Storage of buffy-coat-derived platelets in additive solution: *in vitro* effects on platelets of the air bubbles and foam included in the final unit

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Background. The air bubbles and foam that develop during the preparation of platelet units have traditionally been considered to interact with the platelets, causing activation and release reactions. However, there actually seems to be no data available concerning the platelet damage that may occur as a result of air bubbles and foam present in the final unit. In this *in vitro* study we, therefore, investigated the effects of not removing air bubbles/foam from final platelet units, by measuring *in vitro* parameters during a 7-day storage period.

Design and methods. Platelet samples (n=8) from eight pools of 12 buffy-coats were aliquoted and prepared with the OrbiSac system for storage with (test) or without (reference) air bubbles/foam included in the final units. The metabolic, cellular and activation parameters of all units, comprising approximately 30% plasma and 70% SSP+ platelet additive solution, were analysed during the 7-day storage period.

Results. Differences in platelet counts and contents between the test and reference units were detected throughout storage ($p<0.05$ at day 5 and $p<0.01$ at day 7). Lactate dehydrogenase increased during storage in the test units and was significantly higher than in the reference units ($p<0.01$ from day 5). The hypotonic shock response was greater in the reference units ($p<0.05$ on day 2 and $p<0.01$ from day 5). The extent of shape changes was less in the test units ($p<0.05$ until day 5 and $p<0.01$ on day 7). CD62P was higher in the test units ($p<0.05$ on day 7). CD42b decreased in all units but was lower in the test units ($p<0.01$ on day 5). CD41, CD61 and PAC-1 showed no difference throughout storage between the units (p=NS). Aggregates were visible (day 7) and occurred in three of the test units. pH was maintained at >6.8 (day 7) and swirling remained at the highest level (score =2) for all units throughout storage.

Conclusions. This study shows that storage with air bubbles/foam causes considerable enhancement of disintegration of platelets. In addition, various *in vitro* parameters of the platelets remaining seem to be negatively affected. The results of this study suggest that platelets should be stored without air bubbles/foam, given that these cause increased disintegration of platelets.

Keywords: Platelets, additive solutions, storage.

Introduction

The quality of platelets during storage is influenced by various factors such as the preparation method, the composition of the storage bag, additive solutions, the temperature, the platelet count and the requirement for adequate O_2 to maintain aerobic metabolism[1]. Platelets are stored with continuous gentle agitation, which is thought to enhance the transport of gases such as O_2 and CO_2 through the storage container[2]. Although some data suggest that platelets can resist 4 days without agitation[3], most evidence supports the standard that platelets should be routinely stored with

agitation[4]. During the last decade, numerous studies have investigated the effects of various periods without agitation on platelet quality. However, there seems to be no data available concerning the platelet damage that may occur due to air bubbles and foam included in the agitated unit.

Some centres, but not all, remove the air bubbles/ foam from the storage bag preceding storage, as the foam occurring after preparation is suggested to interact with the platelets, causing activation and release reactions. This procedure seems to be performed more often by tradition rather than as a consequence of scientific knowledge.

It is well known that numerous morphological, biochemical and functional derangements occur during platelet collection, processing and storage, with these changes being collectively termed the "platelet storage lesion"[5,6]. These changes are important because, in some cases, they are associated with decreased post-transfusion survival[7,8]. In addition, it has been shown that oxidative metabolism slows when the oxygen tension is lowered, but well above the level of the Pasteur effect (pO_2 approx. 2 mmHg) when there is a shift from oxidative phosphorylation to anaerobic glycolysis[9]. Accordingly, there may be effects on glycolytic flux and/or activation-specific changes and release reactions[10] in platelets exposed to air bubbles and foam during storage.

For these reasons, we evaluated the potential effects of not removing air bubbles and foam from platelet units. We did this by measuring various *in vitro* parameters during a 7-day storage period.

