Platelet transfusions and patient outcomes after cardiac surgery

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CHAPTER 3

3

Comparison of hemostatic function of PAS-C-platelets versus plasma-platelets in reconstituted whole blood using impedance aggregometry and thromboelastography

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Vox Sang. 2017 Aug;112(6):549-556.

Background and objectives

There are concerns about the hemostatic function of platelets stored in platelet additive solution (PAS). Aim of this study was to compare the hemostatic function of PAS-C-platelets to plasma-platelets in reconstituted whole blood.

Materials and methods

In our experiment, whole blood was reconstituted with red blood cells, solvent-detergent (SD) plasma, and either PAS-C-platelets or plasma-platelets (n=7) in a physiological ratio. On storage days 2, 5, 8 and 13 the agonist-induced aggregation (multiple electrode aggregometry), clot formation (thromboelastography), and agonist-induced CD62P responsiveness (flow cytometry) were measured.

Results

Samples with PAS-C-platelets showed significantly lower aggregation than plasma-platelets when induced with adenosine diphosphate, -6 U (95% confidence interval: -8;-4) or thrombin receptor activating protein, -15 U (-19;-10). Also when activated with collagen and ristocetin the PAS-C-platelets showed less aggregation, although not statistically significant. All samples with PAS-C-platelets showed significantly lower agonist-induced CD62P responsiveness than samples with plasma-platelets. However, there was no difference regarding all TEG parameters.

Conclusion

Our findings demonstrate that the function, aggregation and CD62P responsiveness, of PAS-C-platelets in reconstituted whole blood is inferior to that of plasma-platelets, which may have implications in the setting of massive transfusions.

Abstract

Introduction

Platelet transfusions are used to provide hemostatic capacity to patients with a decreased number or functionality of platelets. In the Netherlands both platelet concentrates stored in plasma and platelets stored in a mixture of plasma and platelet additive solution C (PAS-C) are used. Among others, an advantage of PAS-C-platelets is that they cause fewer transfusion reactions.[1, 2]

However, it is unclear whether PAS-C-platelets are as effective as plasma-platelets. A large *in vitro* study, comparing platelets in plasma with platelets stored in different PASs until storage day 8, demonstrated inferior results for PAS-C-platelets compared to plasma-platelets, with regard to pH, lactate production, Annexin A5 binding and CD62P expression.[3] This study was applied in platelet concentrates, in the absence of red blood cells and plasma. Furthermore, the correlation between the analyzed outcomes and clinically relevant endpoints has not been established.

The clinical studies analyzing the effectiveness of PAS-C-platelet transfusions have been performed in hematology patients and have shown conflicting results.[2, 4] The results of these hematological studies are not applicable to surgical and trauma patients, in whom considerable volume replacement is common. Moreover, in these studies the corrected count increment (CCI) is often used as a measure for the efficacy of platelet transfusions. However, the CCI does not provide information on the platelet function. Furthermore, CCI is not applicable in all clinical settings in which platelets are transfused and it is unclear whether and to what extent CCI is correlated with clinical endpoints such as bleeding.[5]

In contrast, multiple electrode aggregometry (Multiplate analyzer) is a platelet function test reported to correlate well with clinically relevant outcomes like bleeding and thromboembolic events in different clinical settings.[6-9] The Multiplate is a point-of-care (POC), impedance aggregometer which measures platelet aggregation in whole blood samples using several agonists. This test is increasingly used in daily clinical practice to assess the hemostatic condition of patients and to guide transfusion and other interventions. Another frequently used assay that has a clear correlation with clinical endpoints is thromboelastography (TEG) which is a whole blood POC test assessing overall clot formation.[10, 11]

The objective of this exploratory study was to compare the hemostatic function of PAS-C-platelets to plasma-platelets in reconstituted whole blood. To compare the *ex vivo* functionality of the two platelet products in a condition that is as comparable as possible to an *in vivo* transfusion, whole blood was reconstituted to create an *ex vivo* model.

