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Frozen platelets

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ABSTRACT

The technical limitations on platelet shelf life and storage have driven research for alternatives, including cryopreservation of platelets. Over the past 60 years, product development has adopted freezing platelets in 6% dimethyl sulfoxide (DMSO) and storage in mechanical freezers at −80 °C for up to 2 years. Frozen platelets show a primed, hypercoagulable in vitro phenotype post-thaw when assayed using morphology, flow cytometry for marker expression, and thrombin capacity. In vivo studies show a role for frozen platelets in the maintenance of hemostasis and data from limited clinical trials show frozen platelets are safe and appear beneficial. As research continues to address the functional role of in vitro assays for clinical outcomes, frozen platelet product development represents a good alternative to room temperature platelets for many applications.

1. Introduction

Platelets for transfusion have a limited room temperature (RT) storage from five to seven days, generating a high percentage of platelet waste due to outdating. This coupled with required agitation and pathogen testing places a large burden on inventory management and the blood bank's ability to supply platelets. To reduce product waste and provide a stable supply of platelets, mechanisms to extend platelet shelf life by freezing have been active areas of research for over 60 years.

Clinical study of frozen platelet transfusion dates to 1956 [1]. Platelet concentrates were frozen in plasma (at either −15 °C or −30 °C) and stored for up to six weeks at −15 °C prior to transfusion to treat children (2–15 years old) with thrombocytopenia. Transitory hemostasis was observed in 62% of the transfusions, with no thrombosis or serious side effects [1]. This was followed in 1958 by another group showing hemostatic effects of frozen platelets in the absence of maintained circulating platelet counts [2]. Both results for frozen platelets were promising, however there were other reports with lackluster hemostatic benefits from cold platelets [3]. At this time, research emphasis was on efforts to improve circulation time and decreasing the number of transfusions; therefore, development of frozen platelet products slowed.

2. Frozen platelets

The intracellular ice crystals formed when freezing apheresis platelet units (APU) without cryoprotectants results in severe damage to the platelet and renders them useless for standard transfusion. The success of adding glycerol prior to freezing bull semen sparked studies of cryopreservation additives for platelets. Initial additive cocktails included dimethylacetamide (DMAC), dimethyl sulfoxide (DMSO), glycerol, and sugars (dextrose, lactose, levulose, maltose, sucrose, and xylose) at varying percentages [4–11]. As these cryopreservation techniques advanced platelet viability post-thaw, researchers observed at least a moderate circulating presence and post transfusion recoveries at 1 day in several animal models: 30% in dog [10], 78% in rabbit [5], and 70–87% in rat [4,6]. Additionally, clinical studies with frozen platelet products showed recovery rates at 1 day around 30% [7,8]. Noteworthy in these reports is the emphasis on platelet circulation time above any other benefits of frozen platelets, the priority in the 1960's.

During the 70's and early 80's a program developed aiming to stock autologous frozen platelets for transfusion of alloimmunized leukemia patients refractory to donor platelets under the direction of Charles Schiffer [12–15]. This body of work included more than 700 frozen platelet transfusions for more than 150 patients from 1976 to 1981 with ~50% post-transfusion recovery [15]. The 5 years of clinical data

Abbreviations: BCRC, Baltimore Cancer Research Center; BEST, Biomedical Excellence for Safer Transfusions; CABG, coronary artery bypass graft surgery; cGMP, current good manufacturing practice; CLIP, cryopreserved platelets versus liquid platelets trial; CPB, cardiopulmonary bypass; CPP, cryopreserved platelet product; DMAC, dimethylacetamide; DMSO, dimethyl sulfoxide; DoD, US Department of Defense; FDA, Food and Drug Administration; GPIbα, glycoprotein Ibα; ISBT, International Society for Blood Transfusion; LSP, liquid stored platelets; NBRL, Naval Blood Research Laboratory; PBB, Peripheral Blood Banks; RT, room temperature; US, United States; WB, whole blood

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generated by Schiffer's group showed that frozen platelet transfusions were safe and effective in treating chemotherapy induced thrombocytopenia.

