

High-yield Platelet units revealed immediate pH decline and delayed mitochondrial dysfunction during storage in 100% plasma as compared with storage in SSP+

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Vox Sanguinis

Background and Objectives Initial elevated and prolonged high carbon dioxide levels related to mitochondrial dysfunction are recently being suggested as a contributing factor to rapid pH decline in platelet (PLT) units. The use of different storage environments may influence this phenomenon. This study has two objectives (i) to investigate the relationship of mitochondrial function and apoptotic events with different storage environments capability of pH control and (ii) to examine the cause and relationship between pH decline in PLT units, carbon dioxide levels and mitochondrial function.

Materials and Methods Platelet units were prepared for storage in (A) 70% SSP+, 300–400 × 10⁹/unit, (B) 70% SSP+, 550–600 × 10⁹/unit, (C) 100% plasma, 550–600 × 10⁹/unit, and (D) additional 100% plasma, >600 × 10⁹/unit. *In vitro* variables including mitochondrial function (JC-1), reactive oxygen species (ROS) and caspase 3 activity were analysed on days 2, 5 and 7.

Results Glucose/lactate was higher, pH, ATP, Hypotonic shock response (HSR) and extent of shape change (ESC) decreased ($P < 0.001$ on day 7), CD62P ($P < 0.001$ on day 7) increased, the JC-1-positive PLTs were lower ($P < 0.001$ on day 7), and ROS was higher ($P < 0.001$ days 2–7) in the plasma (C) units as compared with the SSP+ (A) and (B) units. All plasma (D) units showed rapid pH and pCO₂ decline from day 2 but by means of >80% maintenance of mitochondrial function until day 7.

Conclusions The use of SSP+ instead of plasma may reduce the risk of triggering pro-apoptotic events in high-yield PLT units. A rapid decline in pH in PLT units cannot be explained with initial elevated and prolonged high carbon dioxide levels and mitochondrial dysfunction.

Key words: mitochondrial function, platelets, platelet storage.

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Introduction

It has long been recognized that platelet (PLT) viability and function decline over time. Numerous morphologic, biochemical and functional derangements occur during PLT collection, processing and storage. This phenomenon is well

known as the 'PLT storage lesion' (PSL) [1, 2] and may be associated with decreased post-transfusion survival. Some of the morphological and biochemical changes that characterize the PSL are reminiscent of cell death by apoptosis [3]. Platelets do contain all proteins required for apoptosis, including cytochrome C, procaspase 9, procaspase 8, procaspase 3 and the formation of active caspase 3 [4–6], illustrating that at least the intrinsic pathway of apoptosis seems to be functional [7].

There is substantial evidence supporting the view that caspase activity may cause numerous of the biochemical

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and morphological features of apoptosis [8]. Additionally, PLTs also contain 'death receptors' [9], several death regulators of the Bcl-2 family and the adaptor molecule Apaf-1 [5, 10]. However, apoptosis is initiated and promoted by a wide variety of intra- and extracellular stimuli but the events that serve as triggers for death pathways and its role during PLT storage are largely unknown. Employing various environments to stress-stored platelets, we attempted to discern whether pH decrements resulting from accelerated glycolysis contribute to the initiation of apoptosis via oxidative cell damage or depolarization of the mitochondrial membrane potential (MMP) or if alternately, oxidative damage and/or mitochondrial membrane depolarization result in a metabolic shift towards anaerobic glycolysis.

Reactive oxygen species (ROS) participate among several other well-defined regulatory factors, such as nitric oxide (NO), prostacyclin (PGI₁) and adenosine in the regulation of PLT activation [11]. In case of activation, several ROS, including O₂⁻, OH⁻ and H₂O₂, are reported to be generated by the PLT themselves [12, 13], which may contribute to oxidative stress, a condition in which the PLTs are exposed to excessive levels of either molecular oxygen or chemical derivatives of oxygen. ROS form as a natural by-product of the normal metabolism of oxygen, but during the time of environmental stress, ROS levels may increase dramatically. Moreover, as ROS play an important role in apoptosis induction [14], occurrence of ROS in the storage environment may be one of several related events that contribute to the detrimental morphological and biochemical *in vitro* changes observed in unsuccessful PLT units. By the release of free radicals, an accelerating circle of oxidative stress, induction of apoptotic pathways with subsequent damage to cellular structures will be the inevitable consequence of such hypothesis. Though, by detection of either immediate or delayed apoptotic and/or oxidative stress-like processes, the role of such events during PLT storage may be further explained.