The hypothesis is that Multiplate-derived aggregation, TEG-measured clot formation, and flow cytometric CD62P responsiveness of PAS-C-platelets are inferior to those of plasma-platelets in reconstituted whole blood.

Materials and methods

Blood products

All blood products were produced and stored by the Dutch Blood Bank Sanquin according to national standards. The platelet concentrates were prepared from five pooled buffy coats from five whole blood donations, and were pre-storage leukoreduced using filtration. The platelets were resuspended either in 100% plasma from one of the five whole blood donations (plasma-platelets) or in 65% platelet additive solution C (also known as PAS-III or Intersol, Fresenius Kabi, Emmer-Compascuum, The Netherlands) and 35% plasma (PAS-C-platelets). To establish a more accurate estimation of the trend over storage time the platelet concentrates were stored and analyzed for 13 days. However, the current maximal storage time in clinical practice is 7 days, so only the results of storage up to 7 days apply to clinical practice. Both the whole period of 13 days and the period up to 8 days are analyzed separately. The platelets were stored at 20-24°C under constant agitation in a PVC-butyryl trihexyl citrate (BTHC) container (C5000, Fresenius Kabi, Bad Homburg, Germany). To obtain RBC units whole blood was centrifuged to remove the buffy-coat and plasma where after RBCs were resuspended in 110 ml saline-adenine-glucose-mannitol (SAGM), and then filtered to remove leucocytes to obtain a standard RBC product. For this experiment the RBC units were stored less than 3 days, at a temperature of 2-6°C, before being mixed with the other components. To use a standardized product, the plasma product that was used was a pooled solvent-detergent-treated plasma (Omniplasma, Octapharma, Vienna, Austria). The plasma was stored in aliquots below -30°C until use.

Whole blood reconstitution

To compare the *ex vivo* functionality of the two platelet products in a condition that is as comparable as possible to an *in vivo* transfusion, whole blood was reconstituted to create an *ex vivo* model. The reconstituted blood samples were composed of AB0-matched RBCs, plasma, and either PAS-C-platelets (n=7) or plasma-platelets (n=7) (Figure S1a). On day 2, 5, 8 and 13 a sample was taken from the stored platelet concentrates to make reconstituted whole blood. The intended ratio of the blood components in the reconstituted whole blood samples was physiological, in other words, a platelet count of 200 * 10^9/l and a hematocrit of 40%. Based on the platelet count of the platelet unit, the volume necessary to achieve a platelet count of 200 * 10^9/l in the whole blood sample was calculated. A sample was taken from a unit of RBCs and centrifuged at 1851

g for 10 minutes (Rotina 420R, Hettich, Geldermalsen, the Netherlands) to reduce the amount of SAGM. The concentrated RBCs and plasma were mixed with the sample of the platelet unit in the aforementioned ratio. Subsequently, the platelet aggregation capacity, agonist-induced CD62P responsiveness and clot formation were assessed in the reconstituted whole blood samples with multiple electrode aggregometry (Multiplate analyzer, Roche Diagnostics Ltd, Rotzkreuz, Switzerland), flow cytometry (FACSCanto, BD Biosciences, Breda, the Netherlands) and thromboelastography (TEG, Haemonetics, Braintree, MA, USA) respectively. To check the composition of the reconstituted whole blood, the platelet concentration and hematocrit were determined (Sysmex XT2000i, TOA, Tokyo, Japan).

