Improved Viability of Previously Frozen Platelets

By R. I. Handin and C. R. Valeri

The main feature of this new approach to platelet freezing is a controlled rate of addition and postthaw removal of 5% dimethylsulfoxide (DMSO). When DMSO was removed from fresh non-frozen platelet concentrates before transfusion, no adverse effect on posttransfusion survival was observed. The $^{51}$Cr recovery of autologous fresh platelet concentrates in vivo was $65\% \pm 5\%$, while that of autologous platelets previously frozen with DMSO and washed prior to transfusion was $47\% \pm 3\%$. The lifespan values were similar for fresh platelets and previously frozen washed platelet concentrates. When approximately $95\%$ of the DMSO was removed from previously frozen washed platelets before transfusion, the $^{51}$Cr recovery was about $70\%$ of the value for fresh platelet concentrates, and the lifespan value was similar to that of fresh platelet concentrates. No adverse side effects were observed. The hemostatic effectiveness of these platelets has yet to be studied.

The transfusion of fresh platelets has been effective in the treatment and prevention of thrombocytopenic hemorrhage. Results with the transfusion of previously frozen platelets have been poor. Baldini, Costea, and Dameshek and Cohen and Gardner, using glycerol as a cryoprotective agent, observed $^{51}$Cr recoveries of $20\%$ after the autotransfusion of previously frozen platelets to normal volunteers. Djerassi et al. transfused platelets frozen with a combination of 5% dimethylsulfoxide (DMSO) and 5% dextrose to thrombocytopenic leukemic children. The rise in platelet count was only $30\%$ of that achieved with fresh platelets. In addition, the large amount of infused DMSO caused nausea, vomiting, and phlebitis in many of the recipients.

For platelet cryopreservation to be satisfactory, a minimum amount of the cryoprotective agent should be present at the time of transfusion and a high yield of circulating and functioning platelets should be realized. Experience with red cell freezing suggests that this can be achieved by careful attention to the addition and removal of the cryoprotective agent. This paper reports an improved method for platelet freezing using a new procedure to add and remove DMSO from human platelets.

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MATERIALS AND METHODS

Four hundred fifty milliliters of blood was 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., Norwalk, 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 for 1 hr at 22°C in 10-15 ml of plasma in order to resuspend them without clumping. Three hundred μCi Na251CrO4 (Squibb Chromotope) was then added, and the platelet concentrate was incubated for 15 min at 22°C. Ten percent DMSO in plasma was prepared by adding 27 ml platelet-poor plasma to 3 ml sterile, analytical grade DMSO. (Fisher Scientific Co., Medford, Mass.) 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/min through a 7° arc. Thirty milliliters of 10% DMSO in plasma was then added over a 5-mm period. The 60 ml volume of platelet concentrate containing DMSO with a final concentration of 5% was transferred to a freezing Hemoflex bag which can withstand exposure to liquid nitrogen (Union Carbide Corp., Chicago, Ill.). The platelets were then frozen to 1°C/min in a controlled-rate liquid nitrogen freezer (Linde BF-4-2, Cryogenics-East, Inc., Burlington, Mass.) to —40°C. The rate of freezing was controlled by a thermocouple inserted into a 5% DMSO platelet-poor plasma blank of identical volume and geometry which was placed in the freezing chamber. The rate was maintained at 1°C/min through the “heat of fusion” by manual control of the freezing by adding more liquid nitrogen. The platelets were removed and stored in the vapor phase of a liquid nitrogen freezer usually for up to 1 mo before use.

The platelets were thawed for 1–2 min in a 37°C water bath. One hundred milliliters of 2% DMSO in plasma was added to the concentrate followed by 16 ml of ACD, after which the platelets were packed by centrifugation at 4500 g for 5 min. The platelets were then resuspended in 30 ml of plasma free of DMSO and were transfused within 4 hr.

Platelet survival was performed as described by Murphy and Gardner. Samples were collected 15 min, 1 hr, and 2 hr posttransfusion, and then daily for 8 days. In this procedure the 51Cr-labeled platelet concentrate was washed once with about 100 ml of nonradioactive platelet-poor plasma and 10 ml of acid-citrate-dextrose, Formula A. Duplicate 0.5 ml aliquots of each washed 51Cr platelet concentrate were diluted with 1% ammonium oxalate followed by 0.9% saline. The platelets were then sedimented and resuspended in 3 ml of saline for counting. During the 8 day posttransfusion period, 15 ml aliquots of blood were collected from each recipient in 1.2% EDTA in saline. Platelet-rich plasma was prepared and the red cells resuspended in saline and recentrifuged. Platelets from the combined supernatants were concentrated and resuspended in 3 ml of saline for counting.

Platelet morphology was studied on siliconized slides and cover slips with a phase contrast microscope; in each sample 200 platelets were examined (Table 1). Residual DMSO was measured by gas chromatography. (Research Chromatograph, Model 7620A, Hewlett-Packard Co., Lexington, Mass.) Platelets were counted with an electronic particle counter.

An aliquot of each unit was thawed 24 hr prior to transfusion and cultured on blood agar and Sabouraud's agar, and in thioglycollate broth. There was no growth in any of the cultures after 7 days.

