Original article

Platelet concentrates in platelet additive solutions generate less complement activation products during storage than platelets stored in plasma

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Background - Platelet transfusions can be associated with adverse reactions, such as febrile non-haemolytic transfusion reaction (FNHTR). It has been suggested that damage-associated molecular patterns (DAMP) and complement play a role in FNHTR. This study investigated the nature of DAMPs and complement activation products contained in platelet concentrates during storage, with a specific focus on different platelet storage solutions.

Materials and methods - Buffy coats (BC) from healthy donors were pooled (15 BC per pool) and divided into three groups of the same volume. After addition of different storage solutions (plasma, platelet additive solutions [PAS]-C or PAS-E; n=6 for each group), BC pools were processed to platelet concentrates (PC). Leukoreduced PCs were stored on a shaking bed at 20-24°C and sampled on days 1, 2, 6 and 8 after collection for selected quality parameters: platelet activation, DAMPs (High Mobility Group Box 1 [HMGB1], nucleosomes), and complement activation products.

Results - During storage, equal levels of free nucleosomes and increasing concentrations of HMGB1 were present in all groups. Complement activation was observed in all PC. However, by day 8, the use of PAS had reduced C3b/c levels by approximately 90% and C4b/c levels by approximately 65%.

Discussion - Nucleosomes and HMGB1 were present in PCs prepared in plasma and PAS. Complement was activated during storage of platelets in plasma and in PAS. The use of PAS is associated with a lower amount of complement activation products due to the dilution of plasma by PAS . Therefore, PC in PAS have less complement activation products than platelets stored in plasma. These proinflammatory mediators in PC might induce FNHTR.

Keywords: *platelet transfusion, platelet storage, complement activation products, nucleosomes, HMGB1.*

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INTRODUCTION

In haematology patients, low platelet counts (a consequence of myeloablative chemotherapy) are associated with increased morbidity and mortality due to bleeding complications.

Prophylactic platelet transfusions have been shown to be effective in preventing, or at least reducing, the frequency of bleeding complications[1]. However, platelet transfusions can also be associated with immunological and infectious adverse reaction (AR). Immunological ARs include allergic reactions, febrile non-haemolytic transfusion reactions (FNHTR), transfusion-related acute lung injury (TRALI), and platelet refractoriness due to anti-HLA and -HPA antibodies, respectively[2-4]. Infectious complications mainly include systemic infections due to bacterial product contamination, and, in very rare cases, infection by blood born viruses[5-7]. Taken together, these immunological and infectious ARs are responsible for an increase in morbidity, with a subsequent increase in the length of hospital stay, and in the possibility of fatality after platelet transfusion[5,6,8]. Several safety measures have recently been implemented, such as donor testing for hepatitis C virus (HCV) and the human immunodeficiency virus (HIV) nucleic acid test (NAT), donor screening for antibodies to hepatitis B core antigen (anti-HBc), and improved bacterial detection methods. These have led to a significant decrease in platelet transfusion-transmitted infections[7,9,10]. Leukoreduction has been shown to effectively reduce the incidence of transfusion reactions after platelet transfusions[11-13]. Furthermore, identification of IgA- and haptoglobin-deficient recipients reduced both their occurrence as well as the severity of allergic reactions in the context of platelet transfusions[14,15].

The role of the storage solution in the pathogenesis of transfusion reactions after platelet transfusion has been extensively studied[16,17]. Plasma as storage solution for platelets has been widely used. Plasma may induce allergic reactions and contain cytokines, chemokine, other soluble immunomodulatory factors, and anti-HLA and -HPA antibodies resulting in TRALI[18]. Therefore, storage solutions with a significantly smaller amount of plasma may reduce AR after platelet transfusion. Plasma has recently been replaced by platelet additive solution (PAS) to lower the risk of storage lesions. This results in a significantly reduced amount of plasma (approx. 35% plasma remaining) in the platelet concentrate[16]. Over recent years, PASs has been optimised to improve their platelet storage properties and reduce AR caused by storage lesions[19,20]. Indeed, platelets stored in PAS are associated with a lower incidence of AR (such as allergic reactions) compared to platelet products with plasma[16,17,21]. No significant reduction in TRALI has been observed between platelets stored in PAS or in plasma[16,17]; this is most probably due to the low incidence of these complications[16,17]. Interestingly, the incidence of FNHTR remains unchanged after the introduction of first generation PAS[2].