Multiple electrode aggregometry

The Multiplate was used to assess platelet function. Platelet aggregation was assessed in single-use test cells that incorporate two independent sensor units, on which platelet aggregation occurs. According to the manufacturer's recommendations all samples were recalcified except for the ristocetin-activated samples. Three hundred microlitres of the reconstituted whole blood was mixed with 300 µl 0.9% NaCl with 3 mmol/l $CaCl_2$ (or 0.9% NaCl in ristocetin-activated samples) in the test cell. After 3 minutes of incubation, 20 µl of activator was added to the test cell. Four different activators were used in each sample on every storage day: adenosine diphosphate (ADP; final concentration 6.5 µmol/ml) that stimulates the P2Y-receptor; ristocetin (RISTO; 0.77 mg/ml) that gives binding of von Willebrand factor to the GPIb receptor; collagen (COL; 3.2 µg/ml) which binds to integrin α2β1 and the GPVI receptor, and thrombin receptor-activating peptide (TRAP; 32 µmol/ml) which stimulates the proteinase-activated receptor 1. All reagents were obtained from the manufacturer. After 6 minutes, the area under the curve (AUC) was determined. All measurements were taken at 37^0C.

Thromboelastography

The clot formation of the whole blood samples was measured using TEG. All samples were measured according to the manufacturer's instructions using disposable cups, pins and standard citrated kaolin reagent from the manufacturer. The samples were recalcified before performing the citrate-kaolin test. Details have been described earlier. [12]

Agonist-induced CD62P expression

To analyze platelet responsiveness the agonist-induced CD62P expression was measured using flow cytometry. The method has been reported previously and was applied with the exception that convulxin (CVX; highest concentration of 39 nmol/l), was used instead of collagen-related peptide.[13] The "platelet responsiveness" was calculated by subtracting the percentage of platelets expressing CD62P with lowest agonist level from

the percentages of platelets expressing CD62P at each concentration, like described earlier.[13] Then the area under the curve (AUC) of these percentages of "platelet responsiveness" was determined (Figure S2). Secondly, the CD62P expression with lowest agonist level, "baseline CD62P expression", was compared between the platelet types. Finally, the CD62P expression with highest level of agonist, "maximal CD62P expression", was compared between platelet types.

Additional experiments

PAS-platelets and PAS fluid compared to plasma-platelets and plasma

To visualize not only the influence of the PAS-C-platelets but also the maximal impact of the accompanying PAS-C-supernatant on hemostatic function, an additional experiment was performed. This experiment, shown in Figure S1B of the Supporting Information, was similar to the main experiment expect for the SD plasma that was replaced entirely by platelet concentrate supernatant, either PAS-C or plasma (n=7). As this experiment focused on the influence of platelets and the accompanying storage fluid on the hemostatic function the clot formation was measured using TEG.

Biochemical characteristics of platelet units

To verify our results, we measured previously studied parameters in the platelet concentrates, that is, before the platelets were mixed with plasma and RBCs. On days 2, 5, 8 and 13 after donation the pH, pO2, pCO2, glucose, lactate, unstimulated CD62P expression and Annexin A5 binding were measured as described previously.[3]

Statistical analysis

The data in the figures are presented as mean and 95% confidence interval (CI). The mean platelet concentration and hematocrit of the whole blood samples prepared with PAS-C-platelets were compared with those of the samples containing plasma-platelets using an independent sample t-test. The aggregation and clot formation in the reconstituted whole blood samples over the whole storage time (13 days), were analyzed with generalized linear models with platelet type (PAS-C-platelets or plasma-platelets) and storage time (in days) as independent variables. The influence of the platelet type independent of storage time, and the influence of storage time independent of platelet type, were expressed as the regression coefficient (beta) with 95% CI (results shown in Table 1). If there was a significant difference between the platelet types, the potential interaction between platelet type and storage time was analyzed in a separate model, by adding an interaction term to the generalized linear model. As in clinical practice platelets are stored up to 7 days, additionally a sensitivity analysis was performed on the data of days 2,5 and 8 to check whether the results regarding the influence of the platelet type are in agreement with the analysis including day 13. The same generalized linear models as

Table 1 Influence of platelet type and storage time on platelet aggregation and clot formation

