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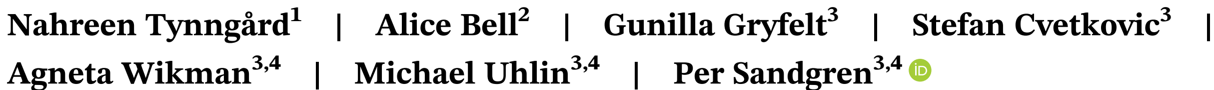
BLOOD COMPONENTS

TRANSFUSION

Cryopreservation of buffy coat derived platelets: Paired in vitro characterization using uncontrolled versus controlled freezing rate protocols



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Abstract

Background: Cryopreserved platelets show a reduced recovery and viability after freezing and thawing including several ultrastructural and phenotypic deteriorations compared with liquid-stored platelets. It is suggested that using Controlled-Rate Freezing (CRF) can reduce variability and optimize the functionality profile for cells. The objective of the study is to compare cellular, metabolic, phenotypic and functional effects on platelets after cryopreservation using different freezing rate protocols.

Study Design and Methods: To evaluate the possible effects of different freezing rate protocols a two-experimental study comparing diverse combinations was tested with a pool and split design. Uncontrolled freezing of platelets in materials with different thermal conductivity (metal vs cardboard) was evaluated in experiment 1. Experiment 2 evaluated uncontrolled vs a controlled-rate freezing protocol in metal boxes. All variables were assessed pre and post cryopreservation.

Results: Directly after thawing, no major differences in platelet recovery, LDH, ATP, $\Delta\psi$, CD62P, CD42b, platelet endothelial cell adhesion molecule and sCD40L were seen between units frozen with different thermal conductivity for temperature. In contrast, we observed signs of increased activation after freezing using the CRF protocol, reflected by increased cell surface expression of CD62P, PAC-1 binding and increased concentration of LDH. Agonist induced expression of a conformational epitope on the GPIIb/IIIa complex and contribution to blood coagulation in an experimental rotational thromboelastometry setup were not statistically different between the groups.

Conclusion: The use of a uncontrolled freezing rate protocol is feasible, creating a platelet product comparable to using a controlled rate freezing equipment during cryopreservation of platelets.

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1 | INTRODUCTION

Platelet concentrates are currently stored at room temperature for a limited time, 5 to 7 days after collection and preparation. Cryopreserved platelets, hyper-concentrated in 5%-6% dimethyl sulfoxide (DMSO) can extend the storage period and thereby bridging inventory shortages of conventionally liquid-stored platelets as well as functioning as backup stock in crisis situations. However, such cryopreserved platelets show a reduced recovery and viability after freezing and thawing including several ultrastructural, phenotypic, and functional deficiencies compared with liquid-stored platelets.[1,4] Given the stakes in the cell-based industry, using uncontrolled freezing protocols might not always provide an adequate level of control for optimal cell performances. Temperature profiles experienced by cells during cryopreservation can be highly variable from vial to vial as well as batch to batch. Such a high level of variability might have detrimental and significant consequences on cell behavior upon thawing.[5]

Using Controlled-Rate Freezing (CRF) might reduce the variability and optimize the functional profile for cells, which is desirable. A programmable freezing profile may also contribute to further optimization of cryopreservation profiles.[6] In the field of cell cryopreservation, a CRF currently provides a controlled platform aimed to achieve accuracy, consistency, and reproducibility at critical stages of a freezing process. However, this procedure is time consuming and requires equipment and staff with specific expertise. In contrast, using an uncontrolled freezing process has the advantages of economy and simplicity and can often be adequate.[7,8]

A general observation in the cryopreservation of cells and other biologic systems is that each system seems to have a specific optimal freezing rate, with decreased survival at freezing rates that are too low (slow-freezing damage) or too high (fast-freezing damage).[9–12] Consequently, the optimal freezing rate seem to fall in a range that is neither too fast nor too slow. In general, the optimal freezing rate of cells seems to be largely determined by their volume and their membrane surface area (volume to surface area ratio), and by the permeability of the membrane to water and to the cryoprotectant.[9,13] However, it has been suggested that the change from a liquid to a crystalline form during the freezing process results in and develops thermodynamic release of fusion heat. This phenomenon may cause detrimental cellular effects and are commonly referred to as the latent heat of fusion.[14] The controlled freezing rate protocol used in this study is therefore aimed to allow enough efflux of water to minimize the chance of intracellular ice formation without maximal dehydration of the platelets and to counteract the risk for the potential phenomenon latent heat of fusion.

To evaluate the possible effects of different freezing rate protocols we set up a 2-experimental study comparing diverse combinations tested with a pool and split design. The objective of the experiments is to compare cellular, metabolic, phenotypic and functional effects on platelets after cryopreservation with different freezing rate protocols. All variables were followed pre and post cryopreservation.

2 | MATERIAL AND METHODS

2.1 | Ethical aspects

Whole blood (WB) is collected from healthy donators who gave their informed consent in accordance with the Karolinska University Hospital Institutional guidelines. In this project, all units were anonymized, thus no code key will remain that can track back the samples to its donor. All material included in the study were used during the set time and were not stored in biobank. Therefore, no additional ethical permit was needed for this project.

