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Buffy coat-derived platelets cryopreserved using a new method: Results from in vitro studies

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A R T I C L E I N F O

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A B S T R A C T

Cryopreservation for the long-term storage of platelets (PLTs) is a useful method to overcome the limits of platelet shortage. This is an in vitro prospective study to evaluate the count, viability, and function of buffy coat-derived pooled platelet concentrates (BC-PLTs), treated with dimethyl sulphoxide (DMSO) and cryopreserved (CRY BC-PLTs) at −80 °C with a modified Valeri method. PLTs were stored in 6% DMSO with a patented kit. Overall, 49 BC-PLTs from 245 healthy volunteer donors were prepared, cryopreserved, and analysed before and after 3, 6, and 9 months of storage. In flow cytometry, a statistically significant reduction in CD 42b (92.7 ± 4.29% at T0 vs. 23.6 ± 27.5% at T3, 16.38 ± 12.54% at T6, and 17.3 ± 9.6% at T9) and PAC-1 (1.9 ± 1.34% at T0 vs. 0.62 ± 0.4% at T3, 0.63 ± 0.83% at T6, and 0.49 ± 0.48% at T9) was observed after storage. CRY BC-PLTs showed a good and stable endogenous thrombin generation potential (nM min): 529.25 ± 98.64 at T0 vs. 533.04 ± 103.15 at T9 months. CRY BC-PLTs showed a good viability in vitro, according to currently accepted criteria for cryopreserved PLTs.

1. Introduction

Currently adopted strategies for platelet storage have been primarily developed for haematological and oncological patients. Platelet transfusion is frequently adopted for bleeding prophylaxis in the setting of cancer or chemotherapy-related severe thrombocytopenia. At present, platelets are stored at room temperature (20 °C–24 °C); this prolongs circulating time, but may negatively affect platelet function [1,2]. Room temperature (RT) stored platelets (PLTs) do indeed have impaired function associated with an unacceptable risk of bacterial contamination [3] that can lead to sepsis, in particular after multiple transfusions. Current regulations allow the administration of RT platelet concentrates up to 5 days after storage; they furthermore require constant agitation to preserve the PLTs aerobic metabolism and gas exchange. RT stored PLTs are adopted to increase the PLT count in thrombocytopenic patients and prevent bleeding. The short shelf life and agitation needed for RT PLT storage may represent important boundaries in some contexts such as rural, isolated, and military fields [4,5]. Studies related to platelet cryopreservation with dimethyl sulphoxide (DMSO) started in the early 1970s [6] with the aim of overcoming these limits. Cryopreservation systems were improved in the following decades to avoid post-thaw processing of PLTs [7]. Further modifications of the cryopreservation technique consisted mainly of suspension of platelets in plasma [8]. Currently available in vitro and in vivo studies on cryopreserved platelet concentrates (CRY-PLTs) report good outcomes. In detail, CRY-PLTs, compared to liquid stored platelets, have shown to exert an increased haemostatic action in vitro mainly due to an increased affinity for coagulation factor V [9] and the release of thromboxane A2 [10]. From this premise, the following aims of the current study are derived: to develop and validate in vitro a new kit for buffy coat derived platelet cryopreservation (CRY BC-PLT) with DMSO.

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2. Methods

2.1. In vitro study

Buffy coat-derived platelet (BC-PLT) concentrates were obtained from 5 buffy coats and pooled. Volunteer blood donors met the requirements established by Italian transfusion centres and took no medication known to affect PLT function for 10 days before donation. Selection criteria to make BC-PLTs available for this study were as follows: pooled PLT concentration $\geq 1250 \times 10^9$/L and blood unit collection time duration shorter than 6 min. In detail, PLTs from 5 donors with the same blood group were assembled in a sterile kit and mixed with an InterSol solution (100 mL) so that at least 30% was constituted by plasma and 70% by InterSol. InterSol was adopted to wash the sacks containing buffy coats and PLT outflow by increasing the volume of supernatant for PLT suspension after centrifuge.

The BC-PLTs combined with InterSol underwent soft centrifugation and, with an automated separator, were then leukoreduced with filtration. The PLT count was assayed from the BC-PLTs after leukodepletion. BC-PLTs were selected for storage only with a PLT count $\geq 1250 \times 10^3$/µL. The final PLT concentrates were transferred to a 650-mL patented cryopreservation kit (Promedical) that allowed the following storage steps without further external manipulation or the use of laminal flow hoods The patented kit avoided one step of external manipulation of the BC-PLTs for cryopreservation [7]. The plastic bag with the BC-PLTs was then connected in sterile conditions to the cryopreservation kit (Promedical) with two plastic bags of 650 mL volume, one connected to a tube and 3 accessory bags for in vitro studies, to avoid repeated freezing/thawing of samples at each time point after cryopreservation.

A total mean amount of 300 mL BC-PLTs was then transferred to one bag of the kit. After connecting a plastic bag containing 75 mL saline solution with 25% DMSO, under constant agitation, the DMSO solution was transferred to the bag containing the BC-PLTs in 5 min. After mixing with the DMSO solution, each bag underwent centrifugation (2000*g* for 10 min). Using a manual separator, supernatant was then completely removed (leaving approximately 10–15 mL) and the BC-PLTs were re-suspended with plasma from 1 of the initial 5 PLT donors to a final volume of 200 mL. The BC-PLTs rested for approximately 45 min and then were agitated for approximately 1 h to allow PLT disaggregation. The mean residual DMSO amount of each plastic bag at freezing was 400 mg. For in vitro studies, obtained BC-PLTs were divided into 40 mL aliquots and stored at −80 °C in a mechanical refrigerator for the assays. For in vivo studies, BC-PLTs obtained using the above method were immediately stored at −80 °C.