Concurrently, programs such as Robert Valeri's in Boston at the Naval Blood Research Laboratory (NBRL) continued to fine tune cryopreservation methods, eventually settling on addition of 6% DMSO and storing in a freezer at −80 °C [16,17]. While appreciation of the effects of cold activation on platelets were decades away [18–21], Valeri's continued research revealed frozen platelets have a partially activated or primed phenotype in vitro [22–24]. Valeri's clinical trials in cardiopulmonary bypass (CPB) surgical patients revealed no adverse thrombotic events and that frozen platelet transfusions were superior to liquid stored platelets (LSP) at reducing blood loss [25]. Another frozen platelet advancement made by this group 5 years later was the development of the no-wash technique where most of the DMSO is removed prior to freezing [26]. The no-wash technique broadened environments for frozen platelet use by eliminating the need for equipment and extensive post-thaw product handling.

3. Cryopreserved platelet product (CPP) manufacturing

Sixty (60) years of efforts to preserve platelet function following cryopreservation and thawing have generated a stable and reproducible product with the addition of DMSO at a final concentration around 6%. In a current program funded by the US Department of Defense (DoD), cryopreserved platelet product (CPP) manufacturing processes use current good manufacturing practice (cGMP) protocols standard for blood processing and International Society for Blood Transfusion (ISBT) labeling for tracking into blood bank data systems in accordance with the Food and Drug Administration (FDA) and AABB standards. APUs under 2 days post-collection are used with the Valeri no-wash method [26]. Most of the DMSO is removed prior to freezing, giving a final CPP at about one-sixth the beginning volume of a standard APU. An APU at approximately 275 ml generates around 25 ml of CPP with a concentration of about 6.5×10^6 platelets/μl (Table 1). Currently manufactured products are stored at −80 °C in a mechanical freezer for up to 2 years. As long-term storage is a driving force behind CPP research and development, studies on the stability of 4-year stored product are in the pipeline.

Table 1
In vitro phenotype for 5-day LSP and CPP.

	5-day LSP	CPP
Platelet concentration (x 10^3/μl)	1521 +/− 144	6295 +/− 816
Microparticles (% of platelet events)	6 +/− 3	42 +/− 9
Morphology (Kunicki scoring) [28]	369 +/− 20	244 +/− 63
P-selectin (% of platelet events)	18 +/− 14	68 +/− 12
Phosphatidylserine exposure (% of platelet events)	7 +/− 4	73 +/− 4
TGA peak thrombin (nM) (SD)	72.3 (10.3)	159.6 (25.7)
time to peak (min) (SD)	21.9 (1.5)	11.6 (1.1)
lag time (min) (SD)	8.5 (0.6)	5 (0.2)
ETP (nM*min) (SD)	1483 (76)	1580 (142)
TEG EXTEM clotting time (sec)	161 +/− 12 *	115 +/− 19 *
maximum clot firmness (mm)	59 +/− 2 *	32 +/− 3 *
Aggregation (ADP + EPI) (%)	75 +/− 24	20 +/− 13
(Collagen) (%)	83 +/− 14	16 +/− 12

For TGA = thrombin generation assay with final concentration of 66×10^3 platelets/μl, 1 pM tissue factor in pooled fresh frozen plasma, ETP = endogenous thrombin potential. For TEG assays: EXTEM = ROTEM thromboelastometry analyzer with tissue factor and phospholipid activators in the presence of heparin inhibition [48]. For aggregation assay final concentrations: ADP = adenosine diphosphate at 10 μM, EPI = epinephrine at 5 μM, collagen at 10 μg/ml and platelet concentrations at 300×10^3 platelets/μl. LPS = liquid stored platelet, SD = standard deviation, *denotes whole blood depleted of native platelets with LSP or CPP added for the assay.