2.1.1 | Preparation of platelet concentrates and general overview of experiment 1 and 2

Whole blood (WB) units with a volume of 450 ± 45 mL were collected from normal blood donors who gave their informed consent in accordance with our institutional guidelines. The blood was stored for at least 2 hours (h) on butanediol cooling plates to reduce the temperature to approximately 20°C before further processing. BC was separated on the day of collection by a hard-spin centrifugation (2700 × g for 10 minutes) on a Macospin centrifuge and a separation step utilizing a Macopress Smart (MacoPharma, Mouvaux, France) following the blood bank standard operating procedures. After overnight storage, BC's from eight donors with identical blood groups were sterile connected to a pooling bag (Kansuk IPP pooling and leucodepletion set). Platelet separation and leucodepletion by filtration were performed using a soft-spin centrifugation (speed 1100 rpm for 9 minutes) on a Macospin centrifuge and a Macopress separator (Macopharma, France). Platelet samples (10 mL) were removed from the double dose (DD)-unit by a sterile connection device (TSCD-II, Terumo BCT) and used to immediately assess the in vitro pre-freezing quality. This manipulation was repeated to create six or eight replicates of DD platelet units that were then split into two equal units of PC's for cryopreservation. In experiment 1, a fast-uncontrolled freezing rate protocol (FU) was compared to

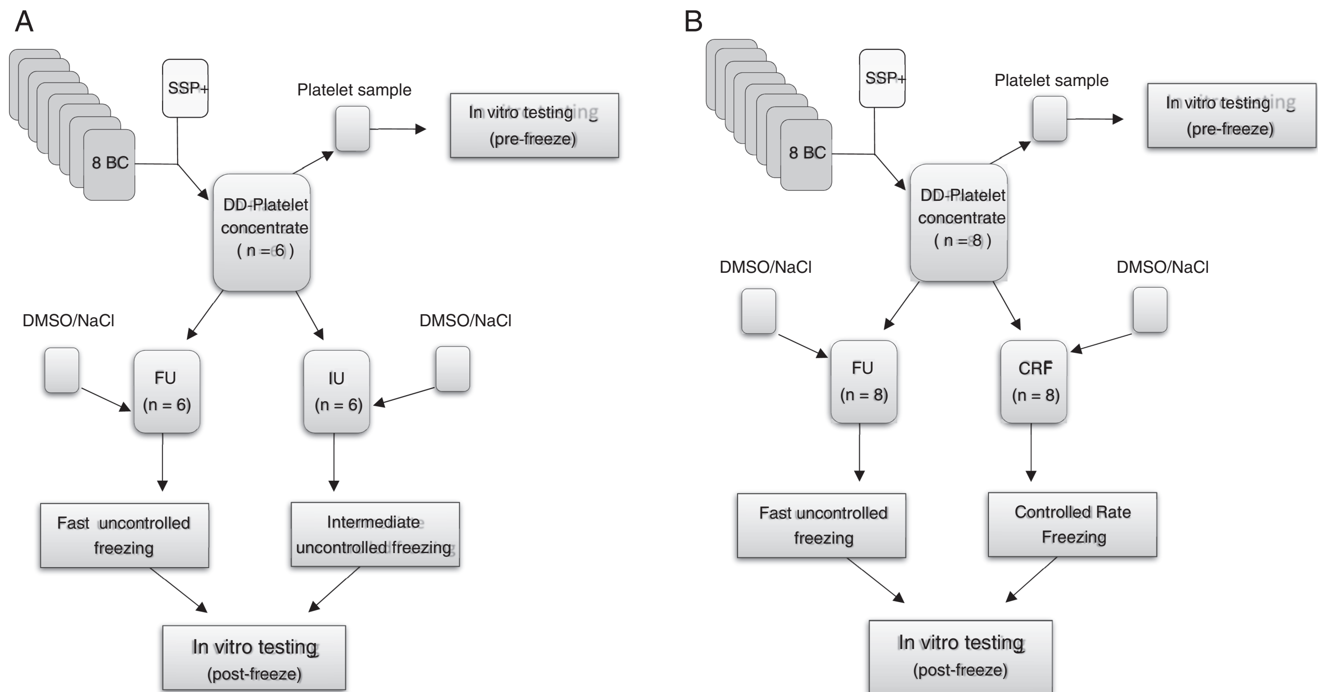

FIGURE 1 General overview of the study designs. (A) Experiment 1. Illustration of six replicates of DD platelet units that were split into two equal units of PC's followed by cryopreservation with a fast-uncontrolled freezing rate protocol (FC) vs an intermediate uncontrolled freezing rate protocol (IC). (B) Experiment 2. Illustration of eight replicates of DD platelet units that were split into two equal units of PC's followed by cryopreservation with a fast-uncontrolled freezing rate protocol (FC) vs a controlled freezing rate protocol (CRF)

an intermediate uncontrolled freezing rate protocol (IU) as shown in Figure. 1A. In experiment 2, a fast-uncontrolled freezing rate protocol (FU) was compared to a controlled freezing rate protocol (CRF) as shown in Figure 1B.

