Freezing expired platelets does not compromise in vitro quality: An opportunity to maximize inventory potential

Lacey Johnson[1], *Lauren Waters*[1], *Sarah Green*[1], *Ben Wood*[1], *and Denese C. Marks*[1,2]

BACKGROUND AND OBJECTIVES: Cryopreservation provides an option for long-term storage of platelet concentrates. While platelets are usually frozen as soon as practical after collection (within 2 days), the ability to freeze units at a later stage of the shelf life may improve inventory management. As such, the aim of this study was to determine the impact of freezing platelets approaching expiry (Day 5/6).

MATERIALS AND METHODS: Two ABO-matched buffy coat–derived platelets (30% plasma/70% platelet additive solution) were pooled and split to produce matched pairs (n = 8 pairs). Platelets were frozen on Day 1 after collection (cryopreserved platelets [CPPs]) or Day 5 or 6 (expired-CPPs) at −80°C with 5% to 6% dimethyl sulfoxide. In vitro platelet quality was tested before freezing and after thawing and reconstitution in plasma.

RESULTS: The majority of prefreeze parameters were equivalent for all platelet units (Day 1 vs. Day 5 or 6). Expired-CPPs had a higher mean postthaw platelet recovery (82 ± 4%) compared to CPPs (75 ± 4%; p = 0.0021). Cryopreservation resulted in a loss of surface glycoproteins (glycoprotein (GP) Ibα, GPIIb, GPVI), an increase in activation markers (phosphatidylserine and P-selectin) and microparticle release, compared to unfrozen platelets. However, the cryopreservation-induced changes were equivalent in CPPs and expired-CPPs. Functionality was measured by thromboelastography and was similar between expired-CPPs (R-time: 5.3 ± 0.3) and CPPs (R-time: 5.4 ± 0.5; p = 0.7094).

CONCLUSION: The phenotype and functional profile of platelets frozen at expiry were similar to platelets frozen 1 day following collection. These data suggest that expired platelets may represent a suitable starting material for cryopreservation.

Conventional room temperature (RT, 20–24°C) storage limits the shelf life of platelet concentrates to between 5 and 7 days. This short shelf life challenges inventory management processes, which may lead to paradoxical wastage due to expiry, as well as the inability to supply platelets at certain times. Further, these effects can be compounded in rural and isolated medical centers or during military operations, where access to platelets is unfeasible due to difficult transport logistics and/or unpredictable clinical requirements.[1]

Platelet cryopreservation is an attractive alternative to RT storage, and international interest in this storage option has grown considerably in the past decade.[2] For cryopreservation, 5% to 6% dimethyl sulfoxide (DMSO) is added to the platelets and they are frozen and stored at −80°C, which extends their shelf life to at least 2 years.[3] Although variations exist in the methods used to cryopreserve platelets internationally, there is consistency in the timing of freezing, where platelets are frozen as soon as logistically possible following collection and mandatory testing, which translates to within 2 days.[2]

Freezing platelets within 2 days of collection reduces the available inventory of liquid-stored platelets and, combined with unpredictable supply and demand, may actually result in the inability to meet demand for this component. As the clinical indications for liquid-stored and cryopreserved platelets (CPPs) are likely to be distinct,[2] a flexible approach to the management of these inventories would be beneficial. Potentially, if platelets

ABBREVIATIONS: ADP = adenosine diphosphate; CPPs = cryopreserved platelets; DMSO = dimethyl sulfoxide; PF4 = platelet factor 4; RT = room temperature.

From the [1]Research and Development, Australian Red Cross Blood Service and [2]Sydney Medical School, University of Sydney, Sydney, New South Wales, Australia.

Address reprint requests to: Dr. Lacey Johnson PhD, Research and Development, Australian Red Cross Blood Service, 17 O'Riordan St, Alexandria, New South Wales, Australia; e-mail: ljohnson@redcrossblood.org.au

Received for publication August 28, 2019; revision received October 30, 2019, and accepted November 12, 2019.

doi:10.1111/trf.15616

TRANSFUSION 2020;60;454–459

could be cryopreserved later in their usable shelf life, following storage at RT, this would maximize utility, as they could be available for standard transfusion and then frozen if not required. Thus, the aim of this study was to assess the quality of platelets frozen at or approaching expiry (5-6 days of RT storage) compared to platelets frozen according to our standard protocol (within 48 hours of collection).