4. CPP in vitro properties

Not surprising, freezing and thawing processes damage CPP; however, approximately a 75% cell recovery is standard. Comparing the in vitro characteristics of post-thaw CPP to room temperature 5-day LSP reveals distinct differences between these two platelet types. Overall, when assayed using current parameters including morphology, flow cytometry for marker expression, as well as thrombin capacity (Table 1), CPP presents with a phenotype primed for clot formation compared to LSP.

4.1. CPP has an activated morphology

The increase in activation status of CPP can be appreciated with transmission electron micrographs (TEM, Fig. 1). As shown in Fig. 1a, LSP cells maintain good intracellular structure with alpha granules and an open canalicular membrane system. These platelets have the discoid shape (Fig. 1a, asterisks), minimal pseudopodia (Fig. 1a, arrows), and, while platelets are touching, there are no aggregates (Fig. 1a). This contrasts with CPP that have more pseudopodia (Fig. 1b, arrows), occasional platelets with centralized granules (Fig. 1b, closed arrowheads) and post-secretion cells with diminished granule content (Fig. 1b, carets). It is known that low temperatures increase platelet microparticle (PMP) production [27], which can be visualized in TEM of 2000 x G supernatant of CPP. Here CPP particles smaller than 500 nm are found (Fig. 1c). PMP flow cytometry assays demonstrate CPP contains a seven-fold increase in PMP (42%, Table 1) compared to LSP (6%, Table 1), consistent with the TEM observations. In contrast, similar preparation of LSP supernatant generated very few particles (data not shown), indicating release of granule, microparticles and platelet membrane fragments specifically in CPP. Functionally important for clinical use, CPP TEM also shows the absence of aggregation (Fig. 1b). CPP samples do contain spherical cells lacking intracellular architecture (Fig. 1b, open arrowheads) and these likely represent the 25% cell loss post-thaw found in CPP.

Using phase-contrast microscopy to appreciate the morphological differences population-wide also reveals an increased abundance of pseudopodia and spherical cells in CPP (Fig. 2b) compared to the primarily discoid shape of LSP (Fig. 2a). These differences can be quantified using the Kunicki system where points are assigned based platelet shape [28]. On this scale, the maximum score is 400 and a score above 300 denotes high viability (see LSP Table 1, Fig. 2a) [28]. While acceptable scores for CPP have not been established, generally scores over 200 are typical of good viability with CPP samples above the threshold at 244 (Table 1, Fig. 2b). These scores are consistent with the swirl test of platelet morphology where most LSPs will have a positive swirl appearance [29]. CPP post-thaw scored as positive or intermediate for swirl represent over 80% of units tested to date (data not shown).

4.2. CPP marker expression indicates activated state

The glycoprotein p-selectin (CD62p, GMP-140, PADGEM) is expressed on platelet alpha granules. With activation, platelet granules are exocytosed leaving p-selectin on the external platelet membrane. The indication of degranulated platelets by p-selectin on the external membrane has been used as a marker of platelet activation in flow cytometry [30]. Consistent with CPP's primed phenotype elucidated with the assays mentioned above, flow cytometry with an antibody to p-selectin, reveals a three-and-a-half-fold increase in activated/p-selectin positive platelets in CPP (68% positive, Table 1) compared to LSP (18% positive, Table 1).

In addition to the uptick of p-selectin in CPP, there is loss of membrane asymmetry and exposure of phosphatidylserine (PS) lipids to the outer membrane leaflet in platelet activation. Using bovine lactadherin to query for PS with flow cytometry [31], CPP presents a tenfold increase in the PS exposure compared to LSP (CPP = 73% and