All cryopreserved platelets were kept frozen in the range of 1 to 3 months. The platelets were then thawed in a thawing bath (Sub Aqua Pro, Nordic Biolabs, Stockholm, Sweden) maintained at 37°C for 1 minute. The thawed platelets were sterile docked and gently reconstituted with either 100% of pooled compatible fresh thawed plasma (experiment 1) or a combination of 40% plasma and 60% SSP+ (experiment 2), all targeted to a final volume of approximately 200 mL. All thawed and reconstituted units were kept at room temperature preceding analysis.

2.1.2 | Experiment 1: Cryopreservation and freezing of platelets in a material with different thermal conductivity for temperature

Platelets were transferred to a freezing bag (Macopharma) using a sterile connection device.

(Terumo BCT). A mixture of 25% DMSO/NaCl (50 mL) was then sterile docked, and the solution added to the platelet concentrates. After centrifugation at 1200 × g for 10 minutes, as much as possible of the supernatant was removed, leaving on average 0.5 mL freezing medium in approximately 10 mL of platelet suspension. The aim of this procedure was to eliminate the need for post-thaw washing of DMSO as previously described.[15] The freezing bags, containing approximately 10 mL of platelet suspension (5% DMSO), were immediately frozen with a fast-uncontrolled freezing rate protocol (FU) or an intermediate uncontrolled freezing rate protocol (IU) (Figure 1A). The uncontrolled freezing protocols comprises the act of placing the platelet suspension either in sheet metal boxes (Ninolab, Stockholm, Sweden) in the FU-arm or cardboard boxes (Nordic Biolabs, Stockholm, Sweden) in the IU arm and then directly deposited in the freezer (−80°C). The uncontrolled freezing rates per min were estimated based on measuring points obtained from equipment (Padwico, Duo Display) which meets the ISO/IEC 17025 requirements. All temperature measurements were pairwise (FU vs IU) performed in triplicate and are reported as mean ± SD in two intervals. Interval I (FU); −9.7°C ± 0.6°C per min from room temperature to − 5°C. Interval II (FU); −6.3°C ± 0.6°C per minute from −5°C to −40°C. In the (IU) arm interval I; −5.3°C ± 0.3°C per min from room temperature to −5°C. Interval II (IU); −4.3°C ± 0.6°C per minute from −5°C to −40°C.

2.1.3 | Experiment 2: Cryopreservation and freezing of platelets using a fast-uncontrolled freezing rate protocol vs a slow-controlled freezing rate protocol

Eight ABO identical BC's obtained from routine production were pooled with the subsequent platelet separation process identical to the one described for experiment 1. This manipulation was repeated to obtain eight DD platelet concentrates for cryopreservation. The units were immediately frozen in sheet metal boxes (Ninolab) at −80°C. One of each DD unit was cryopreserved using the uncontrolled fast freezing protocol (FU) and the other unit cryopreserved in the controlled rate freezer Kryo 560 (Planer), Middlesex, UK in accordance with the manufacturer's instructions, referred to in the study design (Figure 1B) as the controlled freezing rate protocol (CRF). The cryopreservation procedure and the FU protocol was identical as described for experiment 1. The CRF protocol consists of eight controlled rate stages following start temperature at +2°C. Stage I; −1°C per min from +2°C to −5°C. Stage II; Hold 15 minutes at −5°C. Stage III; −25°C per min from −5°C to −47°C. Stage IV; Hold 1 minute at −47°C. Stage V; +10°C per min from −47°C to −13°C. Stage VI; −1°C per min from −13°C to −40°C. Stage VII; −10°C per min from −40°C to −90°C. Stage VIII; Hold 10 minutes at -90°C preceding deposition in the freezer (−80°C).

2.1.4 | In vitro analysis

Metabolic, functional phenotypic properties as well as the degree of pre-apoptotic events of all units were overall analyzed pre-freezing (on Day 2 from collection), and post-freezing on Day 0 (2 hours after thawing). Table 1 shows the in vitro variables investigated in experiment 1 and 2, respectively.

TABLE 1 In vitro variables assessed in experiment 1 and experiment 2

Variable	Experiment 1	Experiment 2
Acidity	pH	pH
Cytoplasmic leakage	LDH	LDH
Metabolic variables	Glucose, ATP, Δψ	Glucose, ATP, Δψ, bicarbonate
BRMs		sCD40L
Phenotypic variables		CD42b, CD62P, GPVI, PECAM-1
Platelet function		ROTEM, PAC-1[a]

Note: Lactate dehydrogenase (LDH), adenosine tri phosphate (ATP), mitochondria transmembrane potential (Δψ).

[a]Platelet function in terms of agonist induced activation of the GPIIb/IIIa recptor was assessed through binding of PAC-1. Spontaneous activation of the GPIIb/IIIa receptor was also assessed by PAC-1 binding.

