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Biochemistry, Function, and Hemostatic Effectiveness of Frozen Human Platelets[1] (37904)

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It has recently been demonstrated (1–3) that human platelets stored after controlled freezing with 5% dimethylsulfoxide (DMSO) in polyolefin bags maintain a nearly normal viability as assessed by their *in vivo* survival after labeling with ^{51}Cr. It is known, however, that a normal survival in the circulation does not always guarantee integrity of platelet function and hemostatic effectiveness (4, 5). The present study was devoted to the measurement of a few parameters of platelet biochemistry and function after storage by freezing and to the proof that the frozen platelets can effectively shorten the bleeding time when infused in thrombocytopenic patients.

Materials and Methods. Preparation and freezing of platelet concentrates. Blood was collected in double blood packs (PA-20, Fenwal Laboratories, Morton Grove, IL) containing acid citrate dextrose (ACD) anticoagulant or in packs containing citrate phosphate dextrose (CPD) anticoagulant (JF-25, Fenwal Laboratories, Morton Grove, IL). Platelet-rich plasma (PRP) was prepared by differential centrifugation (6) at 15° in a Sorvall RC-3 centrifuge (Ivan Sorvall Co., Norwalk, CT). Twelve milliliters of additional ACD solution were added to each 100 g PRP prior to separation of the platelet button. The platelet concentrate containing an average of 72×10^9 platelets suspended in 15 ml plasma had a pH of 6.6–6.8 and was finally transferred to a polyolefin bag (Hemoflex bag, Union Carbide Corp., Chicago, IL). DMSO (Fisher Scientific Co., Pittsburgh, PA or Crown Zellerbach Corp., Camas, Wash.) dissolved in plasma was then gradually added to a final concentration of 5% (2). Slow freezing (1°/min) was done in a controlled freezing bath (Model 11-200, Virtis Co., Gardiner, NY) and the frozen platelets were stored in dry ice–ethanol (−79°) for 1–4 days. After thawing, the DMSO was gradually removed (2) after dilution with 5 vol of plasma. The platelets were finally resuspended in plasma.

Biochemical and functional studies. Biochemical studies included determination of glycogen content, β-glucuronidase (a lysosomal enzyme), and purine nucleoside phosphorylase (a cytoplasmic enzyme). Glycogen was determined before as well as after freezing and thawing, using 1 ml platelet concentrate containing $2–3 \times 10^9$ platelets. The glycogen was extracted with ethanol and hydrolyzed to glucose. The latter was then enzymatically measured (7). It was found that removal of the DMSO before preparation of the extract was unnecessary since this compound did not interfere with glycogen extraction and determination. Beta-glucuronidase activity was measured both in the plasma and in the platelet lysate before and after freezing and thawing. The enzyme was measured according to the method of Fishman *et al.* as modified by Palaice (8). By this method, the enzyme reacts with phenolphthalein mono-β-glucoronic acid (Sigma Chemical Co., St. Louis, MO) and liberates phenolyphthalein which is then measured colorimetrically. One enzyme unit (Fishman unit) corre-

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sponded to one microgram phenolphthalein liberated in one hour. Purine nucleoside phosphorylase activity was estimated spectrophotometrically by the coupled xanthine oxidase method (9). One enzyme unit (international enzyme unit) corresponded to one micromole of substrate (inosine) catalized per minute. Purine nucleoside phosphorylase was also measured before and after freezing and thawing. DMSO in the plasma did not affect determination of either β-glucuronidase or purine nucleoside phosphorylase.

Functional studies of the frozen platelets included measurements of platelet aggregation, reaction to hypotonic shock (reversal reaction (2, 10)), and in vitro serotonin uptake. Measurements were also done on the platelets before freezing with and without DMSO. After addition of the DMSO, an aliquot of the platelet concentrate was maintained at 22° for 30–60 min before the studies were carried out. The other aliquot was frozen after this period of time at 22°. After removal of the DMSO, the platelets were resuspended in plasma. The platelet concentration was 350,000 per mm^3 for measurements of serotonin uptake and aggregation. For the reversal reaction, the platelet count was 1×10^6 per mm^3.

