Platelet Cryopreservation Using a Reduced Dimethyl Sulfoxide Concentration and Second-Messenger Effectors as Cryopreserving Solution

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Cryopreservation of platelets is of great interest since it could extend to years the shelf life of therapeutic platelet concentrates (PCs) and facilitate stockpiling and inventory control in blood banking. We have compared the cryopreservation of PCs by the standard method using 6% Me2SO as cryoprotectant with the method of freezing employing low concentrations of Me2SO (2%) plus ThromboSol, a mixture of second-messenger effectors that protect platelets from cold damage. PC pools were treated either with 6% Me2SO or with ThromboSol and 2% Me2SO and then placed directly in a −80°C freezer or in the vapor phase of a liquid nitrogen freezer (−120°C). After storage for 1 week or for 3 months, samples were removed, thawed, and analyzed. Measurements included cell recovery, biochemical parameters, membrane glycoproteins (GPs), platelet aggregation, and binding of radiolabeled von Willebrand factor (vWF) and fibrinogen. PCs cryopreserved with ThromboSol and 2% Me2SO displayed a platelet recovery (90%) equivalent to those frozen with 6% Me2SO. Following either cryopreservation procedure, platelets showed increased surface expression of P-selectin and moderate loss of GP Ibα in comparison to fresh platelets. The aggregatory response to ristocetin and the binding of vWF were similar in platelets frozen by either procedure. Finally, both methods promoted comparable impairment of the reactivity of platelets to thrombin, aggregation and binding of fibrinogen and vWF, compared to that of fresh platelets. In summary, cryopreservation of PCs using reduced Me2SO concentration and ThromboSol yields platelets with in vitro functional characteristics equivalent to those of cells frozen with the conventional method using 6% Me2SO.

Key Words: platelet cryopreservation; Me2SO; ThromboSol.

The demand for platelet concentrates (PCs) has increased in recent years, due mainly to routine use of chemotherapy, radiotherapy, and transplantation procedures in onco-hematological patients. Today, however, the storage of conventional liquid PCs is limited to only 5 days, since longer storage at 22°C leads to unacceptable lesions of the stored platelets and to an increase in the risk of bacterial contamination. This limitation results in the outdated of units and complicates the management of platelet inventories in blood banking and the supply of this blood product. Therefore, there is considerable interest in developing methods to lengthen the shelf life of PCs (1, 9).

Reports have described attempts to improve and extend the storage of PCs at 22°C by the addition of agents such as protease inhibitors (43), modulators of cellular levels of cyclic 3',5' adenosine monophosphate (cAMP) (5, 7), and metabolic substrates, such as acetate and/or pyruvate (25, 34). In addition, we and others have recently assessed the viability and functional activity of PCs stored at 4°C for up to 9 days, supplemented with the new platelet additive solution, a mixture of second-messenger effectors, named ThromboSol (10, 12, 33). This solution significantly reduced the known defects induced by refrigerated storage of PCs (10, 33) and diminished cytokine accumulation and
bacterial growth, compared to standard PCs (12). Although the findings of those studies are promising, it seems clear that PCs in a liquid state could hardly be stored, either at 22°C or under refrigeration, for periods longer than 2 weeks because of unacceptable risks of bacterial growth and loss/damage of stored platelets.

Alternatively, the technique of cryopreservation would permit the extension of the shelf life of therapeutic PCs for up to 1 year. Additionally, the use of autologous frozen platelets would allow access to HLA/HPA-typed platelets, stockpile autologous platelets for use during the recovery phase of chemotherapy, and therefore, could prevent major complications of allogeneic platelet transfusion, such as alloimmunization or infection with transmissible disease agents. Some studies have shown cellular lesions associated with the cryopreservation of cells, such as disc-spherocyte transformation (11, 37), reduction in hypotonic shock response (27, 40), loss of GP Ib (29), decreased adhesive capacity (28), release of granule contents and LDH (6, 40), and defective aggregation in response to different agonists (4, 36, 37, 41). Nevertheless, there are studies showing that cryopreserved platelets have an acceptable life span in vivo and that they retain hemostatic effectiveness (14, 18, 21, 22, 30, 35, 39), which encourages the development of simplified and improved methods for cryopreservation of platelets. Therefore, efforts have been made during recent years to establish improved techniques for platelet cryopreservation that would allow a more systematic use of frozen platelets.