2.1.5 | Analysis of cell count, recovery, disintegration, and energy generation

Cellular recovery post-freezing of platelet counts was measured using the CA 620 Cellguard (Boule Medical). The extracellular lactate dehydrogenase (LDH) activity, a marker for cell disintegration, was sampled from the supernatant and measured with a spectrophotometric method (Sigma Aldrich kit 063 K6003; Spectrophotometer Jenway 6500). pH (at 37°C) and the extracellular metabolic environment (glucose and bicarbonate) was studied by use of routine blood gas equipment (ABL 800, Radiometer). Total adenosine triphosphate (ATP) concentration, a marker of anaerobic as well as aerobic energy generation, was measured using ATP kit SL 144-401 (BioThema Luminescent Assays). Platelets (in duplicates) were diluted 1:10 0.6% trichloroacetic acid (0.73 M TCA, H_2O Sigma Aldrich) and incubated for 15 minutes on ice. TCA-treated platelets were then diluted 1:81 with Tris-EDTA-buffer (0.1 M Tris[hydroxymethyl] aminomethane, 2 mM EDTA, adjusted to pH 7.75 with acetic acid). All pre- and postfreeze samples were frozen at −60°C and analyzed together in batch. The frozen samples were thawed in room temperature and 40 μL thawed sample was added in triplicates to a 96-well microplate (Biothema) with 120 μL Tris-EDTA-buffer. A total of 40 μL ATP Reagent SL (D-luciferin, luciferase, magnesium ions, inorganic pyrophosphate and bovine serum albumin) was added to all samples. Light emission corresponding to sample ATP levels (μmol/10^{11} platelets) was measured with a luminometer (Orion Microplate, Berthold Detection Systems GmbH, Pforzheim, Germany).

2.1.6 | Analysis of functional, phenotypic, and apoptotic markers

To determine the background activation level preceding stimulation and responses to weak as well as to strong agonists, the expression of a conformational epitope on the GPIIb/IIIa complex of activated platelets was assessed using the FITC-conjugated monoclonal antibody PAC-1 (IgM, Becton Dickinson) as readout. For response purposes, unfixed platelets (10^8/mL) were incubated with 20 μM ADP, or 50 μM collagen (Sigma Aldrich) at 37°C for 15 minutes preceding the staining with PAC-1 at 20°C

for 20 minutes in the dark. Flow cytometric analyses for PAC-1; CD62P (Beckman Coulter); a marker for activation; CD42b (Beckman); a marker of adhesive capability; and PECAM-1 (Sigma), a representative signaling endothelial cell adhesion Molecule, CD61-PE conjugated (Beckman Coulter), a marker detecting the fibrinogen receptor complex and GPVI-FITC conjugated (Pharmingen BD Biosciences, San Jose, USA) for collagen receptor detection) were all demonstrated using a CytoFLEX Flow Cytometer (Beckman Coulter Life Sciences, Indianapolis, USA). Changes in the platelet mitochondria transmembrane potential (Δψ) were measured using the mitochondrial permeability transition detection kit MitoPT JC-1 (Immuno-Chemistry Technologies, LCC, Bloomington, MN, USA). All samples from all groups (1×10^6 platelets/mL) were stained with the MitoPT JC-1 dye reagent at 37°C for 15 minutes. The stained cells were then washed twice in 2 mL and 1 mL 1xAssay-buffer (ImmunoChemistry Technologies) before immediate analysis with CytoFLEX Flow Cytometer (Beckman Coulter). Maintenance of Δψ is expressed as JC-1-positive platelets. In all flow cytometry analysis, a total of 10.000 cells were counted and gating was set to target 100% of cells.

2.1.7 | Thromboelastometry

For assessing platelet function in the coagulation cascade a ROTEM analysis was performed. All platelet samples were diluted in equivalent AB plasma to a concentration comparable to human levels (200×10^9/L) and analyzed using a ROTEM delta 3000 device (TEM International, GmbH, Munich, Germany). Subsequently, EXTEM reagents with tissue factor activation, were analyzed according to the manufacturer's instructions. CT (clotting time) which reflects the time until the clot starts to form; influenced by clotting factors and anticoagulants, clot formation time (CFT), namely the time that clot takes to increase from 2 to 20 mm above baseline and maximum clot firmness (MCF) which reflects the maximum tensile strength of the thrombus were recorded prefreezing and postthawing (within 2 hours).

2.1.8 | Analysis of the biologic response modifier sCD40L

For the BRM analysis, samples from the PCs were collected in citrate theophylline adenosine dipyridamole

TABLE 2 In vitro properties of the platelet units in experiment *1* before and after cryopreservation

Variable	Pre CP	Post CP FU	Post CP IU
Number of PLTs ($\times 10^9$/unit)	264 ± 3	167 ± 30*	172 ± 26*
pH (37°C)	6.91 ± 0.03	7.09 ± 0.04*	7.07 ± 0.03*
Extracellular LDH (%)	5.4 ± 2.7	25.4 ± 3.1*	28.7 ± 3.7*
ATP (μmol/10^{11}PLTs)	7.63 ± 0.26	3.26 ± 0.98*	3.47 ± 0.93*
Δψ (% JC-1+ PLTs)	95 ± 3	58 ± 7*	61 ± 5*
sCD40L (pg/mL)	2714 ± 854	102 798 ± 2339*	118 236 ± 2468*

Note: Data is presented as mean ± SD of n = 6. Results are presented pre cryopreservation (CP) (ie, on day 2 of storage before CP) and post CP 2 hours after thawing for the uncontrolled fast cooling (FU) protocol and uncontrolled intermediate cooling (IU) protocol. The units were resuspended in 100% plasma after CP.
*$P < .05$ compared to pre CP for FU and IU platelets, respectively.