Platelet aggregation was measured in a Chrono-Log aggregometer (Model 300, Chronolog Corp., Broomall, PA) using 50 μM adenosine 5′-diphosphate (ADP; Boeringer Mannheim Corp., New York, NY) and 50 μl of a collagen suspension (Sigma Chemical Co., St. Louis, MO). The amount of collagen added was that used to produce 60–80% maximal aggregation with normal, fresh human platelets. Aggregation was expressed in percentage of the maximum change in optical density produced by the aggregating agent, using platelet-poor plasma for the baseline value.

Measurement of the *in vitro* serotonin uptake was done as previously described (11) with a few modifications. Twenty microliters of a ^{14}C-labeled serotonin solution (17 Ci/1.23 moles/ml of saline; Amersham Searle Corp., Arlington Heights, IL) was added to 1 ml of platelet-rich plasma. After 2 hr of incubation at 37°, the platelets were separated by centrifugation (2500*g* for 15 min) and washed once with 10 ml saline. The platelets were then lysed by the addition of 0.4 ml of 0.3 *N* KOH. Radioactivity of the platelets and of the suspending plasma was measured in a liquid scintillation spectrometer (Tri-Carb, Model 2002, Packard Instrument Co., Downers Grove, IL) using toluene containing 0.4% (w/v) PPO and 0.05% (w/v) POPOP as the scintillation fluid. Two milliliters of Bio-Solv (Formula BBS-3, Beckman Instrument, Inc., Fullerton, CA) were added to 15 ml scintillation fluid in each counting vial. Quenching characteristics were determined by using an internal standard of toluene-^{14}C. Platelet radioactivity was expressed in percent of total radioactivity.

The reversal reaction, i.e., the platelet reaction to hypotonic shock, was measured in a recording spectrophotometer as previously reported (2, 10). The value was expressed in percent of the control made of a freshly prepared platelet suspension.

Measurement of hemostatic effectiveness. Hemostatic effectiveness of the previously frozen platelets was equated to the capacity of the platelets to shorten the prolonged bleeding time in patients with severe and stable thrombocytopenia. The 11 thrombocytopenic patients studied all had severe bone marrow depression due either to idiopathic aplastic anemia or to chemotherapy for acute leukemia. Eight patients were adults and three were children. The platelet counts in the patients were 16,000–40,000 per mm^3 before infusion of the frozen platelets. The bleeding time was measured by the template method as described by Mie'ke *et al.* (12). The normal value by this method was 4–8 min as measured in 12 normal volunteers. In all patients the bleeding time was severely prolonged, 19 min in one patient, 20 min in a second patient, longer than 25 min in two patients, and longer than 30 min in the others. No bleeding time was measured beyond 30 min. The platelet counts were also measured before and after infusion of the frozen platelets. These were done in duplicate (13), and, together with the bleeding-time determinations, they were performed immediately be-

fore and 15 min, 3 hr, and 24 hr after infusion of the frozen platelets. Four units of frozen platelets were infused in each of the adult patients and two units in each of the children. One platelet unit contained an average number of 63×10^9 platelets ($46–76 \times 10^9$).

Results. Biochemistry and function of the frozen platelet. Platelet aggregation with ADP and collagen was studied in seven experiments which demonstrated that exposure to DMSO *per se* did not affect significantly the capacity of the platelets to aggregate, while freezing and thawing caused a moderate reduction of it (Table I). After exposure of the platelet concentrate to 5% DMSO for 30 min at 22° and subsequent dilution and removal of the DMSO, as regularly done with frozen platelets (2), the mean value of platelet aggregation with ADP was 95% of the control and with collagen 85%. The reduction was not significant in both cases. After freezing and storage for 1–4 days at −79°, the average value of platelet aggregation dropped significantly. With ADP it was 68.5% of the control and with collagen 64%. The platelet reversal reaction was not significantly affected by incubation for 30 min at 22° with 5% DMSO in plasma, but freezing and thawing caused a marked reduction to an average value of 44% (Table I).