METHODS

Platelet collection and sampling

Ethics approval was obtained for this study from the Australian Red Cross Blood Service Research Ethics Committee. Whole blood donations were collected from eligible, voluntary, nonremunerated donors. For platelet preparation, four buffy coats were pooled with platelet additive solution (SSP+, MacoPharma) and separated by centrifugation (500 x *g*, 6 min) and extraction with an automated press (MacoPress Smart, MacoPharma). Pooled platelet concentrates were suspended in 30% plasma/70% SSP+, leukoreduced, and stored in polyvinyl chloride bags (ELX; Haemonetics Corporation, Braintree, MA, USA).

On Day 1 following collection, two ABO-matched platelet concentrates were pooled and split to produce matched pairs. Platelet units were randomly allocated to the control (CPP) or expired-CPP group (n = 8 each group). CPPs were sampled for prefreeze testing and then frozen immediately. Expired-CPPs were stored at RT (20-24°C) with constant agitation (Helmer Inc.) until Day 5 or 6 after collection, when they were sampled for prefreeze testing and then frozen. For platelet cryopreservation, 27% DMSO/0.9% saline (DMSO; Sypharma Pty. Ltd.) was added to the platelet units to achieve a final concentration of 5% to 6%. Platelets were hyperconcentrated to approximately 25 mL by centrifugation and supernatant removal, and frozen at −80°C, as previously described.[4]

Cryopreserved platelets were stored at −80°C (for <2 months) until thawed. Thawed platelets were reconstituted in a unit of freshly thawed whole blood–derived plasma (approx. 280 mL). Two plasma units were thawed, pooled, and split to form a matched pair for reconstitution of matched platelet units (CPPs and expired-CPPs). The platelet units were thawed in a 37°C water bath for 4 minutes, agitated at RT for 15 minutes and then sterile welded to a unit of thawed plasma. The platelets were then resuspended in the thawed plasma with gentle mixing.

Laboratory analysis

Platelet count a was assessed using a hematology analyzer (CELL DYN Emerald, Abbott Laboratories). pH was measured using a pH meter at RT (Mettler-Toledo). All platelet units were assessed visually for the presence of swirl and macroaggregates by holding the units up to a light source. Microaggregates were measured by flow cytometry (FACS Canto II, Becton Dickinson), and defined as events with a forward scatter profile larger than single platelets.[5] The data presented were the number of microaggregates per 10,000 events collected.

Platelet supernatant was collected by centrifugation at 1600 × *g* for 20 minutes followed by 12,000 × *g* for 5 minutes at RT and frozen at −80°C until batch analysis was performed. The concentration of soluble P-selectin, RANTES, and platelet factor 4 (PF4) in the platelet supernatant was measured using commercially available enzyme-linked immunosorbent assay kits, according to the manufacturer's instructions (R&D Systems Inc.) and as previously described.[4]

Platelet surface receptor phenotype and microparticle release was assessed by flow cytometry with use of previously described protocols.[6] Briefly, platelets were diluted to 300 × 10^9/L in Tyrode's buffer and stained with anti-CD61-FITC (Dako), anti-GPVI-efluor660 (eBioscience Inc.), anti-CD41a-PE, anti-CD42b-PE, CD62P-PE, and PAC-1-FITC (all from BD Biosciences) for 20 minutes. PAC-1 binding was examined in resting (unstimulated) and activated (20 μmol adenosine diphosphate (ADP) for 5 minutes at 37°C) platelets. Phosphatidylserine exposure was determined by staining 1 × 10^6 platelets with annexin-V-FITC (Biolegend) in calcium-containing buffer for 15 minutes. The absolute number of CD61+/annexin-V+ microparticles was enumerated using tubes containing a defined number of beads (TruCount, BD Biosciences).[7]