TABLE 3 Basic variables of the platelet units in experiment 2 before and after cryopreservation

Variable	Pre CP	Post CP FU	Post CP CRF
Number of PLTs (x 10^9/unit)	307 ± 37	194 ± 31*	211 ± 29*
pH (37°C)	6.91 ± 0.05	7.08 ± 0.02*	7.09 ± 0.02*
Glucose (mmol/L)	7.9 ± 0.7	7.1 ± 0.2	7.5 ± 0.3
Bicarbonate (calculated)	9.3 ± 0.4	6.9 ± 0.4*	7.2 ± 0.7*
Extracellular LDH (%)	4.2 ± 1.8	22.5 ± 5.9*	29.2 ± 6.4*, **
ATP (μmol/10^{11}PLTs)	6.1 ± 1.2	2.9 ± 0.5*	2.5 ± 0.4*

Note: Data is presented as mean ± SD of n = 8. Results are presented pre cryopreservation (CP) (ie, on day 2 of storage before CP) and post CP 2 hours after thawing for the uncontrolled fast cooling protocol (FU) and controlled freezing rate protocol (CRF). The units were resuspended in 40% plasma and 60% SSP+ after CP.
*$P < .05$ compared to pre CP for FU and CRF platelets, respectively.
**$P < .05$ for CRF compared to FU.