Platelet concentrates (PCs) used for transfusion contain a variety of secretory products that impact haemostasis as well as patients' innate immunity. Biological response modifiers in transfusion-grade PC supernatants increase during storage. HMGB1, which exerts a potent inflammatory role through TLR2 and TLR4[18], is released by platelets, and illustrates the central role of platelets in bridging stress as well thrombotic and immune responses. It has been suggested that damage-associated molecular patterns (DAMPs), such as HMGB1, cell-free DNA and DNA-binding proteins, play a part in AR, and particularly in the pathogenesis of FNHTR, by inducing a systemic inflammatory response[22-27]. In addition, recent findings have identified a possible role for complement activation in the pathogenesis of FNHTR[28,29]. DAMPs and complement activation products may be produced during the collection and production process and/or may accumulate during product storage[28-33]. Administration of complement activation products and DAMPs contained in the platelet product may lead to complement activation and may maintain complement activation in the recipient. This may result in a systemic inflammation, which will have a negative impact on the effectiveness of platelet transfusion.

In this study, we investigated the concentration of nucleosomes, HMGB1 and complement activation products in PC made from pooled buffy coats (BC) and stored in plasma, PAS-C (once the standard storage solution in the Netherlands) and PAS-E (the storage solution now used there) shortly after production and during subsequent storage at room temperature. In addition, since white blood cells are present in the BC, and given that the PCs are leukoreduced but not completely leukodepleted, neutrophil activation may occur and constitute a source of DAMPs; accordingly, we also analysed neutrophil activation in these PCs during storage.

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MATERIALS AND METHODS

Blood collection

Approximately 500 mL of whole blood was collected from non-remunerated, informed donors in quadruple bag, bottom-and-top collection systems, containing 70 mL of citrate-phosphate-dextrose (CPD) anticoagulant (Fresenius Kabi, Bad Homburg, Germany). Blood collections were performed in accordance with institutional guidelines and practices. Calibrated blood collection scales equipped with a mixing platform were used. Immediately after collection, samples were mixed at regular intervals, blood flow and bleeding time were monitored over time, the weight of the donation was checked, and the blood was cooled to 20-24°C on butane-1, 4-diol cooling plates (Compocool, Fresenius Kabi). Day of blood collection was considered day 0 of the study.

This study was approved by the medical ethical committees of the institutes taking part, in accordance with the standards laid down in the 1964 Declaration of Helsinki.

Blood processing and preparation of platelet concentrates

Donations meeting the Dutch regulatory criteria of volume (500±50 mL of blood) and bleeding time (<12.5 min) were selected for further processing after being held overnight according to the routine BC procedure as previously described[34] plasma and buffy coat (BC). Briefly, blood processing started with centrifugation in a Sorvall RC12BP centrifuge at 20°C at 4,793 *g* for 8 min. The centrifuged blood was separated into plasma, BC, and red blood cell (RBC) suspensions, using an automated blood component separator (Compomat G5, Fresenius HemoCare, Bad Homburg, Germany). A schematic overview of the process from BC to PC is shown in **Figure 1**. A total of 90 BCs were included in the study for the preparation of PC. Six pools of 15 BCs of the same blood group were formed. After mixing, the pool was divided over 3 pooling bags from C5000 platelet pooling and storage systems (Fresenius Kabi), and 3 pools of equal composition and volume were obtained. Each of these 3 pools was diluted with a plasma unit from one of the corresponding 15 donations, 300 mL of PAS-E (69 mmol/L NaCl, 5 mmol KaCl, 1,5 mmol $MgCl_2$, 10 mmol Na_3-citrate, 26 mmol NaH_2PO_4/Na_2HPO_4, 30 mmol Na-acetate; TPAS+, Terumo BCT, Lakewood, CO, USA), or 280 mL of PAS-C (77 mmol NaCl, 10 mmol Na_3-citrate, 26 mmol NaH_2PO_4/Na_2HPO_4, 30 mmol Na-acetate; Intersol, Fresenius Kabi). After a soft spin, PCs were prepared. During the preparation of PC from the