Design and methods

Preparation and storage of platelets

Platelets were collected from normal blood donors who met standard donation criteria and gave written, informed consent according to institutional guidelines. In an attempt to reduce the risk of interruption in the production-line for blood component preparation, a total of 450 mL of whole blood were drawn into either a CPD/SAG-M quadruple-bag blood container system (Fenwal, La Châtre, France) or the top-and-top blood bag systems with in-line red blood cell filters for white blood cell removal (Imuflex-CRC, Terumo, Tokyo, Japan). After storage at room temperature for 2-6 h, the whole blood units were centrifuged (2,700 *g*) for 10 min at 22 ℃. Automatic equipment was used for the preparation of blood components (Optipress, Fenwal or T-ACE, Terumo), including the buffy-coats. Buffy-coats were prepared with the inclusion of plasma to allow suspension of platelets in SSP+ (Macopharma, Mouvaux, France) at approximately 70%. Buffy-coats (12 units) were stored overnight and combined in a large-volume container to create an ABO-identical primary pool (in total, eight ABO-identical primary pools from prepared from 96 buffy-coat units). The primary pools were split into two equivalent parts for the preparation of platelet units and storage in platelet storage bags (OrbiSac Standard BC set, storage bag, Caridian BCT, Inc, Lakewood, USA). The two buffy-coat units derived from each primary pool were prepared using the OrbiSac system (CaridianBCT, Zaventem, Belgium)[11] to yield platelet units for paired studies. The storage bags used are made of PVC plastic with a citrate-based plasticizer and are intended to contain up to $5x10^{11}$ platelets in 400 mL of plasma.

The air and foam which develop after the preparation of the platelet units were then excluded from the reference units but not from the test units. The platelet units were then stored on a flat bed agitator (60 cycles a minute, model LPR-3, Melco, Glendale, CA, USA) in a temperature-controlled cabinet at 22±2 ℃. Samples were drawn aseptically on days 2, 5 and 7. With exception of pO_2, all sampling was done by sterile connection (TSCD-II, Terumo, Japan) of sampling bags to the respective containers (OrbiSac Standard BC set, storage bag, CaridianBCT, Inc, Lakewood, USA). To avoid disturbing pO_2, pO_2 was measured by direct insertion of a needle in the bag through a sampling site coupler, followed by slow aspiration and immediate analysis in a blood gas analyser.

Analysis of metabolic and cellular parameters

The *in vitro* cellular and metabolic parameters evaluated included platelet counts (per litre and per unit) and mean platelet volume (MPV) using a CA 620 Cellguard (Boule Medical, Stockholm, Sweden). The volume (mL) was calculated by weighing the contents of the storage bag, in grams, on a scale (Mettler PB 2000) and the result, in grams, was divided by 1.01 (1.01 g/mL is the density of the storage medium composed of approximately 70 % SSP+ and 30% plasma). Using a routine blood gas analyser

(ABL 800, Radiometer, Copenhagen, Denmark) we measured the pH, pCO_2, pO_2 (kPa at 37 ℃), glucose (mmol/L) and lactate (mmol/L) conclusions. Bicarbonate (mmol/L) was calculated based on the other measured variables.

Swirling was assessed by inspection and graded according to Bertolini and Murphy[12]. The white blood cell count on day 1 was determined with a Nageotte chamber and a microscope (Zeiss, standard)[13]. Hypotonic shock response (HSR) reactivity as well as extent of shape change (ESC) measurements were determined using a dedicated microprocessor-based instrument (SPA 2000, Chronolog, Havertown, PA, USA) with modifications of the tests described by VandenBroeke *et al.*[14].

The total adenosine triphosphate (ATP) concentration (μmol/10^{11} platelets) was determined with a Luminometer (Orion Microplate Luminometer, Berthold Detection Systems GmbH, Pforzheim, Germany) on the basis of principles described by Lundin[15]. Lactate dehydrogenase (LDH) activity, a marker of disintegration of platelets, was measured with a spectrophotometric method (Sigma Aldrich kit 063K6003; Spectrophotometer Jenway 6500, Staffordshire, UK)[16].

