A Simple Method for Freezing Human Platelets Using 6% Dimethylsulfoxide and Storage at –80°C

By C. R. Valeri, H. Feingold, and L. D. Marchionni

Human platelets were preserved by freeze- ing them with 6% DMSO in a mechanical refrigerator maintained at –80°C. The selection of 6% DMSO was arbitrary. After postthaw washing, the 81Cr recovery in vivo was about 45%, the in vitro recovery was about 75%, and the lifespan was about 8.5 days. The mean residual DMSO of 165 mg produced no adverse side effects. The number of platelets in the circulation 2 hr after the infusion of washed freeze-preserved platelets was about half that found with fresh platelets. The prolonged bleeding time induced by aspirin administration to healthy volunteers was corrected within 24 hr of transfusion of a single unit of previously frozen washed platelets; in nine of 12 studies a 50% reduction in the bleeding time occurred within 2 hr of infusion. We do not know though whether washed freeze-preserved platelets will be as effective in correcting the bleeding diathesis in thrombo- cytopenic patients.

Our laboratory recently reported improved viability of platelets after freeze preservation with 5% dimethylsulfoxide (DMSO) at a controlled rate of freezing at 1°C/min, and storage in the gas phase of liquid nitrogen at –150°C. The main theme of that report was the method for adding and removing DMSO, and no attempt was made to evaluate the hemostatic effectiveness of the preserved platelets. In the previous study controlling the freezing rate proved to be cumbersome and time consuming, so we decided that in this study we would not attempt to control the rate. We evaluated the hemostatic effectiveness in vivo of preserved platelets by their ability to correct the aspirin-induced prolonged bleeding time in healthy volunteers.

MATERIALS AND METHODS

Four hundred fifty milliliters of blood were collected in 63 ml of citrate-phosphate-dextrose (CPD) anticoagulant in a triple blood pack (Fenwal Laboratories, Morton Grove, Ill.). The blood was centrifuged at 4500 g for 3 min at room temperature (22°C) in a Sorval RC-3 centrifuge (Ivan Sorvall Co., Newtown, Conn.), and the platelet-rich plasma was expressed. The platelets were then concentrated by centrifuging at 4500 g for 5 min. The concentrated platelets were stored undisturbed at 22°C for 1 hr in 10 15 ml of plasma in order to prevent the clumping that sometimes occurs upon resuspension. The fresh platelet concentrates were kept at room temperature for up to 4 hr, labeled with 300 μCi Na2 51CrO4 (Squibb Chromotope), and

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washed with autologous platelet-poor plasma before infusion. Other platelet concentrates were labeled with $^{51}$Cr and freeze-preserved with DMSO at $-80^\circ$C for 24 hr. Twelve percent DMSO in plasma was prepared by adding 26.4 ml of platelet-poor plasma to 3.6 ml sterile, analytical grade DMSO (Fisher Scientific Co., Medford, Mass.), and additional platelet-poor plasma was added to adjust the volume of platelet concentrate to 30 ml. The platelet concentrate was placed on a Burrell wrist action shaker (Burrell Corp., Philadelphia, Pa.) oscillating 200 times/mm$^2$ through a 7° arc, and 30 ml of $12^\circ$ DMSO in plasma was added over a 30-min period. The 60-ml volume of platelet concentrate with 6% DMSO was transferred to a Hemoflex bag (Union Carbide Corp., Chicago, Ill. (No. 2030-2)) with a 200-sq cm surface area, and placed in a freezing frame for storage in a $-80^\circ$C mechanical refrigeration unit (Harris Refrigeration Co., Cambridge, Mass.). Although the rate of freezing was not controlled, it was determined to be about 2-3°C/min. In 12 studies the platelets were stored at $-80^\circ$C for 1 day, and in four studies for 4-6 wks.

The frozen platelets were thawed for 1-2 mm in a 37°C water bath. When platelets were stored up to 6 wk, radioactive $^{51}$Cr was added after thawing, and the platelet concentrate was kept at room temperature for 30 min. The platelets were diluted with 100 ml of 2°, DMSO in plasma followed by 16 ml of ACD. The suspension was centrifuged at 4500 g for 5 min, all the visible supernatant was removed, and the platelets were resuspended in 30 ml of autologous plasma. This process takes about 30 min. The washed platelets were stored at room temperature for about 4 hr before transfusion.