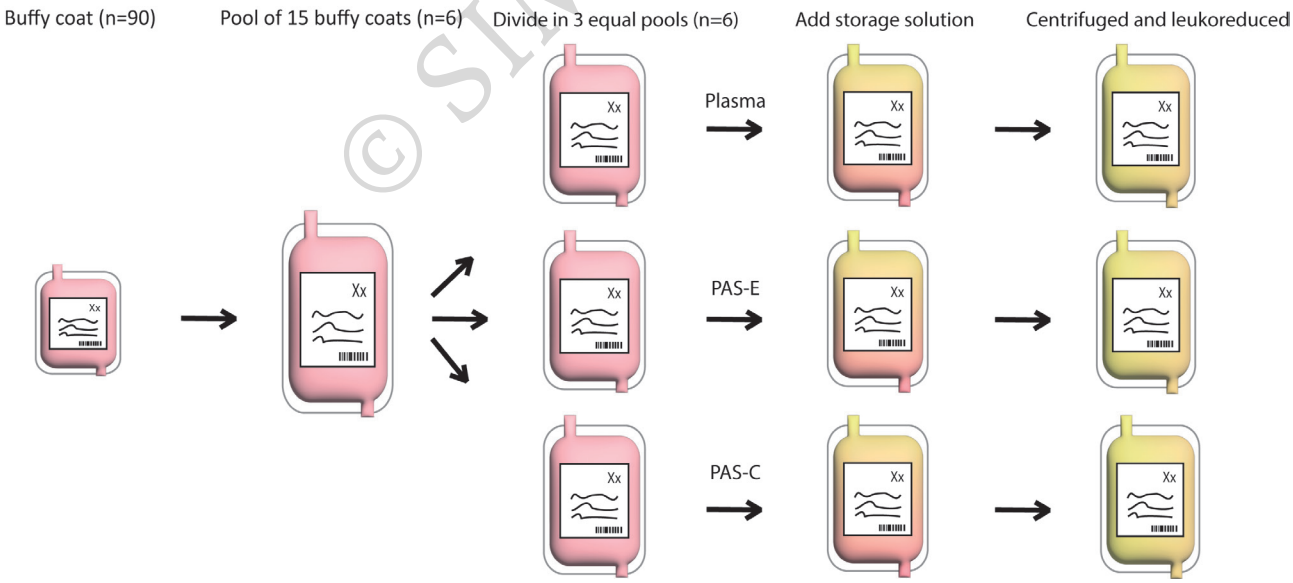

Figure 1 - Schematic overview of study design
A total of 90 buffy coats (BCs) were collected. Six pools were created of 15 BCs each. Each pool was divided into three equal pools and supplemented with storage buffer (plasma of one of the 15 donors, platelet additive solution [PAS]-E or PAS-C). Platelet concentrates were then centrifugated and filtered using a leukoreducing filter.

pooled BC on the Compomat G5, the PCs were filtered using a leukoreduction filter (Compostop CS, Fresenius HemoCare) with an empty 1.3-L PVC-BTHC storage container (Compoflex F730, Fresenius HemoCare) connected to the outlet of the filter.

Storage and sampling

Samples of the different storage solutions (plasma [n=6], PAS-C [n=1] and PAS-E [n=1]) were taken prior to addition to the BC pool. Leukoreduced PCs were stored on a shaking bed, 1 cycle per second, at 20-24°C. Samples (10 mL) were taken on days 1, 2, 6 and 8 after collection using a swan-lock adapter, allowing needle-free aseptical sampling. The adapter was decontaminated with 70% IPA before sampling to measure a selection of quality parameters and soluble factors. Day of blood collection was considered day 0 of the study. At the end of the storage period, PCs were checked for sterility, as previously described[35]. Briefly, both aerobic and anaerobic culture bottles were inoculated with a 7.5 mL sample under aseptic conditions in a laminar airflow cabinet. Culture bottles were incubated at 35°C under the BacT/Alert system either until a positive reaction was detected or for 7 days if negative.

Routine parameters

To determine the *in vitro* quality of the PCs, they were weighed and sampled using a swan-lock adapter (Codan, Lensahn, Germany). The swirling effect was judged and pH, blood gasses, glucose and lactate concentrations were determined with a blood gas analyser at 37°C (Rapidlab 1265; Siemens Healthcare Nederland BV, Den Haag, the Netherlands). Platelet count, and mean PLT volume (MPV) were determined with a blood cell analyser (Advia 2120; Siemens).

Flow cytometric analyses

After sampling the PCs, platelets were stained for CD62p-FITC (P-selectin; Beckman Coulter, Brea, CA, USA Immunotech art n. A07790) in Isotone and Annexine V-FITC (VPS-Diagnostics, Hoeven, the Netherlands, art no. A705) in HEPES buffer (20mM HEPES, 132mM NaCl, 6mM KCl, 1mM $MgSO_4$, 1,2mM KH_2PO_4, 2,5 mM $CaCl_2$, and 5 mM glucose, pH 7.4), After staining, the samples were fixed using 0.5% PFA, and analysed within 2 h by 3 Laser FACS Canto II+HTS (BD Biosciences, Erembodegem, Belgium).

Quantification of sCD62p and CD40L

The levels of soluble CD40L (sCD40L - HCYTOMAG-60K - minimum detectable concentrations: 5.1 pg/mL) and CD62P (sCD62P - HCVD2MAG-67K - minimum detectable concentrations: 0.244 ng/mL) were quantified in PC supernatants using Luminex technology (Millipore, Molsheim, France), according to the manufacturer's instructions, under a Bioplex 200 system (BioplexManager software; Biorad, Marnes-la-Coquette, France), as previously described[36].

Neutrophil activation

Elastase, Elastase-α1-antitrypsin complexes[37], Lactoferrin[38], and nucleosomes were measured by ELISA, as previously described[39].

Complement activation

Levels of C3b/c and C4b/c[40], and IgM concentrations[41] were measured by ELISA, as previously described.