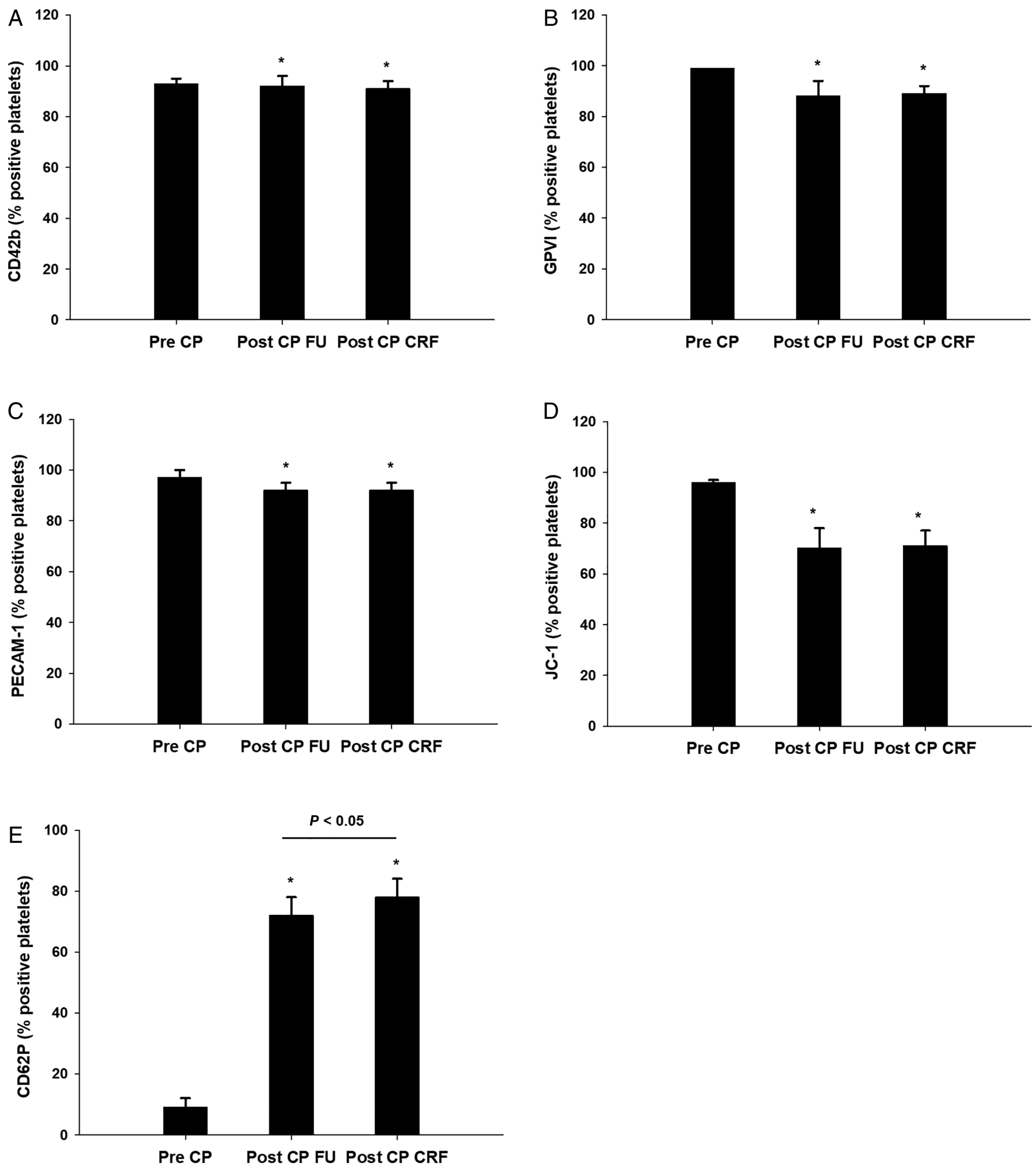

FIGURE 2 Expression of platelet receptors and activation markers in experiment 2. CD42b (A), GPVI (B), PECAM-1 (C), mitochondrial transmembrane potential (Δψ) determined by JC-1 binding (D) and CD62P (E) pre cryopreservation (CP) (ie, on day 2 of storage before CP) and post CP 2 hours after thawing for the fast uncontrolled freezing rate protocol (FU) and controlled freezing rate protocol (CRF). Results are presented as mean + SD (n = 8). $^{*}P < .05$ compared to pre CP for FU and CRF, respectively. Significant differences between FU and CRF platelets are also indicated

(CTAD) tubes, posed on the ice bath. The samples from the collected PCs were then centrifuged at 2500 g per relative centrifugal force (Eppendorf 5810R) for 30 minutes at 10°C, and the supernatants were stored in aliquots at −80°C pending analysis. sCD40L concentrations were determined for experiment 1 with commercial ELISA kit

(Quantikine, DCDL40) in accordance with the manufacturer's (R&D Systems Inc., Minneapolis, MN, USA) recommendations. sCD40L was prediluted 1:5 to fit the standards provided by the manufacturer. No additional samples were taken from the supernatant for detection of potential filtration of extracellular vesicles or from the plasma used for reconstitution. All measurements were performed on the Spectra Max 190 Micro Plate Reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm.

2.1.9 | Statistical analyses

The mean values and standard deviations are given unless otherwise indicated. Repeated measures analysis of variance including post hoc test Fischer's LSD test was performed separately on experiment 1 and 2 to determine the statistical significance of the differences between mean values. The analyses were carried out using Statistica software, v.12 StatSoft, Inc. 1984-2013 (SPSS).

3 | RESULTS

3.1 | Experiment 1: Cryopreservation and freezing of platelets in a material with different thermal conductivity for temperature

This experiment compared two uncontrolled freezing rate protocol by use of metallic (fast uncontrolled, FU) and cardboard boxes (intermediate uncontrolled, IU).

Cryopreservation reduced the number by approximately 30%-40% of platelets for both protocols (Table 2). The platelet recovery was 63 ± 4% for FU and 62 ± 5% for IU. Both protocols resulted in a similar change in the in vitro variables after cryopreservation including a small increase in pH and an increase in extracellular LDH. Cryopreservation with both protocols also caused a reduction in $\Delta\psi$ and ATP (Table 2). The level of the BRM sCD40L was found to increase significantly after cryopreservation with both protocols (Table 2).

3.2 | Experiment 2: Cryopreservation and freezing of platelets using a fast-uncontrolled freezing rate protocol vs a slow controlled freezing rate protocolT

The number of platelets decreased after cryopreservation with both protocols (Table 3). The platelet recovery was 66 ± 7% for FU and 73 ± 4% for CRF protocol. Also, in this experiment, pH was slightly increased after CP. The glucose concentration was not altered by either procedure whereas bicarbonate was decreased by both procedures (Table 3). Both freezing rate protocols resulted in an increase in extracellular LDH, but to a higher degree for CRF platelet units. ATP was decreased to a similar degree for both protocols (Table 3).

The percentage of platelets expressing CD42b, GPVI and PECAM was only slightly decreased after cryopreservation (Figure 2). $\Delta\psi$ was also found to be reduced by cryopreservation and to a similar degree for both cryopreservation protocols (Figure 2). We observed signs of increased activation after freezing and thawing in the controlled freezed platelet units, reflected by increased cell surface expression of CD62P (Figure 2) as well as PAC-1 binding (Figure 3), compared to uncontrolled