The platelet clotting potential was measured by thromboelastography (TEG 5000; Haemoscope Corporation). Platelets, diluted to 200 × 10^9/L in plasma, were recalcified and kaolin activated before addition to a plain cup, as previously described.[7]

TABLE 1. Specifications of platelets, before freezing, when frozen, and after thawing and reconstitution in plasma*

	CPP	Expired-CPP
Platelet concentrate		
Volume (mL)	376 ± 9	373 ± 8
Platelets (10^9/unit)	328 ± 30	329 ± 25
pH (22°C)	7.04 ± 0.04	7.16 ± 0.05†
Swirl	Present	Present
Macroaggregates	Absent	Absent
Thawed platelets		
Volume (mL)	284 ± 14	284 ± 13
Platelets (10^9/unit)	246 ± 14	270 ± 19†
Platelet recovery freeze/thaw (%)	75 ± 4	82 ± 4†
pH (22°C)	7.35 ± 0.18	7.37 ± 0.18
Swirl	Absent	Absent
Macroaggregates	Absent	Absent
Microaggregates (number of events)	161 ± 26	134 ± 34†

* Values shown as mean ± standard deviation; n = 8 in each group.
† $p < 0.01$ compared to CPP.
CPP = cryopreserved platelet; expired-CPP = cryopreserved platelet frozen at Day 5/6 following collection.

Statistical analysis

Data were analyzed using statistical analysis software (Prism version 7, GraphPad Software, Inc.). Significance was calculated using paired, two-sided t tests, or repeated measures analysis of variance comparing fresh and expired units before and after freezing and thawing. Post hoc Bonferroni multiple comparisons were performed to determine specific differences at each time point. A result was considered significant with a p value of less than 0.01.

RESULTS

The volume and platelet content of the components were equivalent at the time of freezing, although the pH of the expired platelets was significantly higher (Table 1). Following thawing, the platelet content and postthaw recovery were higher in the units frozen at expiry (Table 1), compared to the control units frozen on Day 1 following collection. Although aggregates were not macroscopically observable (visible to the eye) in any units upon thawing,