Among the several methods that have been reported for the freezing of platelets (3, 4, 8, 15, 19), the one most widely employed is the use of a plasma and dimethyl sulfoxide (Me₃SO) mixture as the cryopreserving solution. In this procedure, the precooled mixture is added to the PCs at a controlled rate to achieve a final Me₃SO concentration of 5 to 6% and platelets are subsequently frozen and stored either in a deep freezer (−80°C) or in the vapor phase of liquid nitrogen (−120°C). Before use, frozen platelets are thawed rapidly in a water bath (37°C) and must be washed free of Me₃SO, which is potentially harmful for the recipient, and then resuspended in plasma.

Recently, Currie and co-workers (13) have investigated whether the combination of precise second-messenger effectors, reagents that affect signaling transduction by inhibiting specific activation pathways endogenous to platelets, known as ThromboSol (10, 33), might have cryoprotectant properties. In their study, platelets cryopreserved with ThromboSol and 2% Me₃SO exhibited a significantly higher ristocetin-induced aggregation response than those frozen in 2% Me₃SO alone. Additional turbidometric studies (hypotonic shock response, extent of shape change, stirring shape change) also showed higher preservation of functional activities in platelets stored in ThromboSol and 2% Me₃SO than those frozen with 2% Me₃SO alone or with the currently used 6% Me₃SO. In the present study, we increase knowledge of the effects of ThromboSol and reduced concentrations of Me₃SO (2%) by using in vitro tests that better define the overall functionality of platelets, such as ligand binding studies. We also analyze the effect of this cryopreserving method on metabolic parameters and on the expression of surface glycoproteins (GPs), comparing the results to those of cells stored following the conventional 6% Me₃SO cryopreservation procedure.

MATERIAL AND METHODS

Materials

The storage solution ThromboSol, containing 12.5 mM amiloride, 5 mM adenosine, and 2.5 mM sodium nitroprusside [SNP], was kindly provided by LifeCell Corp. (Woodlands, TX) as a 50-fold concentrate in Me₃SO. Von Willebrand factor (vWF), isolated and purified from high-purity FVIII Fandhi concentrates, was kindly provided by Instituto Grifols SA (Barcelona, Spain). Human fibrinogen was from Sigma–Aldrich Quimica SA (Madrid, Spain). Thrombin from human plasma (specific clotting activity 5459 NIH units/mg of protein) was obtained from Calbiochem-Novabiochem AG (Lucerne, Switzerland). Monoclonal antibodies
(MoAbs) against platelet glycoproteins P-selectin (CD62*phycocerythrin [PE]) and granulophysin (CD63*fluorescein isothiocyanate [FITC]) were purchased from Becton–Dickinson (San Jose, CA) and Immunotech (Marseille, France), respectively. The MoAbs anti-GP Iba LJ-P3 and anti-GP IIb/IIa LJ-CP8 were generously provided by Dr. Z. M. Ruggeri (Research Institute of Scripps Clinics, La Jolla, CA). All other chemicals and solvents were of the highest analytical grade commercially available.

Preparation, Freezing, and Thawing of PCs

Whole blood (450 mL) from habitual blood donors was collected into a triple bag system (Karmi series; Kawasumi Lab Inc., Tokyo, Japan), whose primary pack contained 63 mL of CPD anticoagulant. Within 6 h after collection, PCs from platelet-rich plasma (PRP) were obtained as described (32) by centrifugation at 1600 rpm for 9 min at 22°C to obtain PRP. Then PRP was centrifuged at 3500 rpm for 9 min, and 55 ± 5 mL of plasma was automatically left with the platelet pellet by means of a Compomat device (NPBI BV, The Netherlands). PCs were stored overnight at 22°C on an orbital shaker.