Quantification of high mobility group box 1

The levels of soluble high mobility group box 1 (HMGB1) were quantified using ELISA technology (IBL International GmbH, Hamburg, Germany) with minimum detectable concentrations set at 0.313 ng/mL. Absorbance at 450 nm was determined with an ELISA reader (Magellan software and Sunrise; Tecan group Ltd., Lyon, France) and data were expressed in ng/mL, as previously described[27].

Statistical analysis

All statistical analyses and figures were computed with GraphPad Prism software v.9.1.1 (GraphPad Software, La Jolla, CA, USA). Results are represented as median with interquartile range. The Friedman test was performed between groups at different timepoints, and Dunn's test was used for *post-hoc* multiple testing. As only two timepoints were tested, the Wilcoxon test was performed to determine statistical differences between timepoints for glucose consumption and lactate production. $p<0.05$ was considered significant.

RESULTS

Platelets stored in PAS-E show equal or improved storage properties to platelets stored in plasma

Analysis of quality parameters, representative for platelet integrity and metabolic activity during storage, comparing PAS-E and PAS-C to plasma (**Table I**) showed that, over time, platelet quality in PCs stored in PAS-C deteriorated more than that of platelets in plasma, as seen by a significant

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decrease in platelet concentration, a relatively high glucose consumption, and lactate production resulting in a decrease in pH (**Table I**). It should be noted that, because of the decrease in plasma in PCs stored in PAS, these PC have less glucose. Notably, the pH of PCs stored with PAS-E remains stable during the whole storage period (**Table I**). Overall, both PCs in PAS-E and PCs in plasma demonstrate suitable storage properties. Moreover, PCs in PAS-E have a lower glucose consumption and lower lactate production than PCs stored in plasma (**Table I**). Lower lactate production prevents a drop in pH. Thus, PCs stored in PAS-E demonstrated equal or even improved metabolic parameters as compared to those stored in plasma. No bacterial contaminations were found in the PCs included in the study, as determined at the end of the storage period.

Expression of platelet activation markers increased most in platelets stored in PAS-C

To evaluate platelet activation status and apoptosis during storage, we measured migration of CD62p (P-selectin) to the membrane and the binding of Annexin V as a marker for apoptotic cells. All PCs showed a significant increase

Table I - *Storage properties of platelet concentrates (PCs)*

Characteristics	Day	Plasma n=6		PAS-E n=6		PAS-C n=6		Range European and Dutch blood products guidelines
		Median	IQ	Median	IQ	Median	IQ	
Volume (mL)	2	371	360-377	354	353-355	330	311-337[a]	250-400
	6	360	347-364	342	340-343	317	298-324[a]	
	8	349	337-353[d]	331	329-333[d]	307	287-314[a,d]	
PC (PLT×10⁹/L)	2	1,111	994-1,214	1054	949-1,095[a]	1048	946-1,140	700-1,800
	6	1,099	995-1,193	940	856-1,038	862	771-957[a]	
	8	1,072	983-1,171	920	833-1,001[d]	824	737-924[a,d]	
Total platelet count (PLT×10⁹/unit)	2	402	368-452	375	335-387	345	301-376[a]	250-500
	6	389	353-431	321	293-354	273	233-308[a]	
	8	365	340-412[d]	303	278-331[d]	254	215-285[a,d]	
Mean platelet volume (fL)	2	8.2	8.1-8.6	8.5	8.4-86	8.6	8.4-8.8	-
	6	9.2	8.5-9.7[c]	9.1	8.6-9.4	9.0	8.8-9.4	
	8	9.1	9.0-9.2[d]	9.0	8.8-9.1	8.9	8.6-9.1	
pH at 37°C	2	7.21	7.18-7.22	7.13	7.13-7.15	7.13	7.11-7.15[a]	6,3-7,5
	6	7.23	7.22-7.25	7.21	7.20-7.22[c]	7.03	7.03-7.05[a]	
	8	7.16	7.13-7.17	7.19	7.18-7.20	6.94	6.91-6.95[b,d]	
Glucose (mmol/L)	2	18.1	17.7-18.8	6.7	6.7-7.0[a]	6.7	6.7-6.9[a]	>0
	6	14.9	14.7-15.5	4.9	4.8-5.0	3.1	3.0-3.2[a]	
	8	13.2	12.8-13.7[d]	3.6	3.6-3.7[d]	1.0	0.9-1.4[a,d]	
Glucose consumption (mmol/day/10¹² PLT)	6	0.74	0.66-0.80	0.53	0.46-0.57	1.08	0.96-1.19[b]	-
	8	0.79	0.69-0.87	0.59	0.52-0.65	1.12	1.03-1.32[b]	
Lactate (mmol/L)	2	7.1	6.8-8.0	4.4	4.2-4.5[a]	4.9	4.5-5.1	-
	6	12.1	10.7-12.9	7.9	7.5-8.7[a]	10.9	10.5-11.2	
	8	15.4	13.7-16.2[d]	10.0	9.4-10.7[a,d]	14.5	14.0-15.1[d]	
Lactate production (mmol/day/10¹² PLT)	6	1.05	0.95-1.22	1.01	0.83-1.09	1.64	1.55-1.94[a,b]	-
	8	1.19	1.02-1.45	1.04	0.92-1.16	1.91	1.72-2.25[b]	