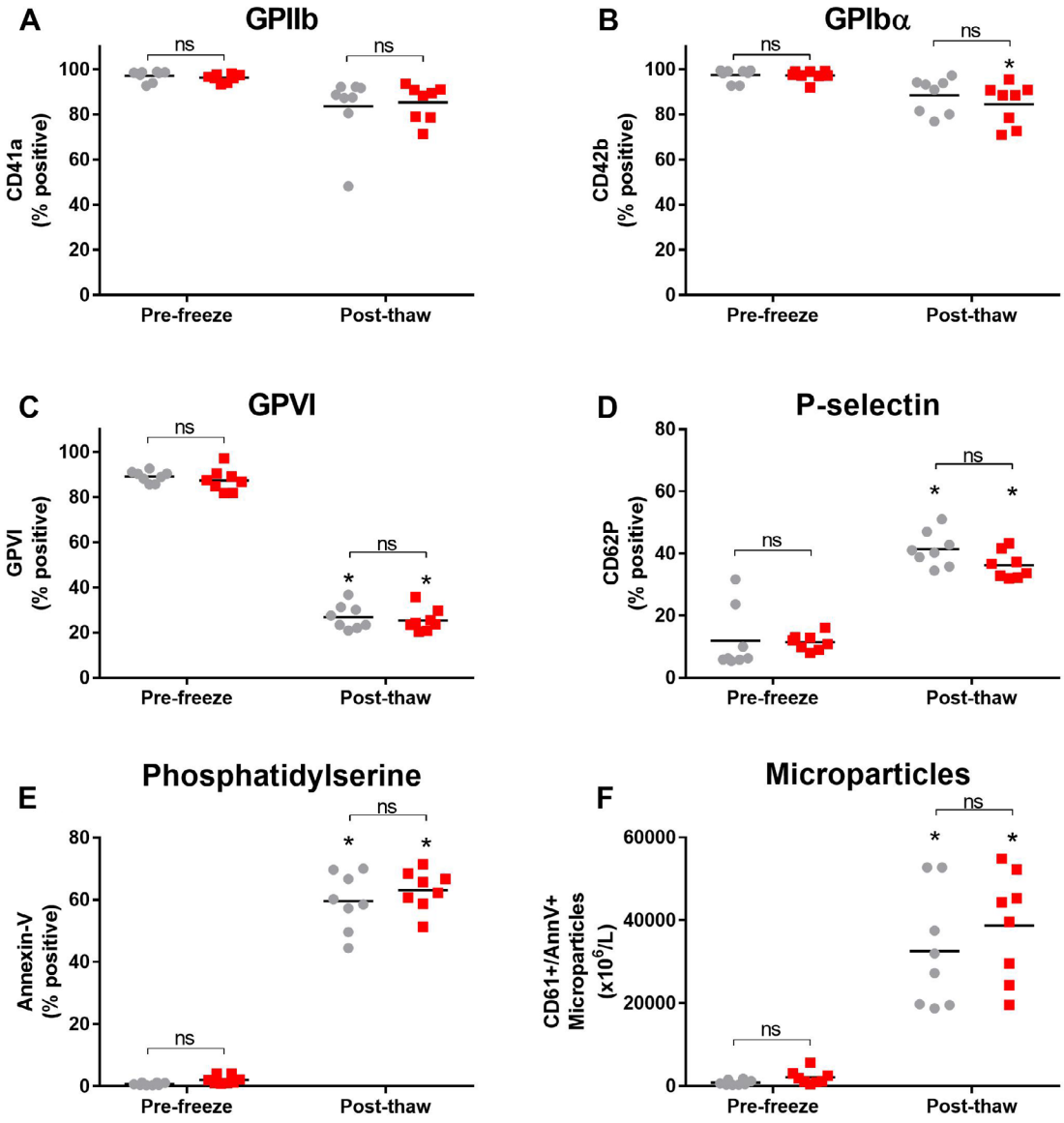

Fig. 1. Cryopreservation of expired platelet components does not negatively impact the thawed platelet phenotype. Platelets were sampled before cryopreservation (pre-freeze) and either cryopreserved on Day 1 (fresh; gray circle) or stored at RT until Day 5 or 6 (expired; red square) before cryopreservation. Cryopreserved platelets were thawed and reconstituted in plasma and sampled immediately (post-thaw). Platelets were stained with (A) CD41a-PE, (B) CD42b-PE, (C) GPVI-eFluor660, (D) CD62P-PE, or (E) annexin-V-FITC, and the proportion of positively stained platelets was measured by flow cytometry. (F) The absolute number of CD61+/annexin-V+ microparticles was enumerated by flow cytometry. Data shown as individual data points (n = 8 in each group) and mean (black line). *p < 0.01 compared to matched pre-freeze group; ns = no statistical difference. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2. Cytokine concentration in CPP components*

	Pre-freeze		Postthaw	
Cytokine	CPP	Expired-CPP	CPP	Expired-CPP
P-selectin (ng/mL)	15.3 ± 2.8	20.1 ± 3.2	56.9 ± 6.1‡	50.5 ± 8.0‡
RANTES (ng/mL)	32.5 ± 5.8	78.4 ± 23.5†	94.8 ± 16.7‡	72.1 ± 14.2
PF4 (ng/mL)	2610 ± 2498	6008 ± 3279†	9047 ± 1243‡	5419 ± 1432†

* Values shown as mean ± standard deviation; n = 8 in each group.
† Indicates $p < 0.01$ comparing CPP and expired-CCP at the same condition.
‡ Indicates $p < 0.01$ comparing prefreeze and postthaw.
CPP = cryopreserved platelet; expired-CPP = cryopreserved platelet frozen at Day 5/6 following collection; PF4 = platelet factor 4.

microaggregates were measureable by flow cytometry, with CPPs containing a significantly higher number than the expired-CPP components (Table 1).

There was no difference in the prefreeze phenotype of CPP or expired-CPP platelets (Fig. 1). Although there was a reduction in the proportion of platelets expressing GPIIb, GPIbα and GPVI following cryopreservation, the loss was equivalent in both groups (Fig. 1A-C). The percentage of platelets with surface expression of CD62P and phosphatidylserine increased following cryopreservation to a similar degree in both groups (Fig. 1D,E). A similar trend was observed regarding microparticles, where an increase in microparticle number was detected following cryopreservation, but the timing of freezing did not impact the number of microparticles generated (Fig. 1F).