In each of six different series of experiments, 10 ABO-compatible PCs were pooled using a sterile device (SCD, Haemonetics, Braintree, MA). Then, a 10-mL sample was removed for analysis of basal platelet parameters, and the remaining volume was redistributed into two transfer bags (Karmi, Kawasumi). One bag was injected via a sampling site coupler (Baxter S.A., Barcelona, Spain) with a single-bolus injection of 50× ThromboSol solution in M2SO at room temperature (1 mL/50 mL of PC), yielding a 2% final concentration of M2SO and the following concentrations of ThromboSol reagents: 0.25 mM amiloride, 0.1 mM adenosine, and 50 μM SNP. The second bag was supplemented with 6% M2SO at a controlled rate (1 mL/min) and at room temperature with constant agitation. Immediately after addition of either cryopreserving solution, 30-mL aliquots of the PCs were transferred into 50-mL polypropylene tubes (114 × 29 mm; Sarstedt SA, Canovelles, Spain) which were placed directly either in a −80°C deep freezer or in the vapor phase of a liquid nitrogen freezer. Following storage for either 1 week or 3 months, the frozen PC aliquots were removed from the correspondant freezers, thawed rapidly in a 37°C water bath, gently mixed, and assayed as described below.

Tests and Assays

The basal parameters of the PCs were assessed in untreated fresh samples, essentially as described below for the frozen–thawed aliquots of PCs.

Immediately after thawing, a sample of each PC was analyzed for cell counts using an electronic particle counter (STKS; Coulter Electronics, Hialeah, FL). The pH and plasma bicarbonate levels were also measured in PCs in a blood-gas analyzer (Radiometer ABL Model, Copenhagen, Denmark).

Platelet-poor plasma (PPP) was obtained by centrifugation of PC samples at 12,000g for 5 min. Glucose and lactic dehydrogenase (LDH) concentrations in these PPP samples were assessed using a Technicon RA-1000 analyzer (Tarrytown, NY).

Flow cytometric analysis of platelet surface expression of P-selectin, CD63, GP Iba, and GP IIb/IIa was performed in duplicate in PC samples which had been previously diluted (1-in-25) with 20 mM Hepes, 0.15 M NaCl, pH 7.35. These experiments were carried out essentially as detailed elsewhere (23), using the appropriate anti-GP MoAbs among those listed above and a fluorescence-flow cytometer (FACScan; Becton–Dickinson, Mountain View, CA). For each sample run, data acquisition of 5000 events was gated on forward and side-angle light scatter with gains adjusted so as to include the platelet population. Then, the fluorescence of stained platelets was analyzed with the CellQuest software (Becton–Dickinson) to obtain both the percentage of positively stained cells and the mean fluorescence intensity (MFI).

For aggregation and binding experiments, thawed PCs were first washed free of ThromboSol, DMSO, and plasma components by the procedure of Mustard et al. (26). Washed platelets were then resuspended in Tyrode’s solution
with albumin (NaH₂PO₄, 0.42 mM; NaCl, 136 mM; KCl, 2.68 mM; NaHCO₃, 11.9 mM; dextrose, 5.4 mM; CaCl₂, 2.0 mM; MgCl₂, 1.0 mM; human serum albumin 0.35%; pH 7.35).

The aggregation response of washed platelets (2.5 × 10⁸ platelets per mL in Tyrode's solution with albumin) to human α-thrombin (0.5 and 1 NIH units/mL) and ristocetin (1 mg/mL) was monitored optically on an Aggregocorder II device (Menarini Diagnostics, Florence, Italy) set at 37°C and 1000 rpm. Pooled normal plasma was added to support ristocetin-induced agglutination. Results were reported as the percentage of maximum change in light transmission for a total time of 5 min, with washed platelet suspensions as the baseline and Tyrode's albumin solution as 100%.