		PAS-C-platelets (vs. plasma-platelets)	storage time (per day)
Multiplate	ADP (U)	-5.6 (-7.7;-3.5)	-1.3 (-1.5;-1.0)
	COL (U)	-1.5 (-4.0;0.9)	-1.1 (-1.4;-0.8)
	RISTO (U)	-2.5 (-6.6;1.6)	-1.2 (-1.7;-0.7)
	TRAP (U)	-14.5 (-18.8;-10.1)	-2.2 (-2.7;-1.7)
TEG	MA (mm)	-0.6 (-3.5;2.2)	-0.1 (-0.4;0.3)
	R-time (min)	0.06 (-0.27;0.39)	-0.07 (-0.11;-0.03)
	angle (^{0})	0.1 (-1.6;1.9)	0.1 (-0.1;0.3)

Main analysis includes data up to storage day 13. Values are generalized linear model regression derived coefficients (95% confidence interval) of platelet type (mean difference between 2 platelet types) and storage time (mean change per day) on aggregation induced with ADP, COL, RISTO and TRAP, and clot strength (MA), initial clot formation (R-time) and clot growth rate (angle). n = 14; * n = 13.

described above were performed on the data of storage days 2, 5 and 8 (results shown in Table 2). Comparisons between the platelet types regarding the AUCs of the CD62P "platelet responsiveness", "baseline CD62P expression" and "maximal CD62P expression" were made with an independent sample t-test (results shown in Table 3).

Table 2 Influence of platelet type and storage time on platelet aggregation and clot formation

		PAS-C-platelets (vs. plasma-platelets)	storage time (per day)
Multiplate	ADP (U)	-5.9 (-8.4;-3.4)	-1.8 (-2.4;-1.3)
	COL (U)	-1.8 (-4.6;0.9)	-1.9 (-2.5;-1.3)
	RISTO (U)	-3.4 (-8.7;1.8)	-2.0 (-3.1;-0.9)
	TRAP (U)	-12.3 (-17.3;-7.3)	-2.5 (-3.5;-1.4)
TEG	MA (mm)	-1.3 (-4.8;2.2)	0.05 (-0.66;0.76)
	R-time (min)	0.04 (-0.39;0.47)	-0.10 (-0.18;-0.01)
	angle (^{0})	-1.1 (-3.0;0.8)	0.5 (0.1;0.8)

Sensitivity analysis includes data up to storage day 8. Values are generalized linear model regression derived coefficients (95% confidence interval) of platelet type (mean difference between 2 platelet types) and storage time (mean change per day) on aggregation induced with ADP, COL, RISTO and TRAP, and clot strength (MA), initial clot formation (R-time) and clot growth rate (angle). n = 14; * n = 13.

Table 3 CD62P expression (%) of plasma-platelets compared to PAS-C-platelets

agonist	day	responsiveness	baseline CD62P expression	maximal CD62P expression
ADP	2	125 (87;163)	-27 (-37;-16)	10 (0;20)
	5	81 (61;101)	-26 (-33;-20)	4 (-5;12)
	8	90 (71;109)	-24 (-30;-18)	7 (1;13)
	13	55 (45;65)*	-6 (-14;2)*	12 (6;19)*
CVX	2	88 (50;127)	-26 (-37;-15)	0 (-1;1)
	5	109 (82;135)	-27 (-34;-20)	2 (1;3)
	8	103 (78;127)	-23 (-29;-17)	4 (3;6)
	13	64 (40;108)*	-5 (-13;3)*	13 (10;17)*
TRAP	2	125 (83;168)	-27 (-37;-16)	0 (-1;1)
	5	124 (91;158)	-27 (-34;-19)	2 (1;3)
	8	115 (93;137)	-23 (-30;-16)*	3 (2;5)
	13	79 (44;114)*	-5 (-13;3)*	12 (8;15)*

Values are mean difference (95% confidence interval) between plasma-platelets and PAS-C-platelets in reconstituted whole blood samples for CD62P responsiveness (%), "baseline CD62P expression": after stimulation with the lowest agonist level and "maximal CD62P expression": after stimulation with highest agonist level. N = 7 in both groups; * n = 6.