Table 1. Phase Microscopy of Fresh, Liquid, and Previously Frozen Platelets With and Without DMSO

<table>
<thead>
<tr>
<th>Preparation</th>
<th>No.</th>
<th>Disks</th>
<th>Spheres</th>
<th>Degranulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>15</td>
<td>92%±4%</td>
<td>8%±2%</td>
<td>0</td>
</tr>
<tr>
<td>4°C, 24 hr</td>
<td>10</td>
<td>16%±3%</td>
<td>79%±6%</td>
<td>5%±2%</td>
</tr>
<tr>
<td>Frozen-thawed in 5% DMSO</td>
<td>20</td>
<td>62%±4%</td>
<td>18%±4%</td>
<td>10%±3%</td>
</tr>
<tr>
<td>Frozen-thawed without DMSO</td>
<td>5</td>
<td>9%±3%</td>
<td>54%±4%</td>
<td>37%±4%</td>
</tr>
</tbody>
</table>
The $^{51}$Cr survivals of fresh and frozen platelets were measured after autotransfusion to normal volunteers (Fig. 1). The recovery of infused radioactivity in normal volunteers with spleens was $65\% \pm 5\%$ (mean $\pm$ SD), with a subsequent lifespan of $8.9 \pm 0.3$ days. The recovery of frozen platelets was $47\% \pm 3\%$, with a lifespan of $8.8 \pm 0.3$ days. The $^{51}$Cr recovery of the frozen platelet concentrates was $70\%$ of the fresh platelets (Fig. 1).

The average unit of blood collected contained $1.1 \times 10^{11}$ platelets. During preparation of the platelet concentrates, $33\%$ of the platelets were lost, so that at the time of infusion fresh units contained $0.73 \pm 0.05 \times 10^{11}$ platelets. During freezing, thawing, and processing, an additional $12\%$ of the original collected platelets were lost. Because of this loss, only $0.59 \pm 0.04 \times 10^{11}$ previously frozen platelets were infused in each unit. The efficacy of the preservation method must take into account both the loss of platelets during freezing and the lower recovery in vivo of previously frozen platelets. Thus, the actual number of previously frozen platelets circulating after transfusion was only $58\%$ of the number of fresh platelets.

The aliquot of previously frozen platelets which was infused had a volume of about $30$ ml of plasma which contained $290 \pm 137$ mg of residual DMSO. The two-step dilution-centrifugation procedure removed $94\% \pm 3\%$ of the DMSO prior to transfusion. None of the volunteers experienced any side effects when this amount was infused. There was no evidence of nausea, vomiting, or local phlebitis as described by other investigators who transfused much larger quantities of DMSO.$^5$

Platelet recovery in vivo was adversely affected by the presence of DMSO at the time of transfusion (Fig. 2). In a volunteer who received previously

Fig. 1. Comparison of $^{51}$Cr survival of autologous platelets prepared as fresh and previously frozen concentrates.
frozen platelets containing 5% DMSO, the $^{51}$Cr recovery was 20% and the lifespan was 9 days. When similarly treated platelets were washed to remove the DMSO the $^{51}$Cr recovery was 43% and the lifespan was 9 days. Furthermore, DMSO could be added and removed from the fresh platelet concentrates without affecting either the recovery in vivo or their lifespan (Fig. 2).

Prior to transfusion the morphology of previously frozen platelets was examined and compared to that of fresh platelet concentrates and platelet concentrates stored at 4°C. Under phase contrast microscopy the majority of fresh platelets were disks. As reported by Murphy and Gardner, platelets stored at 4°C became spherical and irregular. Platelets frozen and thawed without a cryoprotective agent showed a high proportion of swollen, irregular spheres that were devoid of granules. Platelets that were frozen with 5% DMSO, washed and resuspended in plasma showed 50%-60% disks, 10%-20% spheres, and very few irregular, swollen, or degranulated cells (Table 1).

**DISCUSSION**

The data show that 5% DMSO can be added to and removed from platelet concentrates without affecting viability, as measured by both $^{51}$Cr recovery in vivo and the lifespan. In addition, 5% DMSO can partially protect platelets from freeze-thaw injury (Figs. 1, 2). The frozen-thawed platelets were transfused to normal volunteers without detectable side effects, and with recovery in vivo of about 70% of the value observed for fresh platelet concentrates and normal lifespan. The data suggest that the graded addition and removal of DMSO are important aspects of the freezing procedure. Experience with red cell freezing, primarily using glycerol as the cryoprotective agent, suggests that large osmotic stresses produce increases in freeze-thaw hemolysis and decreases in viability. It has been suggested that because DMSO is very permeable across the cell membrane, osmotic gradients are less critical. Data
in Fig. 2 and in two similar experiments suggest that, when previously frozen platelet concentrates contain 5% DMSO, recovery in vivo of the platelets is impaired.

The improved recovery and lifespan of previously frozen platelets noted in the normal volunteers in our study is encouraging. However, further considerations are in order. Reversible changes in the hemostatic effectiveness of platelets during storage at 22°C have been observed; studies to determine whether such changes will occur in previously frozen platelets must be undertaken. The ability of frozen platelets to correct hemorrhage in thrombocytopenic recipients should also be evaluated.

Cryopreservation of blood components is more expensive and complicated than liquid preservation. However, the ability to store platelets in the frozen state for long periods would be useful in supplementing conventional platelet transfusions. Previously frozen platelets could be used on weekends and holidays when fresh platelet concentrates might not be available. At some later date it might prove useful in platelet typing to select platelets for isoimmunized recipients. In selected patients autotransfusions might also be possible in conjunction with planned chemotherapy.

REFERENCES

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