Von Willebrand factor and fibrinogen to be used in the binding experiments were radiolabeled with carrier-free Na⁻¹²⁵¹ (Amersham Ibérica SA, Madrid, Spain) using Iodogen (Pierce Chemical, Rockford, IL) (17). Cold binding experiments for vWF and fibrinogen were performed by mixing washed platelets (1 × 10⁸ cells/mL) with the labeled protein ([¹²⁵¹]vWF 0.1 µg/mL; [¹²⁵¹]fibrinogen 1.5 µg/mL) in the presence of increasing concentrations of the unlabeled counterparts (vWF 0–50 µg/mL; fibrinogen 0–1 mg/mL) plus the appropriate stimulus (33). Binding of vWF to GP Ibα was induced with ristocetin (1 mg/mL), whereas binding of fibrinogen to GP IIb/IIIa was measured following platelet activation with α-thrombin (2.5 NIH units/mL, 5 min, at room temperature). In binding assays designed to evaluate the platelets' thrombin responsiveness, washed cells were activated with graded concentrations of the agonist (0–10 NIH units/mL) before allowing the binding of [¹²⁵¹]vWF (0.1 µg/mL) to GP IIb/IIIa. In all cases, thrombin activation was stopped by the addition of recombinant hirudin (Sigma–Aldrich Quimica SA) at a 20:1 ratio (unit/unit). Binding of ligand was achieved without agitation at room temperature for 30 min, after which platelet-bound and free ligands were separated by centrifugation and counted (LKB, Multigamma, EG&G Wallac, Barcelona, Spain). Binding isotherms were analyzed using the computer-assisted program Ligand (24) to obtain the Bₜₐₙ (total concentration of binding sites). Thrombin dose–response curves were obtained by plotting the amount of [¹²⁵¹]vWF binding versus the concentration of thrombin, and they were analyzed with a nonlinear curve-fitting package (UltraFit; Biosoft, Cambridge, U.K.).

Statistical Analysis

All data are reported as the mean ± SEM of six different series of experiments. Assuming that paired samples follow a normal distribution, statistical differences between fresh platelets and the different groups of frozen platelets in the various parameters investigated were tested by ANOVA and Student's t tests, using the StatView computer program (Abacus Concepts, Calabasas, CA). Differences were considered significant when P < 0.05. For easy identification of statistical comparisons between the distinct groups of PCs in Tables and Figures, we have used the following alphanumeric designations: AO for fresh PCs, A1 and B1 for PCs frozen with the standard 6% Me₂SO concentration and stored in a −80°C deep freezer, A2 and B2 for PCs frozen with 6% Me₂SO concentration and stored in the vapor phase of liquid nitrogen, A3 and B3 for PCs frozen with a mixture of 2% Me₂SO plus ThromboSol and stored at −80°C, and A4 and B4 for PCs frozen with 2% Me₂SO plus ThromboSol and stored in gas nitrogen. A1, A2, A3, and A4 samples were stored under the correspondent conditions for 1 week; B1, B2, B3, and B4 samples were kept frozen for 3 months.

RESULTS

Platelet Count and Biochemical Markers

Table 1 shows the number of platelets recovered from the PCs following storage for 1 week and for 3 months under each storage condition. Compared to the cell numbers in fresh PCs, platelet counts in frozen–thawed PCs were diminished after 1 week and 3 months of storage by 7 ± 3 and by 11 ± 6%, respectively. As shown, cryopreservation with 6% Me₂SO yielded
<table>
<thead>
<tr>
<th></th>
<th>Fresh</th>
<th>Frozen, 1 week</th>
<th>Frozen, 3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A0</td>
<td>6% Me₂SO</td>
<td>2% Me₂SO and ThromboSol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>−80°C A1</td>
<td>−80°C A3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>−120°C A2</td>
<td>−120°C A4</td>
</tr>
<tr>
<td>Platelet count (×10³/µL)</td>
<td>1010 ± 44</td>
<td>942 ± 38*</td>
<td>973 ± 39*</td>
</tr>
<tr>
<td>pH</td>
<td>7.20 ± 0.01</td>
<td>7.20 ± 0.01</td>
<td>7.30 ± 0.02</td>
</tr>
<tr>
<td>HCO₃⁻ (mM)</td>
<td>18.0 ± 0.30</td>
<td>17.2 ± 0.24</td>
<td>18.5 ± 0.80</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>359 ± 5</td>
<td>323 ± 8</td>
<td>323 ± 3</td>
</tr>
<tr>
<td>LDH (units/L)</td>
<td>269 ± 24</td>
<td>490 ± 31*</td>
<td>441 ± 26*</td>
</tr>
</tbody>
</table>

*Note.* Data are mean ± SEM (n = 6).

* P < 0.05 vs A0.

⁺⁺ P < 0.05 vs A1 and A2.