The *in vitro* serotonin uptake of the platelets was also significantly reduced by freezing and thawing, while it was not affected by DMSO. After 30 min at 22° in DMSO-plasma, the average value was 85.4% of the control, while after freezing and thawing the average value dropped to 38% (Table I).

Similarly, glycogen content, β-glucuronidase, and purine nucleoside phosphorylase were not affected by DMSO *per se* when the platelets were kept for 30 min at 22° in DMSO plasma, but freezing and thawing caused a mild to moderate reduction to average values of 60, 79, and 87%, respectively (Table II). The enzyme activities lost by the platelets were recovered in the suspending medium.

Effect of the infusion of frozen platelets on platelet count and bleeding time of thrombocytopenic patients. These results are summarized in Fig. 1. Fifteen minutes after infusion of the frozen platelets in thrombocytopenic patients, there was a marked reduction in the bleeding time to an average value of 14 min. The bleeding time remained beyond 30 min in only one patient. In all patients, however, the bleeding time became shorter 3 hr postinfusion with an average value of 10 min which closely approached the normal range (4–8 min). After 24 hr from infusion, the bleeding time became prolonged with a mean value of 19 min in 8 patients while it was beyond 25

TABLE I. Studies of Platelet Function Before and After Freezing. Mean Values ± SD Are Reported and Are Expressed in Percent of the Mean Control Value Obtained with Fresh Platelets Suspended in Plasma. Absolute Control Values Are Reported in Parentheses (Left Portion).

		5% DMSO in plasma	
Parameters	Control	Before freezing	After freezing and thawing
Aggregation	100%	95 ± 15.1	68.5 ± 11.6
ADP	(49.8 ± 8.2) [a]		
Collagen	100%	85 ± 11	64 ± 6.2
	(70.4 ± 9.7) [a]		
Reversal reaction	100%	85.4 ± 10.1	44 ± 8.5
	(0.082 ± 0.009) [b]		
Serotonin uptake	100%	89 ± 6.0	38 ± 7.6
	(45.2 ± 7.8) [c]		

[a] Percent of difference in optical density between platelet-rich and platelet-poor plasma.
[b] OD change during the first 2 min.
[c] Percent of total radioactivity.

TABLE II. Biochemical Studies of Platelets Before and After Freezing. Mean Values ± SD Are Reported and Are Expressed in Percent of the Mean Control Value Obtained with Fresh Platelets Suspended in Plasma. Absolute Control Values Are Reported in Parentheses (Left Portion).

Parameters	Control	5% DMSO in plasma	
		Before freezing	After freezing and thawing
Glycogen content	100% (0.29 ± .006) [a]	98 ± 9.8	60 ± 16
β-Glucuronidase	100% (24.7 ± 2.6) [b]	99 ± 5.9	79 ± 11
Purine nucleoside phosphorylase	100% (0.153 ± 0.028) [b]	99 ± 7.3	87 ± 14

[a] μmoles of glucose per 10^9 platelets.
[b] Units per 10^9 platelets.