Results

Platelet aggregation (Multiplate)

The reconstituted whole blood with PAS-C-platelets had a mean platelet concentration of 183 (95% CI: 177;190) * 10^9/l and hematocrit of 37.9% (37.1;38.7%), which were not different from those in the plasma-platelets-samples: 182 (174;190) * 10^9/l platelets and a hematocrit of 37.3% (36.8;37.7%). The samples with PAS-C-platelets activated with ADP and TRAP showed significantly lower aggregation than the samples with plasma-platelets (Table 1). Although not statistically significant, when induced with COL and RISTO the PAS-C-platelets also showed less aggregation. In all samples with all activators the aggregation significantly declined over storage time (raw data are shown in Figure 1). The difference between the platelet types remained stable over storage time when aggregation was induced with ADP (interaction term -0.1 U,-0.6;0.5). However, TRAP-induced aggregation declined significantly faster in PAS-C-platelets than in plasma-platelets (interaction term: -1.2 U, -2.2;-0.1). The effect of platelet type detected in the sensitivity analysis was similar to that found in the analysis of 13 days of storage (results shown in Table 2).

Clot formation (TEG)

The clot strength (maximum amplitude, MA), initial clot formation (R-time) and clot growth rate (angle) of the samples with PAS-C-platelets were similar in the samples with

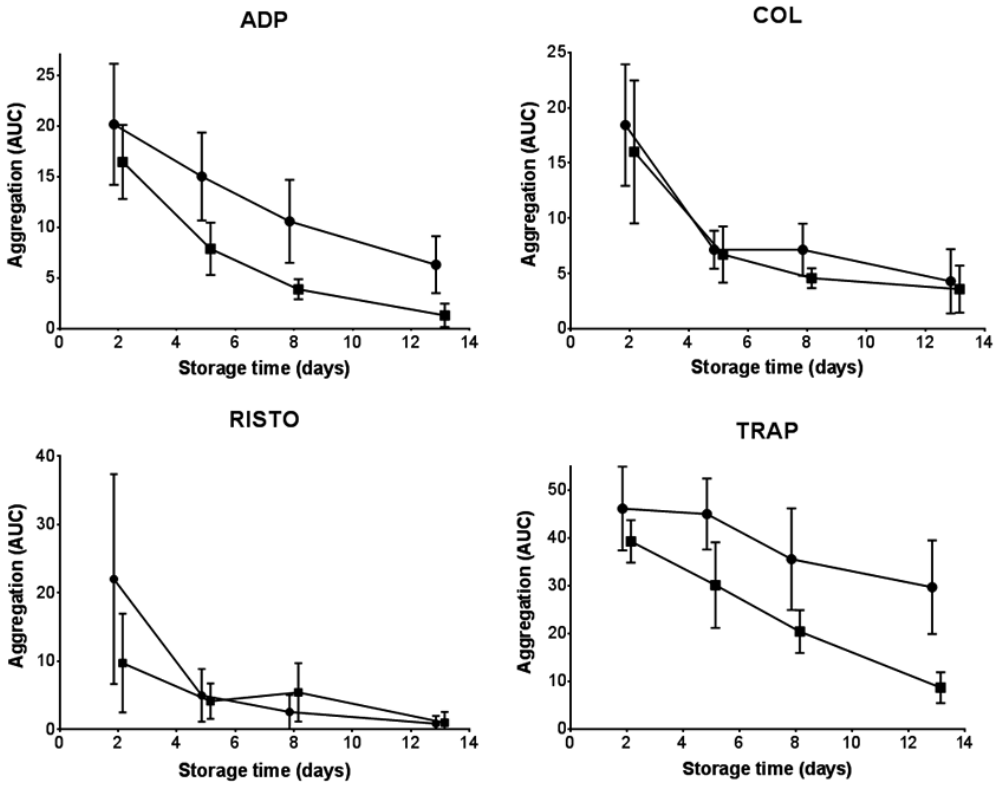

Figure 1 Aggregation (Multiplate) The mean aggregation of plasma-platelets (●) or PAS-C-platelets (■) in reconstituted whole blood, induced by ADP, COL, RISTO and TRAP. Error bars represent 95% confidence intervals.