# P < 0.05 vs B1 and B2.
TABLE 2
Flow Cytometric Analysis of Platelet Surface Glycoproteins in Fresh and Frozen PCs

<table>
<thead>
<tr>
<th></th>
<th>Frozen, 1 week</th>
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<th>Frozen, 3 months</th>
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<tbody>
<tr>
<td></td>
<td>Fresh</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A0</td>
<td>A1</td>
<td>A2</td>
<td>A3</td>
</tr>
<tr>
<td>GP Iba MFI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MoAb LJ-P3</td>
<td>68 ± 7</td>
<td>46 ± 4*</td>
<td>52 ± 8</td>
<td>49 ± 6*</td>
</tr>
<tr>
<td>GP IIb/IIIa MFI</td>
<td>67 ± 8</td>
<td>73 ± 10</td>
<td>68 ± 9</td>
<td>71 ± 9</td>
</tr>
<tr>
<td>MoAb LJ-CP8</td>
<td>20 ± 2</td>
<td>42 ± 3*</td>
<td>38 ± 4*</td>
<td>37 ± 4*</td>
</tr>
<tr>
<td>CD62 MFI</td>
<td>23 ± 6</td>
<td>24 ± 3</td>
<td>21 ± 2</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>% positive platelets</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD63</td>
<td>68 ± 12</td>
<td>52 ± 7</td>
<td>49 ± 6*</td>
<td>52 ± 7</td>
</tr>
</tbody>
</table>

* P < 0.05 vs A0.

Note. Data are mean ± SEM (n = 6). MFI, mean fluorescence intensity.

a slightly, but not significant, higher platelet recovery than freezing with ThromboSol and 2% Me₃SO. In both procedures, the temperature of freezing and storage did not significantly affect the number of cells being recovered. While cryopreservation caused minor changes in plasma pH, bicarbonate, and glucose levels, an elevation of the extracellular LDH concentrations was observed in all frozen–thawed samples (Table 1). As shown, LDH release was significantly lower in 6% Me₃SO-frozen units than in samples cryopreserved with ThromboSol and 2% Me₃SO. In addition, higher LDH levels were found following freezing–storage at −80°C than with cryopreservation at −120°C.

Platelet Glycoproteins

The comparison of the expression of GPs in fresh platelets with that of those cells after cryopreservation is summarized in Table 2. No significant changes in the platelet surface expression levels of GP IIb/IIIa and CD63 were observed following freezing–thawing under either condition. In contrast, membrane GP Iba expression significantly diminished in frozen–thawed platelets, compared to that of fresh cells (mean decrease 26.0 ± 4.5 and 37.7 ± 1.2% for platelets kept frozen for 1 week and 3 months, respectively). This detrimental effect of freezing on GP Iba was equally pronounced in 6% Me₃SO-frozen platelets and in samples cryopreserved using ThromboSol and 2% Me₃SO and slightly higher in samples frozen–stored in a −80°C deep freezer. Cryopreservation of PCs also promoted a significant increase in the number of platelets expressing the CD62 antigen. As shown, under either condition, P-selectin positive platelets in frozen PCs were almost twice those in fresh PCs, indicative of cell activation during the freezing–thawing procedure.

Platelet Functional Response

The functional status of washed frozen–thawed platelets was assessed by means of in vitro aggregation assays and ligand-binding experiments. As summarized in Table 3, for all freezing conditions, no significant differences in the aggregation response to ristocetin (1 mg/mL) was observed between fresh and cryopreserved–thawed platelets. In contrast, cryopreservation significantly impaired the thrombin-induced aggregation response of frozen–thawed
platelets compared to that of fresh platelets (47.2 ± 6.6% decrease for thrombin 1 U/mL in samples kept frozen for 3 months). This effect was slightly more pronounced in platelets cryopreserved using ThromboSol and 2% Me₃SO than in 6% Me₃SO-frozen cells (Table 3).