PCs were stored in plasma, platelet additive solution-E (PAS-E) or PAS-C until day 8 after whole blood collection at 22±2°C, shaken horizontally at 1 cycle/second. Samples were taken aseptically at days 2, 6 and 8 for analysis of quality parameters.
[a]p<0.05 *vs* Plasma ; [b]p<0.05 vs PAS-E; [c]p<0.05 day 2 *vs* day 6; [d]p<0.05 day 2 *vs* day 8.
IQ: interquartile range; PLT: platelet.

in CD62p positive and Annexin V positive platelets during storage (**Figure 2a** and **2b**), which was most pronounced during storage in PAS-C. In addition, there was a significant increase in activation markers sCD62p and sCD40L during storage in all groups (**Figure 2c** and **2d**). PC stored in plasma had lower levels of sCD40L than PCs stored in PAS-C and PAS-E (**Figure 2d**). In addition, PCs stored in plasma had lower levels of sCD62p than PCs stored in PAS-C, and only slightly higher levels than PCs stored in PAS-E (**Figure 2c**).

Cell-free DNA and DNA binding proteins: nucleosomes and HMGB1 are both present in PCs prepared in plasma and PAS

In order to study the influence of storage solution on DAMP release, we measured cell-free DNA in the form of nucleosomes (the basic structural unit of DNA where a segment of DNA is wrapped around histone proteins) and HMGB1 in the supernatants of the concentrates. Nucleosomes can be released by every nucleated cell upon activation and/or after cell death. The amount of nucleosomes in the supernatants of the concentrates was approximately the same regardless of the additive solution. The level of nucleosomes in the PCs was above the median value of the donor plasmas used (**Figure 3a**). In order to investigate whether neutrophils might be a source of the nucleosomes measured in the supernatant, neutrophil activation was assessed by measuring human neutrophil elastase (HNE) α1-antitrypsin complexes and lactoferrin levels. Although these markers of neutrophil activation increased in platelets after the first day of storage in plasma, no significant changes in these markers could be observed in platelet supernatant after storage over time (**Figure 3b** and **3c**). However, we observed a significant difference between PCs stored in plasma and PCs stored in PAS-C and PAS-E (**Figure 3b** and **3c**). Finally, we measured HMBG1 in the supernatants of PCs at days 2, 6 and 8 of storage. In the nucleus, HMGB1 interacts with nucleosomes, transcription factors, and histones. In all PCs, the concentration of HMGB1 that is secreted in the product significantly increased between day 2 and day 8.