plasma-platelets (Table 1). Initial clot formation significantly declined with increasing storage time, but maximum clot strength and clot growth rate remained rather constant with increasing storage time (raw data are shown in Figure S3A of the Supporting Information). The influence of platelet type on the clot formation found in the sensitivity analysis was similar to that found in the analysis of all 13 days of storage (results shown in Table 2).

CD62P responsiveness (flow cytometer)

The platelet CD62P responsiveness after stimulation with increasing concentrations of the agonists ADP, CVX and TRAP for samples containing PAS-C-platelets or plasma-platelets during 13 days of storage are shown in Figure 2. The CD62P expression with minimal agonist concentration, indicating baseline activation, was higher in samples with PAS-C-platelets than with plasma-platelets on every storage day for all three agonists (Table 3). With minimal agonist stimulation, both sample types showed a pattern

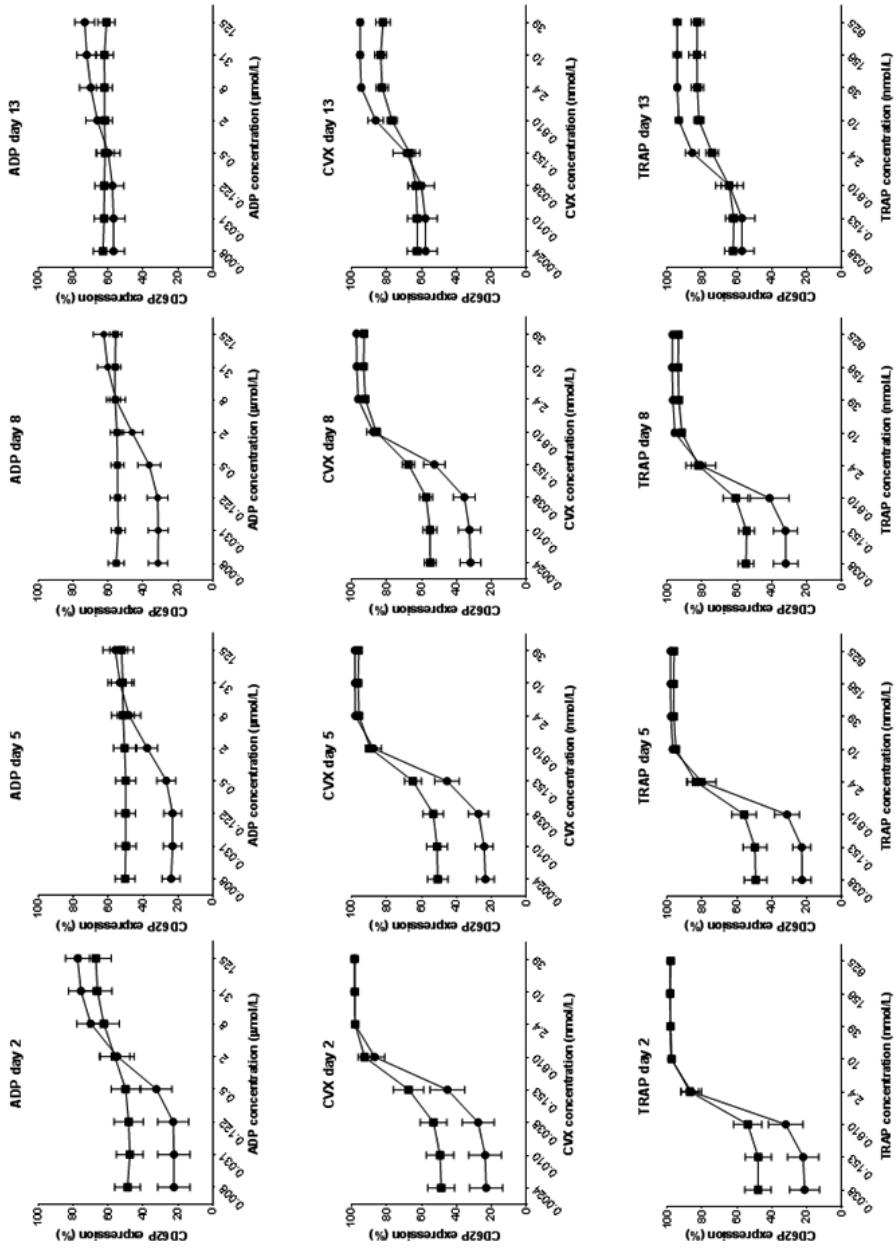

Figure 2 CD62P expression CD62P expression of plasma-platelets (●) or PAS-C-platelets (■) in reconstituted whole blood, induced by 8 different concentrations of ADP, convulxin (CVX) and TRAP on storage day 2, 5, 8 and 13

of increasing percentage of CD62P expression over storage time (raw data are shown in Figure 2). The CD62P expression with highest agonist concentration was lower in samples with PAS-C-platelets than with plasma-platelets on every storage day and with all three agonists (Table 3), suggesting a lower ability to respond to the platelet agonists. The calculated AUC, used as read-out for platelet responsiveness, was higher in samples with plasma-platelets than PAS-C-platelets, for all agonists (ADP, CVX and TRAP) and on all storage days (Table 3).

Additional experiments

PAS-platelets and PAS fluid compared to plasma-platelets and plasma in reconstituted whole blood