The quality of PLTs during storage is influenced by various factors [15]. However, sufficient oxygen supply seems to be a key factor in the maintenance of oxidative phosphorylation necessary for adequate ATP production/levels [16–19]. Previous studies have also reported various plasters and plasticizers permeability to gases [19–21], high PLT counts, carbon dioxide/oxygen levels [18, 22] and mitochondrial dysfunction [23, 24] as factors important for the maintenance of PLT quality during storage. These results illustrate the complexity of the underlying mechanisms leading to reduced PLT viability. Nevertheless, by using optimized synthetic storage media [25, 26] in combination with optimized production systems and storage bags, PLT viability during storage may be improved.

The purposes of this study were therefore (i) to investigate the relationship of mitochondrial function and

apoptotic events with different storage environments capability of pH control and (ii) to examine the cause and relationship between pH decline in PLT units, carbon dioxide levels and mitochondrial function. Progress in the understanding of the underlying and contributing factors that influence the decline in PLT viability may help us to enhance and further optimize storage conditions for the long-term storage of PLTs.

Materials and methods

Preparation and storage of platelets

In this study, non-paired experiments in four arms comparing PAS and plasma storage environment were conducted. The fourth arm (D) was performed only in order to properly study the mechanisms involved in rapid pH decline and searching for answers for the second objective (ii) if these units are included in the group (C), another uncontrolled variable (PLT content) will be present and conclusions cannot be drawn regarding the specific effects of the different storage environments.

Platelets were collected from normal blood donors who met standard donation criteria and gave written informed consent according to the institutional guidelines. A total of 450 ml of non-leucoreduced whole blood (WB) was 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 inline red-blood-cell (RBC) filters for white-blood-cell (WBC) removal (Imuflex-CRC, Terumo, Tokyo, Japan). After storage at room temperature for 2–6 h, WB units were centrifuged (2700 *g*) for 10 min at 22°C. Automatic equipment was used for the preparation of blood components (Optipress, Fenwal or T-ACE, Terumo, Mont-Saint-Guibert, Belgium), including buffy coat (BC). Buffy coats (in total, 179–195 divided into four arms) were kept overnight and were then selected on the basis of the PLT concentration in donor blood for the production of PLT units by using the Orbisac system (Caridian, Zaventem, Belgium) [27] with subsequent storage either in 30% plasma and 70% SSP+ (MacoPharma, Mouvaux, France) or in 100% plasma. The study design is as follows:

- (A) Platelets stored in 70% SSP+, 300–400 × 10⁹/unit (reference) *n* = 8.
- (B) Platelets stored in 70% SSP+, 550–600 × 10⁹/unit (SSP+ high yield) *n* = 8.
- (C) Platelets stored in 100% plasma, 550–600 × 10⁹/unit (plasma high yield) *n* = 8.
- (D) Platelets stored in 100% plasma, >600 × 10⁹/unit (plasma very high yield) *n* = 5.

To avoid disintegration and adverse negative effects on the PLTs [28], the air and foam were excluded from all

units. All 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 $22 \pm 2^\circ\text{C}$. Approximately 10 ml sample was drawn aseptically on days 2, 5 and 7 for immediate measurements of cellular, metabolic, functional and activation parameters, MMP, extracellular superoxide dismutase (SOD) and markers of downstream cellular apoptosis. All sampling was done by sterile connection (TSCD-II, Terumo, Japan) of sampling bags to the respective containers. (OrbiSac Standard BC set, storage bag; Caridian BCT, Inc, Lakewood, CO, USA). The storage bag is made of PVC plastic with a citrate-based plasticizer (BTHC) and intended to contain up to 500×10^9 PLTs in 400 ml of plasma (Caridian BCT). To avoid disturbing $p\text{O}_2$, sampling was not done using a sample bag, but by direct insertion of a needle in the bag through a sampling site coupler, followed by slow aspiration and immediate analysis on the blood gas analyser.