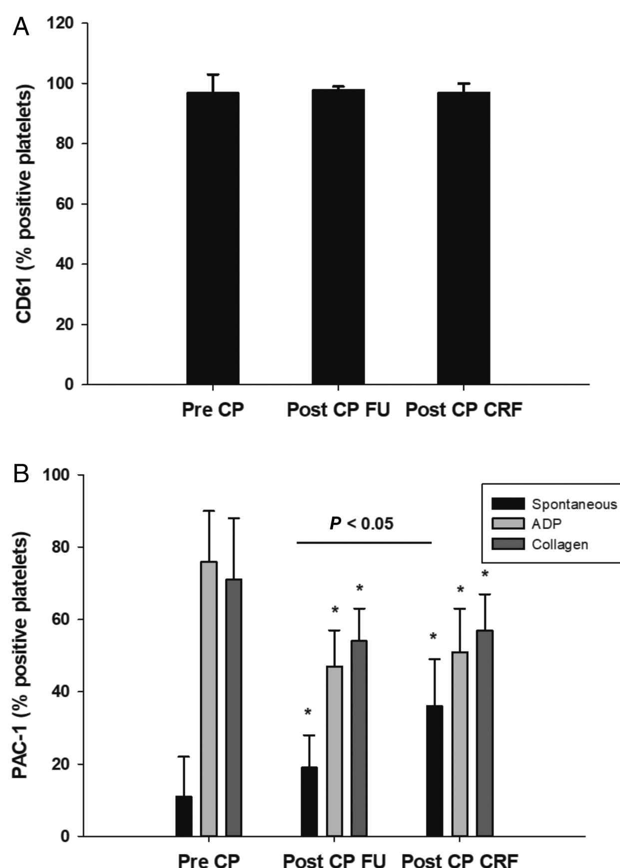

FIGURE 3 Expression of platelet integrin receptor GPIIb/IIIa in experiment 2. CD61 binding to GPIIb/IIIa (A) and PAC-1 binding to the active conformation of GPIIb/IIIa spontaneously or after activation with ADP or collagen (B) pre cryopreservation (CP) (ie, on day 2 of storage before CP) and post CP 2 hours after thawing for the fast uncontrolled freezing rate protocol (FU) and controlled freezing rate protocol (CRF). Results are presented as mean + SD (n = 8). *$P < .05$ compared to pre CP for FU and CRF, respectively. Significant differences between FU and CRF platelets are also indicated

TABLE 4 Viscoelastic properties of the platelets in experiment 2 before and after cryopreservation

Variable	Pre CP	Post CP FU	Post CP CRF
EXTEM CT (s)	46 ± 1	37 ± 1*	38 ± 1*
EXTEM CFT (s)	39 ± 3	130 ± 41*	150 ± 34*
EXTEM MCF (mm)	62 ± 2	28 ± 3*	28 ± 1*

Note: Data is presented as mean ± SD of n = 8. Results are presented pre cryopreservation (CP) (ie, on day 2 of storage before CP) and post CP 2 hours after thawing for the uncontrolled fast cooling protocol (FU) and the controlled freezing rate protocol (CRF).

*$P < .05$ compared to pre CP for FU and CRF platelets, respectively.

freezed platelet units ($P < .05$). Cryopreservation reduced the platelets ability to respond to ADP and collagen as assessed by measurement of the active conformation of GPIIb/IIIa (Figure 3). The overall expression of CD61 was not affected by either CP procedure (Figure 3).

Cryopreservation with both the FU and CRF protocols shortened the EXTEM induced CT ($P < .05$) and prolonged the CFT significantly and reduced MCF, supporting a somewhat impaired platelet function compared to the prefreezing data (Table 4). The changes after CP was similar for both protocols. Taken experiment 1 and 2 together, all aggregates were dissolved before analysis and the swirling phenomenon could be faintly discerned a few hours after reconstruction regardless of freezing protocol.

4 | DISCUSSION

It is well known that freezing and thawing heavily affect platelets,[16–19] and much work has been carried out towards establishing the optimum method for cryopreservation of platelets.[15,20–22] Cryoinvestigations include a number of specific events that can affect platelet response during the process. The extent to which these events compromise platelet function is not fully known, and differences observed between studies may be partly due to different centers using different materials (metal vs cardboard boxes for freezing), freezing rates or equipment. In an attempt to improve the process, it is, however, important to characterize such events in which aberrant platelet response may occur during cryopreservation. In our center, we had an uncontrolled freezing method using metal boxes where we experienced potential for improvement. In the current absence of CRF, we hypothesized whether freezing in other material (cardboard boxes) could lead to any improvements. Given the evidence for equivalent in vitro outcome in experiment 1, we did not change the prevailing method because freezing in metal boxes has several practical advantages. When a later opportunity arose to access CRF, the uncontrolled freezing method using metal boxes were tested in a comparison with CRF in the following experiment 2.

Earlier studies have shown that when cells are cooled to the freezing point very slowly, the risk of intracellular ice formation is minimal.[10,11,13] However, the favorable effects of slow freezing can be ruled out by the possibility that dehydration of the cells may be maximal, which seem to be nonpreferable.[10,11,13] In our controlled rate protocol, the platelets are initially slowly cooled down followed by a rapid decrease in temperature. Since most of the water present in cells will freeze at approximately −2°C to −5°C it is suggested that this phase change from a liquid to a crystalline form releases energy and warming the sample until the equilibrium freezing point is reached.[14] A rapid drop in temperature may minimize potential detrimental effects of this phenomenon.[14] Despite this consideration in our CRF protocol, we did not observe any improvement as compared to the uncontrolled freezing protocol. Our data contrasts with the previous study of Balint et al. which indicates superior results when freezing platelets in a controlled rate system.[23] The differences in outcome between studies indicate that controlled cryobiological settings may be more or less optimized for platelets.