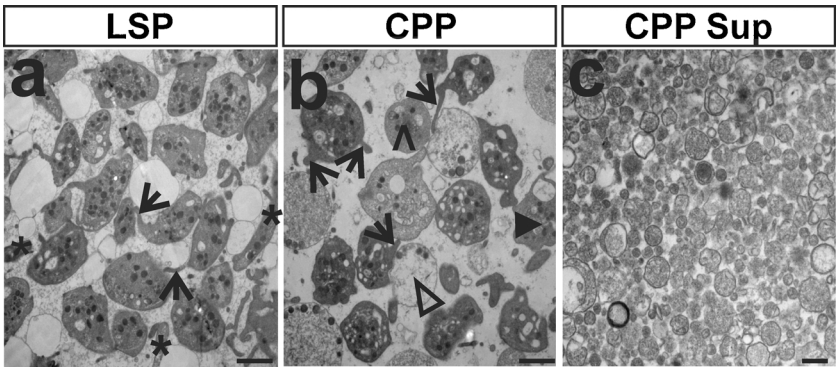

Fig. 1. Transmission electron microscopy of LSP and CPP and CPP supernatant reveal the increased activation morphology of CPP.
Transmission electron micrograph of (a) LSP at 7000×, (b) CPP at 7000× and (c) the CPP supernatant following high centrifugation to visualize microparticles at 195,000×. Plateles are marked with: asterisks = discoid shape, arrows = pseudopodia, closed arrowheads = centralized granules, carets = post-secretion, open arrowheads = spheres with little intracellular structure. Bar = 2 μm in a and b and 500 nm in c. Images from [34].

LSP = 7%, Table 1). The increase in negative phospholipids provides a scaffold that supports the assembly of tenase and thrombinase enzyme complexes central to platelet mediated thrombin generation. Consistent with this, there is an associated acceleration of thrombin generation in CPP discussed below (Table 1).

4.3. Thrombin generation capacity of CPP

Markers of platelet activation are elevated in CPP compared to LSP, however it is not known if this corelates with a functional increase in platelet behavior. Using TGA (thrombin generation assay), the maximum thrombin peak height is more than double for CPP (at nearly 160 nM compared to ˜75 nM for LSP, Table 1). In addition to the increased thrombin peak height, there is a corresponding acceleration in the time to peak height, where CPP takes half as long (11 min) as LSP (nearly 22 min), along with shortened reaction lag time (Table 1). While these readouts of TGA alluded to a primed, hypercoagulable CPP, the endogenous thrombin potential (ETP) of CPP (1580 nM*min) is only slightly higher than that of LSP (1483 nM*min, Table 1) suggesting cumulative clotting effects of CPP are not different than LSP. These data may point to the activated state of CPP which generates faster initial reaction progress, however the internal capability for thrombin generation of platelets in either form remains the same whether beginning in the activated CPP or 'native' LSP state. Future research will clarify this point.

Interestingly, there are functional data points potentially contradicting the concept of CPP being hypercoagulable. When looking at the thrombelastograph (TEG EXTEM) assay, CPP maintains an accelerated clotting time (CPP at 115 s compared to LSP at 161 s, Table 1), however the clot firmness generated by CPP is about half of LSP (CPP at 32 mm vs LSP at 59 mm, Table 1). Additionally, in aggregation tests for both ADP + EPI (adenosine diphosphate and epinephrine CPP = 20%, LSP = 75%) and collagen (CPP = 16% and LSP = 83%) agonists, CPP has a fourfold and fivefold diminished aggregation response respectively (Table 1). These contradictions may simply represent differences in the tests for platelet function in vitro. Further research into the translational significance of in vitro platelet clotting assays are needed.

To query clotting potential in a system more representative of in vivo physiology, research examining the phenotype of CPP under shear flow over rabbit aorta in a flow chamber (with CPP restored, platelet-depleted whole blood, WB) revealed CPP maintained half of the platelet deposition capability of WB restored with 5-day LSP under medium shear (CPP at 10% and LSP at 23% percent platelet coverage, Table 2, Fig. 3). Fibrin coverage is equivalent between both cell types at 21–25% (Table 2, Fig. 3), however CPP supported an almost three-fold higher prothrombin cleavage which is maintained over 10 min of shear flow (CPP at 606 pmol/L compared to LSP 221 pmol/L, Table 2, Fig. 3). Again, the functional use of CPP in vitro readouts such as this and how these correlate to clinical outcomes needs further characterization.