Analysis of cellular, metabolic, functional and platelet surface variables

Cellular and metabolic *in vitro* parameters include measurements of PLT counts ($10^9/\text{l}$ and $10^9/\text{unit}$) and mean PLT volume (MPV) using 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; Mettler-Toledo, Albstadt, Switzerland), and the result, in grams, was divided by either 1.01 (1.01 g/ml is the density of the storage medium composed of approximately 70% SSP+ and 30% plasma) or 1.03 (density 100% plasma). By the use of routine blood gas equipment (ABL 800; Radiometer, Copenhagen, Denmark), we also measured the pH (37°C), $p\text{CO}_2$, $p\text{O}_2$ (kPa at 37°C), glucose (mmol/l) and lactate (mmol/l). Bicarbonate (mmol/l) was calculated from pH and CO_2 partial pressure measurements. The pH of all samples was measured at 37°C . Therefore, Rosenthal's factor of 0.0147 unit/ $^\circ\text{C}$ was used to correct pH to the temperature of sampling (22°C). This factor gives an approximation to the change in the pH of the sample per degree centigrade when it is warmed anaerobically from the collecting temperature 22 – 37°C .

According to Bertolini and Murphy [29], the assessment of swirling was scored as 0, 1 and 2. The white-blood-cell count (WBC) on day 1 was determined using a Nageotte chamber and a microscope (Zeiss, standard, Chester, VA, USA) [30]. Hypotonic shock response reactivity (HSR) and the extent of shape change (ESC) were measured using a dedicated microprocessor-based instrument (SPA 2000; Chronolog, Havertown, PA, USA) with the modifications of these tests described by VandenBroeke *et al.* [31]. The total adenosine triphosphate (ATP) concentration ($\mu\text{mol}/10^{11}\text{PLTs}$) was determined using a luminometer

(Orion Microplate Luminometer; Berthold Detection Systems GmbH, Pforzheim, Germany) on the basis of principles described by Lundin [32]. The extracellular lactate dehydrogenase (LDH) activity (% of total), a marker for the disintegration of PLTs, was measured using a spectrophotometric method (Sigma Aldrich kit 063K6003; Sigma Aldrich, St Louis, MO, USA; Spectrophotometer Jenway 6500; Jenway, Staffordshire, UK) [33]. CD62P, CD42b and PAC-1 analyses were performed as been described in earlier work [28, 34, 35].

Mitochondrial membrane potential (MMP)

Changes in the MMP, a marker of pro-apoptotic events, were measured using the mitochondrial permeability transition detection kit MitoPT JC-1 (ImmunoChemistry Technologies, LCC, Bloomington, MN, USA). All samples from all groups (1×10^6 PLTs/ml) were stained with the MitoPT JC-1 dye reagent at 37°C for 10–15 min and analysed using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Depolarized mitochondria (positive control) were prepared by incubating PLTs with $5 \mu\text{M}$ CCCP for 30–60 min at 37°C . This proton gradient uncoupling agent quickly reduces the electrochemical potential across the inner mitochondrial membrane, resulting in a rapid intracellular mitochondrial depolarization event. Maintenance of MMP is expressed as JC-1-positive PLTs.

Superoxide dismutase (SOD) activity

Samples from the PLTs were collected in citrate theophylline adenosine dipyridamole (CTAD) tubes. The samples were centrifuged at 2500 g (Eppendorf 5810R) for 30 min at $+10^\circ\text{C}$, and the supernatants were stored at -80° pending analysis. Superoxide dismutase (SOD) concentrations were determined using commercial kits (SOD, PromoKine PK-CA577-K335; PromoCell GmbH, Heidelberg, Germany) in accordance with the manufacturer's (PromoCell GmbH, Heidelberg, Germany) recommendations. All measurements were taken in duplicate on HT3 Microtiter Plate Reader (Anthos Labtec Instruments GmbH, Salzburg, Austria) at 466 nm. Superoxide dismutase (SOD) catalyses the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity and inhibited by SOD. The results for the SOD activity are given in inhibition rate (%).

Caspase 3 activity

Caspase-3 activity in cell lysates ($50 \mu\text{l}$) was determined using a colorimetric assay kit (ab39401) in accordance with the manufacturer's (Abcam, Cambridge, UK)

recommendations. All measurements were taken in duplicate. The assay is based on the spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labelled substrate DEVD-*p*NA. Reactions were incubated for 2 h in a flat-bottom 96-well plate at 37°C before the *p*NA light emission was quantified using the HT3 Microtiter Plate Reader (Anthos Labtec Instruments GmbH) at OD_{405 nm}.