As expected, an increase in soluble mediators (P-selectin, RANTES, PF4) was observed in the supernatant during platelet storage at RT (Table 2). Cryopreservation resulted in an increase in these three proteins in the CPP group, but the same trend was observed only for soluble P-selectin in the expired-CPPs. Interestingly, the concentration of RANTES and PF4 in the expired-CPPs was similar before and after cryopreservation (Table 2).

The length of storage before freezing did not negatively affect platelet function. The responsiveness of platelets to ADP stimulation was assessed by measuring PAC-1 binding. There was a reduction in agonist responsiveness during storage at RT for up to 6 days (Fig. 2A). However, while the anticipated loss of platelet responsiveness to agonist stimulation was observed following cryopreservation, it was not exacerbated by prolonged storage at RT. The hemostatic potential of the platelets was assessed using thromboelastography (Fig. 2B). The time to clot formation (R time) was reduced by cryopreservation, but the length of prefreeze storage time had little impact on this functional outcome. The kinetics of clot development (α-angle and K time) and the strength of the clot formed (maximum amplitude; MA) were not affected by storage at RT (data not shown).

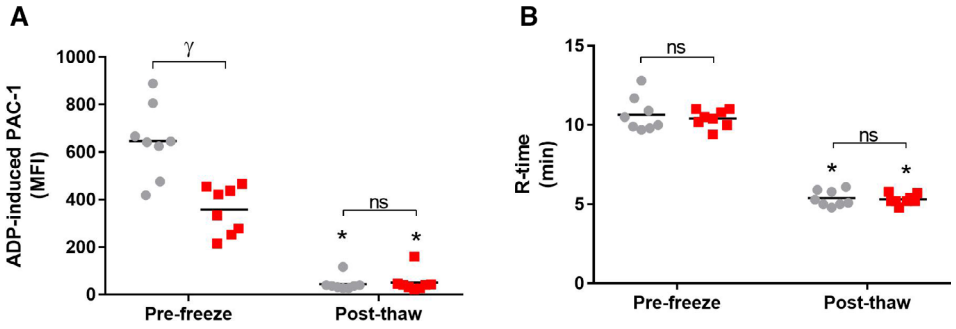

Fig. 2. Cryopreservation of expired platelet components does not negatively impact the functional responses of thawed platelets. Platelets were sampled before cryopreservation (pre-freeze) and either cryopreserved on Day 1 (fresh; gray circle) or stored at RT until day 5 or 6 (expired; red square) before cryopreservation. Cryopreserved platelets were thawed and reconstituted in plasma and sampled immediately (post-thaw). (A) Platelets were stimulated with ADP (20 µmol/L), stained with PAC-1-FITC, and the median fluorescence intensity (MFI) was measured by flow cytometry. (B) The R time (reaction time; time until clot formation) was measured using thromboelastography following recalcification and activation with kaolin. Data shown as individual data points (n = 8 in each group) and mean (black line). *$p < 0.01$ compared to matched pre-freeze group; γ = $p < 0.01$ compared to Day 1; ns = no statistical difference. [Color figure can be viewed at wileyonlinelibrary.com]

DISCUSSION

Platelet cryopreservation significantly extends the useable shelf life of platelets to years, thus providing an opportunity to stockpile platelets for future use. However, the requirement to freeze platelets as soon as practicable following collection, coupled with the short shelf life of liquid-stored platelets, may result in shortages of this component if unanticipated demand for platelet components occurs. The results of this study demonstrate that the standard in vitro quality indicators of CPPs are not adversely affected by freezing platelets following RT storage for their useable shelf life (5-6 days). In fact, the postthaw recovery was actually improved in the units that were expired at the time of cryopreservation.