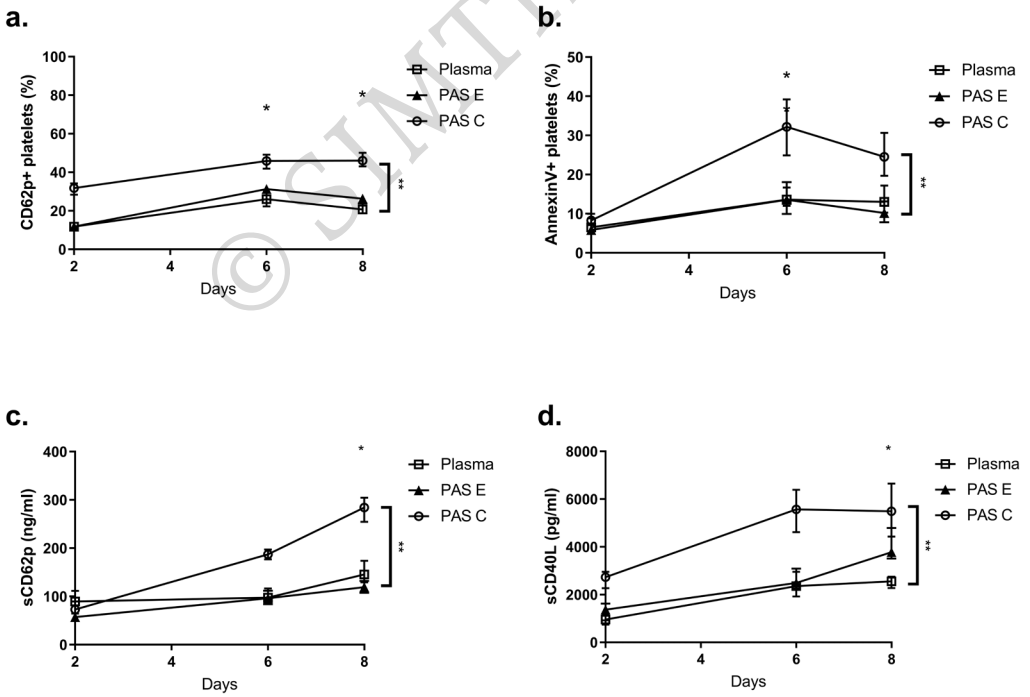

Figure 2 - More platelets express activation markers in platelet additive solution-C (PAS-C) than in PAS-E or plasma
Platelet concentrates (PCs) (n=6) stored in plasma, PAS-E, or PAS-C were sampled at multiple timepoints and the percentage of (a) CD62p+, (b) AnnexinV+ platelets, (c) sCD62p, and (d) sCD40L levels was measured over time. Median and interquartile ranges are shown. Significant differences between days 2 and 6 or 8 are shown above the trendlines. Differences between groups are indicated by the capped line on the side. *p<0.05; **p<0.01.

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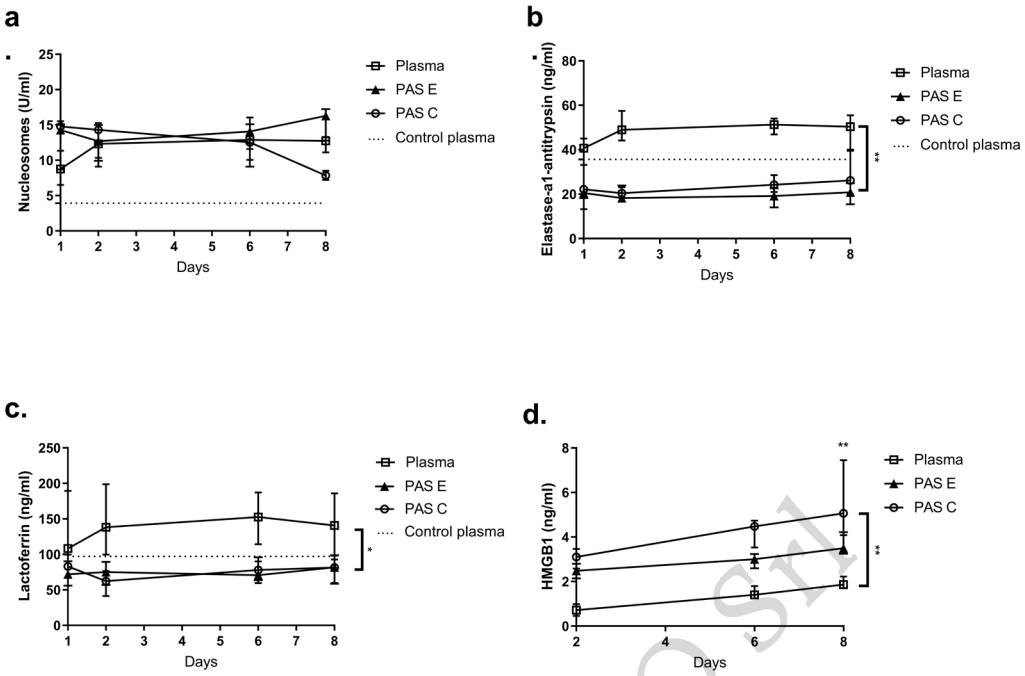

Figure 3 - Nucleosomes and HMGB1 are contained in platelet concentrates (PCs)
PCs (n=6) stored in plasma, platelet additive solution-E (PAS-E), or PAS-C were sampled at multiple timepoints and (a) nucleosome levels, (b) elastase-a1-antitrypsin complexes, (c) lactoferrin levels, and (d) HMGB1 contained in the product were followed over time. Dotted lines represent level of donor plasma used as storage solution in these products. Median and interquartile ranges are shown (a-c). Significance between days 1 and 6 or 8 are shown above the trend lines. Differences between groups are indicated by the capped line on the side. *p<0.05; **p<0.01.

In addition, at all timepoints, the concentration HMGB1 was significantly higher in PAS-C than in plasma (**Figure 3d**). HMGB1 levels were also higher in PAS-E, although the difference was not significant (**Figure 3d**).

