cases), their correlation and the agreement between different assays all suggest that these changes are significant and reproducible. Although the technology of the experiment made it necessary to handle plasma product samples slightly differently from whole blood collected before the procedure, the effects were clearly greater than those possible due to pre-analytical difference[14]. We did not study whether the differences were donor-specific (i.e. reproducible on the following procedures) or random, sample-specific. Further studies are also needed to learn how the effects depend on the specific apheresis procedure and apparatus used and whether there any particular steps of the procedure responsible for hypercoagulation. We only tried to characterise plasma obtained by the whole plasmapheresis cycle and ready for transfusion. Additional research is also required to reveal the biochemical nature of the apheresis-induced hypercoagulation, although the presence of spontaneous clots in thrombodynamics (Figure 1A) and lack of changes in the coagulation factor concentrations (Table I) suggest that the mechanism is contact activation leading to the appearance of long-lived activated factors[13] rather than changes in the concentrations of circulating coagulation factor pre-cursors.

From a practical point of view, the data of this study indicate that the coagulation state of collected plasma can in some cases (5-10%) significantly differ from the normal state. Additional research is required to make valid suggestions on how dangerous this might be, and how this hypercoagulation (if it is found dangerous) can be prevented or, at least, detected early.

Conclusions

Plasma obtained by plasmapheresis is statistically significantly more procoagulant compared with plasma obtained from donors' blood prior to plasmapheresis. Global assays of haemostasis are sensitive to this hypercoagulation. In a fraction of donors (~10%) this hypercoagulation is very pronounced (e.g. ~100% increase of clot growth velocity in thrombodynamics) suggesting it could be clinically relevant and raising safety concerns.

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Authorship contributions

SS planned and performed experiments, analysed the data and wrote the paper. RO performed experiments and analysed the data. IV, EK and VK planned the research, worked with subjects and analysed the data. MP analysed the data and wrote the paper. TV oversaw the project, performed experiments, analysed the data and edited the paper.

Disclosure of conflicts of interest

SS, RO, MP and TV are/were employees and/or founders of HemaCore LLC which holds several patents on the use of thrombodynamics assays. All other Authors declare that they have no conflicts of interest.

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