Comparison of the absorbance (OD_{405 nm}) of *p*NA from the high-yield units with the reference population (non-apoptotic cells) allows determination of the fold increase in caspase 3 activity.

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 PLT samples after incubation for 24 h at 35°C [36].

Statistical analyses

The mean values and standard deviations ($n = 8$) are usually given unless otherwise indicated. A repeated-measure ANOVA including *post hoc* test Bonferroni's adjustment was performed. Three different groups, (A) reference, (B) SSP+ high yield and (C) plasma high yield, were studied over time (Days). 'Days' was the repeated factor, and 'Group' was a between factor. The results of Bonferroni's test are presented in Tables 2 and 3, in subsequent figures and in the result section. The *P*-value represents the difference between groups at specific time-points and was considered statistically significant at $P < 0.001$. The analyses were carried out using the Statistica software, version 9 StatSoft, Inc 1984–2007 (SPSS, Chicago, IL, USA).

Results

In this study, PLTs in plasma or SSP+ were studied over a 7-day storage period. The PLT counts and content for all

Table 1 Platelet (PLT) content and PLT concentration in reference PLT units stored in SSP+ vs. high-yield PLT units stored in either SSP+ or plasma on day 2^a

Platelet units	Platelet Volume (ml)	Platelet count (10 ⁹ /l)	Platelet content (10 ⁹ /unit)
(A) Reference (SSP+)	381 ± 14	975 ± 107	371 ± 35
(B) SSP+ high yield	403 ± 16	1384 ± 81	557 ± 20
(C) Plasma high yield	397 ± 22	1431 ± 102	566 ± 18
(D) Plasma very high yield	384 ± 2	1622 ± 68	622 ± 26

^aResults are expressed as mean ± SD ($n = 8$).

groups on day 2 are given in Table 1. The leucocyte content on day 2 was $<0.2 \times 10^6/l$ in all units. The parameters for group A–C are listed in Tables 2 and 3, and the results from the additional (D) group are presented in Fig. 1 and Table 4.

Cellular, metabolic, functional and platelet surface variables

Mean PLT volume (MPV) increased more in the (C) units ($P < 0.001$ on day 7) vs. units (A) and (B), and the extracellular LDH activity in % of total remained stable at low levels in those units, but increased in the (C) units ($P < 0.001$ on day 7). Throughout storage, the glucose concentration ($P < 0.001$ from day 2) and the production of lactate ($P < 0.001$ from day 5) were higher in the (C) units as

Table 2 *In vitro* effects on platelets (PLTs) ($n = 8$) stored for 7 days in SSP+ and plasma: Values are reported as mean ± standard deviation (SD)

Parameter	Day		
	2	5	7
MPV (fl)			
(A) SSP+ reference	9.0 ± 0.4	9.1 ± 0.4	9.0 ± 0.4
(B) SSP+ high yield	8.5 ± 0.3	8.9 ± 0.5	8.9 ± 0.5
(C) Plasma high yield	8.6 ± 0.4	9.4 ± 1.0	9.8 ± 1.3 ^{*S}
LDH (extracellular % of total)			
(A) SSP+ reference	4.2 ± 2.5	5.7 ± 1.7	4.8 ± 1.9
(B) SSP+ high yield	5.4 ± 3.1	4.1 ± 0.8	3.9 ± 1.9
(C) Plasma high yield	5.6 ± 1.7	8.6 ± 2.9	10.4 ± 5.7 ^{*S}
Glucose (mmol/l)			
(A) SSP+ reference	5.9 ± 0.4	4.5 ± 0.5	3.5 ± 0.6
(B) SSP+ high yield	5.1 ± 0.5	2.1 ± 1.3	1.0 ± 0.9
(C) Plasma high yield	14.9 ± 1.2 ^{*S}	9.5 ± 2.1 ^{*S}	6.5 ± 3.3 ^{*S}
Lactate (mmol/l)			
(A) SSP+ reference	7.4 ± 0.6	9.6 ± 0.8	11.7 ± 1.0
(B) SSP+ high yield	10.5 ± 0.8	16.1 ± 2.9	18.1 ± 2.3
(C) Plasma high yield	12.8 ± 1.4 ^{*S}	22.3 ± 3.8 ^{*S}	27.6 ± 6.7 ^{*S}
pH (22°C)			
(A) SSP+ reference	7.21 ± 0.04	7.32 ± 0.05	7.34 ± 0.04
(B) SSP+ high yield	7.08 ± 0.05	7.07 ± 0.17	7.12 ± 0.15
(C) Plasma high yield	7.12 ± 0.06	6.92 ± 0.19 ^{*S}	6.72 ± 0.35 ^{*S}
pO ₂ (kPa at 37°C)			
(A) SSP+ reference	9.85 ± 2.26	11.96 ± 1.57	12.24 ± 2.15
(B) SSP+ high yield	6.35 ± 0.87	6.77 ± 1.11	8.04 ± 1.10
(C) Plasma high yield	5.17 ± 0.44	8.09 ± 1.81	10.27 ± 4.58
pCO ₂ (kPa at 37°C)			
(A) SSP+ reference	3.76 ± 0.41	2.64 ± 0.36	2.37 ± 0.32
(B) SSP+ high yield	4.91 ± 0.27	3.45 ± 0.21	3.06 ± 0.19
(C) Plasma high yield	8.59 ± 0.67 ^{*S}	5.27 ± 0.50	3.83 ± 0.90