Complement is activated during storage of platelets

Previous studies reported that complement activation occurred in PCs prepared from apheresis during storage in plasma[29]. Therefore, we examined activation of complement in PCs prepared from pooled BCs stored in plasma, PAS-C and PAS-E. In PCs prepared in plasma, complement activation was observed during storage, as evidenced by an increase in C3bc and C4bc levels (**Figure 4a** and **4b**). In PCs stored in PAS, levels of C3b/c were approximately 65% lower than those in plasma on day 2 and approximately 90% lower on day 8, while C4b/c levels were approximately 65% lower at day 8. In order to exclude

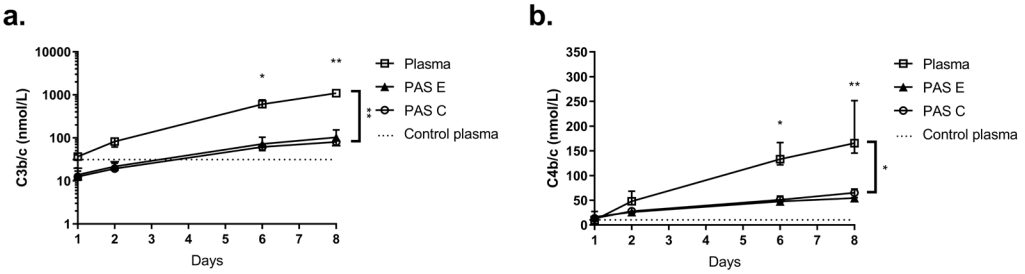

Figure 4 - Complement activation products are detected in platelet concentrates
PCs (n=6) stored in plasma, platelet additive solution (PAS-E), or PAS-C were sampled at multiple timepoints. (a) C3b/c levels and (b) C4b/c levels contained in the product were followed over time. Dotted lines represent level of donor plasma used as storage solution in these products. Median and interquartile ranges are shown. Significance between days 1 and 6 or 8 are shown above the trendlines. Differences between groups are indicated by the capped line on the side. *p<0.05; **p<0.01.

the possibility that changes in volume during storage could have caused an increase in complement activation products, or the impact of any differences in storage media, IgM and C1-inhibitor concentrations during storage were measured. Both protein concentrations remained stable during storage in the products with different storage solutions (*Online Supplementary Content*, **Figure S1a** and **S1b**). Together, these data demonstrate that thrombocytes prepared with plasma as storage solution resulted in significantly higher complement activation as compared to PCs stored in PAS.

DISCUSSION

Due to potential adverse effects, such as allergic reactions, plasma as a storage solution for platelet concentrates has recently been replaced by PAS. PCs stored in PAS contain a significantly reduced amount of plasma (35%), and hence are associated with a decreased incidence of allergic reactions. In this study, we demonstrate that PAS-E (similar to PAS-C with the addition of potassium and magnesium), is either equal or superior to plasma as storage solution in maintaining the quality of platelets during storage. In addition, we demonstrate that platelet storage results in an increase in complement activation products and HMGB1 in the platelet products. However, the increase in complement activation products was less evident in products using PAS as compared to plasma as storage solution.

Although the rate of allergic AR to platelet transfusion significantly decreased after the introduction of PAS, recent data have shown that the incidence of FNHTR after platelet transfusion using platelets stored in PAS remains unchanged. Although multiple factors have been proposed to play a potential role in adverse transfusion reactions, the mechanism behind FNHTR remains unclear. It has been suggested that DAMPs, such as cell-free DNA, may be involved in the initiation of FNHTR[22-26]. Here we demonstrate that cell-free DNA in the form of nucleosomes and HMBG1 is present in the PCs independent of the storage solution, and that the levels of nucleosomes are higher than in plasma of healthy individuals. Given the stable concentration of nucleosomes during storage, and the fact that the PCs are leukoreduced, white blood cells present in the BCs seem the most probable source from which nucleosomes are released. Neutrophil activation in PCs has been assessed by measuring human neutrophil elastase (HNE) α1-antitrypsin complexes and lactoferrin levels. We observed a slight increase in neutrophil activation in PCs stored in plasma, whereas this increase was absent in PCs stored in either PAS. Given that especially elastase complexes and nucleosomes in PC stored in plasma have a comparable lapse time, neutrophil activation as an additional source for nucleosomes cannot be excluded. The release of nucleosomes will probably, therefore, mainly take place between blood collection and processing, whereas release during storage from the few contaminating white blood cells seems less likely. In addition, we demonstrate that HMGB1 levels were significantly higher in PCs stored in PAS-C and that they increased during storage. This suggests that HMGB1 is released as platelets are activated upon storage; this is supported by an increase in CD62p expression in PCs stored in PAS-C. Considering that HMGB1 is a DNA-binding protein, it is also possible that the source of HMGB1 are PMNs or dead cells. However, as we did not observe an increase in nucleosomes, and that there was an increase in CD62p, we hypothesise that the main source of HMGB1 are platelets.