In vitro assays were performed before freezing (T0) and at 3 (T3), 6 (T6), and 9 (T9) months after thawing. Before assay, CRY BC-PLTs were thawed in a water bath at 37 °C for 10 minuets and diluted in plasma to adjust to 300×10^9/L PLTs. Fresh BC-PLTs underwent the same dilution to adjust to 300×10^9/L PLTs. The following parameters were assayed: PLT count (PC), mean platelet volume (MPV), PLT swirl, pH, bacterial contamination (BC), flow cytometry (FACS) expression of CD41a, CD42b, CD61a, CD62p, PAC-1, annexin V PLT surface antigens, thromboelastography (TEG), and thrombin generation. The PC and MPV were determined using a Sysmex XN-2000 platelet counter.

PLT swirling was evaluated immediately after thawing as reported by others [11]. Reagents for flow cytometry were purchased from BD Biosciences PharMingen, San Diego, CA, USA. PLT preparations were labelled according to standard procedures. Appropriate colour compensation was established for FITC and PE with single-labelled controls. PLTs were identified on the basis of their characteristic log forward and side light scatter and binding of CD41a-APC (GPIIb). A minimum of 10,000 events were collected per sample, and the data were saved.

TEG analysis (TEG 5000 Thrombelastograph Analyser, Haemonetics Corporation, Braintree, MA, USA) measured the kinetics of clot formation and clot stability and strength [12]. Clot formation was triggered with a recombinant TF (RecombiPlasTin, Instrumentation Laboratory, Orangeburg, NY, USA) at a final concentration of 3 pM. The analysed TEG parameters were reaction time to clot initiation, the speed of clot propagation (α angle), final clot strength as maximum amplitude, time to maximum amplitude, and the maximum elastic modulus.

The haemostatic potential of CRY BC-PLTs at baseline and after thawing was assayed using a thrombin generation assay [13] with a calibrated automated thrombogram (CAT) (Thrombinoscope, Maastricht, Netherlands). The trigger reagents (platelet poor plasma [PPP], platelet rich plasma [PRP], and MP) contained several tissue factors (TFs) and phospholipids resulting in final concentrations of 1 pM TF/4 µM PL, 1 pM TF/no phospholipid, and no TF/4 µM phospholipid, respectively. Following the addition of fluorescent thrombin substrate and calcium chloride, CAT generates a thrombin generation curve that provides information on several parameters, including the lag time (min), representing the time until initial thrombin had formed, the thrombin peak height (nM thrombin), the time to peak (ttPeak, min), and the endogenous thrombin potential (ETP, which reflects the area under the curve). Thawing rates were constant for all samples. Before assay, the CRY BC-PLT samples were thawed in a bath at 37 °C for 5 min, agitated, and evaluated promptly. All the tests were performed according to the current European recommendations. Results were expressed as mean ± standard deviation (SD). Differences in results obtained at T0 and at 9 months were considered as significant for P values < 0.05 at ANOVA analysis. Repeated measures analysis of variance (ANOVA) was adopted, sphericity test [14] to determine the equality of variances of the differences between measurements was also applied. Pairwise comparisons table was adopted to compare different measurements. Bonferroni correction for multiple comparisons was applied for P-values and confidence intervals. For P-value < 0.05, it is concluded that there is a significant difference between the different measurements. Data were analysed using MedCalc Statistical Software version 14.8.1 (MedCalc Software bvba, Ostend, Belgium).

3. Results

3.1. In vitro study

A total of 49 BC-PLTs from 245 healthy volunteer donors (145 males and 100 females, mean age: 48.16 ± 18.91) were prepared, cryopreserved, and analysed up to 9 months after storage. All the 245 donors were healthy volunteers who did not take any medication known to affect PLT function. Mean DMSO concentration in each bag was 0.945 g/L (SD = 0.538), the mean percentage of removed DMSO from each bag was 93.135% (SD = 3,93). In vitro cell parameters assayed are reported in Table 1. Platelet recovery was acceptable even if a deficit of in vitro platelet quality was recorded. After freezing, PC was only slightly reduced in CRY BC-PLTs while MPV was significantly increased. The mean pH of the cryopreserved platelets was 6.9 (range: 6.8–7.2), which was significantly lower than that of the fresh platelets (7.3, range: 7.1–7.4; $P < 0.0001$). PLT swirl was observed in all samples and graded as 4 for excellent (N = 15) and 3 for good (N = 34). BC was absent. At FACS assays, there were no differences between the groups in CD41a (98.5 ± 1.94 at T0 vs 98.1 ± 3.07 at T3, 98.3 ± 1.24 at T6, and 97.96 ± 3.1 at T9), CD62p (59.0 ± 11.02 at T0, 71.1 ± 14.6 at T3, 76.89 ± 8.65 at T6, and 70.9 ± 7.4 at T9), and annexin V expression, while a statistically significant reduction in CD 42b (92.7 ± 4.29 at T0 vs. 23.6 ± 27.5 at T3, 16.38 ± 12.54 at T6, and 17.3 ± 9.6 at T9), PAC-1 (1.9 ± 1.34 at T0 vs 0.62 ± 0.4 at T3, 0.63 ± 0.83 at T6, and 0.49 ± 0.48 at T9) in CRY BC-PLTs was observed (Figs. 1 and 2).

Cryopreserved PLTs showed a good thrombin generation potential, stably maintained up to 9 months (Table 1) after cryopreservation; ETP (nM min): 529.25 ± 98.64 at T0, 558.82 ± 114.67 at T3, 548.57 ± 93.38 at T6, and 533.04 ± 103.15 at T9 months,

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