4.4. Other notable in vitro characteristics of CPP

The platelet receptor for von Willebrand factor binding, glypcoprotein 1bα (GP1bα, CD42b) is highly expressed on platelets and storage time results in a redistribution [32]. LSP maintains high expression (Fig. 4a Hi), however the cryopreservation process consistently generates a lower GP1bα expressing population, with approximately 50% of platelets falling within this gate (marked GP1bα Low in Fig. 4b). Other cryopreserved platelet products have a similar GP1bα Low population of cells generated post-thaw [33]. The significance of this downward shift in GP1bα is unknown. Based on the in vitro phenotype of CPP characterized to date, there appears to be little correlation with this population of platelets and in vitro function. Additional research is required to appreciate the primed phenotype of CPP with respect to marker expression, GP1bα low cells and the functional consequence of platelet behavior in both in vitro assays and ultimately for clinical applications.

5. CPP 24-h recovery and survival in transfusion

Autologous recovery and survival of CPP in healthy subjects has been evaluated compared to fresh autologous platelets transfused simultaneously following the method recommended by the Biomedical Excellence for Safer Transfusions (BEST) Collaborative, with some modifications for CPP [34–36]. As expected with an activated platelet product, CPP 24-hour recovery was 52% of fresh platelets (Table 3); however, they circulated for 7.5 days or 89% of fresh platelets [35]. This is consistent with good clinical outcomes previously reported when similar frozen platelet products were used. CPP recoveries are lower and could result in the need for increased transfusions, but it is also possible that CPP may allow physicians to manage patients with a lower platelet count due to the improved hemostatic potential. Further studies are needed to evaluate CPP clinical utility and these are currently in progress.

6. CPP phase 1 dose escalation

Advancing CPP into clinical trials has been challenging. In phase 1 clinical trials (Safety Study of Dimethyl Sulfoxide Cryopreserved Platelets; ClinicalTrials.gov Identifier: NCT02078284), patients with a WHO bleeding score ≥2 received from 0.5 to 3 units of CPP (n = 24) or 1 unit of LSP (n = 4). There were no related thrombotic or other serious adverse events and 5 mild transfusion-related adverse events. Among the CPP recipients 14/24 (58%) had improved bleeding scores, including 3/7 (43%) patients who had intracerebral bleeding. CPP post transfusion platelet increments were significantly less than LSP (Fig. 5); however, days to the next transfusion for CPP or LSP were the same [37]. A phase II trial in cardiac surgery patients undergoing coronary artery bypass surgery (CABG) is planned for the near future.