There was no (statistically) relevant difference between the mean platelet count and hematocrit of the samples containing PAS-C-platelets and accompanying PAS-C-supernatant and those with plasma-platelets and accompanying plasma. The samples containing both PAS-C-platelets and PAS-C-supernatant from the same unit showed a maximum amplitude that was on average lower, an R-time that was longer and an angle that was smaller than the samples with plasma-platelets and accompanying plasma when analyzed in the TEG (Figure S3B of the Supporting Information).

Biochemical characteristics of platelet units

The pH, pO_2, pCO_2, glucose, lactate, unstimulated CD62P expression and Annexin A5 binding of the platelet concentrates showed patterns comparable to earlier observed results (shown in Figure S4).

Discussion

The aggregation of PAS-C-platelets was lower than the aggregation of plasma-platelets. Also, the agonist-induced CD62P responsiveness of PAS-C-platelets was significantly lower than the responsiveness of plasma-platelets. Clot formation, as assessed with TEG, of the reconstituted whole blood showed no differences between the two platelet types.

To the best of our knowledge, this study is the first comparing the hemostatic function of PAS-C-platelets to plasma-platelets using whole blood platelet aggregometry and thromboelastography in reconstituted whole blood. By studying reconstituted whole blood instead of unmixed platelet concentrates, the interaction with RBCs and plasma, resembling the clinical situation of a transfused patient more closely, is also taken into account in our study. Multiple *in vitro* and *in vivo* studies have indicated that RBCs and

plasma affect platelet function and hemostasis.[14-18] When stored platelets are *ex vivo* mixed with whole blood, the agonist-induced CD62P expression and aggregation improve,[19, 20] which implies the influence of RBCs and/or plasma on platelet function. Even while the PAS-C-platelets and plasma-platelets were mixed with RBCs and plasma, in our experiment, which may partially recover platelet function, we still observed noticeable differences.