To determine its effect on platelet survival, $^{51}$Cr labeling was performed on the platelet concentrates before freezing and again after thawing in two of the volunteers. The platelets were kept frozen for 24 hr.

Survival studies of autologous platelets were performed according to the technique of Aster and Jandl. Samples were collected 1 and 2 hr posttransfusion, and then daily for 8 days. To estimate the $^{51}$Cr radioactivity in the infused platelets, duplicate 0.5-ml aliquots of each washed $^{51}$Cr platelet concentrate were diluted with 1% ammonium oxalate and 0.9° saline. The platelets were then sedimented and resuspended in 3 ml of saline for counting. For 8 consecutive days after transfusion, a 15-ml aliquot of blood was collected in 1.2°, EDTA in saline from each recipient. Platelet-rich plasma was prepared from this, and the red cells were resuspended in saline and recentrifuged. Platelets from the combined supernatants were concentrated and resuspended in 3 ml of saline for counting.

In each sample the morphology of 200 platelets was studied on siliconized slides and on cover slips with a phase-contrast microscope. Residual DMSO was measured by gas chromatography (Model 7620A, Hewlett-Packard Co., Lexington, Mass.). Platelets were counted with an electronic particle counter. Platelet recovery in vitro was measured in the whole blood, and after freezing, thawing, and washing. The percentage of the collected platelets that circulated 2 hr after infusion was estimated by multiplying the $^{51}$Cr recovery in vivo by the recovery in vitro (including isolation of platelets from whole blood and recovery after freezing, thawing, and washing). Cultures on blood and Sabouraud’s agar and in thioglycollate broth were done on each unit of thawed, washed platelets.

Function of Preserved Platelets

The ability of autologous preserved platelets to correct the prolonged bleeding time produced by administration of 650 mg of aspirin to healthy male volunteers was an indication of their hemostatic effectiveness. The ages of the volunteers ranged from 21 to 45 yr, and no medication was taken during the study periods. Bleeding time was measured in duplicate by the procedure described by Mielke et al. prior to and again 24 hr after aspirin ingestion, and the mean value is reported. Each volunteer was given a single unit of autologous previously frozen washed platelets 24 hr after aspirin ingestion. Measurements of bleeding time were repeated 2, 24, 48, and 72 hr after transfusion. In each volunteer the bleeding time was measured for up to 96 hr after aspirin ingestion to determine the spontaneous correction of the prolonged bleeding time.

RESULTS

$^{51}$Cr survivals were measured after autotransfusion of fresh and frozen platelets to healthy volunteers (Fig. 1). When fresh platelet concentrates were infused into healthy volunteers with spleens the recovery of radioactivity was
Fig. 1. $^{51}$Cr survival values of autologous fresh platelets and previously frozen washed platelets. Platelets were frozen with 6% DMSO and stored at -80°C for 24 hr.

65% ± 5% (mean ± SD), and the lifespan was about 8.5 days. The mean recovery in vivo of $^{51}$Cr freeze-preserved washed platelets was 46% ± 11%, and the lifespan was 8.5 days. The $^{51}$Cr recovery in vivo of the freeze-preserved washed platelet concentrates was 70%, that of the fresh platelets (Fig. 1).

About 35% of the platelets were lost during preparation of the platelet concentrates from CPD whole blood (mean in vitro recovery 62.8% ± 8.6%), and an additional 25% during freezing, thawing, and processing (mean in vitro recovery after the freeze-thaw-wash procedure 76% ± 11%). In evaluating the efficacy of any freeze-preservation method both the loss of platelets during the freeze-thaw-wash procedure and the lower recovery in vivo must be taken into account; thus, the actual number of previously frozen platelets in the circulation was about 50% that of fresh platelets. About 40% of the original platelets circulated 2 hr after infusion of fresh platelet concentrates compared to about 22% for the freeze-preserved platelets. The morphologic study showed 50%–60% discs, 20%–30% spheres, and only a few irregular degranulated cells in the previously frozen washed platelets resuspended in plasma. In the fresh platelet concentrate 90% ± 4% were discs and 8% ± 2% were spheres. There was no bacterial or fungal growth in any of the cultures.