Monoclonal antibody labelling

Platelet concentrate samples, fixed by adding an equal volume of 1% paraformaldehyde-phosphate buffered saline (pH 7.2-7.4) at 22 ℃ for 15 minutes, were stained for 20 minutes at the same temperature in the dark by incubation with 20 mL of fluorochrome-labelled monoclonal antibodies per approximately 20 x 10^6 platelets. Phycoerythrin (PE)-conjugated (IgG1 isotype) CD62P (P-selectin/GMP-140/PADGEM; clone CLB Thromb/6) and fluorescein isothiocyanate (FITC)-conjugated (IgG1 isotype) CD41 (clone P2), CD42b (clone SZ2) and CD61 (clone SZ21) monoclonal antibodies were used for single-colour staining (Immunotech, Beckman Coulter, Marseille, France). Control specimens were processed as above, but incubated with a PE- or FITC-conjugated monoclonal antibody (IgG1 isotype) with irrelevant specificity, again from Immunotech (Beckman Coulter, Marseille, France). After incubation with fluorochrome-conjugated antibodies, the samples were washed twice by adding 2.0 mL filtered phosphate-buffered saline-ethylenediamine tetra-acetic acid 0.33%, pH 6.9, with 0.1% Na-azide, and centrifuged at 2,760 *g* (Eppendorf 5810R) for 10 min at +18 ℃.

Expression of a conformational epitope on the GPIIb/IIIa complex of activated platelets was assessed by using the FITC-conjugated monoclonal antibody PAC-1, immunoglobulin M (BD340507, Beckton Dickinson, San Jose, CA, USA). To initiate PAC-1 binding, unfixed platelets (10^8/mL) were incubated with 20 μL ADP at 37 ℃ for 3 min without stirring. The specificity of the PAC-1 binding was demonstrated by staining in the presence of RGDS[17].

Flow cytometry analysis

A total of 100,000 platelet events were acquired on a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with 15 mW argon ion lasers and Cellquest pro software. Daily controls of optics and fluorescence intensity were performed using standardized beads (Calibrite, BD). The flow cytometer settings were optimised for the acquisition of platelets by logarithmic signal amplification in all four detectors (forward and side scatter channels and fluorescence channels FL1 and FL2). For analysis, the gate was set around the intact platelet population as defined by forward and side scatter characteristics. The percentage of positive platelets of the total platelets expressing activation markers CD62P, PAC-1 and surface membrane glycoproteins (CD41, CD42b and CD61) at levels above those of the background (negative controls) as well as the mean fluorescence intensity (MFI) was recorded.

Bacterial detection

Bacterial contamination was assessed on day 7 using the eBDS system. This system indicates the presence of bacteria through a decrease in oxygen tension, as measured in platelet samples after incubation for 24 hours at 35 ℃ [18].

Statistical analyses

The mean values and standard deviations (n=8) are given unless otherwise indicated. A repeated measure ANOVA was performed including a post-hoc test, Fischer's LSD. Two different groups (the reference and test units) were studied over time (days). "Days" was the repeated factor and "Group" was a

between factor. Results of the Fischer's LSD are presented in the results section as well as in Tables I and II. The difference was considered statistically significant at $p<0.05$ and $p<0.01$.

The analyses were carried out using the Statistica software, version 9.0, StatSoft, Inc 1984-2007 (SPSS, Chicago, IL, USA).

Results

In this paired-study, platelets stored with (Test) or without (Reference) air bubbles/foam in the final unit were studied. We found statistically significant differences in a variety of cellular, functional and flow cytometry parameters (Tables I and II).

Cellular assays

There were statistically significant differences in platelet counts and contents between the groups throughout storage ($p<0.05$ on day 5 and $p<0.01$ on day 7). Mean platelet volume differed significantly ($p<0.01$ from day 5). With exception of a slight increase on day 7, the extracellular LDH activity, as a

Table I - *In vitro* effects on platelets stored for 7 days with (Test) or without (Reference) air bubbles and foam in the final unit: mean±standard deviation (SD) (n=8). Differences were considered statistically significant at $p<0.05$* and $p<0.01$**.