Both Multiplate and TEG are whole blood assays, so during the measurement all plasmatic and cellular components of blood are present. TEG is intended to measure the overall hemostasis, while Multiplate aims to measure only the platelet aggregation. In multiple clinical studies, both Multiplate and TEG have been proven to identify clinically relevant thrombocytopathies and coagulopathies, respectively. Both techniques seem to have added value in guiding transfusion policy in several clinical settings.[11, 21-23] Agonist-induced CD62P expression is correlated to bleeding outcome in ITP patients [24] also suggesting that our results may be clinically relevant. Further research is needed to verify whether the observed differences between PAS and plasma stored platelets are also found *in vivo*, as also vessel walls, blood flow and other patient factors affect clot formation.

The whole blood**,** reconstituted with PAS-C-platelets had a platelet concentration and hematocrit which were not different from those of the plasma-platelet-samples, and are therefore not expected to affect Multiplate and TEG results.[25, 26] We found that the aggregation capacity of PAS-C-platelets was lower than of plasma-platelets. Also, the agonist-induced CD62P responsiveness of PAS-C-platelets was significantly lower than the responsiveness of plasma-platelets. These results show that besides the dilution of coagulation factors by the PAS-fluid, also the platelet function itself may be a concern for the hemostatic function of PAS-platelets. This study is to our knowledge the first to demonstrate a difference in platelet function using Multiplate and CD62P responsiveness between PAS-platelets and plasma-platelets. However, the TEG-derived parameters showed no differences between reconstituted whole blood with PAS-C-platelets and reconstituted whole blood with plasma-platelets. These results suggest that the differences between the platelet types in agonist-induced aggregation and CD62P responsiveness do not result in a difference in overall clot formation. The explanation for these TEG findings could be that the platelets are activated via other pathways than ADP and TRAP, which were affected in the Multiplate, and that although the "responsiveness" is diminished, it does not affect overall clot formation.

With increasing storage time there was a reduction in aggregation capacity for both platelet types with all agonists. These results are in agreement with those of others.[19, 27, 28] In addition, our findings show that the difference between the aggregation

capacity of plasma-platelets and PAS-C-platelets exists despite of the addition of RBCs and plasma and remains with increasing storage time. The clot formation capacity of the samples showed minimal changes over storage time, which is in agreement with an earlier study analyzing TEG parameters of platelet concentrates in the absence of RBCs and plasma.[27] We can only speculate that this is indicative of an actual platelet transfusion,[20] showing that after transfusion the platelet quality remains affected despite of the interaction with RBCs and plasma. More studies are needed in this respect. In our study, a storage time of 13 days was analyzed, but platelets stored more than 7 days are not transfused in clinical practice. The 13-day storage time shows that the pattern of the first 7 days is consistent with the downward trend of the subsequent storage days.

The results of our experiment show that both the aggregation and the CD62P responsiveness of PAS-C-platelets were lower than those of plasma-platelets. Moreover, transfusion of substantial amounts of PAS-C-fluid may aggravate the impairment of hemostasis, as the additional experiment suggests. It should be noted that in our experiment the plasma fraction of the reconstituted whole blood was completely replaced by PAS-C-supernatant, 65% PAS and 35% plasma, which is unlikely to occur to this extent in clinical practice. In the situation of a massive transfusion also other (blood) products are administered and the platelet products that are transfused can have various storage times. Therefore, the difference observed in this study is larger than expected in clinical practice. Nevertheless, clinicians should be aware of the potential impairment of platelet function and hemostasis with massive transfusion of PAS-C-platelets and accompanying PAS-C-fluid. This is in agreement with the recommendations, based on the results of the PROPPR-trial, to avoid PAS-platelets in resuscitation of trauma patients with massive bleeding.[29] In conclusion, our results demonstrate that the function of PAS-C-platelets is inferior to that of plasma-platelets in reconstituted whole blood. These results may have implications in the setting of massive transfusions.

Acknowledgements

Besides, we would like to thank J. Lorinser, D. Sijbrands, S. Groot, H. Korsten and B. Daal (Sanquin Blood Bank, Department of Product and Process Development), for their excellent technical assistance. Also, the authors would like to thank Roche that provided the Multiplate analyser for the study.

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