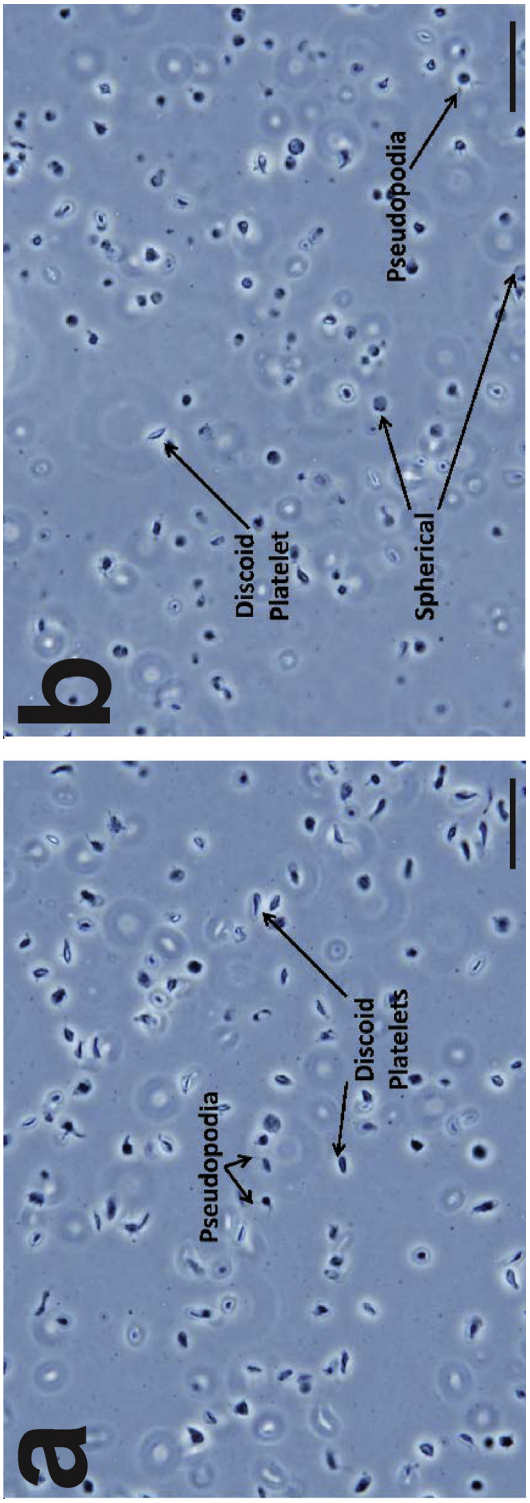

Fig. 2. Phase-contrast microcopy of LSP and CPP show morphological differences.
Phase-contrast microscopy of LSP (a) and CPP (b) at 100×. Cell shapes and components are marked as discoid, pseudopodia or spherical. Bars = 10 μm.

Table 2
In vitro clot properties for 5-day LSP and CPP hemostatic effects under shear.

	5-day LSP[a]	CPP[a]
Platelet coverage (%)	22.9 +/− 4.1	10.7 +/− 3.1
Fibrin coverage (%)	21.6 +/− 4.6	24.8 +/− 5.7
F1 + 2 Pre (pmol/L)	123 +/− 16	181 +/− 24
F1 + 2 increment (pmol/L)	221 +/− 67	606 +/− 216

[a] Platelets for both LSP and CPP were added to platelet depleted whole blood. Data from [48].

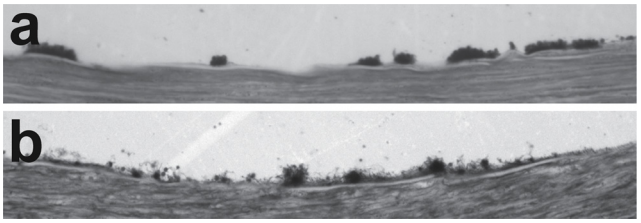

Fig. 3. Micrographs of platelet adhesion under flow conditions (Baumgartner model).
Microscopic fields of perfusion experiments for platelet depleted whole blood substituted with 200 × 10^9 platelets/L of 5-day LSP (a) or CPP (b) reveals CPP with decreased platelet coverage compared to LSP under shear (22 ml at 600/sec for 10 min). Images from [48].

7. International programs

7.1. Netherlands

In response to the pressures of blood banking, the Netherland Military Blood Bank (MBB) initiated a blood cryopreservation program in 2001 [38]. Frozen platelets, in addition to red blood cells (RBCs) and plasma were stored at −80 °C to replace WB transfusions. Development of this program heightened with the conflict in Afghanistan in 2006. The MBB used the −80 °C stored cells to generate Peripheral Blood Banks (PBB) which were mobilized to four military treatment facilities. Under this system clinicians from the Netherlands, Australia, Canada, Germany, Singapore, the United Kingdom, and the US used this resource. Retrospective analysis showed a decrease in hospital mortality from 44% (historic controls) to 10.5% with frozen platelet transfusion [38], providing evidence that use of frozen platelets is safe and effective.