LDH, lactate dehydrogenase; MPV, mean PLT volume.

* $P < 0.001$ vs. (A) ^S $P < 0.001$ vs. (B) [†] $P < 0.001$ vs. (C).

Table 3 *In vitro* effects on platelets (PLTs) ($n = 8$) stored for 7 days in SSP+ vs. plasma: Values are reported as mean \pm standard deviation (SD)

Parameter	Day		
	2	5	7
Bicarbonate (mmol/l)			
(A) SSP+ reference	6.4 \pm 0.2	5.8 \pm 0.4	5.5 \pm 0.6
(B) SSP+ high yield	6.3 \pm 0.6	4.5 \pm 1.4	4.5 \pm 1.4
(C) Plasma high yield	12.0 \pm 0.8 [§]	4.9 \pm 1.7	2.6 \pm 2.0 [§]
ATP ($\mu\text{mol}/10^{11}$ platelets)			
(A) SSP+ reference	7.83 \pm 0.71	7.78 \pm 0.66	7.83 \pm 0.51
(B) SSP+ high yield	7.02 \pm 0.68	7.04 \pm 0.92	7.07 \pm 0.88
(C) Plasma high yield	6.40 \pm 0.19	6.16 \pm 1.20	5.41 \pm 2.33 [§]
HSR (%)			
(A) SSP+ reference	63.8 \pm 3.9	58.2 \pm 5.9	55.1 \pm 6.4
(B) SSP+ high yield	61.7 \pm 7.0	56.1 \pm 10.6	50.9 \pm 11.9
(C) Plasma high yield	64.0 \pm 6.2	50.7 \pm 15.7	36.0 \pm 17.7 [§]
ESC (%)			
(A) SSP+ reference	23.1 \pm 4.4	19.1 \pm 3.4	17.6 \pm 3.1
(B) SSP+ high yield	22.2 \pm 3.1	20.4 \pm 5.0	16.6 \pm 4.2
(C) Plasma high yield	25.8 \pm 2.0	19.2 \pm 3.7	12.9 \pm 8.8
CD62P (%)			
(A) SSP+ reference	21.47 \pm 3.56	25.35 \pm 4.38	25.29 \pm 4.31
(B) SSP+ high yield	23.34 \pm 7.95	28.24 \pm 10.01	31.41 \pm 5.68
(C) Plasma high yield	17.50 \pm 6.01	26.66 \pm 9.30	41.64 \pm 19.46 [§]
PAC-1 (%)			
(A) SSP+ reference	29.53 \pm 10.26	22.31 \pm 5.04	16.21 \pm 4.91
(B) SSP+ high yield	22.34 \pm 7.30	16.51 \pm 7.34	11.58 \pm 8.72
(C) Plasma high yield	22.97 \pm 8.54	16.76 \pm 1.45	10.73 \pm 8.59
CD42b (%)			
(A) SSP+ reference	89.55 \pm 7.19	85.61 \pm 7.99	84.08 \pm 6.65
(B) SSP+ high yield	93.89 \pm 1.89	88.21 \pm 5.69	87.46 \pm 6.60
(C) Plasma high yield	94.40 \pm 4.67	89.64 \pm 9.66	88.27 \pm 10.30

ESC, extent of shape change; HSR, Hypotonic shock response.