Though, when freezing rates are increased too much, the dehydration may not be fast enough to prevent intracellular ice nucleation.[10,11,13] Fast-freezing damage can also be caused by other factors. For instance, it has been proposed that rapid water flow through membrane pores could lead to an uneven distribution of pressure on the membrane.[24,25] Fast-freezing damage could also result from the very sudden changes in size, shape and ultrastructure, caused by the rapid efflux of water.[11,13] Our data on fast-freezing obtained from both experiments confirm these observations, to some extent since nearly 70% of the platelets survive after cryopreservation. Surprisingly, the FU protocol exhibits no differences in cell recovery and ($\Delta\psi$) after thawing and reconstitution compared to the IU as well as the CRF protocol indicating that the favorable effects of a slower freezing rate seem to negligible, hypothetically ruled out by the effects of the solute. However, all protocols appear to correspond to previous observations on cryopreserved platelets.[2,26–28] Taken experiment 1 and 2 together, we confirm the reduced recovery and the appearance of different deteriorations after cryopreservation but without any clear links to the specific freezing process. This outcome justifies interest in hypothesizing whether alternate cryoprotectants,[20,29,30] addition of second messenger effectors,[31] the use of ice recrystallization inhibitors,[32,33] or addition of a calcium chelator[34] prior to cryopreservation in combination with various freezing rate protocols may improve outcome.

Regardless of freezing protocol used, our findings suggest a relative limited redistribution, clustering or ligation of the surface molecules CD61, PECAM-1 and GPVI surface molecules after freezing and thawing, the sharply reduced response to agonists may, therefore, on one hand suggest defects in intracellular signaling pathways[35] or impaired signaling capacity.[27] On the other hand, the reduced ADP and collagen induced PAC-1 binding may be a direct reflection of the higher level of spontaneous platelet activation after freezing and thawing. A third option underlaying reduced response capacity after cryopreservation may suggest impaired mitochondria-based respiration and lower ATP.[8,36]

A large variety of factors is released from platelets after cryopreservation and thawing.[8,37] Because of the potentially harmful effects[38–41] of platelet derived factors, it may be of importance that cryopreservation protocols do not exacerbate such negative events. Though, it has also been suggested that high levels of BRMs may be beneficial for therapeutic transfusions[26,37,42,43] Given the evidence for equivalent activation in experiment 1, it was of interest if the platelets had been equivalent triggered to release immunomodulatory factors post-thaw. To test if this equivalence persists, we chose to test sCD40L as a general marker for the issue because the release of BRMs after cryopreservation is recently described (Tynngård et al). Nevertheless, the data presented show no difference in sCD40L concentration post-thaw. Though, the increase in the activation levels after cryopreservation per platelet population agrees with the previously observed relationship between activation levels and cytokine release. [8] Surprisingly, we noted that the increased concentration of extracellular LDH, commonly used as a marker of cell disintegration [44] found in the CRF units do not seem to correspond to an increased reduction in the platelet count as previously described for room-temperature stored platelets.[45] However, it is not always clear what LDH release signifies with respect to the extent of damage or the survival of the cell [46] and it is suggested that apparently viable cells appear to be able to release low amount of LDH without obvious disadvantage.[46] In this set-up, it is, however, not possible to determine whether LDH loss from practically all cells is due to the disintegrated fraction releasing their soluble factors on an "all or nothing" basis or whether it is due to many viable cells leaking small quantities of LDH.

All our ROTEM results confirm that cryopreserved and thawed platelets supported clot formation in this setup, regardless of freezing rate. However, cryopreservation shortened the clotting time (CT) and decreased clot strength (MCF). It can be ruled out that differences in platelet concentration or differences in the plasma content might have contributed to the changes observed after freezing and thawing since all platelet samples were diluted in equivalent AB plasma to a concentration comparable to human levels (200×10^9/L) preceding all ROTEM runs. The shortened CT might have been caused by release of substances promoting hemostasis from the procoagulant platelets as was also found in a previous study.[8] Functional GPIIb/IIIa are necessary for the clot strength. Thus, the decreased MCF found after cryopreservation could have been due to reduced capacity to transform the GPIIb/IIIa receptors into the active conformation, which is supported by the findings from the flow cytometry measurements. Noteworthy, there were some avoidable limitations in our study design. First, two separate studies were performed which means that the data were not compared between the experiments. Second, the possibility of conducting a paired three arm study would undeniably strengthen the basis for our conclusions.

5 | CONCLUSION

In general, our study have confirmed previous data showing that cryopreservation of platelets displays several phenotypic, and functional deteriorations compared with fresh platelets, and we have shown that the use of an uncontrolled freezing protocol is feasible, thus, creating a platelet product comparable to using a controlled rate freezing equipment. In addition, we concluded that freezing platelets in a material with lower conductivity for temperature was found not to affect the in vitro outcome. There is, however, little insight into the possible impact of our observations on the hemostatic behavior after transfusion, warranting an increased attention on new experimental focusing on cryopreserved buffy coat derived platelets function in vivo.

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