Table 4 shows the results of vWF and fibrinogen binding assays in fresh and frozen–thawed platelets. As indicated, platelets kept frozen for 3 months displayed the lowest values of ristocetin-induced binding of [¹²⁵I]vWF, although they did not statistically differ from those of fresh platelets. A similar vWF binding capacity was obtained from platelets cryopreserved using either 6% Me₃SO or ThromboSol and 2% Me₃SO. In agreement with the results of the aggregation experiments, we observed that cryopreservation significantly altered the capacity of platelets to bind [¹²⁵I]fibrinogen upon stimulation with thrombin. Thus, in comparison to the values of fibrinogen binding of fresh platelets, frozen cells exhibited a 46.0 ± 11.2 and 51.2 ± 10.1% decrease after storage for 1 week and for 3 months, respectively. A greater, though not statistically significant, reduction of thrombin-induced fibrinogen binding capacity was observed in ThromboSol and 2% Me₃SO-frozen cells, compared to platelets cryopreserved using 6% Me₃SO (Table 4). This detrimental effect of cryopreservation in thrombin responsiveness was also evidenced by means of the experiments illustrated in Fig. 1. As shown, frozen platelets displayed a significant decrease in the thrombin-induced binding of [¹²⁵I]vWF to platelets, compared with fresh cells. In these assays, a greater, though not statistically significant, impairment in the thrombin reactivity was observed in platelets cryopreserved using 2% Me₃SO and ThromboSol, compared to those cryopreserved using the standard 6% Me₃SO procedure.

**DISCUSSION**

Cryopreservation of platelets extends the shelf life of PCs, from the current 5-day storage limit to 1 year, therefore potentially solving some of the problems associated with inventory management and the stockpiling of HLA-HPA
TABLE 4
Ristocetin (1 mg/mL)-Induced Binding of [125I]vWF and Thrombin (2.5 units/mL)-Induced Binding of [125I]Fibrinogen in Fresh and Frozen–Thawed Platelets

<table>
<thead>
<tr>
<th></th>
<th>Fresh, 1 week</th>
<th>Frozen, 3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6% Me₂SO</td>
<td>2% Me₂SO and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ThromboSol</td>
</tr>
<tr>
<td></td>
<td>2% Me₂SO</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A0</td>
<td>A1</td>
</tr>
<tr>
<td>Control</td>
<td>−80°C</td>
<td>−120°C</td>
</tr>
<tr>
<td></td>
<td>20715</td>
<td>20485</td>
</tr>
<tr>
<td>Ristocetin-induced binding of [125I]vWF (molecules/platelet) ± SEM</td>
<td>4598</td>
<td>4941</td>
</tr>
<tr>
<td>Thrombin-induced binding of [125I]fibrinogen (molecules/platelet) ± SEM</td>
<td>28381</td>
<td>18233*</td>
</tr>
</tbody>
</table>

*Note. Mean values ± SEM (n = 6).
* P < 0.05 vs A0.

typed PCs or autologous platelets to be used in the oncology setting. Among the different cryoprotectants being used, Me₂SO, which also exhibits a platelet activation inhibitory effect, has been proven to be superior to other techniques using glycerol or other agents (3, 15, 31). However, the concerns with respect to the dose-dependent toxicity of Me₂SO, which is minor to moderate at doses of 0.3 mL/kg of body weight and significantly more severe at infusion doses of 0.6 mL/kg (16), emphasize the need to minimize Me₂SO exposure. Assuming the standard therapeutic dose of 1 PC/10 kg of body weight (2), a threshold of 0.3 mL/kg of cryoprotectant would be infused from units cryopreserved with 5% Me₂SO. Moreover, transfusion of unwashed cryopreserved PCs which contain a final Me₂SO of approximately 2% results in no adverse effects (22).

Recently, we and others have shown that a new platelet additive solution resulting from the combination of second-messenger effectors, named ThromboSol, biochemically stabilizes platelets, protecting them from cold-induced damage (10, 33). Currie and co-workers (13) first tested the effectiveness of the combination of ThromboSol and reduced concentrations (2%) of Me₂SO in the cryopreservation of platelets. These authors reported some benefits of using the combination of these substances in the preparation of frozen platelets, compared to the use of Me₂SO alone at either 2 or 6% final concentration. More recently, that approach for cryopreservation resulted in higher retention of functional activities of PCs obtained with recombinant human thrombopoietin (rh-TPO)-treated donors than that achieved from individuals not receiving the cytokine (38). In the present study, we provide further insight into the functional activity, metabolic parameters, and surface expression of glycoproteins of platelets frozen using the reported alternative cryopreserving solution containing ThromboSol and 2% Me₂SO.