At the time of infusion the previously frozen platelet concentrates contained 165 mg of DMSO with a standard deviation of ± 110 mg. The two-step dilution-centrifugation procedure removed at least 90% of the DMSO, and infusion of the amount remaining produced no immediate side effects. Other investigators who have transfused much larger quantities of DMSO have reported nausea, vomiting, and local phlebitis, but these were not observed in our study.
Repeat ophthalmologic examinations over a 6-mo period showed no abnormalities.

We compared the spontaneous correction in bleeding time after aspirin ingestion to the correction after transfusion of a single unit of autologous previously frozen washed platelets (Fig. 2). One unit of autologous previously frozen washed platelets significantly reduced the bleeding time within 24 hr of transfusion (Fig. 2). Within 2 hr of the infusion the bleeding time was reduced by about 50% in nine of the 12 studies. Results were not significantly different whether the platelets were labeled with $^{51}$Cr prior to freezing or after thawing (Fig. 3). The $^{51}$Cr survival values were similar with frozen platelets stored for 4-6 wk and those stored for only 24 hr (Fig. 4).

Fig. 2. Bleeding times of 12 healthy volunteers prior to and for 48 hr after ingestion of 650 mg of aspirin. On one occasion 24 hr after aspirin ingestion each of the volunteers received 1 U of autologous platelets frozen with 6% DMSO and stored at $-80^\circ$C for 24 hr. On the other occasion no transfusion was administered.

Fig. 3. Effect of $^{51}$Cr labeling of platelet concentrates prior to freezing and after thawing. The platelets were frozen with 6% DMSO and stored at $-80^\circ$C for 24 hr before transfusion to two volunteers.
Satisfactory freeze preservation of human platelets was accomplished using 6% DMSO and storage at -80°C for at least 6 wk. The concentration of 6% DMSO was chosen arbitrarily. The import of this study is that when platelets are prepared with 6% DMSO, they can be freeze preserved by storing them in a mechanical refrigerator with no attempt to control the rate of freezing. Using a two-step dilutional wash procedure, centrifugation, removal of the supernatant, and resuspension of the platelets in autologous plasma, the DMSO was removed from the thawed platelets with excellent results. This method is far simpler than the one we previously reported using 5% DMSO, freezing at 1°C/min in liquid nitrogen, and storage in the vapor phase (-150°C) of liquid nitrogen.

Evaluating the ability of preserved platelets to correct bleeding in thrombocytopenic patients would yield the most reliable results, but such evaluation is difficult. We therefore elected to study healthy volunteers, and found that a single unit of autologous previously frozen platelets reduced their aspirin-induced prolonged bleeding time. Neither 1 U of platelet concentrate stored at 22°C for 24 hr, nor 1 U of platelet concentrate frozen with 5% DMSO and stored at -150°C for 24 hr corrected the bleeding time 24 hr after infusion. However, the transfusion of 1 U of platelets stored at 4°C for 24 hr, or of 1 U of platelet concentrate frozen with 6% DMSO and stored at -80°C, did correct the bleeding time within 24 hr. Our data indicate that the phlebotomy prior to aspirin ingestion did not affect the endogenous platelet response.

Since changes in hemostasis produced by aspirin are not the same as those from spontaneous hemorrhage and capillary fragility inherent in thrombocytopenia, evaluation in thrombocytopenic patients is imperative. Stuart et al. have reported that the prolonged bleeding time in thrombocytopenic patients
was not corrected by platelet concentrates prepared from blood of healthy volunteers who had taken aspirin within 12 hr of donation, but was corrected after infusion of platelet concentrates obtained 36 hr after aspirin ingestion. This simple procedure makes possible freeze preservation of HL-A-compatible platelets for isoimmunized recipients and recipients with aplastic bone marrow disorders. Autotransfusion of frozen platelets in selected cases can be used in conjunction with planned chemotherapy.

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