Cryopreservation is known to result in the loss of a proportion of platelets, presumably due to cryo damage.[3,8,9] We hypothesize that the higher postthaw recovery observed in the expired-CPPs may be related to the reduced formation of microaggregates in these components. This is supported by the finding that reduced postthaw recovery has been associated with aggregate formation in pathogen inactivation–treated cryopreserved platelets.[4] Mechanistically, (micro)aggregate formation may be mediated by activated GPIIb/IIIa, as has been shown in cold-stored platelets.[5] Interestingly, the activatibility of GPIIb/IIIa before freezing was reduced in expired-CPPs, and was strongly associated with the improved postthaw recovery ($r = -0.5704$, $p < 0.0210$).

Cryopreserved platelets are characterized by specific phenotypic changes.[8–10] Following freezing and thawing, the platelets exhibited the features typical of cryo injury, including a loss of certain glycoproteins (GPIbα, GPIIb, GPVI), an increase in activation markers (phosphatidylserine and P-selectin), the release of microparticles, and reduction in the clotting time. Importantly, the platelets frozen after collection or following room temperature storage for 5 or 6 days were almost indistinguishable in relation to these postthaw quality parameters.

Cryopreserved platelet units have been shown to contain a higher concentration of α-granule–associated proteins in the supernatant compared to liquid-stored components.[11,12] This could occur through several mechanisms, including the release from α-granules, cryo damage during freeze/thawing, or from the plasma used for reconstitution. The trend for CPPs was consistent with previously published data.[12] In contrast, no increase in RANTES and PF4 was observed between prefreeze and postthaw conditions in the expired-CPP group. The mechanism for this may be due of the release of these mediators during the longer period of RT storage, before cryopreservation, thus reducing the internal stores available for postthaw release.

There has been a resurgence in interest in cryopreservation of platelets over the past decade.[2] Although CPPs have been transfused for prophylactic purposes,[2,13,14] the most prevalent indication for CPPs has been the treatment of active bleeding.[15–19] In the future, CPPs could represent one component in a multiproduct inventory where the choice of product is dictated by individual patient requirements. RT-stored platelets would likely remain the product of choice for prophylactic transfusions, while CPPs may be reserved for actively bleeding patients, particularly in locations where an RT inventory is not available, such as remote or military environments. Managing these distinct inventories would require careful planning so that the implementation of a frozen inventory does not negatively impact the availability of liquid-stored components. In most institutes, platelets in the supply chain have a shelf life of 5 to 7 days, whereas the usable shelf life is approximately 1 to 3 days less,[20] after taking into account the time taken for transport, manufacture, mandatory testing, and bacterial contamination screening. In Australia, the short shelf life combined with the unpredictable supply and demand of platelets results in unacceptable wastage rates of approximately 20%, primarily due to expiry.[21] The ability to freeze platelets at the end of the useable shelf life, rather than the start, would enable the continued supply of liquid-stored platelets and prevent wastage by reallocation of platelets approaching expiry to a frozen inventory if supply exceeds demand.

While this study presents some encouraging findings, it is limited to the investigation of in vitro parameters only. As such, clinical studies will be required to confirm the value of the approach. Further, as the shelf life of platelets in many countries is 7 days, rather than 5, investigation of whether platelets stored at RT for 7 to 8 days would be a suitable starting material for cryopreservation may also be worthwhile.

Overall, our data demonstrate that delaying cryopreservation does not negatively impact the in vitro platelet quality parameters assessed in this study. Thus, freezing platelets toward the end of their useable shelf-life may facilitate stockpiling of CPPs in times of excess without placing additional pressure on the donor pool. Additional studies are required to determine whether this logistic gain translates to a clinical benefit.

ACKNOWLEDGMENTS

LJ and DCM designed and supervised the conduct of the study. LJ, LW, SG, and BW performed the experimental work. LJ analyzed the data and wrote the manuscript. All authors contributed to writing, critically revised, and approved the final manuscript.

Sources of support

Australian governments fund the Australian Red Cross Blood Service for the provision of blood, blood products, and services to the Australian community.

Conflict of Interest

The authors have disclosed no conflicts of interest.