7.2. The Australian Red Cross

As one third of Australia's population lives in remote areas with limited emergency services management of blood products is challenging [39]. With this pressure and the insight gained from Valeri's group and the MMB, the Australian Red Cross aims to expand the limited frozen platelet clinical data with a multicenter 'Cryopreserved Platelets Versus Liquid Platelets' (CLIP) Trial. Primary goals are feasibility and safety, with clinical efficacy measured by 28-day mortality, blood loss, transfusion requirement and thromboembolism [40]. While enrollment has been a challenge, 42 cardiac surgery patients have received frozen platelet transfusions with no adverse events [41]. Upon completion of the CLIP trial, the Australian Red Cross intends to move on to a pivotal phase 3 trial for frozen platelets.

Lacey Johnson and colleagues have characterized the in vitro phenotype of Australian Red Cross frozen buffy-coat-platelets. Like CPP from the US, Australian Red Cross's frozen product has a similar cell recovery post-thaw at around 73% and appear spherical in phase-contrast microscopy [42,43]. PMP is also increased in these cryopreserved platelets, although to higher extent at nearly twenty-fold [44], compared to CPP's seven-fold increase. These platelets, like CPP, also present with increased p-selectin expression, increased PS exposure and

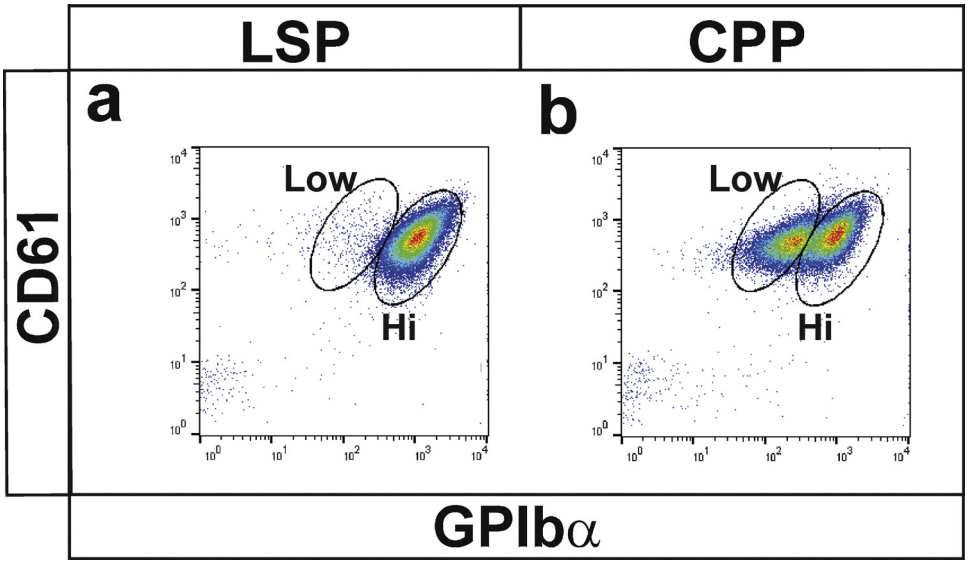

Fig. 4. GP1bα platelet populations.
Flow cytometry of LSP (a) and CPP (b) with CD61 and GP1bα antibodies reveal the generation of a GP1bα low fluorescent population specifically in CPP (marked Low in b).

Table 3
Transfused platelet recovery and survival.

	Platelet recovery (%)	Platelet survival (d)
Fresh	63 +/− 9	8.6 +/− 1.1
CPP	33 +/− 10	7.5 +/− 1.2
P	< .0001	< .0001
% of fresh	52 +/− 12	89 +/− 15

Data reported as mean +/− standard deviation, n = 32.

the generation of a GP1bα low cell population [42,43]. Furthermore, the in vitro cell behavior of frozen buffy-coat-platelets showed similar trends found in CPP where ADP and collagen aggregation are decreased, and in thrombelastography, the time to clot formation and clot strength are diminished [45]. These similarities in post-thaw cell behavior of buffy-coat and APU cryopreserved cells along with current clinical data underpins the comparability and stability of these products for clinical use. To this end, Biomedical Excellence for Safer Transfusion (BEST) Collaborations such as the study 'Resuspension of Cryopreserved Platelets in PAS' under the direction of Dumont and Johnson aim to further development of frozen platelet products [46].