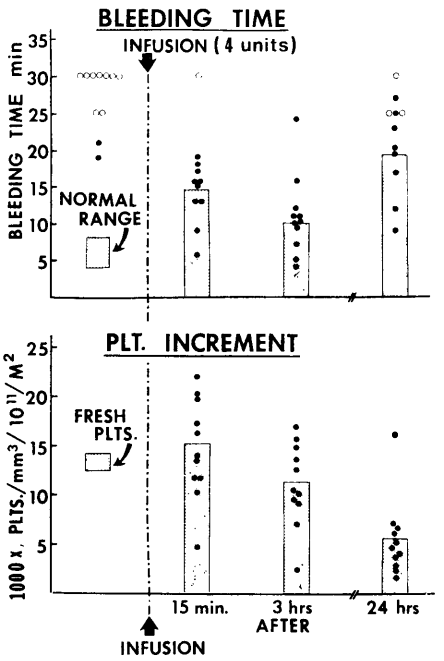

FIG. 1. Hemostatic effectiveness of frozen platelets studied in 11 thrombocytopenic patients. Bleeding-time values before and 15 min, 3 hr, and 24 hr after infusion of frozen platelets are reported in the upper portion, while increments in platelet counts are in the lower portion of the chart. Open circles indicate bleeding times longer than 25 or 30 min. Hatched columns represent mean values. The shortest mean bleeding time was obtained 3 hr postinfusion, while the highest increment in platelet count was observed soon after infusion. These mean values were similar to those obtained with fresh platelets (left portion of chart).

min in 1 patient and beyond 30 min in 2 patients.

Platelet increments after infusion of the frozen platelets are reported in the lower portion of Fig. 1. Values are expressed as increments of platelet number per mm^3 and proportioned to the infusion of 10^{11} platelets per m^2 of body surface. Before infusion, the platelet counts varied between 16,000 and 40,000 per mm^3 with a mean value of 23,000 per min^3. The mean platelet increment 15 min after infusion was 14,700 (11,800–22,000) per mm^3 which was very similar to what is regularly observed with the infusion of freshly prepared platelet concentrates (14, 15) (left column, Fig. 1). The values observed soon after infusion were the highest and were decreased 3 hr and, even more, 24 hr postinfusion.

Discussion. These significant conclusions can be drawn from the data obtained: (a) Human platelets subjected to freezing with 5% DMSO can not only survive in the circulation, as we previously demonstrated (1, 2), but are also capable of sustained hemostatic effectiveness. In fact, the considerable shortening of bleeding time in the thrombocytopenic recipients was of a degree almost similar to that usually obtained with freshly prepared platelets (Fig. 1). (b) The effect of the infused platelets on hemostasis was more pronounced 3 hr than 15 min after infusion, while increments in platelet count were highest soon after infusion. The discrepancy between increase

in platelet count and effect on shortening of the bleeding time is not seen with fresh platelets and indicates to us that the frozen platelets carry a functional lesion which temporarily impairs their hemostatic effectiveness without shortening their survival. Three hours postinfusion, increments in platelet count were lower, but shortening of bleeding time was more pronounced, clearly indicating that the platelets were recovering from the storage lesion. Analysis of the individual results showed no difference between platelets stored for 1 day and those stored for 2–4 days at −79°, indicating that freezing and thawing *per se* rather than storage were causing the platelet lesion. That cell damage indeed occurred during freezing and thawing was also demonstrated by the reduction in aggregation, in the reaction to hypotonic shock, and in the uptake of serotonin. It is therefore concluded that human platelets frozen with 5% DMSO have a complex cellular lesion causing reduced hemostatic effectiveness from which they can, however, recover within a few hours in the circulation. That human platelets and erythrocytes partially damaged in their biochemical and functional integrity by storage can recover in the circulation has already been demonstrated by others (16, 17) and our new findings with frozen platelets are in line with these observations.

The template technique for measurement of the bleeding time (12) gave in our experiments data reproducible within a narrow range. These results also showed that the bleeding time was markedly and invariably prolonged when the platelet count was below 25,000 per mm^3 and that it was only moderately prolonged when the platelet count was 30,000–40,000 per mm^3. The close relationship between platelet count and bleeding time confirmed previous experience obtained by others with fresh platelets (18).