REFERENCES

1. Holley A, Marks D, Johnson L, et al. Frozen blood products: clinically effective and potentially ideal for remote Australia. Anaesth Intensive Care 2013;41:10-9.
2. Cohn CS, Dumont LJ, Lozano M, et al. Vox Sanguinis International Forum on platelet cryopreservation. Vox Sang 2017;112:e69-85.
3. Valeri CR, Srey R, Lane JP, et al. Effect of WBC reduction and storage temperature on PLTs frozen with 6 percent DMSO for as long as 3 years. Transfusion 2003;43:1162-7.
4. Waters L, Padula MP, Marks DC, et al. Cryopreservation of UVC pathogen-inactivated platelets. Transfusion 2019;59: 2093-102.
5. Getz TM, Montgomery RK, Bynum JA, et al. Storage of platelets at 4 degrees C in platelet additive solutions prevents aggregate formation and preserves platelet functional responses. Transfusion 2016;56:1320-8.
6. Wood B, Johnson L, Hyland RA, et al. Maximising platelet availability by delaying cold storage. Vox Sang 2018;113:403-11.
7. Johnson L, Coorey CP, Marks DC. The hemostatic activity of cryopreserved platelets is mediated by phosphatidylserine-expressing platelets and platelet microparticles. Transfusion 2014;54:1917-26.
8. Johnson L, Reade MC, Hyland RA, et al. In vitro comparison of cryopreserved and liquid platelets: potential clinical implications. Transfusion 2015;55:838-47.
9. Dumont LJ, Cancelas JA, Dumont DF, et al. A randomized controlled trial evaluating recovery and survival of 6% dimethyl sulfoxide–frozen autologous platelets in healthy volunteers. Transfusion 2013;53:128-37.
10. Six KR, Delabie W, Devreese KMJ, et al. Comparison between manufacturing sites shows differential adhesion, activation, and GPIbalpha expression of cryopreserved platelets. Transfusion 2018;58:2645-56.
11. Tynngard N, Wikman A, Uhlin M, et al. Haemostatic responsiveness and release of biological response modifiers following cryopreservation of platelets treated with amotosalen and ultraviolet A light. Blood Transfus 2019:1-8. [In press].
12. Johnson L, Tan S, Jenkins E, et al. Characterization of biologic response modifiers in the supernatant of conventional, refrigerated, and cryopreserved platelets. Transfusion 2018;58:927-37.
13. Gerber B, Alberio L, Rochat S, et al. Safety and efficacy of cryopreserved autologous platelet concentrates in HLA-alloimmunized patients with hematologic malignancies. Transfusion 2016;56:2426-37.
14. Schiffer CA, Aisner J, Wiernik PH. Clinical experience with transfusion of cryopreserved platelets. Br J Haematol 1976;34:377-85.
15. Khuri S, Healey N, Macgregor H, et al. Comparison of the effects of transfusions of cryopreserved and liquid-preserved platelets on hemostasis and blood loss after cardiopulmonary bypass. J Thoracic Cardiovasc Surg 1999;117:172-83.
16. Slichter SJ, Dumont LJ, Cancelas JA, et al. Safety and efficacy of cryopreserved platelets in bleeding patients with thrombocytopenia. Transfusion 2018;58:2129-38.
17. Noorman F, Van Dongen TT, Plat MJ, et al. Transfusion: −80 degrees C frozen blood products are safe and effective in military casualty care. PLoS One 2016;11:e0168401.
18. Bohonek M, Kutac D, Landova L, et al. The use of cryopreserved platelets in the treatment of polytraumatic patients and patients with massive bleeding. Transfusion 2019; 59:1474-8.
19. Reade MC, Marks DC, Bellomo R, et al. A randomized, controlled pilot clinical trial of cryopreserved platelets for perioperative surgical bleeding: the CLIP-I trial (Editorial, p. 2759). Transfusion 2019;59:2794-804.
20. Blake JT. Determining the inventory impact of extended-shelf-life platelets with a network simulation model. Transfusion 2017;57: 3001-8.
21. Fedele PL, Polizzotto MN, Grigoriadis G, et al. Profiling clinical platelet and plasma use to inform blood supply and contingency planning: PUPPY, the prospective utilization of platelets and plasma study. Transfusion 2016;56:2455-65.