* $P < 0.001$ vs. (A) [§] $P < 0.001$ vs. (B) [§] $P < 0.001$ vs. (C).

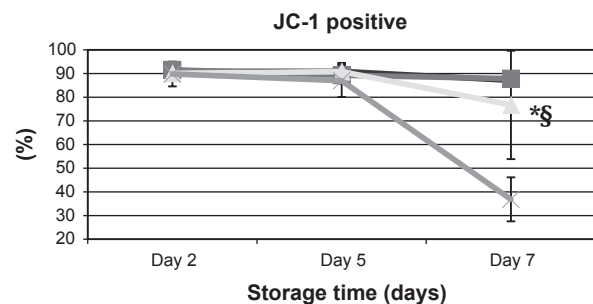


Fig. 1 Maintenance of mitochondrial membrane potential (MMP) expressed as JC-1-positive PLTs in (A) reference PLTs ($n = 8$) in SSP+ \blacklozenge (B) high-yield PLTs ($n = 8$) in SSP+ \blacksquare (C) high-yield PLTs ($n = 8$) in plasma Δ (D) very-high-yield PLTs ($n = 5$) in plasma \times stored for 7 days: Values are reported as mean \pm standard deviation (SD). * $P < 0.001$ vs. (A) [§] $P < 0.001$ vs. (B) [§] $P < 0.001$ vs. (C). The (D) units ($n = 5$) were not statistically calculated.

Table 4 *In vitro* effects on very-high-yield platelets (PLTs) ($n = 5$) stored for 7 days in plasma: Values are reported as mean \pm standard deviation (SD)

Parameter	Day		
	2	5	7
MPV (fl)			
(D) Plasma very high yield	8.9 \pm 0.0	10.5 \pm 1.3	11.1 \pm 1.5
Glucose (mmol/l)			
(D) Plasma very high yield	13.7 \pm 1.1	5.6 \pm 1.6	0.6 \pm 0.6
Lactate (mmol/l)			
(D) Plasma very high yield	14.5 \pm 1.6	29.2 \pm 2.7	39.6 \pm 1.8
pH (22°C)			
(D) Plasma very high yield	7.02 \pm 0.06	6.51 \pm 0.26	6.04 \pm 0.14
pO ₂ (kPa at 37°C)			
(D) Plasma very high yield	5.29 \pm 0.59	8.02 \pm 2.44	15.0 \pm 3.08
pCO ₂ (kPa at 37°C)			
(D) Plasma very high yield	9.57 \pm 0.54	4.71 \pm 0.52	2.79 \pm 0.92
Bicarbonate (mmol/l)			
(D) Plasma very high yield	10.6 \pm 1.4	1.5 \pm 1.4	0.0 \pm 0.0
HSR (%)			
(D) Plasma very high yield	56.9 \pm 2.4	49.0 \pm 24.2	11.1 \pm 9.1
ESC (%)			
(D) Plasma very high yield	25.1 \pm 1.8	13.5 \pm 6.2	3.2 \pm 3.7
CD62P (%)			
(D) Plasma very high yield	41.37 \pm 5.14	49.21 \pm 17.44	74.41 \pm 10.23

ESC, extent of shape change; HSR, Hypotonic shock response; MPV, mean PLT volume.

compared with the (A) and (B) units. The glucose consumption rate (0.49 ± 0.01 mmol/day/ 10^9 PLTs in the (A) units, 0.60 ± 0.01 mmol/day/ 10^9 PLTs in the (B) units and 1.2 ± 0.04 mmol/day/ 10^9 PLT in the (C) units) and the lactate production rate (0.09 ± 0.01 mmol/day/ 10^9 PLTs in the (A) units, 0.11 ± 0.03 mmol/day/ 10^9 PLTs in the (B) units and 0.20 ± 0.07 mmol/day/ 10^9 PLTs in the (C) units) were higher in the (C) units. During storage, the calculated glucose/lactate rate was found to be consistent in all units.

Subsequently, pH decreased more in all (C) units and was lower ($P < 0.001$ from day 5) with levels below 6.4 (22°C) in two of the eight (C) units. The partial pressures of oxygen showed no difference between the groups. In the (C) units, the levels of carbon dioxide and bicarbonate were initially higher ($P < 0.001$ on day 2) and decreased during storage with following reductions in ATP levels, HSR reactivity and increased expression of CD62P ($P < 0.001$ on day 7) as compared with the (A) and (B) units.