It has recently been reported (19) that DMSO is a potent inhibitor of platelet aggregation. We could not see this inhibition because in our experiments DMSO was removed (2) before aggregation was measured. We can, however, confirm that when we added 1% DMSO to the suspending plasma, aggregation was indeed severely depressed.

The simultaneous reduction of glycogen content, β-glucuronidase, and purine nucleoside phosphorylase in the frozen platelets indicated to us that the damage caused by the freezing procedure involved both cytoplasmic and granular components. The fact that these platelets can still survive normally, or nearly so (2), signifies that either the cellular damage incurred during freezing and thawing is not severe enough to curtail cell viability or that the platelet capacity to circulate and survive is dependent on platelet structures and functions (e.g., membrane structure and function) different from those measured in our experiments.

Summary. Biochemistry, function, and hemostatic effectiveness of human platelets preserved by freezing with 5% DMSO (1, 2) were studied. Determination of platelet glycogen, β-glucuronidase (a lysosomal enzyme), and purine nucleoside phosphorylase (a cytoplasmic enzyme) showed that freezing and thawing caused a complex cell lesion. Hemostatic effectiveness of the frozen platelets was measured from the shortening of the bleeding time (template method) upon infusion of the platelets in patients with severe and stable thrombocytopenia due to bone marrow depression. The frozen platelets produced shortening of the bleeding time to a degree almost similar to that obtained with fresh platelets; however, the bleeding time was shorter 3 hr after infusion than soon after it, while increments in platelet count were greatest immediately after infusion. This finding indicates that frozen platelets carry a functional lesion from which they can rapidly recover in the circulation.

In fact, this research demonstrates that frozen platelets are valuable in arresting hemorrhage due to thrombocytopenia.

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1. Kim, B. K., and Baldini, M. G., Cryobiology 9, 329 (1972).

2. Kim, B. K., and Baldini, M. G., Proc. Soc. Exp. Biol. Med. 142, 345 (1973).

3. Handin, R. I., and Valeri, C. R., Blood **40**, 509 (1972).

4. Johnson, S. A., VanHorn, D. L., Pederson, H. J., and Marr, J., Transfusion **6**, 3 (1966).

5. Lundberg, A., and Murphy, S., Scand. J. Haematol. **9**, 222 (1972).

6. Morrison, F. S., and Baldini, M. G., Vox Sang. **12**, 90 (1967).

7. Pfleiderer, G., *in* "Methods of Enzymatic Analysis" (H. M. Bergmyer, ed.), p. 59. Academic Press, New York (1965).

8. Plaice, C. H. J., J. Clin. Pathol. **14**, 661 (1961).

9. Kim, B. K., Cha, S. M., and Parks, R. E., Jr., J. Biol. Chem. **243**, 1763 (1968).

10. Kim, B. K., and Baldini, M. G., Fed. Proc. **29**, 717 (1970).

11. Ozge, A., Baldini, M. G., and Goldstein, R., J. Lab. Clin. Med. **63**, 378 (1964).

12. Mielke, C. H., Jr., Kaneshiro, M. M., Maher, I. A., Weiner, J. M., and Rapaport, S. I., Blood **34**, 204 (1969).

13. Brecher, G., and Cronkite, E. P., J. Appl. Physiol. **3**, 365 (1950).

14. Zucker, M. B., Pert, J. H., Lundberg, A., Yankee, R. A., and Henderson, H. S., Vox Sang. **16**, 373 (1969).

15. Cavins, J. A., Farber, S., and Roy, A. J., Transfusion **8**, 24 (1968).

16. Murphy, S., and Gardner, F. H., J. Clin. Invest. **50**, 370 (1971).

17. Beutler, E., and Wood, L., J. Lab. Clin. Med. **74**, 300 (1969).

18. Harker, L. A., and Slichter, S. J., New Eng. J. Med. **287**, 155 (1972).

19. Holtz, G. C., and Davis, R. B., Proc. Soc. Exp. Biol. Med. **141**, 244 (1972).

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