There is much controversy over the proinflammatory properties of nucleosomes and their causal pathogenic role in systemic inflammation[42]. It has been demonstrated that nucleosomes themselves are not cytotoxic, as evidenced both *in vivo* in a study in mice and *in vitro* with cultured endothelial cells[43,44]. Nonetheless, nucleosomes are composed of DNA wrapped around histones, which are considered highly cytotoxic even at low concentrations, mostly due to their strong positive charge. The lack of cytotoxicity of nucleosomes is explained by their structure, resulting in balanced electrostatic charges, in particular, neutralising cationic effects of histones. However, even a slight distortion of the nucleosome structure, e.g. by bacterial DNases, can induce the exposure of potentially toxic parts of attached histones, resulting in cytotoxicity[45]. In addition, measuring histones in plasma is difficult, since cell-free and DNA-free histones are immediately degraded by factor VII-activating protease[45]. Since most of the circulating cell-free histones are bound to cell-free DNA, nucleosome measurement seems to be a reliable marker for histones[42].

HMGB1 as a DNA-binding protein is often bound to cell-free DNA and may act as a proinflammatory mediator due to its cytokine-like function[22,46,47]. Activated platelets upregulate and release HMGB1 in various inflammatory conditions[48]. Moreover, nucleosomes (protein-free DNA as well as HMGB1) may ligate Toll-like receptors 2, 4 and 9, thereby inducing and/or modulating an inflammatory response[42,49]. Platelets also have a major inflammatory and immune function, essentially through their Toll-like receptors (TLRs) and sialic acid-binding immunoglobulin-type lectin (SIGLEC)[50]. Autocrine activation of platelets from PCs due to DAMPS release and Pattern Recognition Receptor expression cannot be excluded, as is the case for CD40/CD40L[51].

Together, this suggests that potentially cell-free DNA in the form of nucleosomes and HMGB1 might be involved in the pathogenesis of FNHTR. Although the amount of nucleosomes contained in the PC is rather low compared to the levels measured during systemic inflammation in patients, it cannot be excluded that they may induce an inflammatory response. In addition, patients receiving PC transfusion often suffer from neutropenic fever, which may camouflage clinical signs of an inflammatory response induced by platelet transfusion.

It has previously been suggested that complement activation plays a role in the initiation of FNHTR[29]. Interestingly, we found complement activation products in all PC products independent of the storage solution used, albeit in lower concentrations in PCs with PAS as compared to plasma; the lower concentration in the PAS products is most likely due to dilution of plasma by PAS. Nonetheless, complement activation products accumulated during storage in PCs prepared in both plasma and PAS. Although we measured complement activation by the generation of C4bc and C3bc, also C3a and, considering the downstream activation of C5, C5a will also be released[31]. The anaphylatoxins, C5a and to a lesser extent C3a, although rapidly degraded through carboxypeptidases in plasma, may activate platelets in the products[52-56]. C3a and C5a are key mediators of inflammation and exert their function through binding to their receptors[57]. In addition, the C3a and C5a receptors are expressed by many leukocytes as well as by endothelial cells. Upon platelet transfusion, the complement activation products, and probably anaphylatoxins contained in the product, are administered to the patient. One could hypothesise that these transfused complement activation products may induce further complement activation in the patient's circulation. This may subsequently induce an inflammatory response in the recipient through the activation of immune cells, and exert vasoactive effects on endothelial cells. Here it is important to stress that also cleaved forms of anaphylatoxins, e.g. C5adesArg, may retain their vasoactive properties; however, the effects on vascular permeability are 100 times weaker than the non-cleaved forms. In summary, these active complement products contained in the PCs may induce an inflammatory response in the recipient, becoming clinically overt as FNHTR.

An advantage of the current study design was that the platelets from the PCs designated to the different groups (plasma, PAS-E and PAS-C) all originated from the same BC pool. This meant that any possible confounding factors arising from different donor-pool compositions were eliminated. On the other hand, one limitation is the small number of clinical products, which decreases the power of the study. Another limitation is that we did not study the effects of these products in recipients. Finally, for this design, 15 BCs were needed to produce a pool large enough to supply the three study arms. Under standard practice in the Netherlands, 5 donors are used to produce one buffy-coat PC. Thus, using a larger pool may have concealed donor effects which would have been more discernible in a smaller pool, and these could have affected the outcome.

CONCLUSIONS

To summarise, we have demonstrated that cell-free DNA in the form of nucleosomes and HMGB1 is present in PCs. We also established that HMGB1 levels increase over time and is significantly higher in PCs stored in PAS-C. In addition, we observed a marked increase in complement activation products in the concentrates. Both DAMPs, such as nucleosomes and HMGB1, and complement may potentially induce systemic inflammation in the recipient upon transfusion and hence be involved in the pathogenesis of FNHTR. Further research is needed to investigate the role of these complement activation products in the pathogenesis of FNHTR.

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AUTHORSHIP CONTRIBUTIONS

AtB and SSZ share senior authorship. All Authors contributed to the design of the study, acquisition, analysis, and interpretation of data. YW wrote the manuscript and JL, DK, FC, AB, and SS critically revised the work for important intellectual content. All Authors approved the final version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of it are appropriately investigated and resolved.

The Authors declare no conflict of interest.

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