The expression of the conformational epitope on the GpIIb/IIIa determined by using PAC-1 and the expression of CD42b decreased during storage but without any significant differences between the groups. With exception of one unit in group (B) (level 1 at day 7), swirling remained at the

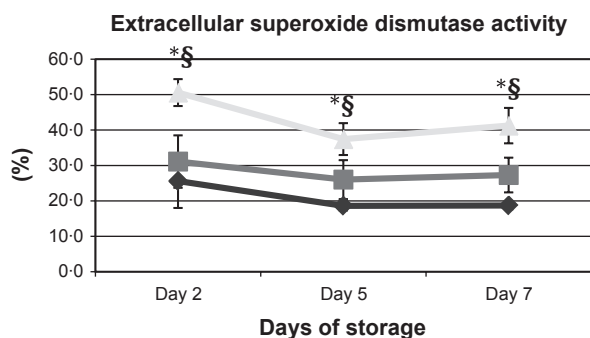


Fig. 2 Extracellular superoxide activity in (A) reference PLTs ($n = 8$) in SSP+ ♦ (B) high-yield PLTs ($n = 8$) in SSP+ ■ (C) high-yield PLTs ($n = 8$) in plasma Δ stored for 7 days: Values are reported as mean \pm standard deviation (SD). * $P < 0.001$ vs. (A) [§] $P < 0.001$ vs. (B) [†] $P < 0.001$ vs. (C).

highest level at all times in all (A) and (B) units. In contrast, a total loss of swirling was detected in four of the eight (C) units on day 7 (data not shown).

Mitochondrial membrane potential (MMP)

Throughout storage, the MMP (JC-1-positive PLTs) in all (A) and (B) units was maintained at similar high levels (NS). In contrast, the maintenance of MMP in the (C) units was less ($P < 0.001$ on day 7) by means of $<50\%$ maintenance of MMP in two of the eight (C) units (Fig. 1).

Superoxide dismutase and Caspase 3 activity

The extracellular SOD activity (Fig. 2) decreased slightly during storage in all units, but was higher ($P < 0.001$ on days 2, 5 and 7) in the (C) units as compared with the (A) and (B) units. The caspase 3 activity remained at low levels at all times in all units without any significant differences between the groups (Fig. 3).

Analysis of additional (D) very-high-yield PLT units stored in plasma ($n = 5$)

All plasma (D) units showed a rapid pH and pCO₂ decline from day 2 but by means of $>80\%$ maintenance of mitochondrial function until day 7. On day 7, all pH (22°C) levels were below 6.4 and $<50\%$ maintenance of MMP (Fig. 1) with subsequent negative effects on various measured *in vitro* variables as presented in Table 4. A total loss of swirling was observed in these units on day 7.

Bacterial contamination

Bacterial contamination was not detected in any of the units.

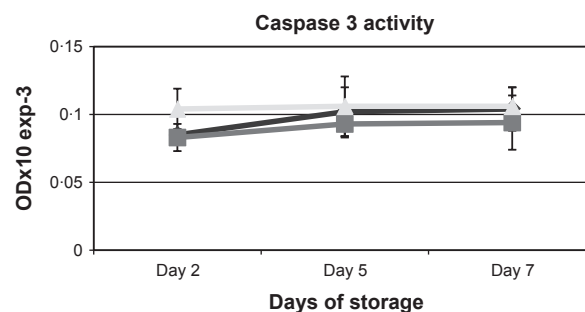


Fig. 3 Caspase 3 activity in (A) reference PLTs in SSP+ ♦ (B) high-yield PLTs in SSP+ ■ (C) high-yield PLTs in plasma Δ stored for 7 days. Values are reported as mean \pm standard deviation (SD) ($n = 8$).