In vitro measure	Day 2	Day 5	Day 7
Platelets, x 10^9/L			
Test	956 ± 84	886 ± 96*	833 ± 106**
Reference	992 ± 91	998 ± 92	982 ± 103
Platelets, x 10^9/unit			
Test	355 ± 26	329 ± 33*	309 ± 35**
Reference	361 ± 28	364 ± 28	358 ± 33
Mean platelet volume (fL)			
Test	9.0 ± 0.3	8.5 ± 0.2**	8.3 ± 0.3**
Reference	9.3 ± 0.3	9.1 ± 0.3	9.1 ± 0.2
LDH (%)			
Test	7.4 ± 2.1	11.6 ± 2.1**	19.1 ± 6.4**
Reference	5.0 ± 1.9	5.3 ± 2.3	7.4 ± 2.4
Glucose (mmol/L)			
Test	6.2 ± 0.4	4.9 ± 0.4	3.9 ± 0.5
Reference	6.2 ± 0.4	4.8 ± 0.4	3.7 ± 0.4
Lactate (mmol/L)			
Test	6.4 ± 0.3	8.8 ± 0.4	10.6 ± 0.7
Reference	6.4 ± 0.4	8.9 ± 0.4	10.8 ± 0.7
pH (kPa at 37 ℃)			
Test	7.025 ± 0.019	7.125 ± 0.021	7.140 ± 0.026
Reference	7.032 ± 0.020	7.126 ± 0.021	7.145 ± 0.019
pCO_2 (kPa at 37 ℃)			
Test	3.89 ± 0.22	2.66 ± 0.18	2.29 ± 0.19*
Reference	3.76 ± 0.25	2.81 ± 0.21	2.56 ± 0.18
Bicarbonate (mmol/L)			
Test	7.2 ± 0.4	6.3 ± 0.5	5.6 ± 0.6*
Reference	7.1 ± 0.4	6.7 ± 0.5	6.4 ± 0.6
ATP (mmol/10^{11}platelets)			
Test	9.05 ± 0.37	8.63 ± 0.36	7.78 ± 0.70*
Reference	8.84 ± 0.46	8.80 ± 0.24	8.39 ± 0.58
pO_2 (kPa at 37 ℃)			
Test	14.20 ± 2.11**	12.58 ± 1.40**	14.73 ± 1.15**
Reference	10.00 ± 1.01	11.18 ± 1.42	12.51 ± 1.14

Table II - In vitro effects on platelets stored for 7 days with (Test) and without (Reference) air bubbles and foam included in the final unit: mean±standard deviation (SD) (n=8). Differences were considered statistically significant at p<0.05* and p<0.01**.

In vitro measure	Day 2	Day 5	Day 7
HSR (%)			
Test	58.4 ± 7.4*	54.5 ± 5.5**	51.3 ± 4.2**
Reference	65.5 ± 3.0	63.0 ± 6.7	58.8 ± 5.1
ESC (%)			
Test	18.4 ± 4.2*	14.4 ± 4.2**	10.5 ± 2.5**
Reference	22.0 ± 2.3	19.2 ± 1.3	16.8 ± 1.1
CD62P (%)			
Test	21.25 ± 2.81	22.52 ± 3.79	25.41 ± 3.04*
Reference	19.37 ± 3.62	21.88 ± 4.49	21.16 ± 1.98
CD42b (%)			
Test	89.84 ± 3.02	81.31 ± 4.72**	76.60 ± 5.85**
Reference	90.73 ± 3.19	87.76 ± 4.54	86.29 ± 5.17
CD41a (%)			
Test	96.95 ± 0.84	96.68 ± 1.02	96.78 ± 0.77
Reference	97.04 ± 1.88	97.52 ± 0.99	97.84 ± 0.42
CD61 (%)			
Test	94.25 ± 4.03	95.24 ± 3.06	94.44 ± 3.99
Reference	94.61 ± 4.11	93.85 ± 4.99	94.73 ± 4.35
PAC-1 (%)			
Test	29.27 ± 3.30	23.05 ± 7.15	15.34 ± 3.65
Reference	28.93 ± 4.70	22.45 ± 6.35	14.84 ± 4.35

percentage of total, remained stable at low levels in the reference units. In contrast, LDH increased during storage in the test units and was significantly higher from day 5 (p<0.01).

Metabolic assays

No significant differences in metabolic rate (data not shown), glucose concentration, production of lactate or pH were detected. On day 7, pCO_2, bicarbonate and ATP levels were significantly higher in the reference units (p<0.05). In contrast to the reference units, pO_2 decreased in the test units from day 2 (p<0.01). This was followed by an increase in the oxygen tension from day 5 (p<0.01).