The first in vitro characteristic that we analyzed was the retention of cell numbers and metabolic parameters. We found an excellent numerical recovery of platelets (90%) following the cryopreservation process with 2% Me₂SO and ThromboSol, comparable to that with the conventional method using 6% Me₂SO. While both procedures were similar in terms of maintenance of plasma pH, bicarbonate, and glucose levels, a greater elevation of extracellular LDH in samples frozen with reduced Me₂SO concentration and ThromboSol was observed. That in-
crease in the release of the cytosolic enzyme, indicative of unfavorable cell integrity, is not an intrinsic effect of the addition of ThromboSol components (33). Therefore, injurious sequels attributable to the reduction of the concentration of Me₂SO, not being compensated by the addition of the combination of second-messenger inhibitors, are presumed.

The second trait that we analyzed was the expression of the activation markers P-selectin and granulophysin, consequences of the externalization of the contents from α-granules and lysosomes, respectively. Consistent with previous studies (6, 29), we observed that cryopreservation caused a significant increase in surface expression of the platelet activation marker P-selectin, being similar in 6% Me₂SO frozen platelets and in cells processed with 2% Me₂SO and ThromboSol. This finding is in contrast with those previously reported (13) showing a lower percentage of cells expressing P-selectin in PCs cryopreserved with ThromboSol and 2% Me₂SO than with 6% Me₂SO. Such a discrepancy might be related to differences in the flow cytometric procedure used in each study. While Currie et al. (13) assessed P-selectin expression in formalin-fixed cells, we stained the platelets with MoAbs against antigens, immediately after their thawing and before the washing and fixing steps. With respect to granulophysin, no changes between fresh and cryopreserved platelets in the expression of the antigen were observed, a fact that might be reflecting the stronger activatory stimulus necessary for the release of lysosomes compared to that of α-granules (20).

The assessment of the major adhesive membrane GP, GPIIb/IIIa, in cryopreserved platelets showed a 30% decrease from levels present in fresh cells, while there was no measurable loss of the principal aggregatory protein, GPIIb/IIIa,
during the freezing/thawing process. Those results are in accordance with previous data using 5% Me₃SO (29) and in our study, irrespective of the freezing procedure being utilized. Despite the reduction in immunoreactive levels of GPIIbα in cryopreserved platelets, the response to ristocetin, analyzed by agglutination and by the binding of [¹²⁵I]vWF, was preserved, compared to fresh cells. This is consistent with the drastic reduction of more than 50% in the vWF receptor, necessary to affect platelet adhesion under flow conditions (42). It is worth noting that aggregation and binding assays in washed platelets involved a normalized population of cells which might in part have excluded, during the washing procedure, those more severely damaged and/or activated. The in vitro analysis of the thrombin-induced responses (aggregation, binding of [¹²⁵I]fibrinogen and [¹²⁵I]vWF) showed a significant decreased activity of cryopreserved platelets toward the agonist. The reduced platelet aggregation in response to thrombin has been previously reported (36), as well as an impairment in the reactivity of conventionally stored PCs at 22°C toward the agonist (23), regardless of the preservation of immunoreactive levels of GPIIb/IIIa. However, and in contrast to what has been previously described (13), in our study the functional activity of ThromboSol–2% Me₃SO frozen platelets was not significantly superior to that displayed by cells cryopreserved with 6% Me₂SO.

In summary, in this study, platelets cryopreserved with ThromboSol and 2% Me₂SO have viability, metabolic characteristics, and functional status similar to those frozen using the conventional procedure with 6% Me₂SO. The simplification of the cryopreservation process by the addition of the cryoprotectant as a single bolus injection and the reduction in the concentration of Me₂SO being used, with the potential direct transfusion of the product without post-thawing steps, argues in favor of the use of the former procedure. Further in vivo studies are expected, analyzing the behavior of platelets cryopreserved by this newly proposed freezing technique combining ThromboSol and 2% Me₂SO.

ACKNOWLEDGMENTS

We thank Dr. Z. M. Ruggeri for providing MoAbs against GP Ib/IX and IbbIIIa, and acknowledge Instituto Grifols SA, for giving free purified vWF.

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