Discussion

Platelets express several distinctive apoptotic features during storage [37, 38], but the role of potential apoptotic features during storage is largely unknown. Conceptually, increased channelling of metabolism via glycolysis that leads to rapid pH decline may activate apoptotic pathways as a means to eliminate dysfunctional cells or being a consequence of such events. Apoptotic events can be initiated via two major signalling pathways: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. Mitochondria and caspases are central components of the regulatory mechanisms of apoptosis. Although no increase in the caspase activity was found in our study, our results suggest that buffer exhaustion with consequent deleterious pH decrements in several of the plasma units may contribute to stimulate such death pathways, as demonstrated by the relationship between certain low pH levels (<6.4) and delayed depolarization of MMP. As previously mentioned ROS is suggested to play a role in triggering receptor-mediated apoptosis [14] through the tumour necrosis factor (TNF) receptor and PLT, mitochondria most likely play a role in the generation of apoptotic events as well as being a target of oxidative stress [39].

However, our results suggest that extracellular generation of ROS is unlikely to account for triggering pro-apoptotic events through the extrinsic death receptor pathway, as no cause and relationship between extracellular ROS activity, depolarization of MMP and caspase activity was detected throughout storage. Mitochondrial dysfunction and apoptotic activity therefore do not seem to be the underlying or the contributing factor to rapid decline of pH with the subsequent morphological and biochemical changes that characterize the lesion effects [40–42]. Though, it can be thought that the increased concentration of ROS in the plasma units may be related to a condition in which these PLTs are exposed to excessive levels of chemical derivatives of oxygen, but the functional importance of these findings needs to be demonstrated.

Evidence from a different experimental approach including the assessment of intracellular ROS formation and its influence on PLT function would be needed. On the other hand, ROS can also be anti-apoptotic [14] and storage-induced derangements have also been associated with necrotic cell death and para-apoptotic cell death, which share the characteristics of apoptotic and non-apoptotic cell death [43]. These data add the complexity of PLT derangements during storage.

The recent data suggesting mitochondrial dysfunction in the presence of initial elevated and prolonged high carbon dioxide levels as an explanation for the metabolic shift towards anaerobic glycolysis [24] are not in line with our results. In our study, the shift towards anaerobic glycolysis with increased glucose consumption, lactate production and pH decline in the several of the plasma units seems to be the immediate effects rather than influenced by depolarization of the PLT MMP, initial elevated and prolonged high carbon dioxide levels in combination with low oxygen levels, as demonstrated by delayed depolarization of the MMP, a decrease in carbon dioxide and increased oxygen levels in these units. The rise in oxygen concentration in these units may be explained by a lower oxygen consumption of PLTs with mitochondrial dysfunction. Thus, our results indicate no further support for a causal relationship between initial elevated and prolonged high levels of carbon dioxide, a lower oxygen content, mitochondrial dysfunction and rapid pH decline but cannot exclude the possibility of a connection between the initial elevated levels of carbon dioxide and poor storage conditions. However, the initial driving force leading to increased channelling of metabolism via glycolysis with pH decline in PLT units seems to be unclear.

Conversely, the capability of pH control in all SSP+ units is probably influenced by the effects of potassium and magnesium [25, 26], the effects of acetate and phosphate [44] in combination with an optimized production system and storage bag. Consequently, using SSP+ instead of plasma may reduce the risk of triggering pro-apoptotic events in high-yield PLT units, as demonstrated by the maintenance of MMP and pH control in all SSP+ units throughout storage.

Probably as a consequence of the SSP+ solution capability to counteract negative effects on the metabolism, higher ATP concentrations and better response to HSR in combination with lower expression of CD62P were observed in these units towards the end of storage time. Although the differences were found in the CD62P expression between the plasma and the SSP+ units, no significant differences were observed in PAC-1 binding. One explanation for this situation may be that storage in synthetic storage media induces deterioration of the PLT responsiveness to ADP [45].

Recent published data show that PLTs in plasma or in different PASs were equivalent regarding *in vitro* characteristics after 7 or 9 days of storage [46, 47]. Our data may indicate an advantage from a quality standpoint to use SSP+ in high-yield PLT units.

To summarize, our study suggests that high-yield PLT units stored in SSP+ maintained metabolic and cellular *in vitro* characteristics to a great extent during 7 days of storage. In contrast, high-yield PLT units stored in plasma are to some extent associated with pH decline and subsequent negative effects on the various *in vitro* variables including increased generation of extracellular ROS activity. In addition, our results suggest that rapid pH decline cannot be explained with initial elevated and prolonged high carbon dioxide levels and mitochondrial dysfunction.

However, because this is an unpaired study, interdonor variations may introduce an uncontrolled variable affecting the outcome of the results.

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Conflict of interest

The study was independently performed without any other interest than useful scientific knowledge.

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