HSR, ESC, swirling and aggregates

The HSR reactivity of platelets over 7 days was significantly higher in the reference units (p<0.05 on day 2 and p<0.01 from day 5). During storage, ESC was lower in the test units (p<0.05 until day 5 and p<0.01 on day 7). Swirling remained at the highest level (score = 2) for all units throughout storage (data not shown). Aggregates were visible (day 7) and occurred in three of the test units (data not shown).

Flow cytometry analysis

The percentage of platelets expressing the activation marker CD62P and mean fluorescence intensity (data not shown) increased slightly during storage in all units and became significantly higher in the test units on day 7 (p<0.05). The mean expression in all preparations was below 20% in platelet units on day 2 and below 30% in 7-day-old platelet units. CD42b decreased in all units but was significantly lower in the test units from day 5 (p<0.01). The percentage of platelets expressing the surface membrane glycoproteins CD41, CD61 and the conformational epitope on the GPIIb/IIIa complex, determined by using PAC-1, showed no significant differences throughout storage between the units.

Bacterial contamination was not detected in any of the units.

Discussion

This study describes the *in vitro* quality of platelets exposed to air bubbles and foam during storage. We found significant differences in a variety of cellular and flow cytometry parameters between platelets stored in the presence or absence of air bubbles/foam. With regards to the cellular variables, the extent of change during the 7-day storage period was fundamentally different between the groups and appeared to be significant with regards to current *in vitro* standards.

In this study, disintegration of platelets caused by exposure to air bubbles and foam seems to be considerably enhanced, as evidenced by a decrease in platelet count and increase in the LDH release. Increased extracellular LDH activity has been shown to be closely related to increased cytokine release into the storage bag[19,20]. This situation is undesirable during platelet storage, as cytokines may be associated with febrile non-haemolytic transfusion reactions[21, 22].

For most markers of platelet metabolism in the remaining cells, however, the degree of change was not or only slightly different between the groups, as evidenced by similar rates of glucose consumption, lactate accumulation and pH levels. The slight decrease in the ATP levels in the test units cannot, therefore, be explained by a greater demand for ATP due to increases in glycolytic flux and/or oxidative metabolism[23]. The slight decrease in ATP levels in the test units seems to be related to the decreased HSR, a reduction in ESC values and increased platelet activation, as evidenced by a higher expression of CD62P[24]. These effects indicate less advantageous storage conditions preceding transfusion[25].

Although differences were found, all HSR scores were above the level for which poor *in vivo* viability has been predicted to occur[5]. In contrast to the findings for HSR, all ESC values were below this level. One explanation for this situation may be that storage in platelet additive solution induces deterioration of the platelet responsiveness to ADP[26]. To preserve responsiveness toward ADP, addition of apyrase to the platelets may increase the response to the ESC test[26].

Glycoprotein (GP) Iba is the receptor for von Willebrand factor and a high affinity receptor for thrombin[27]. We found a significant difference in CD42b expression between the groups, and our findings accord with those of others who have shown that storage of platelets reduces the expression of GP1ba on the surface of the platelets[28-30]. Consequently, the greater reduction of CD42b on the surface of the test platelets may impair the ability of these platelets to become activated by thrombin to a greater extent[29].

In contrast to CD42b, the effects of air bubbles/foam on platelet membrane protein αIIbβ3 seem to be modest. The monoclonal antibody used (CD41/61) recognises only the intact complex of αIIbβ3[31] and was found to bind equally well to test as well as reference platelets. We also found that the regulatory conformation within the complex[32] seems to be affected to the same degree, as evidenced by a similar binding capacity to PAC-1[33] during storage. The overall low levels of PAC-1 binding may again be explained with platelet additive solution-induced deterioration of platelet responsiveness to ADP[26].

In conclusion, we found that the storage of platelets in bags containing air bubbles/foam enhanced disintegration of the platelets to a considerable extent. Furthermore, various *in vitro* variables of the platelets remaining seemed to be negatively affected. Given the increased disintegration of platelets, the results of our study suggest that platelets should be stored without air bubbles/foam.

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