7.3. Other international programs developing frozen platelet products

In addition to efforts in the US and Australia, there is ongoing research and development globally for successful cryopreservation and transfusion of platelets. Under the direction of Milos Bohonek, a program in the Czech Republic has transfused 163 units of frozen APU to 53 heavily bleeding patients in a randomized clinical trial [47]. Interestingly in this study to achieve hemostasis, the quantity of platelets transfused was significantly higher when using conventional LSP than the frozen counterpart [47]. Poland generates and transfuses between 11,000–13,000 frozen platelet units annually [47]. Other countries generating frozen platelet products on a smaller scale include: Switzerland with 40 autologous units for 9 patient transfusions over 10 years, Spain making 24 units and using 3 in transfusions and France maintains a reserve of frozen platelets for alloimmunized patients [47]. Belgium, Brazil, Canada and Singapore manufacture frozen platelets,

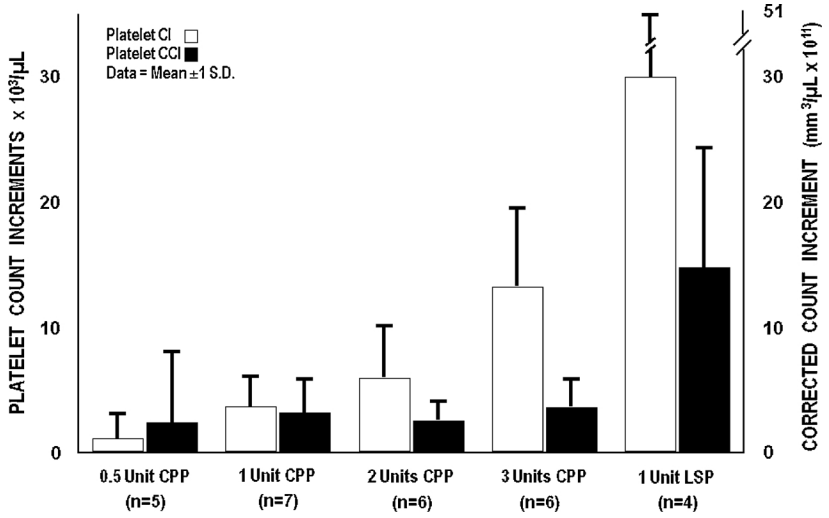

Fig. 5. CPP dose escalation.
Platelet count increments (left axis) and corrected count increment (right axis) for Phase I CPP dose escalation trial [37].

however currently use is exclusively for research purposes [47]. The cumulative data from all these efforts for processes to manufacture frozen platelets, storage conditions and times, resuspension protocols and transfusion practices will contribute to the generation of much-needed alternate platelet components for LSP transfusions.

8. Summary

The storage requirements for platelets and the short shelf life is a rich area of research for the development of alternative cell therapies. One approach over the past 60 years is in cryopreservation techniques. These have been met with heightened interest following twenty first century military conflicts generating international involvement in research and frozen platelet product development. Frozen products from the US and Australia show similar in vitro 'activated' phenotypes using current assays including morphology, marker expression assessed by flow cytometry and thrombin potential. In vivo animal studies support a role for frozen platelets in maintaining homeostasis. In the clinical setting, transfusion of frozen platelets has been safe and represents a promising approach for the development of life-saving alternatives to cumbersome and limited room temperature stored platelets.

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Conflict of interest

LJD has received research support from the US Army Medical Research and Material Command for the development and support of cryopreserved platelets. KK has declared no conflict of interest.

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